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REVIEWS

Review

Diagnostic Challenges in the Era of Canine *Leishmania infantum* Vaccines

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The diagnosis of canine leishmaniosis (CanL) is complex due to its variable clinical manifestations and laboratory findings. The availability of vaccines to prevent CanL has increased the complexity of diagnosis, as serological tests may not distinguish between naturally infected and vaccinated dogs. Current practices of prevaccination screening are not sufficiently sensitive to detect subclinically infected dogs, resulting in the vaccination of infected animals, which may lead to disease in vaccinated dogs that are also infectious to sand flies. This review evaluates the current techniques for diagnosing CanL, and focuses on new challenges raised by the increasing use of vaccines against this disease. Important gaps in knowledge regarding the diagnosis of CanL are underscored to highlight the need for novel diagnostic test development.

Diagnosis of CanL in a Moment

The diagnosis of **canine leishmaniosis** (see Glossary), due to *Leishmania infantum*, is complex because the spectrum of clinical signs and clinicopathological abnormalities is broad and often nonspecific [1]. Subclinical infections provide an added complication, as the prevalence of infection in healthy dogs is often several times higher than that of clinical leishmaniosis [2]. The use of vaccines against CanL in endemic areas poses an additional diagnostic challenge if the vaccine promotes seroconversion detected by conventional diagnostic tests, as well as in some cell-mediated immunity assays. Figure 1A displays the different canine clinical scenarios that exist in areas where vaccination is extensive or in regions where vaccination does not occur. Two vaccines for CanL (Leish-Tec®, Hertape Calier; and Leishmune®, Zoetis) have been marketed for several years in Brazil [3], and two other vaccines (CaniLeish®, Virbac; and LetiFend®, Leti) are currently marketed in Europe [4,5].

The cumulative experience obtained with the use of these vaccines allows a reflection on their impact on the current diagnosis of CanL. Leishmune® was available commercially in Brazil from 2004 to 2014, after which it was removed from the market. LetiFend® has recently been registered in Europe, but there are limited publications available regarding the large-scale use of this vaccine.[i] It is important to highlight that vaccinated infected dogs have been shown to be infectious to sand flies [6]. The presence of an increasing number of anti-*Leishmania* vaccines on the global market makes CanL a diagnostic challenge for the veterinary practitioner, the clinical pathologist, and public health authorities in endemic countries and nonendemic regions where imported infection is of concern [7]. The aim of this review is to update and discuss

Trends

Canine leishmaniosis (CanL) is an important zoonotic disease which is associated with the long history of companionship between dogs and humans. The recent introduction of commercial vaccines against CanL, and their wide use to prevent the disease, have created new challenges in the management of this infection.

These challenges include finding diagnostic tests to reliably detect subclinical infection, in order to ensure that infected dogs are not vaccinated, and also to separate naturally infected dogs from vaccinated dogs.

Serology for *Leishmania infantum* may be positive in both naturally infected and vaccinated dogs. Tests for cell-mediated immunity are currently not sufficient to distinguish natural infection from vaccination.

Vaccinated infected dogs may still be infectious to phlebotomine sand flies, the insect vectors of *Leishmania* spp.

Vaccinated seropositive dogs with suspected CanL should be tested for the presence of *L. infantum* parasites or leishmanial DNA.

