

## RESEARCH ARTICLE

# Efficacy of vaccines based on chimeric or multiepitope antigens for protection against visceral leishmaniasis: A systematic review

Karine Ferreira Lopes<sup>1\*</sup>, Mariana Lourenço Freire<sup>2</sup>, Silvane Maria Fonseca Murta<sup>1</sup>, Edward Oliveira<sup>1\*</sup>

**1** Genômica Funcional de Parasitos, Instituto René Rachou—Oswaldo Cruz Foundation, Belo Horizonte, Minas Gerais, Brazil, **2** Pesquisa Clínica e Políticas Públicas em Doenças Infecciosas e Parasitárias, Instituto René Rachou—Oswaldo Cruz Foundation, Belo Horizonte, Minas Gerais, Brazil

\* [karine.lopes@aluno.fiocruz.br](mailto:karine.lopes@aluno.fiocruz.br) (KFL); [edward.oliveira@fiocruz.br](mailto:edward.oliveira@fiocruz.br) (EO)



## Abstract

### Background

Visceral leishmaniasis (VL) is an infectious parasitic disease caused by the species *Leishmania (Leishmania) infantum* in the Mediterranean Basin, the Middle East, Central Asia, South America, and Central America, and *Leishmania (Leishmania) donovani* in Asia and Africa. VL represents the most severe and systemic form of the disease and is fatal if left untreated. Vaccines based on chimeric or multiepitope antigens hold significant potential to induce a highly effective and long-lasting immune response against infections by these parasites. This review systematically compiles data on the efficacy and protective capabilities of chimeric and multiepitope antigens, while also identifying potential immunogenic targets for vaccine development.

### Methodology

A systematic search was conducted by independent reviewers across four databases to assess the efficacy of vaccines based on chimeric or multiepitope antigens against VL. The review included original studies that reported parasite load or positivity rates in animals immunized with these vaccines and subsequently challenged or exposed to *L. infantum* infection in preclinical and clinical studies. Key information was extracted, tabulated, and analyzed, with the risk of bias being assessed using the SYRCLE Risk Tool.

### Principal findings

A total of 22 studies were selected, with only one being a randomized clinical trial. Most of the studies were conducted with mice, followed by dogs and hamsters. The reduction in parasite load varied from 14% to 99.6% and from 1.7 to 9.0 log orders. Limiting dilution was the most used method for assessing parasite load, followed by quantitative real-time polymerase chain reaction (qPCR). Most domains had an uncertain risk of bias due to insufficient information described.

## OPEN ACCESS

**Citation:** Lopes KF, Freire ML, Murta SMF, Oliveira E (2024) Efficacy of vaccines based on chimeric or multiepitope antigens for protection against visceral leishmaniasis: A systematic review. PLoS Negl Trop Dis 18(12): e0012757. <https://doi.org/10.1371/journal.pntd.0012757>

**Editor:** Bradford S. McGwire, The Ohio State University, UNITED STATES OF AMERICA

**Received:** August 30, 2024

**Accepted:** December 4, 2024

**Published:** December 31, 2024

**Peer Review History:** PLOS recognizes the benefits of transparency in the peer review process; therefore, we enable the publication of all of the content of peer review and author responses alongside final, published articles. The editorial history of this article is available here: <https://doi.org/10.1371/journal.pntd.0012757>

**Copyright:** © 2024 Lopes et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Data Availability Statement:** All relevant data are within the manuscript and its [Supporting Information](#) files.

**Funding:** KFL received PhD scholarship from the Coordination for the Improvement of Higher Education Personnel-CAPES-Brazil (Grant: 88887.481786-2020-00), SMFM and EO were supported by researcher fellows from the Conselho Nacional de Desenvolvimento Científico e Tecnológico- CNPq (Grants: 309994-2023-3 and 301555/2022-2). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing interests:** The authors have declared that no competing interests exist.

## Conclusions

Vaccine formulations containing various chimeric or multiepitope antigens have been developed and evaluated in different preclinical trials, with only one advancing to clinical trials and commercialization. However, the findings of this review highlight the promising potential of chimeric and multiepitope antigens as vaccine candidates against VL. The evidence presented could play a crucial role in guiding the rational development of new studies focused on using these antigens for vaccination against VL.

## Author summary

Visceral leishmaniasis (VL) is primarily caused by *Leishmania (Leishmania) infantum* and *Leishmania (Leishmania) donovani*. Vaccines based on chimeric or multiepitope antigens have demonstrated significant potential for eliciting effective and long-lasting immune responses against these parasites. This review systematically evaluates the efficacy of these antigens and identifies potential immunogenic targets for vaccine development. A comprehensive search across four databases identified 22 studies, including one randomized clinical trial. Most research was conducted with mice, with additional studies with dogs and hamsters. The reduction in parasite load ranged from 14% to 99.6% and from 1.7 to 9.0 log orders. Limiting dilution was the most used method for assessing parasitic load, followed by quantitative real-time polymerase chain reaction (qPCR), imprint, biopsy culture, and smear. These findings underscore the promising potential of chimeric and multiepitope antigens as vaccine candidates for VL and provide valuable insights for guiding future vaccine development.

## Introduction

Leishmaniasis is a group of parasitic diseases caused by different species of the protozoan genus *Leishmania* (Kinetoplastida; Trypanosomatidae) and represents one of the major public health concerns in developing countries, according to the World Health Organization [1]. The disease manifests in two main clinical forms, namely cutaneous (CL) or visceral (VL) leishmaniasis, with the latter being the most severe, systemic form and lethal, if not treated. VL can be caused by *Leishmania (Leishmania) donovani*, which is endemic in Asia, Africa, and the Indian subcontinent, where transmission is anthroponotic. It can also be caused by *Leishmania (Leishmania) infantum*, which occurs in Europe and the Americas, extending from south-central Texas to Central and South America. *Leishmania infantum* is characterized by zoonotic transmission, with the domestic dog being the main reservoir of the parasite [2,3]. An estimated 50,000 to 90,000 new cases of VL occur worldwide each year. In 2022, around 85% of VL cases were reported in seven countries, including Brazil [4].

Control strategies for zoonotic VL involve the diagnosis and treatment of human cases; euthanasia of seropositive dogs, their main reservoir; control of the parasite vector; and health education. However, these strategies often fail to yield satisfactory results, leading to an annual increase in both human and canine cases [4]. Given this scenario, the development of new VL control strategies is extremely important. Among these, vaccines stand out as one of the most cost-effective and efficient methods for controlling and preventing the disease [5,6].

Vaccines based on chimeric or multiepitope antigens demonstrate significant potential in generating a highly effective and long-lasting immune response against *Leishmania* spp. [7,8]. Compared to recombinant protein vaccines, these formulations, by using multiple epitopes, increase the probability of interaction with several MHC haplotypes, optimizing immune recognition. Furthermore, this approach offers a safer and more specific alternative, overcoming some of the complexities and risks associated with formulations using live or attenuated parasites [9,10]. Although various preclinical studies have investigated the use of chimeric or multiepitope antigens in vaccine formulations against VL [11–13], their efficacy is influenced by several variables, including the choice of animal model, the proteins used in their construction, and the type of adjuvant. This review systematically compiles data on the effectiveness and protective efficacy of chimeric and multiepitope antigens, while also identifying potential immunogenic targets for vaccine development.

## Methods

### Protocol and registration

Before study initiation, a search was conducted in the International Prospective Register of Systematic Reviews to identify any equivalent studies that had been completed or were ongoing. Since no similar study protocol was identified, the current one was subsequently registered (PROSPERO: CRD42023449370). This systematic review was conducted following the methodological principles of the Cochrane Handbook [14] and adhered to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines.

### Eligibility criteria

The guiding question of this study was: What is the efficacy of vaccines based on chimeric or multiepitope antigens for protection against visceral leishmaniasis? In this study, chimeric antigens were defined as molecules formed by the fusion of fragments from various distinct antigens, while multiepitope antigens were defined as those designed through the strategic selection of epitopes derived from different antigens. The PICO framework (Population, Intervention, Comparator, Outcome) was employed in the selection process, defined as follows: (P) patients or animals at risk of developing VL caused by *L. infantum*, or in preclinical studies, were considered animals susceptible to *L. infantum* infection; (I) vaccine based on chimeric antigens or multiepitope antigens; (C) placebo or control groups immunized with adjuvant or saline; and (O) parasite load or positivity rate in studies involving natural infection.

This review included studies that reported parasite load or positivity rate in animals immunized with vaccines based in chimeric or multiepitope antigens (synthetic or recombinant antigens) and challenged or exposed to *L. infantum* infection in preclinical and clinical settings. The exclusion criteria were as follows: I) non-original studies, including literature reviews, editorials, brief communications, and case reports; II) evaluations of vaccine formulations against other *Leishmania* spp.; III) evaluations of immune response or other outcomes without reporting parasite load or positivity rate; IV) in vitro studies; V) studies without a control group; VI) vaccines used as immunotherapy; and VII) publications in languages other than Portuguese, English, or Spanish.

### Search strategy

The search was conducted across four electronic databases, namely MEDLINE (PubMed), Embase, Cochrane Central Registry of Controlled Trials (CENTRAL), and the Virtual Health Library (VHL). For each database, search strategies combining keywords related to “visceral

leishmaniasis” and “vaccines” were combined. Details about the strategy used in each database are available in [S1 File](#). Articles published up to July 27, 2023, were considered, with no restrictions on publication date.

### Selection process

For each database, all retrieved articles were imported to Mendeley Reference Management [15] to identify and remove duplicates. The records were then transferred to Rayyan software [16] for title and abstract screening, which was conducted independently by two reviewers (KLF and MLF) according to inclusion and exclusion criteria, with disagreements being resolved by consensus. The selected studies were read in full by two researchers to confirm eligibility or exclude them considering exclusion criteria. Data were also extracted by two independent researchers (KLF and MLF) with disagreements being previously resolved by a third (EO).

### Data extraction

Relevant data were extracted from each selected article including the first author and year of publication, country of study, animal model, immunogen and adjuvant used, route of administration, parasite and strain, and method of parasite load evaluation. Regarding the construction of chimeric or multiepitope antigens, the original antigens were identified, along with the epitope selection method, alleles used for epitope prediction, the use of spacers and the antigen production method. The results were analyzed according to the parasite load assessment method, considering factors that could influence the outcomes, such as immunogen and adjuvant concentrations, number of doses and intervals between them, parasite concentration and infection site, period between last immunization and experimental challenge or exposure to *L. infantum* infection, and period between challenge and evaluation of the result.

Some considerations were made in summarizing the results. For studies that evaluated a vaccine's efficacy at different points [17], the results were presented from the final evaluation. For studies reporting results as the number of positive animals [18,19], the positivity rate was calculated using the following formula: number of positives/total number in the group  $\times 100$ .

### Risk of bias

Risk of bias was assessed using the SYRCLE risk tool, which is specifically designed for preclinical studies. Two researchers independently performed all assessments (KFL and MLF), and any discrepancies were resolved by consensus.

## Results

### Literature search

An initial search identified 3,091 articles in the databases. Screening and selection based on eligibility criteria resulted in 343 studies for potential inclusion. To avoid the exclusion of potentially eligible records, the exclusion of studies based on the type of intervention was conducted only during the full-text review, which resulted in 22 studies that met the inclusion criteria. The PRISMA flow diagram summarizes the study selection process and reasons for exclusion ([Fig 1](#)).

