Reference Point

Guidelines for diagnosis and clinical classification of leishmaniasis in dogs

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In the past decade, several issues have emerged regarding the epidemiology of leishmaniasis in dogs, including the increased incidence of infection in endemic zones, the northern spread of the infection to nonendemic areas of Europe, and the emergence of the disease in North America. Despite the increasing spread of the infection and growing concern for canine health, veterinary approaches to the disease remain heterogeneous. Accordingly, an expert panel, the CLWG, was established in November 2005 in collaboration with the Italian Society of Veterinarians of Companion Animals.

The aim of the CLWG was to develop a scientific-based consensus approach for the management of leishmaniasis in dogs with regard to diagnosis and clinical classification of disease, treatment, and prevention. The guidelines reported here are an updated version of those originally published in Italian in Veterinaria, the official journal of the Italian Society of Veterinarians of Companion Animals, and are based on a thorough review of international literature and, where data are inadequate or incomplete, are supplemented with the experience of CLWG members. These guidelines are intended to assist veterinary practitioners in the diagnosis and clinical assessment of this complex disease.

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ABBREVIATIONS

CLWG Canine Leishmaniasis Working Group
IFAT Indirect immunofluorescent assay test
Th1 T-helper type 1 cell
Th2 T-helper type 2 cell

Leishmaniasis

Leishmaniasis is caused by Leishmania infantum, a parasitic protozoan transmitted by the bite of phlebotomine sandflies. This species was identified early in the 20th century in countries of the Mediterranean basin and was thereafter identified in the Middle East, Central Asia, and China. A species genetically indistinguishable from L. infantum, Leishmania chagasi is the recognized agent of leishmaniasis in the Americas, mainly Brazil and other Latin America countries. Leishmania organisms are members of the Trypanosomatidae family (order Kinetoplastida) and are characterized by the presence of an evident mitochondrial DNA organelle (kinetoplast). The life cycle of Leishmania has long been elucidated. Flagellated forms (promastigotes) multiply in the gut of blood-sucking female phlebotomine sandflies. After the blood meal digestion is completed, the parasites migrate to the foregut, transform into nonreplicative infectious promastigotes (metacyclogenesis), and are deposited into the host’s dermis through the subsequent bite of the insect. Dermal macrophages actively phagocytize promastigotes, which in the phagolysosomal compartment lose the flagellum and become round-shaped (amastigotes). Amastigotes actively replicate in macrophages, leading to cell destruction and progressively infecting more phagocytes.

In endemic areas, transmission of Leishmania spp occurs focally so that broad variations in the prevalence of infection may be apparent in contiguous territories, depending mainly on the relative vector densities. For instance, seroprevalences vary from 2% to 40% in Mediterranean leishmaniasis endemic regions, although findings of studies regarding Leishmania-specific, cell-mediated immunity suggest that the exposure rate of dogs to the parasite is probably much higher. Vectors of leishmaniasis are members of the genus Phlebotomus in the Old World and the genus Lutzomyia in the New World. In endemic settings of the Mediterranean basin, the principal vectors of
Leishmania spp are species belonging to the Larroussius subgenus (eg, Phlebotomus perniciosus, Phlebotomus ariasi, Phlebotomus perfiliewi, Phlebotomus neglectus, and Phlebotomus tobbi), which are characterized by crepuscular and nocturnal activity extending from late spring to late autumn. The main New World vector is Lutzomyia longipalpis, which is active during the entire year. However, dog-to-dog mechanisms of Leishmania transmission have been recently hypothesized to explain leishmaniasis outbreaks among foxhounds in the United States and Canada.15

Leishmania spp have 3 general pathogenic features. First, the parasite's target cell is the macrophage, which is inhibited in terms of antimicrobial activities and becomes the site for parasite replication. Second, establishment of infection and evolution of the disease both depend on the host's immunologic responses. Third, once established, the infection usually persists in tissues. Leishmania tends to localize in all tissues in which mononuclear-macrophagic cells exist in high numbers. Direct diagnostic methods can reveal the presence of Leishmania organisms a few weeks after infection. A proportion of infected dogs may have negative results with some diagnostic tests shortly after a positive finding, even without drug treatment. It is unknown whether such dogs became free of infection, whether the infection lessened to an undetectable degree, or whether the parasite localized in tissues other than those examined at the initial diagnosis.16