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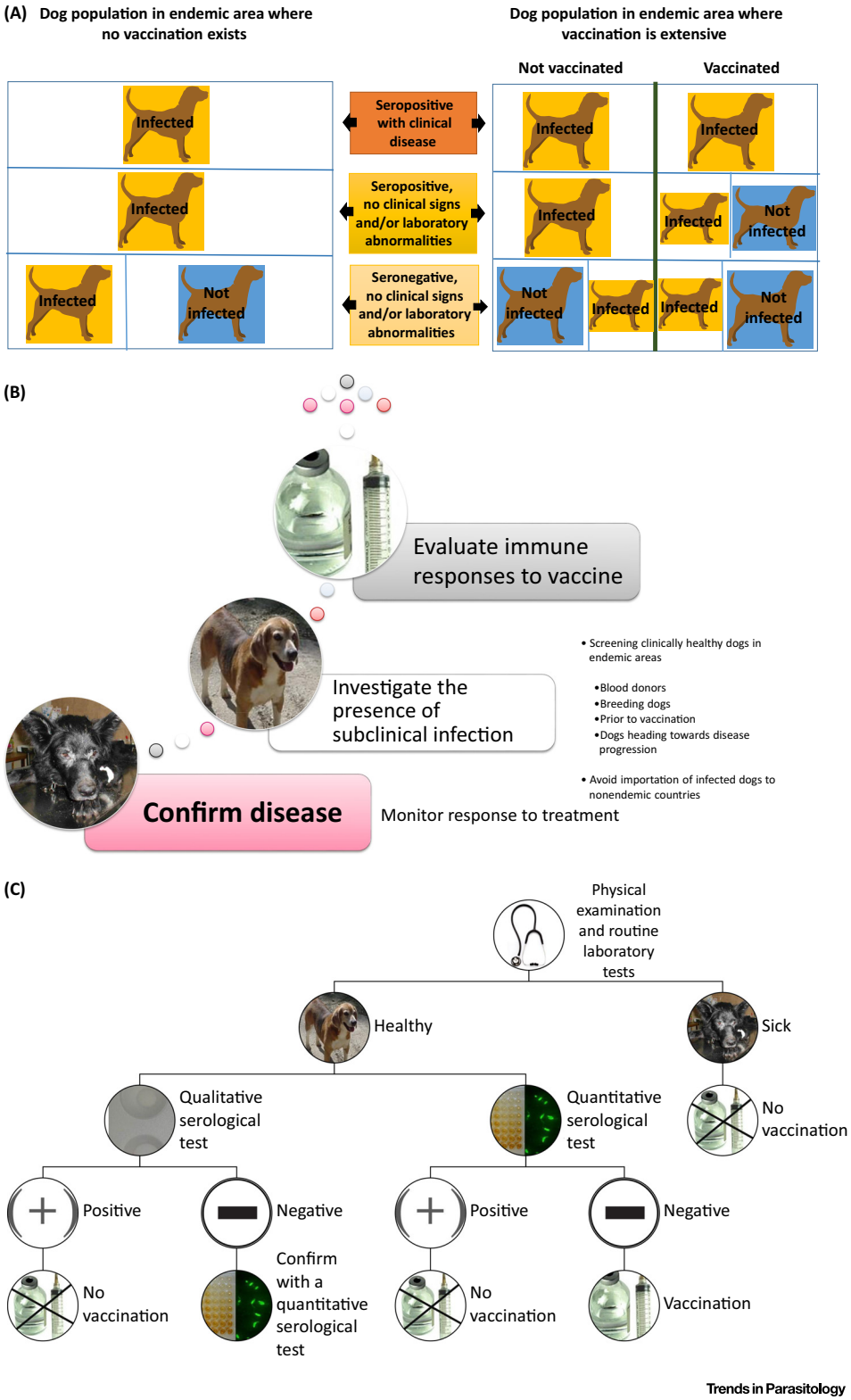

Figure 1. Challenges Raised by the Use of Vaccines against Canine Leishmaniosis. (A) Clinical scenarios in areas where vaccination does not occur (left) or in regions where vaccination is extensive (right). (B) The purpose of diagnosis in dogs. (C) Diagram for determining clinical and serological status prior to vaccination in dogs.

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current knowledge regarding CanL diagnosis caused by *L. infantum* infection, focusing on new challenges raised by the increasing use of vaccines against this disease.

Clinical Staging, Purpose of Diagnosis, and Diagnostic Tools

A clinical staging system for CanL that classifies the disease into four stages based on clinical signs, clinicopathological abnormalities, and measurement of anti-leishmanial antibodies was previously described [1,7]. This system is helpful in making therapeutic decisions and in determining prognosis [1,7], and an updated version of this staging is provided in Table 1. Stage II, moderate disease, appears to be the most common clinical stage of CanL diagnosed in Catalonia (Spain) [8], and this is likely to be similar in other areas of Spain as well as in other countries. The description and frequency of clinical signs and laboratory abnormalities present in dogs at different stages have been recently reported [8].

The various purposes for which *L. infantum* infection diagnosis is performed are listed in Figure 1B. In clinical settings, it is critical to distinguish between subclinical infection, overt disease, and immune responses due to vaccination. It is therefore important to be able to choose the best diagnostic test(s) for each purpose and subsequently to interpret their results accordingly. Accurate diagnosis of CanL requires an integrated approach consisting of a clinicopathological assessment and parasite-specific laboratory tests [9].

Options for the detection of *L. infantum* infection in dogs include parasitological assays, such as cytology, histology with immunochemistry, and culture of the organism in an appropriate medium; molecular tests, such as conventional, nested, and real-time **polymerase chain reaction** (PCR); and serological methods, including qualitative and quantitative antibody tests. In addition, specific cellular immunity tests for *L. infantum* infection have also been developed, but are currently used mainly in the research settings [7]. Isolation of parasites in culture from infected tissues will not provide rapid diagnosis and, likewise, is used mostly for research purposes [7]. However, due to the recent finding of allopurinol resistance in *L. infantum* isolates from dogs [10], parasite culture might help to evaluate therapeutic options in cases of treatment failure. Future development and evaluation of molecular testing to determine the presence of drug resistance would be useful in both human and veterinary medicine [11].

The diagnostic sensitivity of PCR assays relies considerably on the type and number of different tissues evaluated, the amplified DNA target number of copies, as well as the number of times that sampling and PCR is performed. Bone marrow, lymph node, spleen, skin [12], and conjunctival swabs [13,14] allow sensitive detection of *Leishmania* DNA, especially when tested by a PCR targeting kinetoplast DNA (kDNA), in clinically suspected dogs and subclinical infections. PCR assays with whole blood and urine samples are less sensitive than the abovementioned tissues [8,15–17]. Real-time PCR on whole-blood samples appears to be more sensitive with increasing disease severity [8]. Other noninvasive samples, such as oral [14,18], vulvar [19], nasal or ear-cerumen swabs [20,21], and hair [22], have also been evaluated. However, only limited studies are available regarding the diagnostic performance of these noninvasive samples with varying and contradictory results.