### Descriptive analysis of included studies

The main characteristics of the 22 included studies are presented in [Table 1](#). They were published in the period of 2003 to 2023. Ten studies were conducted in Brazil [12,20–28], five in

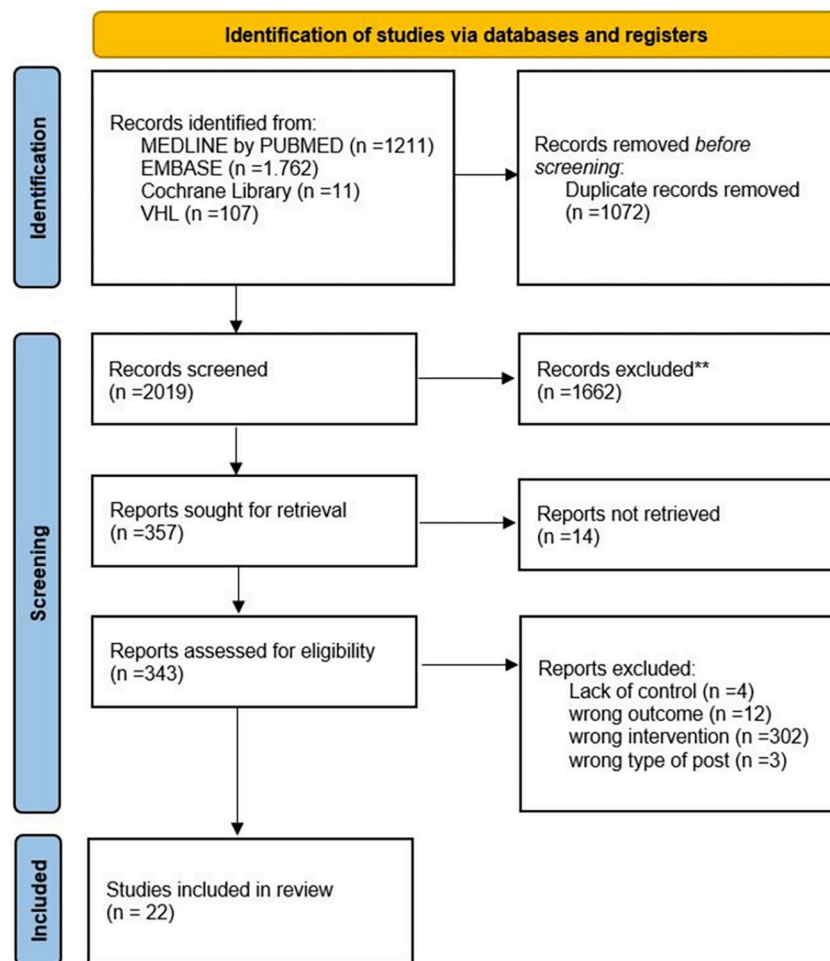


Fig 1. PRISMA flow diagram of the study selection process.

<https://doi.org/10.1371/journal.pntd.0012757.g001>

Spain [17–19,29,30], three in the USA [31–33], two in Greece [34,35], one in Iran [36] and one in Japan [37]. The main animal model used was the mouse (*Mus musculus*), specifically BALB/c (15 out of 22 studies), followed by C57BL (4 out of 22 studies) and B6.Cg-Tg (1 out of 22 studies). Additionally, dogs were evaluated in three studies and hamsters (*Mesocricetus auratus*) in only two.

Most of the included studies were preclinical, with only one randomized clinical trial evaluating natural *Leishmania* infection in dogs previously immunized with a multiepitope vaccine [19]. In total, 19 different vaccine formulations were evaluated, mainly using as adjuvant saponin [12,20–23,30], monophosphoryl lipid A [31,32,34,37], or both [24–27]. In preclinical studies, the subcutaneous route was the most used for vaccine evaluation, whether in the paw, base of the tail or back; intraperitoneal and intramuscular routes were less utilized in the included studies.

Challenge with *L. infantum* occurred predominantly intravenously [17,23,24,27,29–32,34,37], followed by subcutaneously [12,20–22,26,28]. Alternative routes were also used, such as intraperitoneal [25,36], intracardiac [31] and retro-orbital sinus [33], with one study not specifying the route of parasite inoculation [18]. Regarding ethical considerations, only two studies [29,31] included in the review did not explicitly address this aspect for all the different animals models involved in the experiments.

Table 1. The main characteristics of included studies.

Author	Country of study	Animal model (type; age; sex)	Chimeric or multiepitope vaccine *	Adjuvant	Vaccine administration	Challenge: <i>Leishmania</i> species and infection sites	Outcome assessment method
Molano et al. 2003 [17]	Spain	BALB/c; 6-week-old; Beagles; 8 to 9 months old	Protein Q	BCG	IP	<i>L. infantum</i> (M/CAN/ES/96/BCN), IV tail vein	Smear, impression and culture of the spleen
Parody et al. 2004 [29]	Spain	BALB/c; 4 to 6-week-old	Protein Q	CpG-ODN	IP or in the paw	<i>L. infantum</i> , IV	Limiting dilution of the spleen and liver
Coler et al. 2007 [31]	USA	C57BL/6; 6 to 8-week-old; female Hamster LVG; 30–33 days old	Leish-111f	Monophosphoryl lipid A-stable emulsion	SC in the paw and at the base of the tail	<i>L. infantum</i> (MHOM/BR/82/BA-2), IV and intracardiac	Limiting dilution of the spleen and liver
Bertholet et al. 2009 [32]	USA	C57BL/6; 5 to 7-week-old; female	Leish-110	Monophosphoryl lipid A-stable emulsion	SC	<i>L. infantum</i> , IV tail vein	Limiting dilution of the spleen
Carcelén et al. 2009 [18]	Spain	Beagles; 1 to 2 years old; both sexes	Protein Q	No adjuvant	SC on the left flank	<i>L. infantum</i> (M/CAN/ES/96/BCN 150, zimodeme MON-1)	DNA polymerase chain reaction of the skin; biopsy culture and imprint of the spleen and lymph node
Goto et al. 2011 [37]	Japan	C57BL/6; 6 to 8-week-old; female	KSAC	Monophosphoryl lipid A-stable emulsion	SC at the base of the tail	<i>L. infantum</i> (MHOM/BR/82/BA-2), IV tail vein	Limiting dilution of the spleen and liver
Duthie et al. 2017 [33]	USA	C57BL/6 to 8-week-old; female BALB/c	LEISH-F3	GLA-SE	SC at the base of the tail	<i>L. infantum</i> (MHOM/BR/82/BA-2), retro-orbital sinus	DNA polymerase chain reaction of the liver
Athanasiou et al. 2017 [34]	Greece	B6.Cg-Tg (HLA-A/H2-D); 6 to 8-week-old; female	Mix B	Monophosphoryl lipid A	SC	<i>L. infantum</i> (MHOM/GR/2001/GH8), IV	Limiting dilution of the spleen and liver
Martins et al. 2017 [12]	Brazil	BALB/c; 8-week-old; female	Recombinant chimeric protein (RCP)	Saponin	SC in the left hind paw	<i>L. infantum</i> (MHOM/BR/1970/BH46), SC right hind paw	Limiting dilution of the spleen, liver, bone marrow, and draining lymph nodes; DNA polymerase chain reaction of the spleen
Cotrina et al. 2018 [19]	Spain	Dogs	Protein Q (LetiFend)	No adjuvant	SC intrascapular	No challenge	DNA polymerase chain reaction and smear of the of bone marrow and lymph nodes
Dias et al. 2018 [20]	Brazil	BALB/c; 6 to 8-week-old; female	Chimera	Saponin	SC in the left hind paw	<i>L. infantum</i> (MHOM/BR/1970/BH46), SC right hind paw	Limiting dilution of the spleen, liver, bone marrow, and draining lymph nodes; DNA polymerase chain reaction of the spleen
Lage et al. 2020 [21]	Brazil	BALB/c; 8-week-old; female	Chimera T	Saponin	SC in the left hind paw	<i>L. infantum</i> (MHOM/BR/1970/BH46), SC right hind paw	Limiting dilution of the spleen, liver, bone marrow, and draining lymph nodes; DNA polymerase chain reaction of the spleen
Agallou et al. 2020 [35]	Greece	BALB/c; 6 to 8-week-old; female	LiChimera	Addavax	IM	<i>L. infantum</i> (MHOM/GR/2001/GH8), IV tail vein	Limiting dilution of the spleen and liver
Vakili et al. 2020 [36]	Iran	BALB/c; 6 to 8-week-old; female	Vaccine	Freund	SC	<i>L. infantum</i> (MCAN/IR/07/Moheb-gh), IP	Imprint of the spleen
Brito et al. 2020 [23]	Brazil	BALB/c; 6 to 8-week-old; female	Chimera A and Chimera B	Saponin	SC on the back	<i>L. infantum</i> (MCAN/BR/2008/OP46), IV	DNA polymerase chain reaction of the spleen

(Continued)



Table 1. (Continued)

Author	Country of study	Animal model (type; age; sex)	Chimeric or multiepitope vaccine *	Adjuvant	Vaccine administration	Challenge: <i>Leishmania</i> species and infection sites	Outcome assessment method
Martínez-Rodrigo et al. 2020 [30]	Spain	BALB/c; 6 to 8-week-old; female	HisDTC e AK	Saponin	SC in the paw	<i>L. infantum</i> (MCAN/ES/96/BCN150 zymodeme MON-1), IV	Limiting dilution of the spleen and liver
Lage et al. 2020 [22]	Brazil	BALB/c; 8-week-old; female	Chimera T/ Liposoma	Saponin	SC in the left hind paw	<i>L. infantum</i> (MHOM/BR/1970/BH46), SC right hind paw	Limiting dilution of the spleen, liver, bone marrow, and draining lymph nodes; DNA polymerase chain reaction of the spleen
Ostolin et al. 2021 [24]	Brazil	BALB/c; 6 to 8-week-old; female	Chimera A	Saponin and Monophosphoryl lipid A	SC on the back	<i>L. infantum</i> (MCAN/BR/2008/OP46), IV	DNA polymerase chain reaction of the spleen
Lage et al. 2022 [26]	Brazil	BALB/c; 8-week-old; female	ChimT	Saponin or Monophosphoryl lipid A	SC in the left hind paw	<i>L. infantum</i> (MHOM/BR/1970/BH46), SC right hind paw	Limiting dilution of the spleen, liver, bone marrow, and draining lymph nodes; DNA polymerase chain reaction of the spleen
Ostolin et al. 2022 [27]	Brazil	BALB/c; 6 to 8-week-old; female	Poly-T Leish	Saponin and Monophosphoryl lipid A	SC	<i>L. infantum</i> (MCAN/BR/2008/OP46), IV tail vein	DNA polymerase chain reaction of the spleen and liver
Gusmão et al. 2022 [25]	Brazil	Golden hamster; 4 to 6-week-old	Chimera A and Chimera B	Saponin and Monophosphoryl lipid A	SC on the back	<i>L. infantum</i> (MCAN/BR/2008/OP46), IP	DNA polymerase chain reaction of the spleen
Clímaco et al. 2023 [28]	Brazil	BALB/c; 6 to 8-week-old; female	Chimera	Monophosphoryl lipid A	SC	<i>L. infantum</i> (MOM/BR/1970/BH46), SC right hind paw	DNA polymerase chain reaction of the liver

SC—subcutaneous; IP—intraperitoneal; IM—intramuscular; IV—intravenous \* For more detail see Table 2.

<https://doi.org/10.1371/journal.pntd.0012757.t001>

Table 2 summarizes the main characteristics of the evaluated chimeric and multiepitope antigens, highlighting their constituent proteins, epitope selection methods, use of spacers in the sequence design, and antigen production method. In this context, a total of 17 distinct antigens were constructed, including eleven multiepitope antigens, five chimeric antigens, and one pool of three synthetic peptides designed with selected epitopes from *Leishmania infantum* proteins by prediction analyses.

Only one study did not describe the source proteins used in the construction of the multiepitope antigen [28]. Common proteins across different studies include ribosomal and histones proteins (e.g. Lip2a, Lip2b, P0 and H2A), cysteine peptidases (CPA, CPB, and CPC), and hypothetical proteins (LiHy, LiH1, LiH2, etc.). Most studies described the prediction of MHC class I and II epitopes as their method for epitope selection; however, the approach used was not described in some studies [17–19,29,31–33,37]. The use of spacers was relatively uncommon, being reported in only nine of the 22 included studies. In some cases, antigens previously described in other studies were used and, in these cases, the original studies were consulted. Among these, the amino acid sequence “GGPPG” was the most frequently reported spacer [23–25,27,38]. Most of the included studies used chimeric or multiepitope antigens produced by recombinant DNA technology, employing the bacterium *Escherichia coli* as the expression vector. However, the studies by Athanasiou et al. (2017) [34] and Martínez-Rodrigo et al. (2020) [30] used chemically synthesized antigens, while Cotrina et al. (2018) [19] utilized the

Table 2. Characteristics of the chimeric or multiepitope antigens of the evaluated vaccine formulations.

