

## LEISHMANIOSIS

---

### SUMMARY

**Description and importance of the disease:** *Leishmaniosis is not a single entity but comprises a variety of syndromes caused primarily by at least 20 Leishmania species affecting humans, transmitted by phlebotomine sand flies belonging to the genera Phlebotomus and Lutzomyia. Leishmaniosis is associated with and limited by the geographic distribution of its sand fly vectors. Fifteen nosogeographical entities have been classified worldwide, of which 13 have an established or putative zoonotic nature. In recent years, the number of regions becoming Leishmania-endemic has grown significantly, accompanied by an increased number of animal and human cases.*

*In humans, the clinical spectrum ranges from asymptomatic infections to those with high mortality, with three distinct forms being classically described: visceral (VL), cutaneous (CL) and mucocutaneous (MCL). Dogs are commonly affected by L. infantum and L. chagasi (now regarded as synonyms), which causes a chronic viscero-cutaneous disease in this host (canine leishmaniosis, CanL). Asymptomatic infection in dogs is widespread and contributes to maintaining the long-term presence of the parasite in endemic regions. The clinical appearance and evolution of leishmaniosis is a consequence of complex interactions between the parasite and host immune response. The outcome of infection depends on the ability of host macrophages to effectively destroy the parasite.*

**Detection of the agent:** *When clinical signs and characteristic lesions are present in affected humans and animals, the demonstration of the parasites in stained smears of splenic, bone marrow and lymph node aspirates, of skin scrapings, and in tissue biopsies, gives a positive diagnosis. If the infection is low grade, detection of parasites is possible only by attempting in-vitro isolation or by polymerase chain reaction (PCR). As there are very few morphological differences among various species, the identification of any isolated Leishmania organism relies on biochemical and/or molecular methods. Several centres throughout the world are presently using isoenzyme and DNA characterisation to identify the agent.*

**Serological tests:** *Serology is the preferred method for diagnosis of VL and CanL, even during the early stages of the disease. In subclinical forms, seropositive cases are confirmed by parasitological diagnosis or PCR. Serology is of less value for CL and MCL. Of the several serological techniques available, the indirect fluorescent antibody test and the enzyme-linked immunosorbent assay are the most suitable. Crude antigens for serodiagnostic tests can be prepared in the laboratory from cultured parasites. Tests based on recombinant antigens may also be used and have a high specificity although can be less sensitive.*

*The rapid immunochromatographic assay is easy to carry out and can be performed in veterinary clinics, but has lower diagnostic efficiency than the indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA).*

**Requirements for vaccines:** *There is no effective vaccine available worldwide at present for use in dogs or humans. A number of vaccines for use in animals are under evaluation, and four have been authorised for use in dogs (two in Brazil and two in Europe). Beyond other issues to be evaluated, the use of these vaccines is posing present and future challenges to the fields of diagnosis, epidemiology and surveillance of the parasite, especially in countries where the parasite does not occur. Leishmanin skin test antigen is no longer available worldwide and lacks standardisation.*

### A. INTRODUCTION

**Description of the disease:** Leishmaniosis comprises a variety of syndromes caused by members of the protozoan parasite *Leishmania* (Kinetoplastida: Trypanosomatidae), which are transmitted to mammal hosts by the bite of

infected phlebotomine sand flies belonging to the genera *Phlebotomus* (Old World)<sup>1</sup> and *Lutzomyia* (New World). The disease results from the multiplication of amastigote forms in macrophages of the reticuloendothelial system. The epidemiology and clinical manifestations of the diseases are largely diverse, being usually grouped into two main entities: zoonotic leishmanioses, where domestic or wild animal reservoirs are involved in the transmission cycle and humans play a role as an accidental host, and anthroponotic leishmanioses, where humans are the sole reservoir and source of the vector's infection (Esch & Petersen, 2013). T lymphocytes and cytokines, play a crucial role in determining whether infection evolves toward a protective immune status or a progressive and manifest disease. In inbred mouse models, two different T helper (Th) cell subsets are involved, termed Th1 and Th2, which differ in their profile of secreted cytokines by a polarised activation; the Th1 response confers protection, whereas the Th2 response renders the host susceptible to infection. However, in human patients and dogs with clinically apparent infections, Th1 and Th2 type responses are not characteristically polarised, as both activating (e.g. interferon gamma, interleukin 12) and suppressive cytokines (e.g. interleukin 10, interleukin 13, interleukin 4, TGF beta) are detected (Murray *et al.*, 2005).

Various forms of clinical manifestations of human leishmaniosis have been described and divided into three major clinical entities: visceral leishmaniosis (VL, kala azar), cutaneous leishmaniosis (localised, diffuse or disseminated CL, oriental sore, uta, pian bois, chiclero's ulcer) and mucocutaneous leishmaniosis (MCL, espundia) (World Health Organization [WHO], 2010). The diseases are mainly zoonoses with two exceptions, that of CL due to *L. tropica* in urban areas of the Middle East, and that of VL due to *L. donovani* in the Indian sub-continent (northern India, Nepal and Bangladesh) and in some parts of Eastern Africa (e.g. Ethiopia and Sudan). Canine leishmaniosis (CanL) is a chronic viscero-cutaneous disease caused by *L. infantum*, for which the dog acts as the source reservoir. Resistant asymptomatic dogs may exceed >50% of the infected canine population. Typical external signs recorded in susceptible dogs that evolve towards full-blown disease are lymph node enlargement, weight loss, exfoliative dermatitis, onychogryphosis, alopecia, ulcers and ocular alterations. Feline leishmaniosis (FeL) caused by *L. infantum* appears to be an emerging feline disease; in the past two decades it has been more and more frequently reported in endemic areas and sporadically also seen in non-endemic areas in re-homed cats (Pennisi *et al.*, 2015; Richter *et al.*, 2014; Rüfenacht *et al.*, 2005). Sporadic tegumentary cases have been reported in equids and cattle (Gramiccia *et al.*, 2011). Likewise, wild canid (wolf, fox and jackal) populations can show similar infection rates as in dogs, but the prevalence of progressive clinical signs in these species is substantially lower and thus they may not be as infectious as dog populations to supporting a primary transmission cycle. In an outbreak of visceral leishmaniosis in Spain, hares (*Lepus granatensis*) and rabbits (*Oryctolagus cuniculus*) were described as a reservoir (Jimenez *et al.*, 2014; Molina *et al.*, 2012).

**Causal pathogen:** Approximately 20 recognised *Leishmania* species are agents of human leishmanioses. In the New World, tegumentary forms are caused by *L. braziliensis*, *L. guyanensis*, *L. panamenis*, *L. shawi*, *L. naiffi*, *L. lainsoni*, *L. lindenbergi*, *L. peruviana*, *L. mexicana*, *L. venezuelensis* and *L. amazonensis*; visceral and, more rarely, cutaneous forms, are caused by *L. infantum*. In the Old World, cutaneous forms are caused by *L. tropica*, *L. major* and *L. aethiopica*; visceral and, more rarely, cutaneous forms, are caused by *L. infantum* and *L. donovani*. *Leishmania infantum* and *L. chagasi* have been found to be identical by genotyping and should be regarded as synonyms (Kuhls *et al.*, 2011). In addition, the taxonomic position of other *Leishmania* agents of tegumentary forms (*L. killicki* in the Old World; *L. pifanoi*, *L. garnhami* and *L. colombiensis* in the New World) is still under discussion. Other *Leishmania* species not pathogenic to humans include New and Old World rodent parasites and an agent of cutaneous leishmaniosis in Australian macropods (Dougall *et al.*, 2009). Dogs are mainly affected by *L. infantum*, but, other *Leishmania* species are found in dogs in the New World (*Leishmania amazonensis*, *L. braziliensis*, *L. mexicana*, *L. venezuelensis*) (Pennisi *et al.*, 2015; Solano-Gallego *et al.*, 2009). *Leishmania tropica* and *L. major* are rarely reported in dogs and are found mainly associated with skin or mucocutaneous lesions (Baneth *et al.*, 2017).