Point-of-care molecular assays are being developed to allow rapid detection of infection in clinical settings with minimal equipment [23]. Several techniques have been applied to the diagnosis of *Leishmania* infections in humans and dogs, such as lateral flow biosensors (LFB) [24] or loop-mediated isothermal amplification (LAMP) [25–27].

In North Africa and the Middle East, other *Leishmania* species circulate in addition to *L. infantum* and may infect local dogs and those visiting or travelling from these countries. This is the case for *Leishmania major* [28–30] and *Leishmania tropica* [30–32] infections described in dogs in

Glossary

Booster vaccination: administration of a vaccine dose after initial immunization.
Canine leishmaniosis (CanL): infectious disease of dogs, which is caused by *Leishmania infantum*, a zoonotic protozoan.
Diagnostic sensitivity: the conditional probability that a positive dog (i.e., having a disease or infection) will be correctly identified by a clinical test. The number of true-positive results divided by the total positives (i.e., the sum of true-positive plus false-negative results).
Diagnostic specificity: the conditional probability that a negative dog (i.e., not having a disease or infection) will be correctly identified by a clinical test. The number of true-negative results divided by the total number of negatives (i.e., the sum of true-negative plus false-positive results).
Differentiating between infected and vaccinated animals (DIVA): a DIVA vaccine does not elicit an antibody response (or the antibodies produced are different from those of natural infection) and allows the vaccination of a susceptible canine population without compromising the future serological identification of animals that become sick or subclinically infected.
Direct agglutination test (DAT): Serological test used to directly (i.e., with no intermediate steps) detect antibodies to whole promastigotes of *Leishmania* spp.
Enzyme-linked immunosorbent assay (ELISA): serological test used to detect antibodies to soluble, purified, or recombinant antigens of *Leishmania* spp.
Fast agglutination screening test (FAST): serological test similar to the DAT, but allowing faster reading times.
Immunofluorescence antibody test (IFAT): serological test used to indirectly detect antibodies to whole promastigotes of *Leishmania* spp.
***Leishmania* promastigote antigen (LPA):** whole parasites, purified fractions, or recombinant products which are used to detect antibodies specific to *Leishmania* spp.
Leishmanin skin test: intradermal technique that may induce a delayed-type hypersensitivity (DTH) response, which is readable when observing the skin.

these areas. The same occurs in South America, where *Leishmania braziliensis* and other *Leishmania* spp. infect dogs [33]. Routine veterinary diagnostic methods for CanL are not currently tuned to distinguishing between species of *Leishmania*. Nevertheless, that goal can be further achieved by a PCR coupled with subsequent restriction fragment length polymorphism (RFLP) [34], PCR high-resolution melt (HRM) analysis, or DNA sequencing [28,31,35]. Because PCR confirms infection but not disease, information provided by this test should be combined with the data obtained from the clinical examination, clinicopathological, and serological evaluation for a comprehensive assessment of the state of *Leishmania* infection [1]. The diagnostic tools used for monitoring sick treated dogs or clinically healthy infected dogs are shown in Table 2.

Polymerase chain reaction (PCR): molecular biology technique that amplifies nucleic acids, including those of *Leishmania* spp., increasing the sensitivity of detection.
Primary vaccination: initial administration of a vaccine (one or more doses) for inducing immunity.
Promastigote: one of the two main development stages of *Leishmania* spp., the other being the amastigote form. Promastigotes, found in the digestive tract of the vector hosts, are elongated extracellular forms with a free flagellum and measure 15–30 μm in length and 2–3 μm in width.

Serological Testing in the Age of *Leishmania* Vaccines

The introduction of commercial vaccines against CanL in Brazil and Europe has raised concerns regarding the use of serological testing for the diagnosis of this important zoonotic disease. The administration of a vaccine may elicit antibody production that could be detected by standard serological diagnostic techniques. This may not allow '**differentiating between infected and vaccinated animals**', a concept abbreviated as DIVA [36]. However, the majority of commercially available vaccines against CanL are not DIVA. In addition, another important question is how to screen dogs in endemic areas prior to vaccine administration

Table 1. Clinical Staging of Canine Leishmaniosis (CanL) Based on Serological Status, Clinical Signs, Laboratory Findings, and Type of Therapy and Prognosis for Each Clinical Stage