In susceptible mammals, L. infantum causes infections that are usually chronic and are sometimes subclinical and evolve toward overt clinical disease. Immune responses play a pivotal role in tipping the balance from infection to disease because of T-helper CD4+ lymphocytes, which can induce the immune system to shift toward a humoral (Th2) or cell-mediated (Th1) response. In infected dogs, there are 2 extremes of clinical expression: healthy dogs, characterized by mild or no Th2 response and the presence of a robust Th1-specific response against Leishmania, and severely sick dogs, characterized by an exaggerated Th2 response and an absent or mild Th1 response.17

Resistance to disease in dogs appears to be associated with a Th1-Th2 mixed-type immune response, with the predominance of Th1 cytokines, whereas susceptibility to disease appears to be related to reduced production of cytokines, mainly of the Th2 type.1,17–23

In these situations, continuous antigenic stimulation and excess antibody production cause chronic hypergammaglobulinemia, resulting in formation and deposition of immune complexes that may cause glomerulonephritis, vasculitis, polyarthritis, uveitis, and meningitis, in addition to the production of autoantibodies against platelets and RBCs.1 Moreover, sick or subclinically infected dogs with a pronounced humoral response are characterized by parasite dissemination in the body, a low number of CD4+ T-helper cells, and immunosuppression.1,23 The immunopathologic background of each infected dog is strictly linked with the fate of the disease, as the number of CD4+ T-helper cells appears to be inversely related and the seropositivity rate directly related to infectivity of Leishmania organisms.22–24

Diagnostic Methods

Diagnosis of leishmaniasis in dogs should be based on an integrated approach considering signalment, history, clinical findings, and results of basic laboratory analyses that detect the parasite or evaluate the immune response of the host.

Signalment and history—Any canine breed can be affected by leishmaniasis, although some breeds (eg, German Shepherd Dog and Boxer) appear to be more predisposed to overt disease than others.22–26 Furthermore, a sex predilection has been noted: females reportedly have a higher risk than males of acquiring leishmaniasis,27,28 as has been reported for humans29 and Syrian hamsters.30 Dogs can be infected at any age, but the prevalence of infection within certain age groups has a bimodal distribution, with a first peak in dogs <3 years of age and a second in dogs from 8 to 10 years of age.1 Because disease distribution varies widely throughout the world, it is essential to find out whether a dog lives in or has traveled to a known endemic area and hence has been potentially exposed to phlebotomine vectors. It is also important to find out whether the dog received preventive treatments that are potentially effective against sandflies or whether treatments were administered that may interfere with immune system efficiency. History taking should include collection of information regarding any signs detected by the owner that are consistent with leishmaniasis, such as weight loss, cutaneous changes, polyuria-polydipsia, and epistaxis.

Physical examination and diagnostic imaging—The most common clinical signs of leishmaniasis in dogs are lymph node enlargement and skin lesions. However, a broad and heterogeneous spectrum of clinical signs and lesions can be detected during physical examination (Table 1).31–34 Some of these changes (eg, hepatosplenomegaly) can be more efficiently detected via diagnostic imaging. Using information on signalment and history, practitioners can decide whether to include leishmaniasis in the list of differential diagnoses; when included, it is always advisable to perform appropriate laboratory tests.

Basic laboratory tests—Basic tests include a CBC, serum biochemical analysis, serum protein electrophoresis, and urinalysis. In leishmaniasis, these tests allow the detection of 1 or more of the changes associated with the disease.31–34 On the basis of the results of these basic tests, additional laboratory assays can be performed (Table 2).32–37

Specific laboratory tests (etologic diagnosis)—To identify the parasite or a patient's response to it, various integrated methods of etiologic diagnosis are available.38 Indeed, finding Leishmania organisms in target tissues such as bone marrow or lymph nodes may not be always indicative of established infection and accordingly does not permit one to conclude that the observed clinical signs are caused by infection with the parasite. However, detection of parasites in tissues that bear lesions consistent with leishmaniasis is good evidence that the parasite caused the lesions. Although most tests used to obtain an etiologic diagnosis should
be performed at reference diagnostic laboratories, the simplest ones (eg, microscopy of stained smears from enlarged lymph nodes or from other damaged tissues or rapid immunochromatographic assay) can be performed in the clinic. Detailed instructions about sample collection, handling, storage, and shipping should be obtained from reference laboratories.