**Zoonotic risk and biosafety requirements:** Direct contact with infected hosts or handling of biological samples and parasite cultures from these hosts is of low risk, because of the sand fly-borne nature of the infections and the lack of resistant forms in the environment. All laboratory manipulations with *Leishmania* spp. should be performed at an appropriate biosafety and containment level determined by biorisk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

**Differential diagnosis:** In dogs, the most common leishmaniosis signs may be confounded with ehrlichiosis, babesiosis and vector-borne or intestinal helminthiasis.

**Role of the vector:** The phlebotomine vectors of leishmaniosis are, in some cases, only permissive to the complete development of the species of *Leishmania* that they transmit in nature, while in other instances they can transmit more than one species of *Leishmania* (Bates, 2007). There are emerging species of *Leishmania* infecting humans and animals for which the vector has not been identified. There are some 500 known phlebotomine species, but only about 30 have been found to transmit leishmaniosis. Only the female sand fly transmits the parasites (WHO, 2010). In temperate areas the transmission of *Leishmania* occurs only during warm months, coinciding with the

<sup>1</sup> In this chapter, the term 'New World' refers to the Americas, and the term 'Old World' refers to Africa, Asia and Europe (WHO, 2010).

activity period of adult sand flies, but due to the variable and lengthy pre-patent period, seasonal variation in the disease incidence in dogs and humans may not be apparent. By contrast, in warmer regions such as much of endemic Brazil, sand fly activity and human and canine transmission of zoonotic VL is year round. Temperature affects the development time and overwintering of sand flies and the extrinsic incubation period, which is likely to be reflected in their duration of infectiousness.

## B. DIAGNOSTIC TECHNIQUES

Leishmaniosis diagnosis must be based on an integrated approach that takes into account signalling, medical history, physical findings, clinical and pathological changes, and results of diagnostic tests. The diagnostic methods currently available are described below.

The list and fitness of each test for different purposes are given in Table 1.

**Table 1.** Test methods available for diagnosis of leishmaniosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
<b>Detection of the agent<sup>2</sup></b>						
<b>Cytological examination</b>	–	–	–	++	–	–
<b>Histological examination</b>	–	–		++	–	
<b>Isolation in culture</b>	–	+		++	–	
<b>Molecular methods</b>	++	+++		++	++	
<b>Detection of immune response</b>						
<b>IFAT</b>	+++	++	–	++	+++	–
<b>ELISA</b>	+++	++		++	+++	
<b>Direct agglutination test</b>	++	++		++	++	
<b>Rapid immunochromatographic assay</b>	–	–		++	+	

Key: +++ = recommended for this purpose; ++ recommended but has limitations; + = suitable in very limited circumstances; – = not appropriate for this purpose.

PCR = polymerase chain reaction; IFAT = Indirect fluorescent antibody test; ELISA = enzyme-linked immunosorbent assay.

### 1. Detection of the agent

#### 1.1. Cytological examination

Cytological examination allows the presence of amastigotes in macrophages or extracellular fluid to be highlighted after appropriate staining (May Grunwald–Giemsa staining). Amastigote are small intracellular rounded or oval body, 1.5–3 × 2.5–6.5 µm in size, found in vacuoles within the cytoplasm of

<sup>2</sup> A combination of agent identification methods applied on the same clinical sample is recommended.

the macrophages. There is no free flagellum. The organism has a relatively large nucleus and a kinetoplast consisting of a rod-like body and a dot-like basal body.

The cytological investigation should therefore be performed on the following samples:

- i) Papular, nodular and ulcerative skin lesions: sampling by fine needle aspiration or impression smear; ischemic ulcerative lesions may, however, be negative;
- ii) Bone marrow and lymph nodes in the presence of clinical signs or clinical pathological changes referable to the involvement of parasites (anaemia, lymphadenomegaly, etc.);
- iii) Other locations: biological fluids taken from sites with lesions (e.g. synovial fluid in the case of arthritis/polyarthritis, cerebrospinal fluid in the case of neurological signs, etc.). In the absence of sampling lesions, the organs or tissues in which parasites are most likely to be found are represented, in decreasing order of diagnostic sensitivity, by spleen, bone marrow, lymph node and blood (Mylonakis *et al.*, 2005; Saridomichelakis *et al.*, 2005).

In the case of a negative cytological result, the material used for cytology can be stored and sent to the laboratory for testing by polymerase chain reaction (PCR) (see Section B.1.4 *Molecular methods*). The specificity of microscopy for the diagnosis of visceral leishmaniosis is high, but its sensitivity varies according to the sampled tissues: 93–99% for the spleen, 52–85% for bone marrow, and 52–58% for lymph node aspirates. However, splenic aspiration is not recommended, given the very high risk of life-threatening bleeding. Bone marrow aspiration is less sensitive but safer and is currently considered the best method for obtaining a sample of tissue to analyse.

## 1.2. Histological examination

The parasite can be highlighted in sections of lesions stained with haematoxylin-eosin. In association with the parasite, the CanL-compatible alterations can also be highlighted, represented by granulomatous inflammation and/or vasculitis affecting different organs, ischaemic dermatopathies, lymphoplasmacytic dermatitis of the dermo-epithelial junction, lymphoid hyperplasia of the spleen and lymph nodes. The use of histological examination is always advisable when, despite a negative cytological examination, the strong suspicion of CanL persists, especially in the presence of dermatitis and in cutaneous forms characterised by focal lesions. If histological alterations such as those described above are detected in haematoxylin-eosin stained sections, but without detectable parasites it is advisable to proceed with immunohistochemical staining for *Leishmania* antigens. If this approach is also negative, the biopsy sample can be used for genetic analyses (PCR, sequencing, loop-mediated isothermal amplification) (see Section B.1.4 *Molecular methods*) (Muller *et al.*, 2003; Roura *et al.*, 1999a).