Clinical stages	Serology[a]	Clinical signs	Laboratory findings	Therapy	Prognosis
Stage I Mild disease	Negative to low positive antibody levels	Dogs with mild clinical signs such as solitary lymphadenomegaly or papular dermatitis	Usually no clinicopathological abnormalities observed Normal renal profile: creatinine <1.4 mg/dl; nonproteinuric: urinary protein:creatinine ratio (UPC) <0.5	Scientific neglect[b]/ Monitoring of disease progression (see Table 2)	Good
Stage II Moderate disease	Low to high positive antibody levels	Dogs which, apart from the signs listed in Stage I, may present, for example: diffuse or symmetrical cutaneous lesions such as exfoliative dermatitis/onychogryphosis, ulcerations (planum nasale, footpads, bony prominences, mucocutaneous junctions), generalized lymphadenomegaly, loss of appetite, and weight loss	Clinicopathological abnormalities such as mild nonregenerative anemia, hypergammaglobulinemia, hypoalbuminemia, serum hyperviscosity syndrome Substage (i) Normal renal profile: creatinine <1.4 mg/dl; nonproteinuric: UPC <0.5 (ii) Creatinine <1.4 mg/dl; UPC = 0.5–1	Allopurinol + meglumine antimoniate or miltefosine	Good to guarded
Stage III Severe disease	Medium to high positive antibody levels	Dogs which, apart from the signs listed in Stages I and II, may present signs originating from immune-complex lesions (e.g., uveitis and glomerulonephritis)	Clinicopathological abnormalities listed in Stage II Chronic kidney disease (CKD) IRIS[c] Stage I with UPC = 1–5 or Stage II (creatinine 1.4–2 mg/dl)[v]	Allopurinol + meglumine antimoniate or miltefosine Follow IRIS guidelines for CKD[vi]	Guarded to poor
Stage IV Very severe disease	Medium to high positive antibody levels	Dogs with clinical signs listed in Stage III. Pulmonary thromboembolism, or nephrotic syndrome and end-stage renal disease	Clinicopathological abnormalities listed in Stage II CKD IRIS Stage III (creatinine 2.1–5 mg/dl) and Stage IV (creatinine >5 mg/dl)[v] or nephrotic syndrome: marked proteinuria UPC >5	Specific treatment should be instaured individually Follow IRIS guidelines for CKD[vi]	Poor

[a]Dogs with negative to medium positive antibody levels should be confirmed as infected with other diagnostic techniques such as cytology, histology/immunohistochemistry and PCR. High levels of antibodies are conclusive of a diagnosis of CanL and are defined as a three- to fourfold increase in a well established laboratory reference cut-off.
[b]Dogs in Stage I (mild disease) are likely to require less prolonged treatment with one or two combined drugs (allopurinol, domperidone, meglumine antimoniate, or miltefosine) or alternatively monitoring with no treatment. There is limited information on dogs in this stage and, therefore, treatment options remain to be defined.
[c]Abbreviation: IRIS, International renal interest society.

Table 2. Monitoring Infected Dogs[a]

Parameters	Sick treated dogs	Clinically healthy infected dogs
	Frequency	
• Clinical history and physical examination • CBC, biochemical profile ± serum electrophoresis • Complete urinalysis ±UPC	• After the first month of treatment and then every 3–4 months during the first year. • Later on, every 6–12 months in dogs fully recovered clinically with treatment	Every 3–6 months
• Quantitative serology[b]	• Not before 6 months after initial treatment and every 6–12 months	
• Real-time PCR (optional)	• At the same time as serology	

[a]Abbreviations: CBC, complete blood count; UPC, urinary protein:creatinine ratio.
[b]Some dogs have a significant decrease in antibody levels (i.e., a more than three twofold dilutions difference between monitoring samples) associated with clinical improvement within 6–12 months of therapy. A marked increase in antibody levels (i.e., a more than three twofold dilutions difference between monitoring samples) should be interpreted as a marker of relapse, especially in dogs following the discontinuation of treatment.

(Figure 1C). All vaccines are recommended solely for clinically healthy and seronegative dogs. A summary of the vaccines available against CanL, their main characteristics, and the problems related with their use and diagnosis are listed in Table 3. Considerations regarding antibodies elicited after vaccination are shown in Box 1.

Table 3. Summary of the Main Vaccines against Canine Leishmaniosis (CanL) in South America and Europe[a]

Name of the vaccine (manufacturer, country)	Composition of vaccine	Vaccine protocol	Antibodies elicited by vaccine	Kinetics of antibodies at primary vaccination (peak and duration)	Diagnostic interference associated with vaccine	Refs
Leishmune® (Zoetis, Brazil)[b]	Fucose–mannose ligand (FML) of *Leishmania donovani*, a glycoproteic complex and \the surface glycoconjugate gp36, its major antigen; saponin-derived Quil-A® adjuvant	Three primary vaccination doses (SC), 21-day intervals; one annual booster	Antibodies to FML only and to LPA	Peak: 90 days after the 1st dose of primary vaccination; duration: 6 months after the vaccination start	Detection of vaccinal antibodies with official tests (DPP®, ELISA, and IFAT). Antibodies not detected after 45 days of first annual booster by FAST or DAT	[43,46,70]
Leish-Tec® (Hertape Calier Saúde Animal, Brazil)	Recombinant A2 antigen of *L. donovani*; saponin adjuvant	Three primary vaccination doses (SC), 21-day intervals; one annual booster	Antibodies to A2 (and potentially to LPA)	Peak: 21 days after the 2nd dose of primary vaccination; duration: decrease at 6 months	Detection of vaccinal antibodies with official ELISA	[3]
CaniLeish® (Virbac Santé Animale, France)	Purified excreted–secreted proteins of *Leishmania infantum* (LiESP); saponin-derived QA-21 adjuvant	Three primary vaccination doses (SC), 21-day intervals; one annual booster	Antibodies to LiESP and LPA (and potentially to Speed Leish K™ kinesins)	Peak: 2 weeks after the 3rd dose of primary vaccination; duration: variable, but may persist for 4–12 months	Detection of vaccinal antibodies with quantitative tests (ELISA and IFAT); rare detection of vaccinal antibodies with Speed Leish K™	[51,52,71]
Letifend® (Laboratorios Leti, Spain)	Recombinant chimerical protein Q (five antigenic fragments of four *L. infantum* proteins [histone H2A, LiP2a, LiP2b, and LiPo]); no adjuvant	One primary vaccination dose (SC); one annual booster	Antibodies to protein Q	Peak: 14 days after primary vaccination; duration: at least, 60 days (not assessed afterwards)	No detection of vaccinal antibodies by quantitative tests (IFAT and ELISA) or rapid tests[iv]	[4]

