Assessment of Vaccine-Induced Immunity Against Canine Visceral Leishmaniasis

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Canine visceral leishmaniasis is an increasingly important public health problem. Dogs infected by *Leishmania infantum* are the main domestic reservoir of the parasite and play a key role in its transmission to humans. Recent findings have helped in the development of novel diagnostic methods, and of control measures such as vaccines, some of which are already commercially available. However, quantitative procedures should be followed to confirm whether these vaccines elicit a cell-mediated immune response. The present work describes the need for this evaluation, and the techniques available for confirming this type of immune response.

**Key words:** vaccine, canine visceral leishmaniasis, immunity, efficacy, *Leishmania infantum*

ZOONOTIC VISCERAL LEISHMANIASIS: A GROWING PUBLIC HEALTH CONCERN

Zoonotic visceral leishmaniasis (ZVL) is a vector-borne disease caused by the protozoan parasite *Leishmania infantum* (syn. *Leishmania chagasi*). In the Old World the parasite is transmitted by the bite of sand flies belonging to the genus *Phlebotomus*; in the New World the members of the genus *Lutzomyia* takes on this role. ZVL occurs in Mediterranean Europe, in North Africa and the Near East, Central Asia, China and Latin America, appearing in foci that coincide precisely with the geographical distribution of the disease vectors. The incidence of human visceral leishmaniasis (HVL) is estimated at 4,500–6,800 cases in the Americas, 1,200–2,000 in Mediterranean countries, and 5,000–10,000 across the Middle East to Central Asia (1).

Dogs, the main reservoir of the parasite, are susceptible to canine visceral leishmaniasis (CVL) (Box 1). The seroprevalence of *Leishmania* for the canine population ranges from 3 to 30% depending on the area and ecological variables (2). It is higher in areas where transmission can occur throughout the year; for example, in the south of Bahia, Brazil, it may be as high as 50.3% (3). However, when PCR-based tools are used for screening, prevalence figure can be even 3 times higher than that detected by serology (4). Indeed, follow-up studies of dogs living in areas where active transmission occurs show virtually all of them to have been in contact with the parasite at some point in their lives (5).

Wild animals such as wolves, jackals and foxes, hares and rabbits have also been described to act as reservoirs of the parasite. However, the proximity of dogs to humans, the high prevalence of infection among them, and the ease with which they transmit the parasite to sand flies, allow for the domestic transmission of *L. infantum* to humans. Actually, several studies have reported a correlation between the incidence of CVL and HVL (6).
CVL is not, therefore, only an important veterinary problem; it is also a major public health concern (7, 8). Infected dogs are directly involved in the spread of ZVL to disease-free areas. The appearance of cases of human leishmaniasis in previously non-endemic areas is usually preceded by the appearance of competent vectors and cases of CVL (9, 10) and northern Argentina (11). The recent PAHO/WHO report on the human leishmaniasis situation in the Americas indicates that while the total number of cases of cutaneous leishmaniasis has remained stable over recent years, the number of cases of HVL has increased by 26.4% in the region, with increases in the fatality rate and number of deaths seen since 2014 (12). The transmission of HVL is increasing in Brazil, where the age-standardized disability-adjusted life years values associated with the disease increased by 83.6% between 1990 and 2016, and the age-standardized incidence rate and the years of life lost increased by 52.9 and 108% respectively over the same period (13).

Preventing the expansion of ZVL and disease transmission to humans requires surveillance of the vector, the implementation of measures to reduce the prevalence and incidence of CVL, and the development of procedures for assessing the impact of such control measures in affected populations.

**PREVENTION OF *L. INFANTUM* INFECTION IN DOGS**

The natural history of CVL is complex and depends on multiple factors like nutritional and immunological state of the animal, age, dog breed, or virulence of the parasite (14). The result is a dynamic spectrum of naturally infected dogs ranging from resistant, asymptomatic animals to those with severe disease (15). The number of *Leishmania*-infected dogs is much higher than the number that actually develops the disease; as a result, the overall burden of infection in the canine population in endemic areas is unknown (16). Some of these *Leishmania*-infected asymptomatic animals may act as “carriers,” especially if they harbor parasites in the skin; some 51% of the dogs from endemic areas are PCR skin-positive (17). The existence of such a significant source of parasites hinders the implementation of effective control measures. Certainly, strategies involving drug treatment for CVL have been shown unsuccessful; chemotherapy does not clear parasites from dogs, and although the majority improve clinically, relapses are frequent and the animals remain infectious to sand flies (5). Eliminating seropositive dogs does not help either; not only is it ethically unacceptable, it has been found to have no influence on disease prevalence (18, 19).