Author	Source Proteins	Epitope Selection Method	Alleles Used for Epitope Prediction	Uses spacers? If yes, which ones?	Multiepitope vaccine	Chimeric or multiepitope antigen production method
Molano et al. 2003 [17]	Acidic ribosomal proteins Lip2a, Lip2b, P0 and the histone H2A protein from <i>L. infantum</i>	NM	NM	No	Protein Q	Expression in <i>E. coli</i>
Parody et al. 2004 [29]	Acidic ribosomal proteins Lip2a, Lip2b, P0, and the histone H2A protein from <i>L. infantum</i>	NM	NM	No	Protein Q	Expression in <i>E. coli</i>
Coler et al. 2007 [31]	<i>L. braziliensis</i> elongation initiation factor (LeIF), <i>L. major</i> stress-inducible protein (LmSTI1), and <i>L. major</i> thiol-specific antioxidant (TSA)	NM	NM	No	Leish-111f	Expression in <i>E. coli</i>
Bertholet et al. 2009 [32]	<i>L. braziliensis</i> elongation initiation factor (LeIF), <i>L. major</i> stress-inducible protein (LmSTI1), and <i>L. major</i> thiol-specific antioxidant (TSA)	NM	NM	No	Leish-110	Expression in <i>E. coli</i>
Carcelén et al. 2009 [18]	Acidic ribosomal proteins Lip2a, Lip2b, P0, and the histone H2A protein from <i>L. infantum</i>	NM	NM	No	Protein Q	Expression in <i>E. coli</i>
Goto et al. 2011 [37]	<i>L. infantum</i> kinetoplastid membrane protein 11 (XM_001468995.1), <i>L. infantum</i> sterol 24-c-methyltransferase (XM_001469795.1), <i>L. donovani</i> A2 (S69693.1), and <i>L. infantum</i> cysteine proteinase B (AJ420286.1)	NM	NM	No	KSAC	Expression in <i>E. coli</i>
Duthie et al. 2017 [33]	<i>L. infantum</i> nucleoside hydrolase (NH), and <i>L. donovani</i> sterol-24-c-methyltransferase (SMT)	NM	NM	No	LEISH-F3	Expression in <i>E. coli</i>
Athanasioi et al. 2017 [34]	Cysteine peptidase A (CPA), histone H1, and e kinetoplastid membrane protein 11 (KMP11) from <i>L. infantum</i>	Prediction of MHC-I and MHC-II epitopes	HLA-A2 (A*0201), HLA-A3 (A*03), HLA-A24 (A*2402), HLA-DRB1, HLA-DPA1, and HLA-DQA1 from human; H2-Db, and H2-Kb from mouse	No	Mix B	Chemical synthesis
Martins et al. 2017 [12]	Hypothetical amastigote-specific protein 1 (XP_001468941.1), LiHyp6 (XP_001568689.1), IgE-dependent histamine-releasing factor (HRF) (CAJ05086.1), and hypothetical protein <i>Leishmania</i> LiHyV (XP_888524.1) from <i>L. infantum</i>	Prediction of MHC-I and MHC-II epitopes	HLA-A2, HLA-A3, and HLA-B7 from human; H-2-Kd, H-2-Ld, H-2-Dd, I-Ad; I-Ed from mouse	No	Recombinant chimeric protein (RCP)	Expression in <i>E. coli</i>
Cotrina et al. 2018 [19]	Acidic ribosomal proteins Lip2a, Lip2b, P0 and the histone H2A protein from <i>L. infantum</i>	NM	NM	No	Protein Q (LetiFend)	Commercial vaccine
Dias et al. 2018 [20]	Hypothetical protein S (XP_001467126.1), small glutamine-rich tetratricopeptide repeat-containing (XP_001467120.1), and prohibitin (XP_001468827.1) from <i>L. infantum</i>	Prediction of MHC-I and MHC-II epitopes	HLA-A2, HLA-A3, and HLA-B7 from human; H-2-Kd, H-2-Ld, H-2-Dd, I-Ad, and I-Ed from mouse	No	Chimera	Expression in <i>E. coli</i>

(Continued)



Table 2. (Continued)

Author	Source Proteins	Epitope Selection Method	Alleles Used for Epitope Prediction	Uses spacers? If yes, which ones?	Multiepitope vaccine	Chimeric or multiepitope antigen production method
Lage et al. 2020 [21]	Prohibitin (XP_001468827.1), Eukaryotic Initiation Factor 5a (XP_001466105.1), <i>Leishmania</i> hypothetical protein 1 (XP_001468941.1), and <i>Leishmania</i> hypothetical protein 2 (XP_001462854.1) from <i>L. infantum</i>	Prediction of MHC-I and MHC-II epitopes	A2, A3, A24, B7 from human; H-2Db, H-2Dd, H-2Kb, H-2Kd, H-2Kk, H-2Ld, H-2IAb, H-2IAd, H-2Ias, H-2IEd, H-2IEb from mouse	Yes, amino acid sequence GG	Chimera T	Expression in <i>E. coli</i>
Agallou et al. 2020 [35]	Cyclophilin 2 (XP_001463094), cyclophilin 40 (XP_001469283), enolase (XP_001468063), dihydrolipoamide dehydrogenase (XP_001468025), mitochondrial chaperonin HSP60 (XP_001467869), and a hypothetical protein (XP_001463461) from <i>L. infantum</i>	Prediction of MHC-I and MHC-II epitopes	H2-Dd, H2-Kd, H2-Ld, H2-IAd, and H2-IEd from mouse	Yes, amino acid sequence AAY, GGPPG, and EAAAK	LiChimera	Expression in <i>E. coli</i>
Vakili et al. 2020 [36]	Histone H1, sterol 24-c-methyltransferase (SMT), and <i>Leishmania</i> -specific antigenic protein (LSAP) from <i>L. infantum</i> , and <i>Leishmania</i> -specific hypothetical protein (LiHy) from <i>L. major</i>	Prediction of MHC-I and MHC-II epitopes and IFN- $\gamma$ inducers	H-2Db, H-2Dd, H-2Kb, H-2Kd, H-2Kk, H-2Ld, H-2IAb, H-2IAd, H-2IAs, H-2IEd, and H-2IEb from mouse	Yes, amino acid sequence AAYKK, GSGSGS, and EAAAK	NI	Expression in <i>E. coli</i>
Brito et al. 2020 [23]	Chimera A: Histone protein (H2A), acid ribosomal protein P2 (LiP2a), acid ribosomal protein P0 (LiP0), <i>Leishmania</i> homologue of activated C kinase (LACK), and Cysteine peptidase C (CPC) from <i>L. infantum</i> Chimera B: Cysteine peptidase A (CPA), cysteine peptidase B (CPB), surface antigenic protein (PSA-50S), and amastigote protein A2 (A2) from <i>L. infantum</i>	Prediction of MHC-I and MHC-II epitopes	HLA-B62, HLA-A*01, HLA-A*02, HLA-A*03, HLA-A*24, HLA-A*26, HLA-B*27, HLA-B*39, HLA-B*44, HLA-B*58, HLA-B*62, HLA-DRB1*0101, HLA-DRB1*0401, HLA-DRB1*0404, HLA-DRB1*0701, HLA-DRB1*0901, HLA-DRB1*1302, HLA-DRB1*1501, HLA-DRB4*0101, and HLA-DRB5*0101 from human; H2-Dd, H2-Dk, and H2-Kd from mouse	Yes, amino acid sequence GGPPG	Chimera A and Chimera B	Expression in <i>E. coli</i>
Martínez-Rodrigo et al. 2020 [30]	Histone H2A, histone H2B, Histone H3, histone H4, A2 protein, and kinetoplastid membrane protein 11 from <i>L. infantum</i>	Prediction of MHC-I and MHC-II epitopes	HLA-A, HLA-B, HLA-C, HLA-E, HLA-DR, HLA-DP, and HLA-DQ from human; H2 from mouse	No	HisDTC and AK	Chemical synthesis
Lage et al. 2020 [22]	Prohibitin (XP_001468827.1), Eukaryotic Initiation Factor 5a (XP_001466105.1), <i>Leishmania</i> hypothetical protein 1 (XP_001468941.1), and <i>Leishmania</i> hypothetical protein 2 (XP_001462854.1) from <i>L. infantum</i>	Prediction of MHC-I and MHC-II epitopes	HLA-A2, HLA-A3, HLA-A24, HLA-B7, and HLA-DR from human; H-2Db, H-2Dd, H-2Kb, H-2Kd, H-2Kk, H-2Ld, H-2IAb, H-2IAd, H-2Ias, H-2IEd, and H-2IEb from mouse	Yes, amino acid sequence GG	Chimera T/ Liposome	Expression in <i>E. coli</i>

(Continued)

Table 2. (Continued)

Author	Source Proteins	Epitope Selection Method	Alleles Used for Epitope Prediction	Uses spacers? If yes, which ones?	Multiepitope vaccine	Chimeric or multiepitope antigen production method
Ostolin et al. 2021 [24]	Histone protein (H2A), acid ribosomal protein P2 (LiP2a), acid ribosomal protein P0 (LiP0), <i>Leishmania</i> homologue of activated C kinase (LACK), and Cysteine peptidase C (CPC) from <i>L. infantum</i>	Prediction of MHC-I and MHC-II epitopes	HLA-B*62, HLA-A*01, HLA-A*02, HLA-A*03, HLA-A*24, HLA-A*26, HLA-B*27, HLA-B*39, HLA-B*44, HLA-B*58, HLA-B*62, HLA-DRB1*0101, HLA-DRB1*0401, HLA-DRB1*0404, HLA-DRB1*0701, HLA-DRB1*0901, HLA-DRB1*1302, HLA-DRB1*1501, HLA-DRB4*0101, and HLA-DRB5*0101 from human; H2-Dd, H2-Dk, and H2-Kd from mouse	Yes, amino acid sequence GGPPG	Chimera A	Expression in <i>E. coli</i>
Lage et al. 2022 [26]	<i>Leishmania</i> hypothetical protein 1 (XP_001468941.1), <i>Leishmania</i> hypothetical protein V (XP_001462854.1), <i>Leishmania</i> hypothetical protein C (XP_001470432.1), and <i>Leishmania</i> hypothetical protein G (XP_001467126.1) from <i>L. infantum</i>	Prediction of MHC-I and MHC-II epitopes	HLA-A*2, HLA-A*3, HLA-A*24, HLA-B*7, and HLA-DR from human; H-2Db, H-2Dd, H-2Kb, H-2Kd, H-2Kk, H-2Ld, H-2IAb, H-2IAd, H-2Ias, H-2IEd, and H-2IEb from mouse	Yes, amino acid sequence GG, and KK	ChimT	Expression in <i>E. coli</i>
Ostolin et al. 2022 [27]	Cysteine peptidase A (CPA), cysteine peptidase B (CPB), surface antigenic protein (PSA-50S), and amastigote protein A2 (A2) from <i>L. infantum</i>	Prediction of MHC-I and MHC-II epitopes	HLA-B*62, HLA-A*01, HLA-A*02, HLA-A*03, HLA-A*24, HLA-A*26, HLA-B*27, HLA-B*39, HLA-B*44, HLA-B*58, HLA-B*62, HLA-DRB1*0101, HLA-DRB1*0401, HLA-DRB1*0404, HLA-DRB1*0701, HLA-DRB1*0901, HLA-DRB1*1302, HLA-DRB1*1501, HLA-DRB4*0101, and HLA-DRB5*0101 from human; H2-Dd, H2-Dk, and H2-Kd from mouse	Yes, amino acid sequence GGPPG	Poly-T Leish	Expression in <i>E. coli</i>
Gusmão et al. 2022 [25]	Chimera A: Histone protein (H2A), acid ribosomal protein P2 (LiP2a), acid ribosomal protein P0 (LiP0), <i>Leishmania</i> homologue of activated C kinase (LACK), and Cysteine peptidase C (CPC) from <i>L. infantum</i> Chimera B: Cysteine peptidase A (CPA), cysteine peptidase B (CPB), surface antigenic protein (PSA-50S), and amastigote protein A2 (A2) from <i>L. infantum</i>	Prediction of MHC-I and MHC-II epitopes		Yes, amino acid sequence GGPPG	Chimera A and Chimera B	Expression in <i>E. coli</i>
Clímaco et al. 2023 [28]	NI	Prediction of MHC-I and MHC-II epitopes	HLA-A*2, HLA-A*3, and HLA-B*7 from human	No	Chimera	Expression in <i>E. coli</i>

NI—Not informed: the original article did not include any information about the specific item; NM—No made: the analysis was not conducted in the studies

<https://doi.org/10.1371/journal.pntd.0012757.t002>

manufactured LetiFend vaccine that contains Protein Q as the active pharmaceutical ingredient.