## 1.3. Isolation in culture

Isolation in culture is the most specific test because the development in culture of vital promastigotes is solely attributable to the genus *Leishmania* in the case of samples taken in endemic areas of the Old World. The parasitological investigation should be performed on the following samples: lymph node aspirate, bone marrow aspirate, skin scrapings and biopsies. In VL, blood can also be used (by taking the buffy coat), with reduced sensitivity. However, isolation in culture has the disadvantage of requiring long execution times and is performed only in specialised laboratories. The choice of the isolation and culture methods will depend on the immediate circumstances and on the technical capability and experience of the laboratory staff (WHO, 2010). Unfortunately, there is still no 'universal' culture medium in which all the different leishmanias will grow easily, and it is almost impossible to predict which medium will be best suited to the growth of a particular isolate of *Leishmania*. Individual laboratories have to find the most suitable medium among biphasic blood agar media and tissue culture media supplemented with fetal calf serum (Evans, 1987). When attempting primary isolation of unknown organisms, a blood agar-based medium should be used – preferably NNN medium (Novy, McNeil and Nicolle), otherwise brain–heart infusion (BHI) agar medium or EMTM (Evans' modified Tobie's medium) should be used. For bulk cultivation of established isolates, suitable media are reported in Section B.1.3 below (see Evans, 1987 for media composition). The organisms from patients with chronic CL and MCL can be very difficult to cultivate. The parasites sometimes die when subcultured, even when the initial isolation is successful. This seems especially common when the initial isolation has been into a rich medium. Often this can be overcome if subcultures are made into less nutritionally rich media, such as NNN, or one of the semisolid media such as 'sloppy Evans' or semisolid Locke blood agar. There are many references available offering new alternative culture media to be used for cultivation of the parasite (Castelli *et al.*, 2014; Santarém *et al.*, 2014).

## 1.4. Molecular methods

PCR methods are available for diagnosis and/or identification of *Leishmania* from different types of human and animal samples. The molecular investigation should be performed on the following samples in decreasing order of sensitivity: bone marrow/lymph node, skin, conjunctiva, buffy coat, peripheral blood. In resistant dogs, *Leishmania* inoculation may not be followed by parasite dissemination therefore, in endemic areas, a positive PCR result on a skin sample in the absence of skin lesions does not necessarily mean that the dog is infected and will develop infection (Gradoni, 2002). Similarly bone marrow PCR positives may subsequently test negative (Oliva *et al.*, 2006). It is always better to use fresh or frozen material or material fixed in 95% ethyl alcohol. Formalin-fixed or paraffin-fixed samples can be used but give lower diagnostic yields.

Essentially, techniques developed either to identify established isolates of *Leishmania* or to detect organisms from several samples, include: (a) digestion of material with proteinase K and DNA extraction. These steps can be either performed using in-house protocols and reagents, or by commercial kits that are widely available; (b) standard PCR amplification using oligonucleotide sequences (primers) selected from the small-subunit rRNA gene (Mathis & Deplazes, 1995), kinetoplast DNA minicircles (Maarten *et al.*, 1992), or other highly repetitive genomic DNA sequences (Bulle *et al.*, 2002; Piarroux *et al.*, 1993); (c) analysis of amplification products by 1–2% agarose gel. PCR is a very sensitive method, especially if the PCR targets are “multi-copy” genomic sequences, those present in high numbers in every single parasite, such as the kinetoplast minicircles DNA (Cortes *et al.*, 2004).

The three most used techniques are:

- i) Conventional or traditional PCR: *Leishmania* DNA is amplified using a pair of primers (complementary base sequences to the target sequence contained in *Leishmania* DNA); (Lachaud *et al.*, 2002; Muller *et al.*, 2003);
- ii) Nested PCR: a modification of traditional PCR, more sensitive but less specific, as increasing the number of steps tends to increase the risk of contamination by foreign DNA and, therefore, of false positive results (Fisa *et al.*, 2001; Roura *et al.*, 1999b);
- iii) Real-time PCR (quantitative): the use of fluorescent molecules or probes makes it possible to quantify the number of DNA copies present in a biological sample. Real-time PCR has a sensitivity similar to the nested PCR, but if performed with ‘closed’ systems, it is more specific because the sample undergoes a smaller number of manipulations and is therefore less prone to contamination. Real-time PCR can also provide information useful in the monitoring phase, e.g. number of parasites (Manna *et al.*, 2008).

Studies have also demonstrated the potential of loop-mediated isothermal amplification (LAMP) as a point of care diagnostic technique for CanL particularly in resource limited endemic areas (Nzelu *et al.*, 2019).

## 2. Serological tests

Seroconversion occurs within a few months of infection: on average 5 months (range 1–22) for natural infections and 3 months (range 1–6) for experimental infections (Moreno & Alvar, 2002). The diagnostic techniques available vary. Some, such as Western blotting, while showing excellent diagnostic performance, are not used on a large scale because of time constraints and cost. The most widely available are indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), direct agglutination tests (DAT), and the rapid immunochromatographic assay (dipstick or strip-test).

### 2.1. Indirect fluorescent antibody test

The IFAT is widely used because it is easy to perform. The test is genus specific, although significant cross-reactions have been reported in individuals infected with *Trypanosoma cruzi*. For these subjects, serological tests based on specific recombinant *Leishmania* antigens would be more appropriate (see Sections B.2.2.2 and B.2.2.4 below). In Chagas’ disease-free areas, the IFAT for the diagnosis of clinical VL or CanL has a sensitivity of 96% and specificity of 98%, which is similar to the ELISA. Although amastigotes from frozen sections or smears of infected organs can be used as antigen, cultured promastigotes represent the commonest antigen source.

#### 2.1.1. Antigen preparation

- i) Harvest 3–4 ml of the liquid media of a 3-day-old culture showing flourishing promastigote growth (see Section B.1 for culture media).

- ii) Wash the organisms three times with phosphate-buffered saline (PBS), pH 7.2–7.4, by centrifugation at 350 **g** for 15 minutes at room temperature.
- iii) Resuspend the final cell pellet in PBS and adjust the promastigote concentration to approximately  $4 \times 10^6$ /ml with the aid of a haemocytometer.
- iv) Distribute 30  $\mu$ l of the promastigote suspension on to each circle of a multi-spot slide and allow to dry at room temperature.
- v) Fix the promastigotes in cold acetone for 10 minutes, then put the slides into a plastic box and keep in a deep freezer ( $-35^\circ\text{C}$ ) for no longer than 2–3 months.

### 2.1.2. Test procedure

- i) Wash the frozen antigen-coated slides in PBS and allow to dry at room temperature.
- ii) Inactivate the sera for 30 minutes in a water bath at  $56^\circ\text{C}$ .
- iii) Make doubling dilutions of test sera from 1/80 to 1/10,240 for human VL, and from 1/40 to 1/5120 for CanL. Positive and negative control sera, at dilutions of 1/80 and 1/160 for human VL, and of 1/40 and 1/80 for CanL, are also included in the test. No standard sera are available, but internal standards should be prepared and titrated.
- iv) Distribute 30  $\mu$ l of diluted serum samples on to each slide circle and incubate for 30 minutes at  $37^\circ\text{C}$ .
- v) Remove the serum samples by vigorous washing in PBS, followed by immersion of the slides in PBS for 10 minutes. Allow the slides to dry.
- vi) Distribute 30  $\mu$ l of diluted fluorescein isothiocyanate (FITC)-conjugated anti-immunoglobulin on to each slide circle and incubate for 30 minutes at  $37^\circ\text{C}$ . FITC-conjugated anti-human and anti-dog immunoglobulins are commercially available. Follow the instructions for the appropriate dilution.
- vii) Repeat step v and mount with a cover-slip in a few drops of PBS/glycerol (50% [v/v] of each).
- viii) Read the slides under a fluorescent microscope. The highest dilution showing fluorescent promastigotes is taken to be the antibody titre.