In this complex scenario, the best option for the efficient control of CVL is prevention, both of sand flies biting dogs (20), and of dogs developing the disease (21). Several insecticides and repellents are available in the form of collars, lotions and pipettes that impregnate the animal’s skin and prevent sand fly bites. Controlled trials of these products have returned good results (22), but their effectiveness may decrease if dog owners fail to maintain their use (23).

Vaccines against CVL can provide dogs with specific, internal protection against developing clinical disease. Strong, specific and permanent immunity can be induced, preventing the multiplication and dispersion of the parasite, ruling out the development of CVL. The prophylactic vaccines currently available represent a clear advance in the control of this disease. Given their ease of use, and their cost/benefit ratio, prophylactic vaccines are usually the most effective prevention and control tools at our disposal (15).

**Development of Vaccines for CVL: A Challenging Task**

Parasites are complex eukaryotic unicellular and multicellular pathogens. Most have very complicated life cycles that include the infection of intermediate invertebrate hosts; their morphological and genetic complexity makes them challenging targets for vaccines; and the parasites have evolved to resist the host immune response by evading effectors or preventing their production (24).

Early studies showed that parasites may trigger immediate-type hypersensitivity or delayed-type hypersensitivity, both mediated by CD4+ T helper (Th) cells (25). This reaction
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dichotomy was explained following the discovery that CD4+ T cells could be classified into multiple subsets depending on their cytokine expression profile, i.e., Th1 cells expressing interferon(IFN)-γ, interleukin(IL)-12 and/or tumor necrosis factor (TNF)-α (inducing delayed -type hypersensitivity), and Th2 cells expressing IL-4, IL-5, and IL-13 (promoting immediate-type hypersensitivity) (26). In the case of the Leishmania protozoan (an obligate intracellular parasite that infects mammalian host macrophages), this reaction dichotomy was demonstrated when BALB/c and C57BL6 mice experimentally infected with Leishmania major promastigotes developed either a Th2 or Th1 response associated respectively with either exacerbation or healing of the infection (27) (Figure 1). To prevent parasite multiplication and dissemination, an efficient cell-mediated immune response is required, involving dendritic cell-primed CD4+ (Th1 type) and CD8+ T lymphocytes that produce IFN-γ, and/or IL-12, and/or TNF-α. These cytokines activate infected macrophages to produce nitric oxide and reactive oxygen species, which lead to the physical elimination of the parasite (28). Leishmania has, however, evolved sophisticated mechanisms that help it prevent these responses (29). It also induces the expression of immunosuppressive IL-10, interferes with the production of Leishmania-specific antibodies, and stops the expansion of parasite-specific CD8-T cell clones, as well as disables the antigen presenting capacity of macrophages (30). Different high throughput techniques have shown that Leishmania infection affects the expression of a multitude of host genes (31), with the progress of the infection dependent on the balance struck between the virulence of the parasite and the host’s innate and adaptive immune responses (32). The dichotomy in the reaction to Leishmania is seen in naturally infected dogs from the same disease-endemic area developing either severe CVL or remaining asymptomatic. The complexity of host-parasite interactions hinders the development of effective vaccines against CVL, certainly making it very difficult to identify a single hallmark of protection against leishmaniasis. All the factors involved in the response to infection by Leishmania must therefore be studied in detail (15, 33).

For many years, dogs were thought the most susceptible host in the transmission cycle, and that they had no possibility of recovery (14). This idea developed because of the high percentage of seropositive dogs that developed the disease, the large number of cases of CVL in endemic areas compared to HVL, and the only partial effectiveness of chemotherapy in sick animals. In contrast, when active HVL is successfully treated, cell-mediated immunity toward Leishmania spp. is developed (34, 35).

The observation that dogs naturally infected by L. infantum could actually develop a protective response to the disease was the proof of concept needed to show that the induction of immune protection against canine leishmaniasis was feasible.
Several experimental infection trials later demonstrated that it was possible to induce this protective response experimentally (5). Nevertheless, obtaining an effective vaccine against CVL, capable of inducing a safe, strong and prolonged Leishmania-specific protective response in the dog, is a challenging task. Eliciting this type of cell-mediated response by vaccination is more difficult than obtaining a humoral response, especially given the antigenic complexity of the pathogen and its possession of evasion mechanisms (37).