[a]Abbreviations: DAT, direct agglutination test; DPP®, dual-path platform (CVL rapid test, Bio-Manguinhos Institute, Fiocruz, Brazil); ELISA, enzyme-linked immunosorbent assay; FAST, fast agglutination screening test; FML, fucose–mannose ligand; IFAT, immunofluorescence antibody test; LiESP, *Leishmania infantum* excreted–secreted proteins; LPA, *Leishmania* promastigote antigen; NA, not available/determined; SC, subcutaneous; Speed Leish K™ (BVT, France).
[b]Withdrawn from the market in 2014.

Box 1. Considerations Regarding Humoral- and T Cell-Mediated Immunity Elicited by Vaccination

(i) Differences in immune responses to *Leishmania* antigens, including those elicited by vaccines, can be caused by:
- Age, sex, and breed;
- Factors influencing the immune status, including coinfections (with other agents), other noninfectious concomitant diseases, malnutrition, congenital and acquired immunodeficiencies;
- Individual genetic differences in the determination of humoral and cellular immune responses, resulting in high responders, low responders, or nonresponders.

(ii) Performance of different serological tests shows variability, depending on the test employed and the time after vaccination. For instance:
- Serological assays based on the detection of antibodies reactive with recombinant proteins are usually less sensitive for the recognition of antibodies elicited by vaccination than those based on whole-parasite antigens [46,52,53].
- Quantitative serological techniques are frequently capable of detecting antibodies elicited by vaccination, while rapid serological tests are usually less sensitive [46].

(iii) It is important to develop new serological techniques that will differentiate between antibodies due to natural infection and those elicited by vaccination with vaccines for which this discrimination is not currently possible.

Leishmania Vaccines and the Use of Serological Tests in Brazil

Brazil became the first country in the world to offer commercially available vaccines to immunize dogs against *L. infantum*. In 2003, the Leishmune® vaccine, originally marketed by Fort Dodge Animal Health and later by Zoetis, was licensed for the prevention of CanL by the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA). It was sold on the market from 2004 to 2014. In 2007, the MAPA licensed the use of the Leish-Tec® vaccine (Hertape Calier Saúde Animal, Brazil), which is currently the only commercial vaccine available against CanL in Brazil.

During the past decade, the official criteria for identifying dogs infected with *Leishmania* spp. recommended by the Brazilian Ministry of Health were based on an initial screening by an **enzyme-linked immunosorbent assay** (ELISA) and confirmation by an indirect **immunofluorescence antibody test** (IFAT) [37]. Testing prior to dog culling is now based on a dual-path platform (colloidal gold-based immunochromatography assay; DPP® CVL rapid test, Bio-Manguinhos Institute, Fiocruz, Brazil), which uses a recombinant K28 protein of *L. infantum* as antigen, and an indirect ELISA (with antigens of *L. major*-like **promastigote** forms; Bio-Manguinhos Institute), for screening and confirmation, respectively [38].

Discrimination of Antibodies to Natural Infection from Those Elicited by Vaccination in Brazil

Antibody responses detected by standard diagnostic techniques in vaccinated dogs may be interpreted as natural infection with *L. infantum*, potentially requiring removal for euthanasia in Brazil [39]. Given the importance of serodiagnosis in veterinary practice and epidemiological surveillance, CanL vaccines should ideally not induce antibodies detectable by serological tests used for the diagnosis of infection [3].

There is considerable controversy regarding the distinction between Leishmune®-vaccinated and naturally infected dogs through the application of serodiagnostic tests. Although dogs may test serologically negative immediately after vaccination against CanL with Leishmune®, subsequent seroconversion, antibody peak, and positivity of up to 6 months may lead vaccinated dogs to be falsely identified as having natural infection [39]. Attempts to use IgG1/IgG2 ratios to distinguish Leishmune®-vaccinated from infected dogs have been reported as contradictory [39–41]. However, these contradictory results are probably due to the specificity of the commercially available polyclonal antisera used to detect the canine IgG1 and IgG2 subclasses, as the inability to distinguish between canine IgG subclasses is well recognized unless monoclonal antibodies are used [42]. Lastly, in a vaccination study, none of 71 Leishmune®-vaccinated dogs tested seropositive by the **fast agglutination screening test** (FAST) or the **direct agglutination test** (DAT) after 45 days of the first annual **booster vaccination**. Therefore, these tests have been suggested to be useful in cases where Leishmune®-vaccinated dogs have shown seropositivity by other tests [43].