Diagnostic methods are grouped into 2 main categories: direct (cytologic evaluation, histologic evaluation, organism culture, PCR assay, and xenodiagnosis) and indirect (serologic testing and evaluation of cellular immune response).

**Cytologic evaluation**

Cytologic evaluation permits microscopic detection of *Leishmania* amastigotes in macrophages within affected tissues. In smear preparations, heavily infected cells may burst so that parasites can be found extracellularly. Microscopic evaluation of biopsy specimens or aspirate smears may also permit evaluation of cytologic changes consistent with leishmaniasis, such as lymphoplasmacytic or granulomatous-pyogranulomatous inflammation, lymph node reactive hyperplasia, myeloid hyperplasia, or erythroid hypoplasia. Cytologic examinations should involve fine-needle aspiration of the following tissues or lesions: papular, nodular, and ulcerative skin lesions (ulcerative lesions can also be evaluated via impression smears); bone marrow and lymph nodes when clinical signs (eg, anemia and lymphadenopathy, respectively) suggest their involvement; and any biological fluids that can be obtained from affected sites (eg, synovial fluid when arthritis or polyarthritis is present or CSF when neurologic signs are present).

In the absence of clinical signs that can be attributed to or that involve a particular organ or tissue, samples should be obtained from tissues in which parasites are more likely to be detected, such as bone marrow, lymph node, spleen, and buccal coat from peripherally obtained blood, in descending order of diagnostic sensitivity.

The material sampled for cytologic evaluation can be stored and, in the situation of negative findings, can be sent to reference laboratories for molecular diagnostic tests (eg, PCR assay for detection of *Leishmania* DNA).

**Histologic evaluation**

Parasites from infected dogs can be detected in tissue sections obtained from lesions and prepared with routine H&E staining, although morphological identification of *Leishmania* organisms is more challenging than with cytologic smears. As for cytologic detection of infection, pathological changes consistent with leishmaniasis in dogs can also be detected in histologic preparations, including lymphoplasmacytic or granulomatous-pyogranulomatous inflammations or vasculitis in several organs, ischemic skin disease, lymphoplasmacytic dermatis of dermoepithelial junctions, or lymphoid hyperplasia of spleen and lymph nodes. When leishmaniasis is strongly suspected despite negative results of cytologic testing, histologic examination is always advisable, particularly when dermatis or skin disease characterized by focal lesions is present. Moreover, in the event that routine histologic evaluation reveals the aforementioned microscopic changes but not parasites, immunohistochemical staining can be performed by use of antibodies against *Leishmania* antigens, with some limitations because of serologically cross-reacting agents. If this approach also yields negative results, tissue sections can be further examined by use of molecular assays.

**Organism culture**

Culture of *Leishmania* organisms is the most specific assay because of the development of viable promastigotes, a flagellated stage of the life cycle that is exclusive to *Leishmania* spp. However, the blood agar–based media needed for diagnostic cultures is not available commercially, and thus such cultures are performed only at specialized laboratories. Commercial liquid media such as RPMI or Schneider *Drosophila* medium, although useful in the maintenance of culture-adapted promastigotes, are poorly effective for parasite isolation. Promastigote culture also has the disadvantage of requiring a long period (up to 30 days) before results are obtained.