Many strategies for inducing immunity against Leishmania infection have been tested in murine models (with greater or lesser success). These have been based on the use of killed Leishmania parasites, attenuated parasites, different antigen fractions, purified proteins, recombinant proteins, synthetic peptides, non-protein antigens, bacterial and virus-expressed parasite immunogens, and even “bare” parasite DNA (including the DNA of plasmids or linear vectors) (38–40). Only a few have been tried in dogs, however, because of the technical difficulties involved in handling the experimental animals and the high costs involved. Certainly, very few vaccine candidates for CVL have been tested in double-blind randomized field trials.

Partial protection against CVL has been reported after immunization with sonicated or autoclaved promastigotes (41, 42), with purified and recombinant Leishmania proteins (43–46), with parasite DNA (47, 48), and with attenuated Leishmania strains (49). A review summarizing the few efficacy studies performed in dogs, involving different types of Leishmania antigen (purified proteins, recombinant proteins or DNA), adjuvants and post-vaccination Leishmania infantum challenge, indicated different levels of protection to be obtained by the different vaccine candidates (50). Later, the attenuated L. donovani centrin-deleted strain [LdCen(-/-)] (when used as a vaccine) was found to reduce the parasite burden of subsequently infected dogs by up to 87.3% at 18 months post-challenge (51). The immunogenicity of, and partial protection afforded by, recombinant non-pathogenic Leishmania tarentolae expressing the A2 and cystein proteinases A and B proteins has also been reported (52). Alum-precipitated, autoclaved Leishmania major mixed with Bacillus Calmette-Guérin (BCG) and imiquimod was shown in a field trial to be of low efficacy in dogs (53) (Table 1).

To date, three vaccines against CVL have been approved, one in Brazil and two in Europe:

- Leish-Tec (Hertape Calier, Brazil), based on the recombinant protein A2, with saponin as an adjuvant (54, 55),
- Canileish (Virbac, France) made with L. infantum excreted/secreted antigens, with QA-21 as an adjuvant (56), and
- Letifend (Laboratorios Leti, Spain), based on the fusion protein Q, formulated without adjuvant (57).

### Table 1: Vaccine trials for canine visceral leishmaniasis.

| Vaccine formulation | Type of trial | Cell immunity test | Efficacy | References |
|---------------------|--------------|--------------------|---------|------------|
| *L. braziliensis* sonicated promastigotes + BCG | Experimental infection 2.3 × 10⁶ promastigotes IV | CPA | Partial | (41) |
| Purified fucose mannose ligand + QuilA saponin | Phase III—Natural infection | LST | 80.0% | (43) |
| Recombinant fusion protein Q | Experimental infection 500,000 promastigotes IV | LST | 90% | (44) |
| Alum precipitated *L. major* autoclaved promastigotes + BCG | Phase III—Natural infection | LST | 69.3% | (42) |
| Recombinant proteins H1, HASPB1 + Montanide. Polyprotein MML + MPL-SE | Experimental infection 1 × 10⁶ promastigotes IV | CPA | Partial | (45) |
| Plasmid with CPA and CPB — recombinant protein CPa, CPB + CpG ODN + Montanide. Prime—boost vaccination | Experimental infection 5 × 10⁶ promastigotes IV | CPA, LST | Cytokine analysis | Not determined | (47) |
| DNA-LACK plasmid followed by rVaccinia virus containing the same gene (rVV-LACK), Prime-boost vaccination | Experimental infection 10⁶ promastigotes IV | CPA | Cytokine analysis | Partial | (48) |
| Excreted/Secreted proteins + QA-21 saponin | Experimental infection 10⁶.5 promastigotes IV | CPA, ELISpot IFN-γ CMLA | Partial | (46) |
| Attenuated line *L. infantum* H-line), established by culturing promastigotes in vitro under gentamicin in pressure | Phase III—Natural infection | None | 93% (estimated) | (49) |
| Live attenuated *L. donovani* parasites LdCen(-/-) | Experimental infection 10⁷ promastigotes IV | CPA | Cytokine analysis | Not determined | (51) |
| Alum precipitated autoclaved *L. major* mixed with BCG and imiquimod | Phase III—Natural infection | LST | 40.4% | (53) |
| *L. tarentolae* expressing the A2 and cystein proteinases A and B proteins | Experimental infection 4 × 10⁷ promastigotes IV | LST | Cytokine analysis | Partial | (52) |

CPA, Cell Proliferation Assay; LST, Leishmanin Skin Test; CMLA, Canine Macrophage Leishmanicidal Assay.
The level of protection and efficacy in the prevention of disease reported for all three vaccines was similar (92–98 and 68–72%, respectively) (54, 58, 59) (Table 2). However, Leish-Tec was mainly assessed via the expression of induced antigen-specific IgG2 antibodies (60), while Letifend was assessed via the cellular immunity detected by the leishmanin skin test (57). In contrast, Canileish was found to induce a specific humoral response as well as specific cellular immunity, as confirmed by (i) the appearance of Leishmania-specific Th1 cell clones able to produce IFN-γ upon stimulation with leishmanial antigens, (ii) the induced leishmanicidal activity of macrophages, and (iii) the increased expression of iNOS and NO (which finally kills the parasite) (56). The specific cell-mediated immune response against the parasite was strong and remained effective against experimental challenge at 1 year (46).