The second vaccine used in Brazil, Leish-Tec®, has been reported to induce antibody responses detectable by specific A2-ELISA, but no seroconversion as determined by ***Leishmania* promastigote antigen** (LPA)-ELISA and DPP standard tests for CanL [44]. However, in another study, it was shown that Leish-Tec® did induce seroconversion in 30.9% of the vaccinated animals, as found by the LPA–ELISA, with IgG levels peaking on the 21st day after the second dose of this vaccine [45]. In addition, a comparative trial showed no significant differences with regard to IgG seropositivity in dogs vaccinated with Leishmune® or Leish-Tec® and exposed to infection for 11 months in a visceral leishmaniosis-endemic area [44]. Thus, so far, distinguishing vaccinated dogs from naturally infected ones by the routine serological methods in Brazil has not been proven to be reliable [46].

A prototype flow cytometry test was unable to detect serological reactivity in dogs vaccinated with Leishmune® or Leish-Tec® [47]. However, the sampling time after vaccination was unknown in this study. Moreover, this test showed cross-reactivity with canine pathogens other than *L. infantum* [47]. Dog culling based on serological results alone may lead to the killing of vaccinated dogs, misdiagnosed as naturally infected by *Leishmania* spp. Therefore, other testing methods such as molecular approaches should be used to provide a more reliable diagnosis and to avoid the elimination of noninfected dogs [46].

Variable Diagnostic Performance among Different Serological Tests in Brazil

The performance of serological tests is greatly affected by the antigen used in the technique [47]. Antibodies to *Leishmania* may be detectable in vaccinated dogs for months [39,46]. In fact, up to 6 months after the first dose of Leishmune®, vaccinated dogs were found to be positive by an 'in-house' ELISA and by three official Brazilian tests for the detection of antibodies to *Leishmania*, that is, an ELISA, IFAT, and the DPP® CVL rapid test [46]. The in-house ELISA was the serological test which identified the highest number of seropositive dogs over time, reaching 100% at 42 and 90 days, and 88.8% of the dogs at 6 months after the first vaccine dose. By contrast, a decline in the number of seropositive dogs was observed at 6 months after the first dose, with 88.8%, 33.3%, 11.1%, and 5.5% of dogs remaining seropositive to *Leishmania* by the in-house ELISA, the official ELISA, DPP® CVL rapid test, and IFAT, respectively [46].

The CaniLeish® Vaccine and Serological Screening Test Speed Leish K® in Europe

The CaniLeish® vaccine (Virbac, France) was authorized in the European Union in 2011. This vaccine has shown efficacy in decreasing the incidence of clinical disease due to natural *L. infantum* infection in dogs [5].

Since dogs should be seronegative prior to CaniLeish® vaccination, a serological screening test should always be performed before vaccinating dogs. The Speed Leish K® (BVT, France), produced by a company affiliated with the manufacturer of the CaniLeish® vaccine, has been recommended for prevaccination screening of *L. infantum* infection before the use of the CaniLeish® vaccine, and the manufacturer recommends vaccination only of those dogs with a negative Speed Leish K® test result. This is a rapid serological test that detects circulating antibodies directed against a kinesin of *L. infantum*.[ii] Its **diagnostic sensitivity** and **specificity** have been reported to be good in the majority of sick dogs with high *L. infantum*-specific antibody levels confirmed by IFAT [48] or quantitative ELISA [49]. However, the diagnostic sensitivity of this assay was low compared with IFAT [48] and quantitative ELISA [49], when dogs with low antibody levels were tested. It is important to highlight that a screening serological test should have a very good diagnostic sensitivity. The Speed Leish K® does not seem to have an optimal diagnostic performance when used as a screening test. The use of this test may result in vaccinating dogs that are infected with *L. infantum* and seropositive by other tests – or even sick dogs which are seronegative when tested with the rapid Speed Leish

K® test [49]. The consequences of vaccinating seropositive dogs are unknown, but there is a risk that vaccinated and previously infected seropositive dogs would develop clinical leishmaniosis. A report on vaccinated dogs that developed clinical leishmaniosis found that the majority of these animals were diagnosed only a few months after vaccination [50]. This highlights the importance of using accurate screening diagnostic tests prior to vaccination in dogs living in endemic areas [50]. In addition, incorrect diagnosis of infected dogs may have important veterinary medicine and public health implications that should be considered.