**PCR assay**

Polymerase chain reaction techniques allow amplification of specific sequences of the *Leishmania* ge-
The method is very sensitive, particularly when multicopy DNA sequences, such as small subunit rRNA genes or kinetoplast DNA minicircles, are targeted for amplification. Thus, it is possible to detect very low amounts of protozoan DNA in biological materials. The 3 most commonly used techniques are conventional or traditional PCR assay, nested PCR assay, and quantitative PCR assay. In a conventional PCR assay, Leishmania DNA is amplified by use of specific primers (short sequences complementary to target Leishmania DNA sequences). In a nested PCR assay, which is a modification of the traditional PCR assay, 2 consecutive PCR assays with 1 or 2 internal primers are performed. This technique is more sensitive than the conventional method but is prone to lower specificity because more laboratory steps are used, increasing the risk of foreign DNA contamination. In a quantitative PCR assay (eg, real-time PCR assay), fluorescent probes are used to quantify the number of Leishmania DNA copies present in a biological sample, starting from a predefined DNA standard. Depending on the targeted sequence, the quantitative test can be as sensitive as a nested PCR assay, but because it is performed in a closed system, the quantitative assay is less prone to false-positive results. According to published findings, the quantitative PCR assay is also useful in monitoring the effectiveness of treatment. Therefore, real-time PCR assays may be considered a first-line diagnostic approach when offered by a reference laboratory.

Molecular techniques can be easily applied to several biological samples. In addition to specifically injured tissues, samples with the highest chance of containing leishmanial DNA include, in descending order of sensitivity, bone marrow or lymph nodes, skin, conjunctiva, buffy coat, and whole peripherally obtained blood. However, in dogs with efficient immune responses against Leishmania, infection may not be followed by parasite dissemination; hence, in endemic geographic regions, positive PCR assay results for skin samples when skin lesions are not apparent are not nec-

![Table 2—Results of basic and advanced laboratory tests consistent with a diagnosis of leishmaniasis in dogs.](image-url)
necessarily associated with established infection or disease development. Similarly, positive PCR assay results for bone marrow samples obtained during or shortly after a dog’s exposure to the organism may be followed by restoration of negative values.

For PCR analyses, the material of choice is fresh or frozen samples or samples after fixation in 95% ethanol alcohol. Formalin-fixed, paraffin-embedded samples are poorer diagnostic specimens. As mentioned, it is advisable to request a PCR test when cytologic and histologic evaluations yield negative results despite strong clinical evidence of leishmaniasis.

**Xenodiagnosis**

Xenodiagnosis consists of allowing laboratory-bred phlebotomine vectors to feed on a dog suspected of having leishmaniasis, which is sedated and put into a holding cage. The flies are then examined after blood digestion for the presence of promastigotes in the gut. The method is highly specific and sensitive, and indeed, the use of competent vectors can be regarded as the most efficient culture medium available even though it is not applicable for routine clinical practice.

**SEROLOGIC METHODS**

Seroconversion may occur months following infection. For natural infection, the interval to seroconversion can range from 1 to 22 months (median, 5 months), and for experimental infection, it ranges from 1 to 6 months (median, 3 months). Only in dogs in which the parasite has disseminated do antibody titers tend to be high or increase with time. Several serologic techniques are available. Some (eg, western blotting) yield a high diagnostic accuracy but are of limited use because of poor standardization or high cost. The most commonly available serologic methods include the IFAT, ELISA, and rapid immunochromatographic strip test.

An IFAT is performed by placing serial serum dilutions onto slides coated with *Leishmania* promastigotes. Specific antibody binding and relative concentration (antibody titer) is revealed by use of fluorescent anti antibodies. Evaluation of fluorescence intensity by microscopy is prone to subjective interpretation, which is a limit of the assay. Because the test’s sensitivity and specificity are high in several endemic settings, the IFAT is recommended by the World Organization for Animal Health (OIE) as the reference serologic method. Although antibody titers are not always associated with severity of clinical signs, they are useful to distinguish subclinically infected dogs, which usually have low titers, from those with parasite dissemination and disease, which usually have high titers. The terms low titer and high titer should be considered with caution and should always be compared with the threshold positive value indicated by the reference laboratory. Furthermore, sequential samples should be examined by the same laboratory by use of the same assay when findings need comparative evaluation (eg, pretreatment vs posttreatment samples). There is no consensus among laboratories on the IFAT threshold titer, which may vary from 1:40 to 1:320. Considering the broad variations in intertest and intratest agreement that characterize serologic studies, it is reasonable to consider as high titer only those values that are 2- to 4-fold higher than the threshold positive value indicated by the reference laboratory and as low titer any other titer equal to 1- to 2-fold higher than the threshold positive value indicated by the reference laboratory. The assay specificity may be affected by serologically cross-reacting infections or, more rarely, metabolic disorders. Antibodies against *Trypanosoma cruzi* have long been recognized as the main cause of cross-reactivity in IFATs to detect *Leishmania* infection in areas endemic for both diseases, namely in Central and South America, and in some parts of the southern United States. Improvements in fluorescence detection systems may help to discriminate heterologous from homologous reactions; however, methods proposed are impractical for routine diagnosis. Serologic methods other than IFAT and recombinant *Leishmania*-specific antigens should be used as an alternative in routine situations.