**Assessing Immunity Against CVL**

Usually, the assessment of the immunogenicity and efficacy of CVL vaccines consists of the clinical, serological and parasitological follow-up of vaccinated animals. However, several procedures can be followed to quantify the level of cell-mediated immunity (T cell memory) induced by natural infection and experimental immunization (Table 3). Most are based on the specific recognition of parasite antigens by Leishmania-specific T cell clones. Tests should be periodically re-performed, since in dogs the immune response to Leishmania can change (15).

**The Leishmanin Skin Test—LST**

This involves the inoculation of phenolized promastigotes into the epidermis and the measurement of the corresponding intradermal reaction—a delayed-type hypersensitive (DTH) response that can be examined under field conditions. This test provides a physiological means of assessing the development of Leishmania-specific cell-mediated immunity associated with the state of “resistance” to the parasite. In humans, the LST is a good detector of acquired protective immunity to Leishmania, becoming positive after effective therapy for HVL (35, 61). Further, the LST+ rate is inversely associated with the incidence of HVL (e.g., in populations with an LST+ rate of >45%, few cases of VL are seen).

LST is also a good method to assess anti-Leishmania specific DTH cellular responses in dogs, particularly under field conditions (62, 63). Most dogs that develop cell-mediated immunity do so early in infection, although some dogs with a positive LST result do develop clinical leishmaniases (64, 65) (note that asymptomatic dogs show stronger LST+ reactions than do symptomatic ones). The LST+ reaction reflects a lack of progression of the disease, making it one of the most useful test for evaluating Leishmania-specific cell-mediated immunity (66–69). An LST+ reaction may indicate that the immune system is controlling the infection, even in animals that have a positive spleen culture (70). The use of LST in vaccine trials is limited to phases IIB and III, when it becomes necessary to confirm a Leishmania-specific cell-mediated response.

### The in vitro Cell Proliferation Assay—CPA

This technique involves the *in vitro* stimulation of peripheral blood mononuclear cells (PBMCs) with leishmanial soluble antigen to confirm the presence of circulating Leishmania-specific memory T cell clones. The degree of cell proliferation (measured via the synthesis of DNA in the culture, or by cell division), indicates the degree of specific cell-mediated immunity against the parasite. Such testing has been used to assess the antigenicity of Leishmania proteins in humans (71).

In dogs, the intensity of the CPA and LST responses are correlated (52, 72), and a Leishmania-specific CPA+ response is associated with recovery after treatment for CVL. Relapse in treated animals is associated with the lack of, or the disappearance of, a positive CPA result (73).

Unlike the LST, *in vitro* cell stimulation is a very useful way of assaying the antigenicity of vaccine candidate proteins, and for detecting specific cell responses after immunization. In experimentally infected dogs, the CPA has successfully been used to examine the antigenicity of different Leishmania proteins, such as P-8 (74) HSP-70, KMP-11, PFR-2 (75) and PSA (76). Stimulation with the specific proteins included in the vaccine formulation also allows one to determine their capacity to induce T cell memory clones (45, 56, 77–79), and to assess the duration of the immunity produced (80).

CPA can be complemented with the analysis of the cytokines (i.e., those involved in the Th1 response, but mainly IFN-γ, the effector cytokine involved in the activation of macrophages to kill the parasites) secreted into the supernatant, allowing for a better characterization of the cellular responses activated. CPA thus becomes an IFN-γ release assay (IGRA)—the type of cell assay used to demonstrate immunity to intracellular pathogens (51, 81–84).