Here, parasite load was considered the main outcome of interest to understand the vaccine efficacy of different formulations. However, there was no consensus on the evaluation methodology, the evaluated organs, or the best way to present the results. Therefore, Tables 3 to 5 shown the outcomes according to the methodology used to evaluate parasite load.

Limiting dilution was the most frequently used methodology (12/22) to assess parasite load in the spleen, liver, bone marrow, and lymph nodes (Table 3). In these studies, parasite load was mainly evaluated after three doses of vaccine with 14 days between them. For the challenge, parasite dose in the infection ranged from  $5 \times 10^5$  to  $1 \times 10^7$ . These studies compared the parasite load of organs of animals immunized with chimeric or multiepitope antigens with the load of animals immunized with saline, adjuvant, or non-immunized. Parasite load reduction was demonstrated through a percentage, ranging from 14% [35] to 99.6% [31], or through log orders, ranging from 1.7 [26] to 9.0 [21]. However, some studies only presented qualitative descriptions of the results without quantifying the reduction in parasite load [12,20,30,32].

Several studies independently evaluated formulations containing a multiepitope antigen and those containing the multiepitope antigen associated with adjuvants. The addition of adjuvants is generally associated with a greater reduction in parasite load [12,29,35]. Animals immunized with Protein Q, Leish-111f, or Chimera T, experienced a reduction in parasite load of more than 90%, particularly in the spleen and liver, compared to animals immunized with saline [17,22,31]. Notably, Chimera T was the only immunogen that achieved this high reduction in parasite load without the need for an adjuvant [22].

The studies that assessed parasite load using qPCR are described in Table 4. Among these, only one used dog as an animal model, immunizing them with one or two doses of Protein Q, and observed variable positivity, ranging from 14.4% to 57.1%, in the skin detected by qPCR after experimental infection. Most of the studies evaluated vaccines in mice or hamsters and used three doses with intervals of 14 or 21 days.

The spleen was the most frequently evaluated organ [12,20,22–26], followed by the liver [28,33], or both organs [27], in addition to the skin [18]. Kinetoplast minicircle DNA (kDNA) was the most employed molecular target for quantifying parasite load, used in nine studies. Only two studies utilized different targets, namely genomic repeat region specific to *Leishmania* species [33] and DNA polymerase [23]. The reduction in parasite load ranged from 41.9% [12] to 96% [25], or was reduced by 2 to 7 times [26].

A limited number of studies employed alternative methodologies to assess parasite load, including imprinting [17,18,36], biopsy culture [17,18] and organ smears [17] (Table 5). Parasite load reduction in murine model ranged from 72% to 81%, while in dogs the antigen protein achieved a protective efficacy of 90% [17]. Furthermore, Carcelén et al. (2009) [18] presented results based on the number of negative dogs, while the effectiveness of the tested formulation was not quantified in Vakili et al. (2020) [36], although a significant reduction was found.

Table 6 presents the data from the only clinical trial included. The LetiFend vaccine, based on Protein Q, was evaluated in 168 dogs compared to a placebo group, with both groups being exposed to a natural infection and receiving a single vaccine dose. The results were analyzed based on animal infection, assessed by qPCR and bone marrow and lymph node smears. The infection positivity rate in the placebo group was 16.1%, while in the vaccinated group it was 7.15%, as detected by qPCR. These results were consistent with the smear findings, where the positivity rate was 5.35% in the vaccinated group and 13.9% in the placebo group [19].

Table 3. Parasite load assessed by limiting dilution reported by the different included studies.

Authors	Animal model	Formulation with chimeric or multiepitope chimeric vaccine	Number of doses (interval between doses)	<i>L. infantum</i> concentration and infection site	Days between last immunization and challenge	Days between infection and outcome assessment	Parasite load assessment compared to control group:		
							Saline	Adjuvant	Non-immunized
Parody et al. 2004 [29]	Mouse (BALB/c)	Protein Q (2 µg)	2 x (15 days)	1x10 <sup>6</sup> IV	21	28	↓ 50% in the spleen and in the liver	NA	NA
	Mouse (BALB/c)	Protein Q (2 µg) + CpG-ODN (20 µg)	2 x (15 days)	1x10 <sup>6</sup> IV	21	28	↓ 99% in the spleen and in the liver	NA	NA
	Mouse (BALB/c)	Protein Q (2 µg) + CpG-ODN (20 µg)	2 x (15 days)	1x10 <sup>6</sup> IV	98	28	↓ 89% in the spleen	NA	NA
Coler et al. 2007 [31]	Mouse (C57BL/6)	Leish-111f (10 µg) + MPL-SE (20 µg)	3 x (21 days)	5x10 <sup>6</sup> IV	30	35	↓ 91.7% in the liver	NA	NA
	Hamster LVG	Leish-111f (10 µg) + MPL-SE (20 µg)	3 x (21 days)	5x10 <sup>6</sup> intracardiac	30	30	↓ 99.6% in the spleen; NSr in liver	NA	NA
Bertholet et al. 2009 [32]	Mouse (C57BL/6)	Leish-110 (10 µg) + MPL-SE (20 µg)	3 x (14 days)	5x10 <sup>6</sup> IV tail vein	NI	28	SR in the spleen	NA	NA
Goto et al. 2011	Mouse (C57BL/6)	KSAC (10 µg) + MPL-SE (20 µg)	3 x (21 days)	5x10 <sup>6</sup> IV tail vein	21	28	↓ 66% in the spleen and 93% in the liver	NA	NA
Athanasίου et al. 2017 [34]	Mouse B6.Cg-Tg (HLA-A/H2-D)	Mix B (6 µg) + MPLA (3 µg)	3 x (14 days)	2x10 <sup>7</sup> IV	14	30	NA	NA	↓ 61.98% in the spleen e 72.91% in the liver
	Mouse B6.Cg-Tg (HLA-A/H2-D)	Mix B (6 µg) + MPLA (3 µg)	3 x (14 days)	2x10 <sup>7</sup> IV	14	60	NA	NA	↓ 73.64% in the spleen and 64.4% in the liver
Martins et al. 2017 [12]	Mouse (BALB/c)	Recombinant chimeric protein (RCP) (25 µg) + saponin (25 µg)	3 x (14 days)	1x10 <sup>7</sup> SC right hind paw	30	70	SR in the spleen, liver, bone marrow and draining lymph nodes	NA	NA
	Mouse (BALB/c)	Recombinant chimeric protein (RCP) (25 µg)	3 x (14 days)	1x10 <sup>7</sup> SC right hind paw	30	70	NSr in the spleen, liver, bone marrow and draining lymph nodes	NSr in the spleen, liver, bone marrow and draining lymph nodes	NA
Dias et al. 2018 [20]	Mouse (BALB/c)	Chimera (25 µg) + saponin (25 µg)	3 x (14 days)	1x10 <sup>7</sup> SC right hind paw	60	60	SR in the spleen, liver, bone marrow and draining lymph nodes	SR in the spleen, liver, bone marrow and draining lymph nodes	NA
	Mouse (BALB/c)	Chimera (15 µg)	3 x (14 days)	1x10 <sup>7</sup> SC right hind paw	60	60	NSr in the spleen, liver, bone marrow and draining lymph nodes	NSr in the spleen, liver, bone marrow and draining lymph nodes	NA

(Continued)

Table 3. (Continued)

Authors	Animal model	Formulation with chimeric or multiepitope chimeric vaccine	Number of doses (interval between doses)	<i>L. infantum</i> concentration and infection site	Days between last immunization and challenge	Days between infection and outcome assessment	Parasite load assessment compared to control group:		
							Saline	Adjuvant	Non-immunized
Lage et al. 2020 [21]	Mouse (BALB/c)	Chimera T (15 µg) + saponin (15 µg)	3 x (14 days)	1x10 <sup>7</sup> SC right hind paw	30	45	↓ ~6.0–9.0 log orders of magnitude in the spleen, liver, bone marrow and draining lymph nodes	↓ ~6.0–9.0 log orders of magnitude in the spleen, liver, bone marrow and draining lymph nodes	NA
Agallou et al. 2020 [35]	Mouse (BALB/c)	LiChimera (10 µg) + Addavax (40 µL)	2 x (14 days)	1x10 <sup>7</sup> IV tail vein	14	56	↓ 73% in the spleen and 98% in the liver	NA	NA
	Mouse (BALB/c)	LiChimera (10 µg)	2 x (14 days)	1x10 <sup>7</sup> IV tail vein	14	56	↓ 46% in the spleen and 98% in the liver	NA	NA
	Mouse (BALB/c)	LiChimera (10 µg) + Addavax (40 µL)	2 x (14 days)	1x10 <sup>7</sup> IV tail vein	14	84	↓ 83% in the spleen and 88% in the liver	NA	NA
	Mouse (BALB/c)	LiChimera (10 µg)	2 x (14 days)	1x10 <sup>7</sup> IV tail vein	14	84	↓ 14% in the spleen and 37% in the liver	NA	NA
Martinez-Rodrigo et al. 2020 [30]	Mouse (BALB/c)	HisDTC (50 µg) + saponin (25 µg)	3 x (15 days)	5x10 <sup>5</sup> IV	30	42	SR in the spleen and in the liver	SR in the spleen and in the liver	NA
	Mouse (BALB/c)	AK (50 µg) + saponin (25 µg)	3 x (15 days)	5x10 <sup>5</sup> IV	30	42	SR in the liver; NSr in spleen	SR in the liver; NSr in spleen	NA
	Mouse (BALB/c)	HisDTC (25 µg) + AK (25 µg) + saponin	3 x (15 days)	5x10 <sup>5</sup> IV	30	42	SR in the spleen and in the liver	SR in the spleen and in the liver	NA
Lage et al. 2020 [22]	Mouse (BALB/c)	Chimera T (20 µg)	3 x (14 days)	1x10 <sup>6</sup> SC right hind paw	30	45	↓ 98% in the spleen and in the draining lymph nodes, and 94% in the liver and bone marrow	NA	NA
	Mouse (BALB/c)	Chimera T (20 µg) + saponin (20 µg)	3 x (14 days)	1x10 <sup>6</sup> SC right hind paw	30	45	↓ > 99.5% in the spleen, liver, bone marrow and draining lymph nodes	NA	NA
	Mouse (BALB/c)	Chimera T (20 µg) /Liposome	3 x (14 days)	1x10 <sup>6</sup> SC right hind paw	30	45	↓ > 99.5% in spleen, liver, bone marrow and draining lymph nodes	NA	NA

(Continued)

Table 3. (Continued)

Authors	Animal model	Formulation with chimeric or multiepitope chimeric vaccine	Number of doses (interval between doses)	<i>L. infantum</i> concentration and infection site	Days between last immunization and challenge	Days between infection and outcome assessment	Parasite load assessment compared to control group:		
							Saline	Adjuvant	Non-immunized
Lage et al. 2022 [26]	Mouse (BALB/c)	ChimT (20 µg) + saponin (20 µg)	3 x (14 days)	1x10 <sup>7</sup> SC right hind paw	30	45	NA	↓ 4.0 log orders of magnitude in the spleen, 2.5 in the liver, 1.7 in the bone marrow and 4.7 in the draining lymph nodes	NA
	Mouse (BALB/c)	ChimT (20 µg) + MPLA (20 µg)	3 x (14 days)	1x10 <sup>7</sup> SC right hind paw	30	45	NA	↓ 4.5 log orders of magnitude in the spleen, 3.2 in the liver, 2.5 in the bone marrow and 4.5 in the draining lymph nodes	NA
	Mouse (BALB/c)	ChimT (20 µg)	3 x (14 days)	1x10 <sup>7</sup> SC right hind paw	30	45	NSr in the spleen, liver, bone marrow and draining lymph nodes	No Sr in the spleen, liver, bone marrow and draining lymph nodes	NA

SC—subcutaneous; IV—intravenous; NSr—non-significant results; SR—significant results; NA—not assessed

<https://doi.org/10.1371/journal.pntd.0012757.t003>

## Risk of bias assessment

The risk of bias assessment is presented in Fig 2. Except for detection bias, all other domains predominantly indicate an uncertain risk. This is due to insufficient information or inadequate descriptions in many of the evaluated studies. Conversely, most studies showed a low risk of bias in the detection bias domain.