### 2.1.3. Interpretation of the results

In acute human VL, the threshold titre usually ranges from 1/80 to 1/160. Asymptomatic or subclinical human disease usually results in titres below 1/80. In CanL the threshold titre ranges from 1/40 (indicative of exposure but not necessarily of established infection) to 1/160 (indicative of established infection), whereas a titre of 1/320 or above can be indicative of the disease in clinically suspected dogs (Paltrinieri *et al.*, 2010). As regards other domestic mammals (e.g. cats) no standardised IFA threshold limits are available. As IFAT performance may vary in different laboratories, it is better for each laboratory to define its own threshold titre using defined positive and negative reference sera.

## 2.2. Enzyme-linked immunosorbent assay

The ELISA can be carried out on serum or on a measured volume of blood. The blood is collected by needle-prick on to suitable absorbent paper strips and allowed to dry. The sample is eluted and tested at a single dilution previously determined to give an acceptable sensitivity and specificity. This test can be used for seroepidemiological surveys under field conditions.

In the classical method, the antigen is prepared as follows: promastigotes harvested from cultures are washed four times with PBS, pH 7.2, at 1000 **g** for 15 minutes. The packed promastigotes are resuspended in twice their volume of distilled water, and then sonicated at medium amplitude in an ice bath. The suspension is left at  $4^\circ\text{C}$  overnight to allow the proteins to come into solution. After a final centrifugation at 4000 **g** for 10 minutes to eliminate the cellular debris, the overlay, representing the concentrated soluble antigen, is dispensed into vials and stored at  $-20^\circ\text{C}$  until required. For use in the test, it is reconstituted with PBS to the predetermined optimal protein concentration (around 20  $\mu\text{g}/\text{ml}$ ) as measured by Lowry's method. Enzyme (usually horseradish peroxidase)-conjugated reagents consist of anti-dog goat immunoglobulins or Protein A (Hamarsheh *et al.*, 2012).

The ELISA is useful for the diagnosis of Old and New World leishmaniosis. Cross-reaction with *Babesia canis* and *Trypanosma* spp. has been reported (Gottstein *et al.*, 1988). According to the *Leishmania* strain used, sensitivity of the ELISA can range from 86% to 99%.

A detergent-soluble promastigote antigen has been used in ELISA instead of the crude lysate, for the diagnosis of CanL. The detergent was Triton X-100 and the proteic extract was protected with protease inhibitors. Using this method, ELISA sensitivity increased to 99.5%, while its specificity was comparable with that of the IFA test (97%) (Mancianti *et al.*, 1995).

The ELISA methods described above are based on crude antigenic preparations. A recombinant antigen from a cloned protein of *L. infantum*, called rK39, has been reported to be highly reactive to sera from human and canine visceral leishmaniosis cases when run in an ELISA format. Using 25–50 ng of the antigen, 99% specificity and sensitivity was consistently found for dogs with parasitologically proven disease (Scalone *et al.*, 2002). In HIV-positive patients, K39-ELISA showed higher sensitivity (82%) than the IFA test (54%) (Houghton *et al.*, 1998). The K39 antigen, which shows remarkable stability and reproducibility, is commercially available. More recently, a K9-K39-K26 recombinant chimeric antigen has been evaluated as a single ELISA protocol for serological diagnosis of both human and canine *Leishmania* infections (Daprà *et al.*, 2008). In dogs, test specificity and sensitivity were reported to be 99.5% and 98.5%, respectively, with high concordance (K value: 0.98) with standard IFA test.

### 2.3. Direct agglutination test

The DAT has been described for the diagnosis of VL and CanL. After test improvement, DAT has been validated as a specific and sensitive assay for field investigations (Boelaert *et al.*, 1999; Cardoso *et al.*, 2004; Ozbek *et al.*, 2000). The antigen consists of promastigotes harvested from cultures, washed in PBS, pH 7.2, treated with 0.4% trypsin (for 45 minutes at 37°C and then washed again), and stained with 0.02% Coomassie brilliant blue. Twofold serial dilutions of serum in PBS are made in V-bottomed microtitre-plate wells; 50 µl of antigen preparation is added to each well, and the plate is then carefully shaken by hand and left for 18 hours at room temperature. The test is read visually against a white background. Positive reactions are indicated by typical light-blue aggregates, while negative samples give a clear sharp-edged blue spot.

A modified DAT for detection of specific anti-leishmanial antibodies in canine reservoir hosts is considered to be highly suitable for wide-scale epidemiological and ecological field work and diagnosis of CanL, having 100% sensitivity and 98.9% specificity (Harith *et al.*, 1988; 1989). The reliability of the test was improved by treating the test sera with 0.2 M 2-mercaptoethanol and incubating them at 37°C.

### 2.4. Rapid immunochromatographic assay (dipstick or strip-test)

The rapid immunochromatographic assay is easy to carry out and can be performed in veterinary clinics, but has lower diagnostic sensitivity than the ELISA and IFAT: specificity is medium-high but sensitivity is low (30–70%) (Gradoni, 2002; Mettler *et al.*, 2005; Reithinger *et al.*, 2002) and therefore it can give false negative results. When there is a strong suspicion of a false negative result using a rapid immunochromatographic assay, one of the other serology tests must be used. The value of a positive result is limited because the test does not allow antibody titre to be evaluated, however positive results can be useful for identifying individuals with parasite dissemination and for monitoring therapeutic response.

A rapid immunochromatographic assay using rK39 as antigen (K39 dipstick or strip-test, commercially available) has been evaluated in different endemic settings of VL. The nitrocellulose membrane of the test kit holds an absorbent pad at one end, a band of immobilised anti-protein A antibody (used to detect IgG) at the other (control region), and a band of rK39 antigen in the middle (test region). A protein-A-colloidal gold conjugate is used as the immunochromatographic detection reagent. One small drop (20 µl) of the serum to be examined is placed on the absorbent pad before two large drops (100 µl) of test buffer are added to the pad, and the mixture is allowed to migrate up the strip by capillary action. After 2–10 minutes, the result is positive if two distinct red lines appear (one in the test region and another in the control region), it is negative when no red line appears in the test region, and it is invalid if the control line fails to appear.

In clinical cases of human VL, two commercial brands of K39 dipstick showed 99–100% sensitivity and 95–100% specificity in India (Sundar *et al.*, 2006), 90% sensitivity and 100% specificity in Brazil (Carvalho *et al.*, 2003), and 100% sensitivity and specificity in the Mediterranean basin (Brandonisio *et al.*, 2002). In dogs with both asymptomatic and symptomatic cases of CanL, the sensitivity and the specificity of the K39 dipstick were 97% and 100%, respectively (Otranto *et al.*, 2005).

### 3. *Leishmania* species, subspecies or strains identification

Morphological identification enables identification of *Leishmania* at the genus level, but not at the species or subspecies level. Several techniques may be used to identify the different *Leishmania* species, subspecies or strains. Fifteen recognised *Leishmania* Identification Centres were listed by WHO in 2010.