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**Table 2** Comparison of CVL vaccines currently marketed.

| VACCINE (References) | LEISH-TEC (54) | CANILEISH (58) | LETIFEND (59) |
|----------------------|---------------|----------------|---------------|
| **Vaccine formulation** | A2 + saponin | L. infantum excreted/secreted protein + QA21 | O chimeric protein |
| **Efficacy in the prevention of clinical signs** | 71.0% | 66.4% | 72% |
| **Level of protection** | 96.4% | 92.7% | 98% |
| **Reduction of symptoms** | YES | YES | YES |
| **Reduction of parasite burden after experimental infection (PCR)** | YES | YES | YES |
| **IgG2 expression** | YES | YES | YES |
| **Th1 cells activation** | Not determined | YES | Not determined |
| **IFN-γ expression after stimulation** | YES | Not determined | |
| **Leishmanicidal activity** | Not determined | YES | Not determined |
| **LST / DTH after infection** | Not determined | YES | YES |

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The ELISpot—IFN-γ Test
This is an IGRA-type test that combines the in vitro cell stimulation of PBMCs with the in situ expression of IFN-γ. This allows the direct quantification of the frequency of T cell clones producing IFN-γ after challenge with the leishmanial antigen—or vaccine stimulation—and therefore determines the potency of the immunity induced. This assay has proven useful for determining the immunological condition of Leishmania-infected people who are LST- (85). IFN-γ expression by Leishmania-specific T-cells is key in disease resistance. Several studies report the predominant role of IFN-γ in the activation of macrophages and the stimulation of their leishmanicidal activity in mice [reviewed in (26, 86, 87)]. The same role has been confirmed in dogs; high IFN-γ expression levels in peripheral blood lymphocytes from asymptomatic animals following stimulation with leishmanial antigen indicate a response to vaccination and are associated with the absence of symptoms (74, 88–90). All the latter studies emphasize the importance of T-cell-derived IFN-γ as a hallmark of immunity, and highlight the suitability of this approach when evaluating the efficacy of CVL vaccines. It is important to note that while the IFN-γ levels related to immunity to Leishmania are derived from T cells, the provenance of the same cytokine present at high levels in serum and in infected tissue is unsure, although it appears to be related to an inflammatory response (91). The ELISpot—IFN-γ test has been used to confirm the induction of cell-mediated immunity in vaccinated dogs. PBMCs are stimulated with total leishmanial soluble antigens—not just the antigens included in the vaccine (56).

Canine Macrophage Leishmanicidal Assay—CMLA
This is a complex assay performed in vitro to demonstrate that the cell-mediated immune response elicited is fully functional and can eliminate the parasite; it reveals the capacity of Leishmania-specific T cell clones to induce the leishmanicidal activity of infected macrophages when cultured together. This activity is measured via the reduction in the number of parasites present in cells after 72 h of co-culture. The test can be combined with analyses of the expression of factors such as IFN-γ, NO or iNOS which are directly involved in this leishmanicidal activity (76). This strategy has been used in laboratory studies to confirm immunogenicity after vaccination in dogs, but it is very difficult to use under field conditions (56, 80).

The Whole Blood Stimulation Assay—WBA
This recently developed IGRA-type test can be used to assess asymptomatic Leishmania infection in humans; the results are comparable to those provided by CPA (92). A peripheral blood sample is stimulated with leishmanial antigens and the cytokines/chemokines presents in the plasma determined after 24 h of incubation. IL-2, IFN-γ, IP-10, MIG, and MCP-1 are all associated with a protective immune response (93–95). In humans, this test has been used to confirm full recovery
after treatment (94, 96). Our own epidemiological studies involving dogs have confirmed that this is an easy-to-use, robust field technique, and that it can be used to detect natural asymptomatic infection in dogs. More importantly, it can also be used to assess the Leishmania-specific immunity induced by vaccination (97). The ease of use of this assay makes it appropriate to veterinary clinical practices for determining the level of protection induced by the vaccination against CVL (98). Further, it can be used to analyze vaccination-induced phenotypic changes in circulating immune cells; the increased expression of Toll-like receptors, activation and co-stimulatory molecules, and of inflammation-associated intracytoplasmic cytokines in neutrophils, monocytes and lymphocytes, have all been reported in Leishmune-immunized dogs (99).

CONCLUDING REMARKS AND PERSPECTIVES

CVL is a growing public health concern whose control requires the use of effective measures to prevent infection and the development of the disease. Vaccines for CVL represent an important advance for this control, but the complexity of the protective response that these vaccines have to induce in the host makes it difficult their obtaining and the assessment of their efficacy. The techniques discussed for assessing cell-mediated immunity in humans and dogs have all demonstrated their usefulness in this respect and should be used in order to confirm whether a dog has become protected after vaccination. Tools for testing specific immunity against CVL are important given that different vaccines for CVL are on the market and others are in the pipeline. Comparisons between already registered vaccines should go beyond confirming negative serological and parasitological results, but take advantage of cell-mediated immunity tests. The latter should be used in the different phases of clinical development of CVL vaccines and be incorporated into the follow-up of vaccinated animals involved, and into Phase IV post-marketing trials.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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