## Discussion

The development of a safe, immunogenic, and effective vaccine is crucial for controlling VL. Vaccines based on chimeric or multiepitope antigens represent a promising approach, combining multiple epitopes into a single sequence to enhance the immune response and provide broader protection against the antigenic variability of the parasite [9,10]. Although some review articles on chimeric or multiepitope vaccines against VL have been published [39–42], a systematic evaluation of the tested vaccine candidates has not yet been conducted. The initial finding of the present review highlights the efforts made in this field but reveals a significant gap between preclinical and clinical studies. Out of 22 articles retrieved through the search strategy, only one was identified as a randomized clinical trial [19]. This finding also highlights the absence of a strategic plan for developing vaccines against VL. A recurring pattern emerged among the 22 selected studies, characterized by the repeated use of the same antigens across various approaches. For instance, Protein Q was investigated in multiple studies, both alone and in combination with various adjuvants [17,29]. It was also explored in research assessing different immunization protocols [18]. Protein Q was engineered by fusion of five intracellular antigenic fragments of acidic ribosomal proteins (LiP2a, LiP2b and LiP0) and histone A2 protein. The antigenicity of Protein Q was first evaluated in an enzyme-linked immunosorbent



Table 4. Parasite load assessed by DNA polymerase chain reaction (qPCR) reported by the different included studies.

Authors	Animal model	Formulation with chimeric or multi-epitope vaccine	Number of doses (interval between doses)	<i>L. infantum</i> concentration and infection site	Days between last immunization and challenge	Days between infection and outcome assessment	qPCR target for parasitic load	Parasite load assessment compared to control group:		
								Saline	Adjuvant	Other controls
Carcelén et al. 2009 [18]	Dog (Beagle)	Protein Q (100 µg)	Single dose	5x10 <sup>5</sup>	60	330	Kinetoplast minicircle DNA	NA	NA	Positivity rate = 14.4%
	Dog (Beagle)	Protein Q (100 µg)	2 x (21 days)	5x10 <sup>5</sup>	39	330	Kinetoplast minicircle DNA	NA	NA	Positivity rate = 57.1%
Duthie et al. 2017 [33]	Mouse (C57BL/6)	LEISH-F3 (5 µg) + GLA-SE (5 µg)	3 x (21 days)	1x10 <sup>6</sup> retro-orbital sinus	30	28	genomic repeat region specific to <i>Leishmania</i> species	Sr in the liver	NA	NA
	Mouse (BALB/c)	LEISH-F3 (5 µg) + GLA-SE (5 µg)	3 x (21 days)	1x10 <sup>6</sup> retro-orbital sinus	30	28	genomic repeat region specific to <i>Leishmania</i> species	Sr in the liver	NA	NA
Martins et al. 2017 [12]	Mouse (BALB/c)	RCP (25 µg) + saponin (25 µg)	3 x (14 days)	1x10 <sup>7</sup> SC right hind paw	30	70	Kinetoplast minicircle DNA	↓ 49.8% in the spleen	↓ 41.9% in the spleen	NA
	Mouse (BALB/c)	RCP (25 µg)	3 x (14 days)	1x10 <sup>7</sup> SC right hind paw	30	70	Kinetoplast minicircle DNA	NSr in the spleen	NSr in the spleen	NA
Dias et al. 2018 [20]	Mouse (BALB/c)	RCP (25 µg) + Saponin (25 µg)	3 x (14 days)	1x10 <sup>7</sup> SC right hind paw	60	60	Kinetoplast minicircle DNA	↓ 91% in the spleen	↓ 88% in the spleen	NA
Brito et al. 2020 [20]	Mouse (BALB/c)	Chimera A (10 µg) + saponin (60 µg)	3 x (15 days)	1x10 <sup>7</sup> IV	15	30	DNA polymerase	↓ 82% in the spleen	↓ 82% in the spleen	NA
	Mouse (BALB/c)	Chimera B (10 µg) + saponin (60 µg)	3 x (15 days)	1x10 <sup>7</sup> IV	15	30	DNA polymerase	↓ 87% in the spleen	↓ 87% in the spleen	NA
Lage et al. 2020 [22]	Mouse (BALB/c)	Chimera T (20 µg)	3 x (14 days)	1x10 <sup>6</sup> SC right hind paw	30	45	Kinetoplast minicircle DNA	↓ 45% in the spleen	NA	NA
	Mouse (BALB/c)	Chimera T (20 µg) (20 µg) + saponin (20 µg)	3 x (14 days)	1x10 <sup>6</sup> SC right hind paw	30	45	Kinetoplast minicircle DNA	↓ 65% in the spleen	NA	NA
	Mouse (BALB/c)	Chimera T (20 µg)/ Liposome	3 x (14 days)	1x10 <sup>6</sup> SC right hind paw	30	45	Kinetoplast minicircle DNA	↓ 77% in the spleen	NA	NA
Ostolin et al. 2021 [24]	Mouse (BALB/c)	Chimera A (20 µg) + Saponin (30 µg) + MPLA (12,5 µg)	3 x (14 days)	1x10 <sup>7</sup> IV	14	42	Kinetoplast minicircle DNA	↓ 92% in the spleen	↓ 92% in the spleen	NA
	Mouse (BALB/c)	Chimera A (20 µg)	3 x (14 days)	1x10 <sup>7</sup> IV	14	42	Kinetoplast minicircle DNA	NSr in the spleen	NSr in the spleen	NA

(Continued)

Table 4. (Continued)

Authors	Animal model	Formulation with chimeric or multi-epitope vaccine	Number of doses (interval between doses)	<i>L. infantum</i> concentration and infection site	Days between last immunization and challenge	Days between infection and outcome assessment	qPCR target for parasitic load	Parasite load assessment compared to control group:		
								Saline	Adjuvant	Other controls
Lage et al. 2022 [26]	Mouse (BALB/c)	ChimT (20 µg) + Saponin (20 µg)	3 x (14 days)	1x10 <sup>7</sup> SC right hind paw	30	45	Kinetoplast minicircle DNA	↓ 4x in the spleen	↓ 4x in the spleen	NA
	Mouse (BALB/c)	ChimT (20 µg) + MPLA (20 µg)	3 x (14 days)	1x10 <sup>7</sup> SC right hind paw	30	45	Kinetoplast minicircle DNA	↓ 7x in the spleen	↓ 7x in the spleen	NA
	Mouse (BALB/c)	ChimT (20 µg)	3 x (14 days)	1x10 <sup>7</sup> SC right hind paw	30	45	Kinetoplast minicircle DNA	↓ 2x in the spleen	NA	NA
Ostolin et al. 2022 [27]	Mouse (BALB/c)	Poly-T Leish (10 µg) + Saponin (30 µg) + MPLA (12,5 µg)	3 x (14 days)	1x10 <sup>7</sup> IV tail vein	14	45	Kinetoplast minicircle DNA	↓ 96% in the spleen and in the liver	↓ 96% in the spleen and in the liver	NA
	Mouse (BALB/c)	Poly-T Leish (10 µg)	3 x (14 days)	1x10 <sup>7</sup> IV tail vein	14	45	Kinetoplast minicircle DNA	NSr in the spleen and in the liver	NSr in the spleen and in the liver	NA
Gusmão et al. 2022 [25]	Hamster	Chimera A (20 µg) + Saponin (50 µg) + MPLA (12,5 µg)	3 x (15 days)	2x10 <sup>7</sup> IP	21	60	Kinetoplast minicircle DNA	↓ 87% in the spleen	NA	NA
	Hamster	Chimera A (20 µg)	3 x (15 days)	2x10 <sup>7</sup> IP	21	60	Kinetoplast minicircle DNA	↓ 76% in the spleen	NA	NA
	Hamster	Chimera B (20 µg) + Saponin (50 µg) + MPLA (12,5 µg)	3 x (15 days)	2x10 <sup>7</sup> IP	21	60	Kinetoplast minicircle DNA	↓ 92.5% in the spleen	NA	NA
	Hamster	Chimera B (20 µg)	3 x (15 days)	2x10 <sup>7</sup> IP	21	60	Kinetoplast minicircle DNA	↓ 84% in the spleen	NA	NA
Climaco et al. 2023 [28]	Mouse (BALB/c)	Chimera (10 µg)	3 x (14 days)	1x10 <sup>6</sup> SC right hind paw	30	120	Kinetoplast minicircle DNA	NA	NSr in the liver	NSr in liver compared to MPLA-associated chimeric protein
	Mouse (BALB/c)	Chimera (10 µg) + MPLA (10 µg)	3 x (14 days)	1x10 <sup>6</sup> SC right hind paw	30	120	Kinetoplast minicircle DNA	NA	↓ 73% in the liver	↓ 73% in the liver compared to chimeric protein alone

SC—subcutaneous; IV—intravenous; IP—intraperitoneal; NSr—non-significant results; SR—significant results; NA—not assessed

<https://doi.org/10.1371/journal.pntd.0012757.t004>

assay (ELISA) and Western blotting technique, using canine serum samples. Later, Protein Q was evaluated in an ELISA assay and presented sensitivity of 79 to 93% and specificity of 96 to 100% for canine VL diagnosis [43]. These results, associated with those about the capacity of the Lip2a protein to induce proliferation of naïve splenocytes and induce IFN-γ production

Table 5. Parasite load assessed by other methodologies in the different included studies.

Authors	Animal model	Formulation with chimeric or multiepitope chimeric vaccine	Number of doses (interval between doses)	<i>L. infantum</i> concentration and infection site	Days between last immunization and challenge	Days between infection and outcome assessment	Outcome assessment method	Parasite load assessment compared to control group:		
								Saline	Adjuvant	Other controls
Molano et al. 2003 [17]	Mouse (BALB/c)	Protein Q (2 µg) + BCG (50.000 PFU)	3 x (14 days)	1x10 <sup>5</sup> IV tail vein	14	30	Smears	↓ 80% in the spleen	↓ 72% in the spleen	NA
	Mouse (BALB/c)	Protein Q (2 µg) + BCG (50.000 PFU)	3 x (14 days)	1x10 <sup>5</sup> IV tail vein	14	30	Imprint	↓ 81% in the spleen	↓ 73% in the spleen	NA
	Dog (Beagle)	Protein Q (4 µg/Kg) + BCG (1.000.000 PFU)	3x (21 days; 23 days)	5x10 <sup>5</sup> IV	66	634	Biopsy culture	90% protective efficacy	NA	NA
Carcelén et al. 2009 [18]	Dog (Beagle)	Protein Q (100 µg)	single dose	5x10 <sup>5</sup>	60	330	Biopsy culture	3 dogs with negative spleen culture out of 7 evaluated; 2 dogs with negative lymph node out of 7 evaluated	NA	NA
	Dog (Beagle)	Protein Q (100 µg)	single dose	5x10 <sup>5</sup>	60	330	Imprint	7 dogs with negative spleen culture out of 7 evaluated; 6 dogs with negative lymph node out of 7 evaluated	NA	NA
	Dog (Beagle)	Protein Q + Protein Q (100 µg)	2 x (21 dias)	5x10 <sup>5</sup>	39	330	Biopsy culture	3 dogs with negative spleen culture out of 7 evaluated; 1 dog with negative lymph node culture out of 7 evaluated	NA	NA
	Dog (Beagle)	Protein Q + Protein Q (100 µg)	2 x (21 days)	5x10 <sup>5</sup>	39	330	Imprint	5 dogs with negative spleen culture out of 7 evaluated; 6 dogs with negative lymph node culture out of 7 evaluated	NA	NA
Vakili et al. 2020 [36]	Mouse (BALB/c)	Vaccine (30 µg)	2 x (21 days)	3x10 <sup>6</sup> IP	21	30	Imprint	SR in the spleen	SR in the spleen	NSr compared to the peptide Vaccine + adjuvant

IV—intravenous; IP—intraperitoneal; SR—significant results; NA—not assessed

<https://doi.org/10.1371/journal.pntd.0012757.t005>

[44] led the research group to test Protein Q associated with the Bacillus of Calmette & Guérin (BCG) in mouse and dogs. The study reported an 81% reduction in parasite load in mice and a 90% efficacy in dogs [17]. Following this, Parody et al. 2004 [29] demonstrated that

Table 6. Positivity rate of immunized dogs exposed to a natural infection.