#### 3.1. Isoenzyme characterisation

Also known as multi-locus enzyme electrophoresis (MLEE), isoenzyme characterisation is the reference method for species identification (Rioux *et al.*, 1990; WHO, 2010), although this technique requires cultivation of a large number of parasites ( $5 \times 10^9$ – $1 \times 10^{10}$ ). The principles of enzyme electrophoresis are as follows: soluble enzymes are extracted from the organisms grown in media for bulk cultivation (BHI medium, MEM/FCS/EBLB [minimal essential medium/fetal calf serum/Evans' blood lysate broth] medium, Schneider's *Drosophila* medium). A small amount of the extract is then placed in an inert supporting substance, the matrix, containing a buffer at a fixed pH. The matrix is usually starch gel, but it could equally well be absorbent cellulose acetate, acrylamide or agarose. The pH of the buffer in the matrix is usually chosen so that the isoenzymes are negatively charged. A direct current is passed through the matrix carried by the ions in the buffer. When electrophoresis is completed, most proteins will have moved in the matrix towards the anode, depending on the amount of negative charge. If stained at this stage with a general protein stain, many bands will be seen. However, the high substrate and cofactor specificity of enzymes make it possible to stain only these proteins. Hence, the electrophoretic mobility of one particular enzyme can be compared among several organisms. The stained matrix with its collection of stained isoenzyme bands is known as a zymogram. Normally one or more extracts from reference organisms, in which the enzyme banding patterns are well documented, are included in the gel to aid the interpretation of results. Most enzymes used for characterisation purposes are stained by methods incorporating a dehydrogenase reaction. At least 12 enzymes should be examined; organisms showing identical zymograms are classified into zymodemes of a given species.

#### 3.2. Molecular characterisation

Different molecular techniques have been developed that allow *Leishmania* characterisation at the species or strain level such as (a) PCR-restriction fragment length polymorphism (RFLP) analysis, in which the PCR products are digested by appropriate restriction enzymes and the resulting restriction fragment patterns are analysed (Marfurt *et al.*, 2003; Minodier *et al.*, 1997; Montalvo *et al.*, 2012; Volpini *et al.*, 2004); (b) multi-locus microsatellite typing (MLMT) (Kuhls *et al.*, 2011) and (c) multi-locus sequence typing (MLST) (Mauricio *et al.*, 2006). In these cases, repeated and polymorphic DNA sequences are targeted, such as ribosomal internal transcribed spacer 1 (ITS1), cysteine protease B, kinetoplast DNA minicircles, surface glycoprotein 63, heat-shock protein 70, mini-exons and microsatellites (Reithinger & Dujardin, 2007; Schönian *et al.*, 2008).

## C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

### 1. Vaccines

#### 1.2. Outline of production and minimum requirements for vaccines

Currently there are no available laboratory-made antigens for *Leishmania* vaccine production. There are four licensed inactivated vaccines against CanL, which are patent-protected. The first vaccine was developed in Brazil. Outlines and requirements for production were approved by the Brazilian Ministry of Agriculture, Livestock and Food Supply. The vaccine consists of the glycoprotein-enriched fraction of *L. donovani* known as 'fucose-mannose ligand' (FML). Field studies showed about 80% clinical protection conferred by the antigen administered with QuilA saponin as adjuvant, and also good immunotherapeutic efficacy when used in sick dogs (Palatnik-de-Sousa *et al.*, 2008). A second vaccine was also developed in Brazil, which uses the recombinant A2 antigen of *L. donovani* in association with a saponin adjuvant. It demonstrated 43% protection against a culture positive state in an artificial challenge model. The third vaccine is licensed in Europe. Outlines and requirements for production have been approved by the European Medicines Agency. Purified excreted-secreted proteins of *L. infantum* and with QA-21 saponin significantly reduces the risk of progressing to active infection or overt disease, with a clinical efficacy of 68%. In vaccinated dogs that developed disease and that were exposed to the bites of reared *P. perniciosus* vectors, the reduction in parasite transmission was significant when compared with matched controls. However, vaccine does not enable differentiation of vaccinated from infected animals. In addition, some safety concerns have been reported dogs vaccinated with purified excreted-secreted proteins (Oliva *et al.*, 2014). The fourth vaccine, available in the EU, contains the

active substance protein Q, which is made of different fragments of proteins from *L. infantum*. A dog vaccinated with recombinant protein Q from *L. infantum* MON-1 has five times less risk of developing clinical disease than a non-vaccinated dog. There is a lack of information concerning the interruption of *Leishmania* transmission by dogs vaccinated with recombinant protein Q from *L. infantum* MON-1 is lacking (Cotrina *et al.*, 2018). Surveillance programmes, especially those based solely on serology in dogs should be revised to take into account the impact of vaccination and to ensure epidemiological data is interpreted appropriately.

At present, a number of promising anti-leishmanial vaccines are under experimental evaluation (Coler & Reed, 2005; Das & Ali, 2012). A chimeric antigen generated from three recombinant *Leishmania* antigens screened for their ability to elicit cellular immune responses (known as Leish-111f, patent-protected) and adjuvanted with monophosphoryl lipid A – stable emulsion (MPL-SE), represents the first defined vaccine for leishmaniosis in humans, having completed phase 1 and 2 safety and immunogenicity testing in healthy subjects (Coler *et al.*, 2007). The same polyproteinic antigen and adjuvant failed to protect dogs from *L. infantum* infection in a phase 3 trial (Gradoni *et al.*, 2005), while conferred some protection from disease when used as an immunotherapeutic agent in dogs with mild CanL (Trigo *et al.*, 2010). Recently, a novel chimeric antigen generated from four recombinant antigens (KSAC, patent-protected) has been found promising for vaccine protection in both human VL and CanL (Goto *et al.*, 2011).

## 2. Diagnostic biologicals (antigens for skin tests)

As the skin test is not used for diagnosis of animal infections, and the leishmanin antigen has not been internationally standardised, no recommendations can be made by the OIE.

## REFERENCES

- ALIMOHAMMADIAN M.H., JONES S.L., DARABI H., RIAZIRAD F., AJDARY S., SHABANI A., REZAEI M.A., MOHEBALI M., HOSSEINI Z. & MODABBER F. (2012). Assessment of interferon- $\gamma$  levels and leishmanin skin test results in persons recovered for leishmaniasis. *Am. J. Trop. Med. Hyg.*, **87**, 70–75.
- BANETH G., YASUR-LANDAU D., GILAD M. & NACHUM-BIALA Y. (2017). Canine leishmaniosis caused by *Leishmania major* and *Leishmania tropica*: comparative findings and serology. *Parasit. Vectors*, **10**, 113.
- BATES P.A. (2007). Transmission of *Leishmania metacyclic* promastigotes by phlebotomine sand flies. *Int. J. Parasitol.*, **37**, 1097–1106.
- BOELAERT M., EL SAFI S., JACQUET D., DE MUYNCK A., VAN DER STUYFT P. & LE RAY D. (1999). Operational validation of the direct agglutination test for diagnosis of visceral leishmaniasis. *Am. J. Trop. Med. Hyg.*, **60**, 129–134.
- BRANDONISIO O., FUMAROLA L., MAGGI P., CAVALIERE R., SPINELLI R. & PASTORE G. (2002). Evaluation of a rapid immunochromatographic test for serodiagnosis of visceral leishmaniasis. *Eur. J. Clin. Microbiol. Infect. Dis.*, **21**, 461–464.
- BULLE B., MILLON L., Bart J.M., GALLEGU M., GAMBARELLI F., PORTUS M., SCHNUR L., JAFFE C.L., FERNANDEZ-BARREDO S., ALUNDA J.M. & PIARROUX R. (2002). Practical approach for typing strains of *Leishmania infantum* by microsatellite analysis. *J. Clin. Microbiol.*, **40**, 3391–3397.
- CASTELLI G., GALANTE A., LO VERDE V., MIGLIAZZO A., REALE S., LUPO T., PIAZZA M., VITALE F. & BRUNO F. (2014). Evaluation of two modified culture media for *Leishmania infantum* cultivation versus different culture media. *J. Parasitol.*, **100**, 228–230.
- CARDOSO L., SCHALLIG H.D., NETO F., KROON N. & RODRIGUES M. (2004). Serological survey of *Leishmania* infection in dogs from the municipality of Peso da Regua (Alto Douro, Portugal) using the direct agglutination test (DAT) and fast agglutination screening test (FAST). *Acta Trop.*, **91**, 95–100.
- CARVALHO S.F., LEMOS E.M., COREY R. & DIETZE R. (2003). Performance of recombinant K39 antigen in the diagnosis of Brazilian visceral leishmaniasis. *Am. J. Trop. Med. Hyg.*, **68**, 321–324.
- COLER R.N. & REED S.G. (2005). Second-generation vaccines against leishmaniasis. *Trends Parasitol.*, **21**, 244–249.
- COLER R.N., GOTO Y., BOGATZKI L., RAMAN V. & REED S.G. (2007). Leish-111f, a recombinant polyprotein vaccine that protects against visceral Leishmaniasis by elicitation of CD4+ T cells. *Infect. Immun.*, **75**, 4648–4654.