The Serological Response Elicited by the CaniLeish® Vaccine

The maximum peak of antibodies following vaccination can be detected roughly 2 weeks after the administration of the third dose of the CaniLeish® vaccine during the **primary vaccination** course [51,52]. Antibodies reactive with *Leishmania* antigen have been reported to persist for 4–12 months in dogs vaccinated with CaniLeish® after the end of the primary vaccination protocol[ii],[iii] [52]. Nevertheless, there is a marked decrease of antibody levels over time[ii] [52,53]. In one study, antibody levels determined by IFAT (cut-off of 1:200) were undetectable after 10 months from the first dose of the CaniLeish® vaccine [52]. Some dogs may have low IFAT titers (1:80) at 12 months before annual booster time [53]. Very limited information is available regarding antibodies elicited after the annual boosters of CanL vaccination [53], but the dynamics of IFAT titers seems to be similar [54].

Discrimination between Natural Antibodies and Those Elicited by CaniLeish® Vaccination

Vaccination with CaniLeish® interferes with antibodies measured by quantitative serological assays for *L. infantum*. The rapid serological test Speed Leish K® detects circulating antibodies directed against kinesins of *L. infantum* and might be used to distinguish between antibodies elicited by CaniLeish® vaccination from those derived from a natural infection.[ii] However, the Speed Leish K® might uncommonly detect antibodies elicited by primary vaccination with CaniLeish® in healthy dogs previously seronegative by Speed Leish K® and quantitative serological testing.[iii] In addition, it is probable that other rapid tests based only on recombinant proteins would be less likely to detect antibodies elicited by the CaniLeish® vaccine. Nevertheless, the diagnostic performance of rapid tests is variable, and there are limited serological comparative studies regarding antibodies elicited by this vaccine. Therefore, there is currently no well-documented standardized serological test which is able to discriminate between antibodies elicited by the CaniLeish® vaccine from those derived from natural infection.

Variable Diagnostic Performance among Different Serological Tests in the Detection of Antibodies Induced by CaniLeish®

Limited comparative data have been published regarding the diagnostic performance of different serological tests in the detection of antibodies induced by the CaniLeish® vaccine. IFAT has been shown to detect antibody levels during at least 10 months or more[iii] [52]. As mentioned earlier in this review, quantitative serological tests using the whole parasite would give a better diagnostic performance than a qualitative serological test using recombinant proteins.

The Serological Response Elicited by the LetiFend® Vaccine in Europe

The European Medicine Agency (EMA) has recently authorized an additional CanL vaccine, LetiFend® (Laboratorios Leti, Spain). The basis of this vaccine is Q Protein (QP), a recombinant protein formed by the fusion of five antigenic fragments of *L. infantum* intracellular proteins [4]. Vaccination of dogs with LetiFend® induces a transient increase in anti-QP IgG2 antibodies which peaks 14 days after primary vaccination and declines thereafter [4]. However, evaluation of the kinetics of anti-QP IgG2 antibodies was only followed-up at 60 days postvaccination – when antibodies were not yet below cut-off levels [4]. There is no information available regarding the time when anti-QP IgG2 antibodies achieve baseline levels. In addition, there are currently no published data regarding antibody responses after the annual booster.

Discrimination between Antibody Responses to Natural Infection and Elicited by Vaccination with LetiFend®

Vaccination with LetiFend® does not interfere with antibodies measured by the most widely used *L. infantum* serological quantitative assays[iv] [4]: IFAT, soluble *Leishmania* antigen ELISA [4], whole promastigote ELISAs [49], Leiscan® ELISA (Ecuphar, Belgium), INgezim® ELISA (Ingenasa, Spain), and qualitative serological diagnostic tests (Kalazar *Detect*™, InBios, USA), SNAP® *Leishmania* (IDEXX, USA), Speed Leish K™ and WITNESS® Leishmania (Zoetis, USA). This allows serological discrimination between vaccinated and naturally infected dogs.[iv]

Parasite-Specific Cellular Immunity Elicited by the Available CanL Vaccines

It is well established that a protective anti-*Leishmania* vaccine should elicit parasite-specific, long-lasting, cell-mediated immunity in order to be effective against CanL. Classically, an inflammatory T helper (Th) 1 response, associated with the cytokine interferon-γ (IFN-γ), is thought to provide resistance to the development of disease [55–58], whereas a T regulatory response, conferred by production of the cytokine IL-10, is associated with susceptibility to disease with increased parasite burden and a strong ineffective humoral response [8,59,60]. Parasite-specific T cell-mediated immunity can be assessed in dogs by a **leishmanin skin test**, which induces a delayed-type hypersensitivity (DTH) response [61]. Tests to assess parasite-specific cell-mediated immunity *ex vivo* include lymphocyte proliferation assays, the production of IFN-γ, and measurement of additional proinflammatory cytokines such as tumour necrosis factor-α (TNF-α) by several techniques [8,62–64], and assessment of enhanced macrophage leishmanicidal activity via superoxide analyses [59] or more general reactive oxygen species [52]. CanL vaccines such as Leishmune® [65,66] and CaniLeish® have been reported to elicit parasite-specific cellular immunity which was demonstrated to last at least 1 year [52]. LeishTec® has been shown to induce IFN-γ production [67] and provide protective immunity for at least 9 months, and after annual booster it provided protection through a second year [3].