Author	Animal model	Multiepitope vaccine	Number of doses (interval between doses)	Follow-up days (between the first dose and outcome assessment)	Outcome assessment method	Positivity (%)	
						Vaccinated	Placebo
Cotrina et al. 2018 [19]	Dog	0.5 mL of LetiFend (Protein Q)	2 x (365 days)	730	qPCR (bone marrow and lymph node)	7.15	16.1
	Dog	0.5 mL of LetiFend (Protein Q)	2 x (365 days)	730	Smear (bone marrow and lymph node)	5.35	13.9

<https://doi.org/10.1371/journal.pntd.0012757.t006>

immunizing BALB/c mice with Protein Q plus CpG motifs induced a predominant IgG2a response and provided significant protection against *L. infantum* infection, leading to an 89% reduction in parasite load in the spleen [29]. Subsequently, Protein Q was tested in a double-blind placebo-controlled experiment with dogs, administered as either a single or double dose without adjuvant. Interestingly, the single-dose protocol provided greater protection than the double-dose protocol, with only one out of seven dogs testing positive compared to four out of seven, respectively [18]. The most recent study included in the present review evaluated the effectiveness of Protein Q in a commercial vaccine formulation (LetiFend) in a large-scale, multicenter, randomized, double-blind, placebo-controlled field study [19]. The authors reported a statistically significant reduction in canine VL cases and in the number of parasite-positive dogs in the vaccine group ( $p = 0.0564$ ). In summary, the overall efficacy of Protein Q (LetiFend vaccine) against canine VL was 72% when used for immunizing dogs living in endemic areas. Another notable example is the Leish-111f protein, which is composed of a thiol-specific antioxidant (TSA), *L. major* stress-inducible protein (LmSTI1), and the 26 kDa N-terminal portion of *Leishmania* elongation initiation factor (LeIF), fused in tandem. This antigen was analyzed by Coler et al. (2007) [31], who demonstrated that Leish-111f, when associated with either rIL-12 or 4'-monophosphoryl lipid A plus squalene (MPL-SE), induced significant protection against *L. infantum* infection in mice and hamsters, resulting in a 99.6% reduction in parasite load. However, for production purposes, and due to regulatory concerns, Leish-111f was modified by removing a 6-histidine sequence near the amino-terminal region and by mutating a proteolytic cleavage site, resulting in the Leish-110 protein [32,45]. Notably, Leish-110 combined with MPL-SE also demonstrated a statistically significant reduction in liver parasite load in mice immunized and challenged with *L. infantum*, when compared to both the saline control ( $p < 0.01$ ) and the adjuvant-alone control ( $p < 0.05$ ) [32].

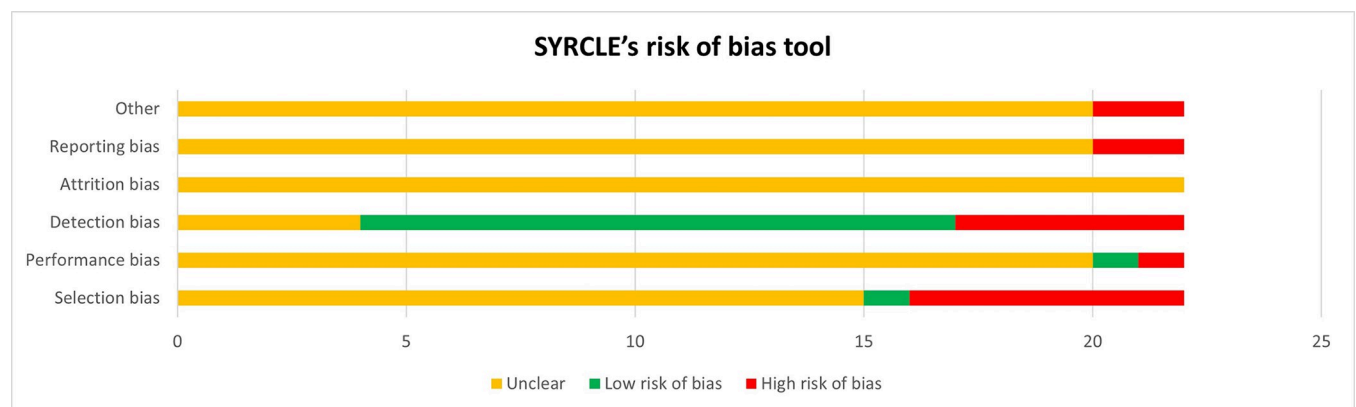


Fig 2. Risk of bias observed in the included studies.

<https://doi.org/10.1371/journal.pntd.0012757.g002>

Chimera A and B antigens have been extensively evaluated in several studies. Chimeric A antigen is composed of epitopes from histone (H2A) protein, acid ribosomal protein (LiP2a), acid ribosomal protein P0 (LiP0), *Leishmania* homologue of activated C kinase (LACK), and cysteine peptidase C (CPC). In contrast, Chimeric B antigen is composed by epitopes from cysteine peptidase A and B antigens (CPA and CPB), surface antigenic antigen (PSA-50S), and specific amastigote antigen A2 (A2), with GP/GPG amino acid sequences intercalated as spacers [23]. Initially, both chimeric antigens were constructed and evaluated in association with the saponin adjuvant. Chimera A resulted in an 82% reduction in parasite load in the spleen, while Chimera B led to an 87% reduction in the same organ [23]. Subsequently, the immune response to different doses of Chimera A was investigated. After determining that the optimal dose was 20 µg, the efficacy of the antigen was evaluated both alone and in combination with the saponin and monophosphoryl lipid A adjuvant system. Although Chimera A alone did not induce a reduction in the parasite load in the spleen, Chimera A associated with the adjuvant system resulted in a 92% reduction [24]. The Chimera B antigen, later renamed Poly-T Leish, was also evaluated with this same adjuvant system and led to a 96% reduction in parasite load in both the spleen and liver [27]. Both chimeric proteins, when tested in a hamster model using the saponin and monophosphoryl lipid A adjuvant system, were able to induce a reduction in parasite load ranging from 76% to 92.5%, corroborating the findings observed in the murine model [25]. In addition, the immunogenicity and vaccine efficacy of the Chimera T antigen, which contains specific T-cell epitopes from *Leishmania* Prohibitin, Eukaryotic Initiation Factor 5a and the hypothetical LiHyp1 and LiHyp2 proteins, have been widely evaluated. Chimera T was evaluated in two studies, one in association with the saponin adjuvant [21] and one in a liposomal formulation [22]. This same research group also developed and evaluated Recombinant Chimeric Protein (RCP) [12], Chimera [20], and ChimT [26]. Although various studies demonstrate promising results, showing significant reductions in parasite load across different organs and assessment methods, there has been limited progress in advancing the technological development process. This includes a lack of implementation of real-world studies and/or randomized clinical trials.

Several studies have utilized bioinformatics tools for the strategic selection of immunodominant epitopes, which had been favored by the improvement of genome and proteome from *Leishmania* spp. in data banks. Among these, at least 14 studies employed *in silico* prediction methods to map immunogenic epitopes [12,20,23,28,30,34,35]. This approach has shown promise in rapidly identifying potential immunogenic epitopes of *Leishmania* spp. through predicted MHC binding and B cell interactions, essential steps toward inducing immune responses. While further *in vitro* and *in vivo* validation is required to confirm that these predicted epitopes effectively trigger protective immunity, this *in silico* methodology represents a substantial advancement, streamlining and accelerating vaccine development [46–48]. Mice, particularly the BALB/c and C57BL strains, were the primary experimental models employed in 18 analyzed studies. These models are widely used due to their well-understood immune responses, ease of handling, and availability of reagents and tools for immunological assays. However, the course of VL in mice does not always fully mirror its manifestation in humans, and different mouse strains exhibit variability in susceptibility to infection, which can complicate direct comparisons of results [49,50].

Regarding the adjuvants, except for the studies by Carcelén et al. (2009) [18] and Cotrina et al. (2018) [19], all other studies utilized adjuvants in conjunction with chimeric or multiepitope antigens. Given the relatively limited antigenic repertoire compared to inactivated or attenuated parasites, these formulations often need adjuvants to elicit a robust and long-lasting immune response [51–53]. The saponin stood out as one of the most employed in association with multiepitope antigens, showing promising results in 7/22 studies. Other adjuvants, such

as MPLA and GLA-SE, were also consistently used and demonstrated potential benefits. The selection of the most suitable adjuvant for a formulation is indeed a critical task in the development of new vaccines, as the immune response to a given adjuvant is highly dependent on the specific characteristics of the vaccine and the immunological context. Importantly various aspects of the formulation must be carefully considered when defining the adjuvant, such as administration routes, the type of immune response desired (humoral or cellular, or both), the nature of the pathogen and antigen, biological characteristics of the vaccine receptors, as well as safety and economic feasibility of the adjuvants [54]. Indeed, in many cases, when multi-epitope antigens were evaluated without adjuvants, they failed to significantly reduce the parasite load in the assessed organs [12,20,24,26,27].

In the studies included in the present review, different routes were used for the challenge with *L. infantum*, especially the intravenous route [17,23,24,27,29–32,34,35,37] and the subcutaneous route [12,20–22,26,28]. Different inoculation routes, along with parasite dose, impact the course of infection and parasite load. Kaur et al. (2008) [55] demonstrated that challenge via the subcutaneous route results in a lower hepatic parasite load compared to intradermal, intraperitoneal, and intracardiac routes in terms of hepatic parasite load. In contrast, intravenous inoculation consistently results in effective infection, regardless of parasite dose, leading to persistent parasite presence in the spleen and liver of the animals [49]. Additionally, a clear dose-dependent effect of the parasites was observed. Mice inoculated subcutaneously with a low number of parasites (i.e.,  $10^3$ ) exhibited a mild infection associated with a Th1 response. In contrast, a higher number of parasites ( $10^7$ ) resulted in a high parasite load in the spleen and lymph nodes, along with a Th2 response [56]. Intracardiac inoculation is linked to the development of a Th2 response, which facilitates the establishment of a persistent infection [55]. Parasite persistence in the spleen can be detected for at least four months post-infection [57]. Therefore, the comparison between different studies is often limited. Different parasite strains and concentrations, the routes used, and the methods employed for quantifying parasite load must all be considered [58]. Ideally, the establishment of a standardized protocol could improve the quality of studies about the development of vaccines for VL.

The most used methods for assessing parasite load were limiting dilution [12,20–22,26,29,31,32,34,35,37] and quantitative PCR (qPCR) [12,18,20,22–28,33]. Limiting dilution is a labor-intensive method that requires multiple processing steps and several days to obtain results, which significantly increases the risk of contamination. Despite these challenges, it remains a sensitive technique capable of specifically detecting live parasites, thereby confirming the presence of active disease [59–61]. In contrast, qPCR provides faster results, with kDNA was the most frequently targeted marker in these studies [12,18,20,22,24–28]. Due to its high copy number in the parasite, kDNA allows the detection of as few as  $10^{-3}$  parasites in a sample [62,63]. However, the persistence of DNA in the host organism complicates the differentiation between viable and non-viable parasites [64,65]. To address this limitation, other markers have been evaluated for detecting viable *Leishmania* parasites. Recent studies suggest that spliced leader RNA (SL-RNA) degrades more rapidly than kDNA and correlates better with microscopic examination, making it a potentially superior marker for monitoring disease progression and evaluating vaccine efficacy [66,67].

The spleen is one of the primary organs affected by the *L. infantum* parasite, exhibiting persistent infection in murine models [68]. Therefore, this organ is crucial for evaluating vaccine efficacy. Generally, all included studies assessed the parasite load in the spleen, in addition to other organs. However, the studies by Duthie et al. (2017) [33] and Clímaco et al. (2023) [28], evaluated only the liver. Although both studies assessed parasite load in the same organ, there was a significant difference in the time interval between infection and outcome evaluation: 28 and 120 days, respectively. The results concerning the reduction of parasite load should be



interpreted with caution, considering the period between the challenge and the evaluation of outcomes, as the dissemination of parasites in the liver is self-limiting. In experimental infection studies, following intravenous infection with *L. infantum*, promastigotes transform into amastigotes and replicate in Kupffer cells and dendritic cells. During the first two weeks, parasite growth is uncontrolled due to an ineffective Th1 response [69]. Consequently, the parasitic load peaks between 2- and 8-weeks post-infection. After this period, hepatic infection generally resolves through the formation of granulomas, with amastigotes being almost absent [70].