- CORTES S., ROLAO N., RAMADA J. & CAMPINO L. (2004). PCR as a rapid and sensitive tool in the diagnosis of human and canine leishmaniasis using *Leishmania donovani* s.l.-specific kinetoplastid primers. *Trans. R. Soc. Trop. Med. Hyg.*, **98**, 12–17.
- COTRINA J.F., INIESTA V., MONROY I., BAZ V., HUGNET C., MARAÑÓN F., FABRA M., GÓMEZ-NIETO L.C. & ALONSO C. (2018). A large-scale field randomized trial demonstrates safety and efficacy of the vaccine LetiFend® against canine leishmaniosis. *Vaccine*, **36**, 1972–1982.
- DAPRÀ F., SCALONE A., MIGNONE W., FERROGLIO E., MANNELLI A., BIGLINO A., ZANATTA R., GRADONI L. & ROSATI S. (2008). Validation of a recombinant based antibody ELISA for diagnosis of human and canine leishmaniasis. *J. Immunoassay Immunochem.*, **29**, 244–256.
- DAS A. & ALI N. (2012). Vaccine development against *Leishmania donovani*. *Front. Immunol.*, **3**, 99.
- DOUGALL A., SHILTON C., LOW CHOY J., ALEXANDER B. & WALTON S. (2009). New reports of Australian cutaneous leishmaniasis in Northern Australian macropods. *Epidemiol. Infect.*, **137**, 1516–1520.
- ESCH K.J. & PETERSEN C.A. (2013). Transmission and epidemiology of zoonotic protozoal diseases of companion animals. *Clin. Microbiol. Rev.*, **26**, 58–85.
- EVANS D.A. (1987). *Leishmania*. In: *In-Vitro Methods for Parasite Cultivation*, Taylor A.E. & Baker J.R., eds. Academic Press, London, UK, 52–75.
- FISA R., RIERA C., GÁLLEGO M., MANUBENS J. & PORTÚS M. (2001). Nested PCR for diagnosis of canine leishmaniosis in peripheral blood, lymph node and bone marrow aspirates. *Vet. Parasitol.*, **99**, 105–111.
- GOTO Y., BHATIA A., RAMAN V.S., LIANG H., MOHAMATH R., PICONE A.F., VIDAL S.E., VEDVICK T.S., HOWARD R.F. & REED S.G. (2011). KSAC, the first defined polyprotein vaccine candidate for visceral leishmaniasis. *Clin. Vaccine Immunol.*, **18**, 1118–1124.
- GOTTSTEIN B., DEPLAZES P., ARNOLD P., MEHLITZ D., REITER I. & ECKERT J. (1988). Immundiagnose der Leishmaniose des Hundes mit ELISA und Mini-Western-Blot. *Schweiz. Arch. Tierheilk.*, **130**, 249–262. (in German)
- GRADONI L. (2002). The diagnosis of canine leishmaniasis. In: *Canine Leishmaniasis: Moving Towards a Solution*, Killick-Kendrick R., ed. Intervet International, Boxmeer, Netherlands.
- GRADONI L., FOGLIA MANZILLO V., PAGANO A., PIANTEDOSI D., DE LUNA R., GRAMICCIA M., SCALONE A., DI MUCCIO T. & OLIVA G. (2005). 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*, **23**, 5245–5251.
- GRAMICCIA M. (2011). Recent advances in leishmaniosis in pet animals: Epidemiology, diagnostics and anti-vectorial prophylaxis. *Vet. Parasitol.*, **181**, 23–30.
- HAMARSHEH O., NASEREDDIN A., DAMAJ S., SAWALHA S., AL-JAWABREH H., AZMI K., AMRO A., AREKAT S., ABDEEN Z. & AL-JAWABREH A. (2012). Serological and molecular survey of *Leishmania* parasites in apparently healthy dogs in the West Bank, Palestine. *Parasit. Vectors*, **5**, 183.
- HARITH A.E., KOLK A.H.J., LEEUWENBURGH J., MUIGAI R., HUIGEN E., JELSMA T. & KAGER P.A. (1988). Improvement of a direct agglutination test for field studies of visceral leishmaniasis. *J. Clin. Microbiol.*, **26**, 1321–1325.
- HARITH A.E., SLAPPENDEL R.J., REITER I., VAN KNAPEN F., KORTE P.D., HUIGEN E. & KOLK A.H.J. (1989). Application of a direct agglutination test for detection of specific anti-*Leishmania* antibodies in the canine reservoir. *J. Clin. Microbiol.*, **27**, 2252–2257.
- HOUGHTON R.L., PETRESCU M., BENSON D.R., SKEIKY Y.A.W., SCALONE A., BADARO R., REED S.G. & GRADONI L. (1998). A cloned antigen (recombinant K39) of *Leishmania chagasi* diagnostic for visceral leishmaniasis in human immunodeficiency virus type 1 patients and a prognostic indicator for monitoring patients undergoing drug therapy. *J. Infect. Dis.*, **177**, 1339–1344.
- JIMENEZ M., GONZÁLEZ E., MARTÍN-MARTÍN I., HERNÁNDEZ S. & MOLINA R. (2014). Could wild rabbits (*Oryctolagus cuniculus*) be reservoirs for *Leishmania infantum* in the focus of Madrid, Spain? *Vet. Parasitol.*, **202**, 296–300. doi: 10.1016/j.vetpar.2014.03.027. Epub 2014 Apr 4.