However, there are limited studies and scientific data available on T cell-mediated immunity for the majority of commercial vaccines available (Box 1). For example, studies of T cell immunity induced after CaniLeish® vaccination were mainly done in small numbers of young Beagle dogs [51,52]. It is likely that the T cell-mediated immune response to vaccination will not be as homogeneous in a large-scale study that includes variable breeds or outbred dogs, and dogs of different ages, as compared to laboratory-bred Beagles [51,52]. As previously mentioned, all *Leishmania* vaccines licensed to date have focused on vaccination of seronegative healthy animals. In dogs that have already been infected with *Leishmania* and are heading toward disease progression, there is likely a subpopulation of dogs with more limited T cell responses, specifically in their ability to produce IFN-γ, but perhaps in other aspects as well [55,59]. These dogs have been characterized as having an immune cell deficit that comes with chronic exposure to infection and the inflammatory response that it induces. This is known as T cell exhaustion [59]. This leads to a lack of responsiveness of T cells, robust production of regulatory cytokines and antibodies, and a proliferation of parasites, all of which eventually lead to antigen–antibody deposits in the kidneys and death of the dog [68]. This exhaustion is characterized by robust surface expression of the inhibitory receptor Programmed-death-1, which increases with progressive disease [59]. Preliminary studies of canine T cells from infected dogs have indicated that the use of Toll-like receptor 7 agonists may be able to reverse these responses in both infected and clinically affected dogs [56].

Diagnosis of Clinical Leishmaniosis in Vaccinated Dogs

Veterinarians practicing in areas where CanL is endemic in southern Europe have sporadically diagnosed clinical disease in previously vaccinated dogs [50,69]. In sick dogs with compatible clinical signs and clinicopathological abnormalities of leishmaniosis, a history of previous

Leishmania vaccination does not rule out CanL. The disease should be included in the list of differentials based on clinical history, physical examination, and/or clinicopathological findings, even when vaccination has been performed. Therefore, it is important to establish how to make the diagnosis in these dogs.

Since there is no sensitive test to discriminate between antibodies to vaccination and to natural infection with *L. infantum*, the use of quantitative serology as the sole diagnostic technique for the detection of CanL in vaccinated dogs is not recommended. Therefore, additional diagnostic techniques based on observation of the inflammatory response in the lesion by cytology or histology, and detection of the parasite or its DNA by PCR or cytology, should be employed in vaccinated dogs. It is essential to know precise details of the vaccination history of evaluated dogs in order to make a correct interpretation of the serological test employed. All the evaluated information, including history, clinical signs, results of general tests such as complete blood count, biochemistry profile, and urinalysis, and those of specific diagnostic tests such as cytology, histology, quantitative serology, and PCR, should be combined for a comprehensive assessment.

Concluding Remarks and Future Perspectives

CanL is a complex multifaceted infection with great zoonotic importance. The advent of vaccination presented important progress in the control of the disease but has also changed and further complicated its diagnosis. Dog owners, veterinarians, and public health professionals should be educated to recognize the disease, select the right diagnostic techniques to be employed for each diagnostic purpose and indication, and also be aware of the possibility of persistently infected vaccinated dogs. Despite the fact that CanL has been known and managed in dogs for about 100 years, there are still gaps in our knowledge about this disease and its management. New diagnostic techniques to improve the detection of infection and to discriminate between vaccinated and naturally infected dogs are warranted. In addition, molecular diagnosis might allow characterizing parasite-specific features (see Outstanding Questions).

Acknowledgments

The authors thank Bayer Animal Health and Virbac for sponsoring the activities of the LeishVet association. The sponsors had no influence on the scientific content of this publication.

Resources

[i]www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/veterinary/medicines/003865/vet_med_000333.jsp&mid=WC0b01ac058008d7a8.

[ii]www.vin.com/Proceedings/Proceedings.plx?CID=BSAVA2012&PID=83631&O=Generic

[iii]http://worldleish2017.org/#/abstracts (C1460, p. 1053)

[iv]www.sevc2016.com/index.php/es/posters/enfermedades-infecciosas/1160-vaccination-with-letifend-a-novel-canine-leishmaniosis-vaccine-does-not-interfere-with-serological-diagnostic-tests

[v]www.iris-kidney.com/guidelines/en/staging_ckd.shtml

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Outstanding Questions

Can qualitative or quantitative serological tests be developed to distinguish between dogs that have been vaccinated against leishmaniosis and naturally infected dogs?

Can the performance of point-of-care serological tests for prevaccination screening be improved?

Can simple tests be developed to discriminate between responses to vaccination and natural infection by evaluating cellular immunity?

Can new biological markers be discovered for differentiating between responses to natural infection and those stimulated by vaccination?

Can markers be found to assess resistance and susceptibility of dogs to leishmaniosis?

Can markers be developed to assess the efficacy of long-term antileishmanial treatment?

Will rapid molecular diagnosis allow identification of *Leishmania* infecting dogs at the species and strain virulence levels?

Can further development of molecular diagnosis determine the presence or formation of drug resistance in parasite strains?

Can molecular techniques be applied in the future for determining the prognosis or selecting the most adequate treatment for dogs with leishmaniosis?

How will coinfections with other pathogens or the presence of concomitant diseases influence the efficacy of vaccination against CanL?

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