Some of the included articles did not provide essential information, such as the strain of the parasite used in experimental infection [29,32], the interval between the last immunization and the challenge of the immunized animals [32], and the route of parasite administration during experimental challenge [18]. Additionally, the absence of details on methodological aspects, like the random allocation of animals to experimental groups or the blinding of researchers to the groups, compromised the ability to assess risk of bias.

## Conclusion

Based on the studies included in this review, no single vaccine formulation emerged as superior among those evaluated. However, the aim was not to compare the studies directly but to use the presented information to guide the rational development of new research involving chimeric or multiepitope antigens as active pharmaceutical ingredients for VL vaccination. Based on the findings of the reviewed studies, immunization via subcutaneous or intradermal routes were the most employed methods, alongside intravenous challenge to better mimic the course of infection, appear to be promising strategies. However, the choice of the optimal adjuvant remains highly context-dependent, varying according to the antigen and its interaction with the immune system. Furthermore, standardized protocols for evaluating immune responses and parasitic load would greatly enhance the comparability and reproducibility of results across studies. However, it is important to note that the collected data supports the feasibility of these antigens for the development of a vaccine against this disease.

## Supporting information

**S1 File. Search strategy used in each database.**  
(DOCX)

## Author Contributions

**Conceptualization:** Karine Ferreira Lopes, Mariana Lourenço Freire, Edward Oliveira.

**Data curation:** Karine Ferreira Lopes, Mariana Lourenço Freire, Edward Oliveira.

**Formal analysis:** Karine Ferreira Lopes, Mariana Lourenço Freire.

**Investigation:** Karine Ferreira Lopes, Mariana Lourenço Freire.

**Methodology:** Karine Ferreira Lopes, Mariana Lourenço Freire.

**Project administration:** Edward Oliveira.

**Supervision:** Edward Oliveira.

**Validation:** Karine Ferreira Lopes, Mariana Lourenço Freire.

**Visualization:** Karine Ferreira Lopes, Mariana Lourenço Freire, Silvana Maria Fonseca Murta.

**Writing – original draft:** Karine Ferreira Lopes, Mariana Lourenço Freire, Silvana Maria Fonseca Murta, Edward Oliveira.

**Writing – review & editing:** Karine Ferreira Lopes, Mariana Lourenço Freire, Silvana Maria Fonseca Murta, Edward Oliveira.

## References

1. Grevelink SA, Lerner EA. Leishmaniasis. *J Am Acad Dermatol*. 1996; 34(21):257–72. [https://doi.org/10.1016/s0190-9622\(96\)80121-6](https://doi.org/10.1016/s0190-9622(96)80121-6) PMID: 8642091
2. Kevric I, Cappel MA, Keeling JH. New World and Old World *Leishmania* Infections: A Practical Review. *Dermatol Clin*. 2015; 33(3):579–93.
3. Burza S, Croft SL, Boelaert M. Leishmaniasis. *Lancet*. 2018; 392(10151):951–70. [https://doi.org/10.1016/S0140-6736\(18\)31204-2](https://doi.org/10.1016/S0140-6736(18)31204-2) PMID: 30126638
4. WHO. Leishmaniasis [Internet]. [cited 2024 Aug 8]. Available from: <https://www.who.int/data/gho/data/themes/topics/gho-ntd-leishmaniasis>.
5. Lee BY, Bacon KM, Shah M, Kitchen SB, Connor DL, Slayton RB. The Economic Value of a Visceral Leishmaniasis Vaccine in Bihar State, India. *Am J Trop Med Hyg*. 2012; 86(3):417. <https://doi.org/10.4269/ajtmh.2012.10-0415> PMID: 22403311
6. Bacon KM, Hotez PJ, Kruchten SD, Kamhawi S, Bottazzi ME, Valenzuela JG, et al. The Potential Economic Value of a Cutaneous Leishmaniasis Vaccine in Seven Endemic Countries in the Americas. *Vaccine*. 2013; 31(3):480. <https://doi.org/10.1016/j.vaccine.2012.11.032> PMID: 23176979
7. Darrah PA, Patel DT, De Luca PM, Lindsay RWB, Davey DF, Flynn BJ, et al. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against *Leishmania major*. *Nat Med*. 2007; 13(7):843–50.
8. de Andrade Ferraz I, Carvalho AMRS, de Brito RCF, Roatt BM, Martins VT, Lage DP, et al. Development of an immunogen containing CD4+/CD8+ T-cell epitopes for the prophylaxis of tegumentary leishmaniasis. *Appl Microbiol Biotechnol*. 2022; 106(12):4627–41. <https://doi.org/10.1007/s00253-022-12033-7> PMID: 35759035
9. Skeiky YAW, Coler RN, Brannon M, Stromberg E, Greeson K, Thomas Crane R, et al. Protective efficacy of a tandemly linked, multi-subunit recombinant leishmanial vaccine (Leish-111f) formulated in MPL adjuvant. *Vaccine*. 2002; 20(27–28):3292–303. [https://doi.org/10.1016/s0264-410x\(02\)00302-x](https://doi.org/10.1016/s0264-410x(02)00302-x) PMID: 12213399
10. Kumari S, Kumar A, Samant M, Sundar S, Singh N, Dube A. Proteomic approaches for discovery of new targets for vaccine and therapeutics against visceral leishmaniasis. *Proteomics Clin Appl*. 2008; 2(3):372–86. <https://doi.org/10.1002/prca.200780017> PMID: 21136840
11. Gradoni L, Foglia Manzillo V, Pagano A, Piantadosi D, De Luna R, Gramiccia M, et al. Failure of a multi-subunit recombinant leishmanial vaccine (MML) to protect dogs from *Leishmania infantum* infection and to prevent disease progression in infected animals. *Vaccine*. 2005; 23(45):5245–51.
12. Martins VT, Duarte MC, Lage DP, Costa LE, Carvalho AMRS, Mendes TAO, et al. A recombinant chimeric protein composed of human and mice-specific CD4+ and CD8+ T-cell epitopes protects against visceral leishmaniasis. *Parasite Immunol*. 2017; 39(1):e12359. <https://doi.org/10.1111/pim.12359> PMID: 27593711
13. Arya A, Arora SK. A T-cell epitope-based multi-epitope vaccine designed using human HLA specific T cell epitopes induces a near-sterile immunity against experimental visceral leishmaniasis in hamsters. *Vaccines (Basel)*. 2021; 9(10). <https://doi.org/10.3390/vaccines9101058> PMID: 34696166
14. Deeks JJ, Bossuyt PM, Leeflang MM, Takwoingi Y. Cochrane Handbook for Systematic Reviews of Diagnostic Test Accuracy. *Cochrane Handbook for Systematic Reviews of Diagnostic Test Accuracy*. 2023;1–408.
15. Mendeley | Free reference manager | Elsevier [Internet]. [cited 2024 Aug 7]. Available from: <https://www.elsevier.com/products/mendeley>.
16. Ouzzani M, Hammady H, Fedorowicz Z, Elmagarmid A. Rayyan—a web and mobile app for systematic reviews. *Syst Rev*. 2016; 5(1):1–10. <https://doi.org/10.1186/s13643-016-0384-4> PMID: 27919275
17. Molano I, García Alonso M, Mirón C, Redondo E, Requena JM, Soto M, et al. A *Leishmania infantum* multi-component antigenic protein mixed with live BCG confers protection to dogs experimentally infected with *L. infantum*. *Vet Immunol Immunopathol*. 2003; 92(1–2):1–13.
18. Carcelén J, Iniesta V, Fernández-Cotrina J, Serrano F, Parejo JC, Corraliza I, et al. The Chimerical Multi-Component Q protein from *Leishmania* in the absence of adjuvant protects dogs against an experimental *Leishmania infantum* infection. *Vaccine*. 2009; 27(43):5964–73.

19. Fernández Cotrina J, Iniesta V, Monroy I, Baz V, Hugnet C, Marañón F, et al. A large-scale field randomized trial demonstrates safety and efficacy of the vaccine LetiFend against canine leishmaniosis. *Vaccine*. 2018; 36(15):1972–82.
20. Dias DS, Ribeiro PAF, Martins VT, Lage DP, Costa LE, Chávez-Fumagalli MA, et al. Vaccination with a CD4+ and CD8+ T-cell epitopes-based recombinant chimeric protein derived from *Leishmania infantum* proteins confers protective immunity against visceral leishmaniasis. *Transl Res*. 2018; 200:18–34.
21. Lage DP, Ribeiro PAF, Dias DS, Mendonça DVC, Ramos FF, Carvalho LM, et al. A candidate vaccine for human visceral leishmaniasis based on a specific T cell epitope-containing chimeric protein protects mice against *Leishmania infantum* infection. *NPJ Vaccines*. 2020; 5(1):1–13.
22. Lage DP, Ribeiro PAF, Dias DS, Mendonça DVC, Ramos FF, Carvalho LM, et al. Liposomal Formulation of ChimeraT, a Multiple T-Cell Epitope-Containing Recombinant Protein, Is a Candidate Vaccine for Human Visceral Leishmaniasis. *Vaccines (Basel)*. 2020; 8(2):289. <https://doi.org/10.3390/vaccines8020289> PMID: 32526867
23. Brito RCF, Ruiz JC, Cardoso JM de O, Ostolin TLVDP, Reis LES, Mathias FAS, et al. Chimeric Vaccines Designed by Immunoinformatics-Activated Polyfunctional and Memory T cells that Trigger Protection against Experimental Visceral Leishmaniasis. *Vaccines (Basel)*. 2020; 8(2):252. <https://doi.org/10.3390/vaccines8020252> PMID: 32471081
24. Ostolin TLVDP, Gusmão MR, Mathias FAS, Cardoso JM de O, Roatt BM, Aguiar-Soares RD de O, et al. A chimeric vaccine combined with adjuvant system induces immunogenicity and protection against visceral leishmaniasis in BALB/c mice. *Vaccine*. 2021; 39(20):2755–63. <https://doi.org/10.1016/j.vaccine.2021.04.004> PMID: 33875268
25. Gusmão MR, Ostolin TLVDP, Carvalho LM, Costa AFP, Moreira GJL, Cardoso JM de O, et al. Immunoprophylaxis using polypeptide chimera vaccines plus adjuvant system promote Th1 response controlling the spleen parasitism in hamster model of visceral leishmaniasis. *Vaccine*. 2022; 40(37):5494–503. <https://doi.org/10.1016/j.vaccine.2022.08.005> PMID: 35963820
26. Lage DP, Vale DL, Linhares FP, Freitas CS, Machado AS, Cardoso JMO, et al. A Recombinant Chimeric Protein-Based Vaccine Containing T-Cell Epitopes from Amastigote Proteins and Combined with Distinct Adjuvants, Induces Immunogenicity and Protection against *Leishmania infantum* Infection. *Vaccines (Basel)*. 2022; 10(7):1146.
27. Ostolin TLVDP, Gusmão RM, Mathias FAS, Cardoso JMO, Roatt BM, Aguiar-Soares RDO, et al. A specific *Leishmania infantum* polypeptide vaccine triggers Th1-type immune response and protects against experimental visceral leishmaniasis. *Cell Immunol*. 2022; 380:104592.
28. Clímaco MC, de Figueiredo LA, Lucas RC, Pinheiro GRG, Dias Magalhães LM, Oliveira ALG, et al. Development of chimeric protein as a multivalent vaccine for human Kinetoplastid infections: Chagas disease and leishmaniasis. *Vaccine*. 2023; 41(37):5400–11. <https://doi.org/10.1016/j.vaccine.2023.07.020> PMID: 37479612
29. Parody N, Soto M, Requena JM, Alonso C. Adjuvant guided polarization of the immune humoral response against a protective multicomponent antigenic protein (Q) from *Leishmania infantum*. A CpG + Q mix protects Balb/c mice from infection. *Parasite Immunol*. 2004; 26(6–7):283–93.
30. Martínez-Rodrigo A, Mas A, Álvarez-Campos D, Orden JA, Domínguez-Bernal G, Carrión J. Epitope Selection for Fighting Visceral Leishmaniasis: Not All Peptides Function the Same Way. *Vaccines*. 2020; 8(3):352. <https://doi.org/10.3390/vaccines8030352> PMID: 32630347
31. Coler RN, Goto Y, Bogatzki L, Raman V, Reed SG. Leish-111f, a recombinant polyprotein vaccine that protects against visceral leishmaniasis by elicitation of CD4+ T cells. *Infect Immun*. 2007; 75(9):4648–54. <https://doi.org/10.1128/IAI.00394-07> PMID: 17606603
32. Bertholet S, Goto Y, Carter L, Bhatia A, Howard RF, Carter D, et al. Optimized subunit vaccine protects against experimental leishmaniasis. *Vaccine*. 2009; 27(50):7036. <https://doi.org/10.1016/j.vaccine.2009.09.066> PMID: 19786136
33. Duthie MS, Pereira L, Favila M, Hofmeyer KA, Reed SJ, Metangmo S, et al. A defined subunit vaccine that protects against vector-borne visceral leishmaniasis. *NPJ Vaccines*. 2017; 2(1):1–9. <https://doi.org/10.1038/s41541-017-0025-5> PMID: 29263878
34. Athanasiou E, Agallou M, Tastsoglou S, Kammona O, Hatzigeorgiou A, Kiparissides C, et al. A poly(lactic-co-glycolic) acid nanovaccine based on chimeric peptides from different *Leishmania infantum* proteins induces dendritic cells maturation and promotes peptide-specific IFN $\gamma$ -producing CD8+ T cells essential for the protection against experimental visceral leishmaniasis. *Front Immunol*. 2017; 8:684.
35. Agallou M, Margaroni M, Kotsakis SD, Karagouni E. A Canine-Directed Chimeric Multi-Epitope Vaccine Induced Protective Immune Responses in BALB/c Mice Infected with *Leishmania infantum*. *Vaccines*. 2020; 8(3):350.