- KUHLS K., ALAM M.Z., CUPOLILLO E., FERREIRA G.E., MAURICIO I.L., ODDONE R., FELICIANGELI M.D., WIRTH T., MILES M.A. & SCHÖNIAN G. (2011). Comparative microsatellite typing of new world *Leishmania infantum* reveals low heterogeneity among populations and its recent Old World origin. *PLoS Negl. Trop. Dis.*, **5**, e1155.
- LACHAUD L., MARCHERGUI-HAMMAMI S., CHABBERT E., DEREURE J., DEDET J.P. & BASTIEN P. (2002). Comparison of six PCR methods using peripheral blood for detection of canine visceral leishmaniasis. *J. Clin. Microbiol.*, **40**, 210–215.
- MAARTEN H.L., DE BRUIJN M.H.L. & BARKER D.C. (1992). Diagnosis of New World leishmaniasis: specific detection of species of the *Leishmania braziliensis* complex by amplification of kinetoplast DNA. *Acta Trop.*, **52**, 45–58.
- MANCIANTI F., FALCONE M.L., GIANNELLI C. & POLI A. (1995). Comparison between and enzyme-linked immunosorbent assay using a detergent-soluble *Leishmania infantum* antigen and indirect immunofluorescence for the diagnosis of canine leishmaniosis. *Vet. Parasitol.*, **59**, 13–21.
- MANNA L., REALE S., VITALE F., PICILLO E., PAVONE L.M. & GRAVINO A.E. (2008). Real-time PCR assay in *Leishmania*-infected dogs treated with meglumine antimoniate and allopurinol. *Vet. J.*, **177**, 279–282.
- MANSON-BAHR P.C. (1987). Diagnosis. In: The Leishmaniasis in Biology and Medicine. Vol. II. Clinical Aspects and Control, Peters W. & Killick-Kendrick R., eds. Academic Press, London, UK, 703–729.
- MARFURT J., NASEREDDIN A., NIEDERWIESER I., JAFFE C.L., BECK H.P. & FELGER I. (2003). Identification and differentiation of *Leishmania* species in clinical samples by PCR amplification of the miniexon sequence and subsequent restriction fragment length polymorphism analysis. *J. Clin. Microbiol.*, **41**, 3147–3153.
- MATHIS A. & DEPLAZES P. (1995). PCR and *in vitro* cultivation for detection of *Leishmania* spp. in diagnostic samples from humans and dogs. *J. Clin. Microbiol.*, **33**, 1145–1149.
- MAURICIO I.L., YEO M., BAGHAEI M., DOTO D., PRATLONG F., ZEMANOVA E., DEDET J.P., LUKES J. & MILES M.A. (2006). Towards multilocus sequence typing of the *Leishmania donovani* complex: resolving genotypes and haplotypes for five polymorphic metabolic enzymes (ASAT, GPI, NH1, NH2, PGD). *Int. J. Parasitol.*, **36**, 757–769.
- METTLER M., GRIMM F., CAPELLI G., CAMP H. & DEPLAZES P. (2005). Evaluation of enzyme-linked immunosorbent assays, an immunofluorescent-antibody test, and two rapid tests (immunochromatographic-dipstick and gel tests) for serological diagnosis of symptomatic and asymptomatic *Leishmania* infections in dogs. *J. Clin. Microbiol.*, **43**, 5515–5519.
- MINODIER P., PIARROUX R., GAMBARELLI F., JOBLET C. & DUMON H. (1997). Rapid identification of causative species in patients with Old World leishmaniasis. *J. Clin. Microbiol.*, **35**, 2551–2555.
- MOLINA R., JIMÉNEZ M.I., CRUZ I., IRISO A., MARTÍN-MARTÍN I., SEVILLANO O., MELERO S. & BERNAL J. (2012). The hare (*Lepus granatensis*) as potential sylvatic reservoir of *Leishmania infantum* in Spain. *Vet. Parasitol.*, **190**, 268–271. doi: 10.1016/j.vetpar.2012.05.006. Epub 2012 May 23.
- MONTALVO A.M., FRAGA J., MAES L., DUJARDIN J-C. & VAN DER AUWERA G. (2012). Three new sensitive and specific heat-shock protein 70 PCRs for global *Leishmania* species identification. *Eur. J. Clin. Microbiol. Infect. Dis.*, **31**, 1453–1461.
- MORENO J. & ALVAR J. (2002). Canine leishmaniasis: epidemiological risk and the experimental model. *Trends Parasitol.*, **18**, 399–405.
- MULLER N., ZIMMERMANN V., FORSTER U., BIENZ M., GOTTSTEIN B. & WELLE M. (2003). PCR-based detection of canine *Leishmania* infections in formalin-fixed and paraffin embedded skin biopsies: elaboration of a protocol for quality assessment of the diagnostic amplification reaction. *Vet. Parasitol.*, **114**, 223–229.
- MURRAY H.W., BERMAN J.D., MARTIN V., DAVIES C.R. & SARAVIA N.G. (2005). Advances in leishmaniasis. *Lancet*, **366**, 1561–1577.
- MYLONAKIS M.E., PAPAIOANNOU N., SARIDOMICHELAKIS M.N., KOUTINAS A.F., CHARALAMBOS B. & VASSILIOS I.K. (2005) Cytologic patterns of lymphadenopathy in dogs infected with *Leishmania infantum*. *Vet. Clin. Pathol.*, **34**, 243–247.
- NZELU C.O., KATO H. & PETERS N.C. (2019). Loop-mediated isothermal amplification (LAMP): An advanced molecular point-of-care technique for the detection of *Leishmania* infection. *PLOS Negl. Trop. Dis.*, **13**, e0007698. <https://doi.org/10.1371/journal.pntd.0007698>