36. Vakili B, Nezafat N, Zare B, Erfani N, Akbari M, Ghasemi Y, et al. A new multi-epitope peptide vaccine induces immune responses and protection against *Leishmania infantum* in BALB/c mice. *Med Microbiol Immunol*. 2020; 209(1):69–79.
37. Goto Y, Bhatia A, Raman VS, Liang H, Mohamath R, Picone AF, et al. KSAC, the first defined polyprotein vaccine candidate for visceral leishmaniasis. *Clin Vaccine Immunol*. 2011; 18(7):1118–24. <https://doi.org/10.1128/CVI.05024-11> PMID: 21632891
38. Agallou M, Margaroni M, Karagouni E. Cellular vaccination with bone marrow-derived dendritic cells pulsed with a peptide of *Leishmania infantum* KMP-11 and CpG oligonucleotides induces protection in a murine model of visceral leishmaniasis. *Vaccine*. 2011; 29(31):5053–64.
39. Joshi S, Rawat K, Yadav NK, Kumar V, Siddiqi MI, Dube A. Visceral Leishmaniasis: Advancements in Vaccine Development via Classical and Molecular Approaches. *Front Immunol*. 2014; 5:380. <https://doi.org/10.3389/fimmu.2014.00380> PMID: 25202307
40. Brito RCF, Cardoso JM de O, Reis LES, Vieira JF, Mathias FAS, Roatt BM, et al. Peptide Vaccines for Leishmaniasis. *Front Immunol*. 2018; 9:1043. <https://doi.org/10.3389/fimmu.2018.01043> PMID: 29868006
41. Palatnik-De-Sousa CB. Nucleoside Hydrolase NH 36: A Vital Enzyme for the *Leishmania* Genus in the Development of T-Cell Epitope Cross-Protective Vaccines. *Front Immunol*. 2019; 10:813. <https://doi.org/10.3389/fimmu.2019.00813> PMID: 31040850
42. Oliveira DS de, Zaldivar MF, Gonçalves AAM, Resende LA, Mariano RM da S, Pereira DFS, et al. New Approaches to the Prevention of Visceral Leishmaniasis: A Review of Recent Patents of Potential Candidates for a Chimeric Protein Vaccine. *Vaccines*. 2024; 12(3):271. <https://doi.org/10.3390/vaccines12030271> PMID: 38543905
43. Soto M, Requena JM, Quijada L, Alonso C. Multicomponent chimeric antigen for serodiagnosis of canine visceral leishmaniasis. *J Clin Microbiol*. 1998; 36(1):58–63. <https://doi.org/10.1128/JCM.36.1.58-63.1998> PMID: 9431920
44. Soto M, Alonso C, Requena JM. The *Leishmania infantum* acidic ribosomal protein LiP2a induces a prominent humoral response in vivo and stimulates cell proliferation in vitro and interferon-gamma (IFN- $\gamma$ ) production by murine splenocytes. *Clin Exp Immunol*. 2000; 122(2):212–8.
45. Moulton G. An Improved Manufacturing Process for a Recombinant Polyprotein Vaccine. *Biopharm Int*. 2008.
46. Brito RCF, Guimarães FG, Velloso JPL, Corrêa-Oliveira R, Ruiz JC, Reis AB, et al. Immunoinformatics Features Linked to *Leishmania* Vaccine Development: Data Integration of Experimental and In Silico Studies. *Int J Mol*. 2017; 18(2).
47. Brito RCF, De Oliveira Cardoso JM, Reis LES, Mathias FAS, Aguiar-Soares RDDO, Teixeira-Carvalho A, et al. Synthetic Peptides Elicit Strong Cellular Immunity in Visceral Leishmaniasis Natural Reservoir and Contribute to Long-Lasting Polyfunctional T-Cells in BALB/c Mice. *Vaccines (Basel)*. 2019; 7(4):162. <https://doi.org/10.3390/vaccines7040162> PMID: 31661776
48. Zandieh M, Kashi T, Taheri T. Assessment of Protection Induced by DNA and Live Vaccine Encoding *Leishmania* MHC Class I Restricted Epitopes against *L. major* Challenge in Balb/c Mice Model. *J Microb Biochem Technol*. 2015; 07(06):427–38.
49. Carrión J, Nieto A, Iborra S, Iniesta V, Soto M, Folgueira C, et al. Immunohistological features of visceral leishmaniasis in BALB/c mice. *Parasite Immunol*. 2006; 28(5):173–83. <https://doi.org/10.1111/j.1365-3024.2006.00817.x> PMID: 16629702
50. Nieto A, Domínguez-Bernal G, Orden JA, De La Fuente R, Madrid-Elena N, Carrión J. Mechanisms of resistance and susceptibility to experimental visceral leishmaniasis: BALB/c mouse versus syrian hamster model. *Vet Res*. 2011; 42(1):1–13.
51. Aguilar JC, Rodríguez EG. Vaccine adjuvants revisited. *Vaccine*. 2007; 25(19):3752–62. <https://doi.org/10.1016/j.vaccine.2007.01.111> PMID: 17336431
52. Purcell AW, McCluskey J, Rossjohn J. More than one reason to rethink the use of peptides in vaccine design. *Nat. Rev. Drug Discov*. 2007; 6(5):404–14. <https://doi.org/10.1038/nrd2224> PMID: 17473845
53. Li W, Joshi MD, Singhanian S, Ramsey KH, Murthy AK. Peptide Vaccine: Progress and Challenges. *Vaccines*. 2014; 2(3):515–36. <https://doi.org/10.3390/vaccines2030515> PMID: 26344743
54. Zhao T, Cai Y, Jiang Y, He X, Wei Y, Yu Y, et al. Vaccine adjuvants: mechanisms and platforms. *Signal transduct. target. ther*. 2023; 8(1):1–24.
55. Kaur S, Kaur T, Garg N, Mukherjee S, Raina P, Athokpam V. Effect of dose and route of inoculation on the generation of CD4 + Th1/Th2 type of immune response in murine visceral leishmaniasis. *Parasitol Res*. 2008; 103(6):1413–9. <https://doi.org/10.1007/s00436-008-1150-x> PMID: 18751727

56. Oliveira DM, Costa MAF, Chavez-Fumagalli MA, Valadares DG, Duarte MC, Costa LE, et al. Evaluation of parasitological and immunological parameters of *Leishmania chagasi* infection in BALB/c mice using different doses and routes of inoculation of parasites. *Parasitol Res*. 2012; 110(3):1277–85.
57. Mukherjee P, Ghosh AK, Ghose AC. Infection pattern and immune response in the spleen and liver of BALB/c mice intracardially infected with *Leishmania donovani* amastigotes. *Immunol Lett*. 2003; 86(2):131–8.
58. Loeuillet C, Bañuls AL, Hide M. Study of *Leishmania* pathogenesis in mice: Experimental considerations. *Parasit Vectors*. 2016; 9(1):1–12.
59. Buffet PA, Sulahian A, Garin YJF, Nassar N, Derouin F. Culture microtitration: A sensitive method for quantifying *Leishmania infantum* in tissues of infected mice. *Antimicrob Agents Chemother*. 1995; 39(9):2167–8.
60. Moreno Castilho T, Marcelo Camargo LA, McMahon-Pratt D, Shaw J. A Real-Time Polymerase Chain Reaction Assay for the Identification and Quantification of American *Leishmania* Species on the Basis of Glucose-6-Phosphate Dehydrogenase. *Am J Trop Med Hyg*. 2008; 78(1):122–32.
61. Srivastava A, Sweat JM, Azizan A, Vesely B, Kyle DE. Real-Time PCR to Quantify *Leishmania donovani* in Hamsters. *J. Parasitol*. 2013; 99(1):145–50.
62. Francino O, Altet L, Sánchez-Robert E, Rodriguez A, Solano-Gallego L, Alberola J, et al. Advantages of real-time PCR assay for diagnosis and monitoring of canine leishmaniasis. *Vet Parasitol*. 2006; 137(3–4):214–21. <https://doi.org/10.1016/j.vetpar.2006.01.011> PMID: 16473467
63. Kocher A, Valière S, Bañuls AL, Murienne J. High-throughput sequencing of kDNA amplicons for the analysis of *Leishmania* minicircles and identification of Neotropical species. *Parasitology*. 2018; 145(5):585–94.
64. Ilboudo H, Camara O, Ravel S, Bucheton B, Lejon V, Camara M, et al. *Trypanosoma brucei* gambiense Spliced Leader RNA Is a More Specific Marker for Cure of Human African Trypanosomiasis Than T. b. gambiense DNA. *J Infect Dis*. 2015; 212(12):1996–8.
65. Nemati Zargaran F, Rostamian M, Akya A, Niknam H. *Leishmania tropica*: The comparison of two frequently-used methods of parasite load assay in vaccinated mice. *Asian Pac J Trop Biomed*. 2020; 10(6):248–53.
66. Keer JT, Birch L. Molecular methods for the assessment of bacterial viability. *J Microbiol Methods*. 2003; 53(2):175–83. [https://doi.org/10.1016/s0167-7012\(03\)00025-3](https://doi.org/10.1016/s0167-7012(03)00025-3) PMID: 12654489
67. Hendrickx R, Melkamu R, Tadesse D, Teferi T, Feijens PB, Vleminckx M, et al. Spliced-Leader RNA as a Dynamic Marker for Monitoring Viable *Leishmania* Parasites During and After Treatment. *J Infect Dis*. 2024; 230(1).
68. Kaye PM, Svensson M, Ato M, Maroof A, Polley R, Stager S, et al. The immunopathology of experimental visceral leishmaniasis. *Immunol Rev*. 2004; 201(1):239–53. <https://doi.org/10.1111/j.0105-2896.2004.00188.x> PMID: 15361245
69. Gomes-Pereira S, Rodrigues OR, Rolão N, Almeida PD, Santos-Gomes GM. Hepatic cellular immune responses in mice with “cure” and “non-cure” phenotype to *Leishmania infantum* infection: importance of CD8+ T cells and TGF- $\beta$  production. *FEMS Immunol Med Microbiol*. 2004; 41(1):59–68.
70. Squires KE, Kirsch M, Silverstein SC, Acosta A, McElrath MJ, Murray HW. Defect in the tissue cellular immune response: Experimental visceral leishmaniasis in euthymic C57BL/6 ep/ep mice. *Infect Immun*. 1990; 58(12):3893–8. <https://doi.org/10.1128/iai.58.12.3893-3898.1990> PMID: 2123825