- OLIVA G., NIETO J., FOGLIA MANZILLO V., CAPPIELLO S., FIORENTINO E., DI MUCCIO T., SCALONE A., MORENO J., CHICHARRO C., CARRILLO E., BUTAUD T., GUEGAND L., MARTIN V., CUISINIER A.M., MCGAHIE D., GUEGUEN S., CAÑAVATE C. & GRADONI L. (2014). A randomised, double-blind, controlled efficacy trial of the LiESP/QA-21 vaccine in naïve dogs exposed to two *Leishmania infantum* transmission seasons. *PLoS Negl. Trop. Dis.*, **8**(10):e3213
- OLIVA G., SCALONE A., FOGLIA MANZILLO V., GRAMICCIA M., PAGANO A., DI MUCCIO T. & GRADONI L. (2006). Incidence and time course of *Leishmania infantum* infections examined by parasitological, serologic, and Nested-PCR techniques in a cohort of naïve dogs exposed to three consecutive transmission seasons. *J. Clin. Microbiol.*, **44**, 1318–1322.
- OTRANTO D., PARADIES P., SASANELLI M., LEONE N., de CAPRARIIS D., CHIRICO J., SPINELLI R., CAPELLI G. & BRANDONISIO O. (2005). Recombinant K39 dipstick immunochromatographic test: a new tool for the serodiagnosis of canine leishmaniasis. *J. Vet. Diagn. Invest.*, **17**, 32–37.
- OZBEL Y., OSKAM L., OZENSOY S., TURGAY N., ALKAN M.Z., JAFFE C.L. & OZCEL M.A. (2000). A survey on canine leishmaniasis in western Turkey by parasite, DNA and antibody detection assays. *Acta Trop.*, **74**, 1–6.
- PALATNIK-DE-SOUSA C.B., BARBOSA ADE F., OLIVEIRA S.M., NICO D., BERNARDO R.R., SANTOS W.R., RODRIGUES M.M., SOARES I. & BORJA-CABRERA G.P. (2008). FML vaccine against canine visceral leishmaniasis: from second-generation to synthetic vaccine. *Expert Rev. Vaccines*, **7**, 833–851.
- PALTRINIERI S., SOLANO-GALLEGO L., FONDATI A., LUBAS G., GRADONI L., CASTAGNARO M., CROTTI A., MAROLI M., OLIVA G., ROURA X., ZATELLI A. & ZINI E. (2010). Canine Leishmaniasis Working Group, Italian Society of Veterinarians of Companion Animals. Guidelines for diagnosis and clinical classification of leishmaniasis in dogs. *J. Am. Vet. Med. Assoc.*, **236**, 1184–1191.
- PENNISI M.G., CARDOSO L., BANETH G., BOURDEAU P., KOUTINAS A., MIRÓ G., OLIVA G. & SOLANO-GALLEGO L. (2015). LeishVet update and recommendations on feline leishmaniosis. *Parasit. Vectors*, **8**, 302.
- PIARROUX R., AZAIEZ R., LOSSI A.M., REYNIER P., MUSCATELLI F., GAMBARELLI F., FONTES M., DUMON H. & QUILICI M. (1993). Isolation and characterization of a repetitive DNA sequence for *Leishmania infantum*: development of a visceral leishmaniosis polymerase chain reaction. *Am. J. Trop. Med. Hyg.*, **49**, 364–369.
- REITHINGER R. & DUJARDIN J-C. (2007). Molecular diagnosis of leishmaniasis: current status and future applications. *J. Clin. Microbiol.*, **45**, 21–25.
- REITHINGER R., QUINNELL R.J., ALEXANDER B. & DAVIES C.R. (2002). Rapid detection of *Leishmania infantum* infection in dogs: comparative study using an immunochromatographic dipstick test, enzyme-linked immunosorbent assay, and PCR. *J. Clin. Microbiol.*, **40**, 2352–2356.
- RICHTER M., SCHAARSCHMIDT-KIENER D., KRUDEWIG C. (2014). Ocular signs, diagnosis and long-term treatment with allopurinol in a cat with leishmaniasis. *Schweiz. Arch. Tierheilkd.*, **156**, 289-294
- RIOUX J.A., LANOTTE G., SERRES E., PRATLONG F., BASTIEN P. & PERIERES J. (1990). Taxonomy of *Leishmania*, use of isoenzymes. Suggestions for a new classification. *Ann. Parasitol. Hum. Comp.* **65**, 111–125.
- ROURA X., FONDEVILA D., SANCHEZ A. & FERRER L. (1999a). Detection of *Leishmania* infection in paraffin-embedded skin biopsies of dogs using polymerase chain reaction. *J. Vet. Diagn. Invest.*, **11**, 385–387.
- ROURA X., SANCHEZ A. & FERRER L. (1999b). Diagnosis of canine leishmaniasis by a polymerase chain reaction technique. *Vet. Rec.*, **144**, 262–264.
- RÜFENACHT S., SAGER H., MÜLLER N., SCHAEERER V., HEIER A., WELLE M.M. & ROOSJE P.J. (2005). Two cases of feline leishmaniosis in Switzerland. *Vet. Rec.*, **156**, 542–545.
- SANTARÉM N., CUNHA J., SILVESTRE R., SILVA C., MOREIRA D., OUELLETTE M. & CORDEIRO-DA-SILVA A. (2014). The impact of distinct culture media in *Leishmania infantum* biology and infectivity. *Parasitology*, **141**, 192–205.
- SARIDOMICHELAKIS M.N., MYLONAKIS M.E., LEONTIDES L.S., KOUTINAS A.F., BILLINIS C. & KONTOS V.I. (2005). Evaluation of lymph node and bone marrow cytology in the diagnosis of canine leishmaniasis (*Leishmania infantum*) in symptomatic and asymptomatic dogs. *Am. J. Trop. Med. Hyg.*, **73**, 82–86.
- SCALONE A., DE LUNA R., OLIVA G., BALDI L., SATTÀ G., VESCO G., MIGNONE W., TURILLI C., MONDESIRE R.R., SIMPSON D., DONOGHUE A.R., FRANK G.R. & GRADONI L. (2002). Evaluation of the *Leishmania* recombinant K39 antigen as a

diagnostic marker for canine leishmaniosis and validation of a standardized enzyme-linked immunosorbent assay. *Vet. Parasitol.*, **104**, 275–285.

SCHÖNIAN G., MAURICIO I., GRAMICCIA M., CAÑAVATE C., BOELAERT M. & DUJARDIN J-C. (2008). Leishmaniasis in the Mediterranean in the era of molecular epidemiology. *Trends Parasitol.* **24**, 135–142.

SHUIKINA E.E., SERGIEV V.P., TRIERS I.I., SHCHERBAKOV V.A. & DIVEEV S.KH. (1968). Experience of antileishmaniosis vaccination with cultures of *Leishmania tropica major* grown in various types of media. *Med. Parazitol. (Mosk.)*, **37**, 648–651 (in Russian).

SOLANO-GALLEGO L., KOUTINAS A., MIRÓ G., CARDOSO L., PENNISI M.G., FERRER L., BOURDEAU P., OLIVA G. & BANETH G. (2009). Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis. *Vet. Parasitol.*, **165**, 1–18,

SUNDAR S., MAURYA R., SINGH R.K., BHARTI K., CHAKRAVARTY J., PAREKH A., RAI M., KUMAR K. & MURRAY H.W. (2006). Rapid, noninvasive diagnosis of visceral leishmaniosis in India: comparison of two immunochromatographic strip tests for detection of anti-K39 antibody. *J. Clin. Microbiol.*, **44**, 251–253.

TRIGO J., ABBEHUSEN M., NETTO E.M., NAKATANI M., PEDRAL-SAMPAIO G., DE JESUS R.S., GOTO Y., GUDERIAN J., HOWARD R.F. & REED S.G. (2010). Treatment of canine visceral leishmaniosis by the vaccine Leish-111f+MPL-SE. *Vaccine*, **28**, 3333–3340.

TURDAY N., BALCIOGLU I.C., TOZ S.O., OZBEL Y. & JONES S.L. (2010). Quantiferon-Leishmania as an epidemiological tool for evaluating the exposure to *Leishmania* infection. *Am. J. Trop. Med. Hyg.*, **83**, 822–824.

VOLPINI A.C., PASSOS V.M., OLIVEIRA G.C. & ROMANHA A.J. (2004). PCR-RFLP to identify *Leishmania (Viannia) braziliensis* and *L. (Leishmania) amazonensis* causing American cutaneous leishmaniosis. *Acta Trop.*, **90**, 31–37.

WORLD HEALTH ORGANIZATION (WHO) (2010). Control of the leishmaniasis, WHO Technical Report Series 949, WHO, Geneva, Switzerland, 1–186.

\*

\* \*

**NB:** There is an OIE Reference Laboratory for Leishmaniosis  
(please consult the OIE Web site:

<https://www.oie.int/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>)

Please contact the OIE Reference Laboratory for any further information on  
diagnostic tests, reagents and vaccines for leishmaniosis

**NB:** FIRST ADOPTED IN 1991 AS LEISHMANIOSIS. MOST RECENT UPDATES ADOPTED IN 2021.