Although some commercial kits are available, most information on the diagnostic performance of the ELISA comes from in-house manufactured assays. Diluted sera are placed in *Leishmania* antigen-coated microplates. When a result is seropositive, a colorimetric reaction appears that can be quantified by spectrophotometry and therefore does not involve subjective evaluation. The ELISA is a specific test with a medium-high sensitivity that increases when multiple antigens are used. Furthermore, the test allows quantification of specific antibody titers.

Several commercial kits are available to perform rapid immunochromatographic strip tests, and each kit differs in the antigen and reagents used. These tests are available in easy-to-use formats that can be used in-clinic, although their diagnostic performance is usually lower than that of the ELISA or IFAT. Although they possess medium-high specificity, sensitivity can be in the low range of 30% to 70%. Hence, the main problem is the occurrence of false-negative results. When the clinical suspicion of leishmaniasis persists despite a serologic negative result, serologic testing must be repeated with more sensitive methods. Furthermore, positive findings do not indicate the titer of specific antibodies. An advantage of immunochromatographic strip tests that involve recombinant *Leishmania*-specific antigens is that they can discriminate serologic reactions to *Leishmania* from those to *T cruzi*.

**EVALUATION OF CELLULAR IMMUNE RESPONSE**

Because resistance or susceptibility to *Leishmania* infection is mediated by cellular immune responses, their evaluation is largely used in scientific research on the immunology and immunopathology of leishmaniasis in dogs. However, most of the tools for evaluation of cellular immune responses are still unavailable for clinical practice and, to date, no reports about the practical usefulness of measuring the cellular immune response in clinical practice have been published. Nevertheless, information on cell-mediated immunologic conditions can be obtained from some tests that are feasible to perform, such as intradermal administration of leishmanin antigen (whole or disrupted promastigotes) or flow cytometric determination of the CD4+ to CD8 ratio in T lymphocytes from peripherally obtained blood samples.
Integration of Diagnostic Methods

As a general rule, leishmaniasis in dogs can be quickly and efficiently confirmed by cytologic, serologic, or PCR analyses in dogs with overt clinical signs or severe alterations of relevant clinicopathologic variables. However, this is not the situation for most dogs living in endemic areas that undergo periodic medical visits and often have early vague and nonspecific signs. The main problem is the demonstration of a cause-effect relationship between the direct or indirect test results and the observed alterations. Without such confirmation, there is a risk of concluding that the disease is present when it may not be. In dogs with a history and clinical signs suggestive of leishmaniasis, the first-line diagnostic approaches should include cytologic analysis of injured tissues and specific serologic assays. Depending on the results, more accurate and specific tests can be performed. Possible combinations of findings and their interpretation can be summarized (Figure 1).

Cytologic findings—When injured tissues (including bone marrow when anemia is detected) contain cytologic evidence of *Leishmania* infection, dogs should be considered as affected by leishmaniasis, regardless of serologic results. It should be pointed out, however, that these dogs usually have high titers of anti-*Leishmania* antibodies, except for sporadic cases of highly localized lesions or rare situations of early infection in which manifestation of clinical signs precedes specific antibody responses.

When tissues do not have cytologic evidence of *Leishmania* infection, serologic testing becomes crucial to decide whether a dog is sick because of leishmaniasis. A high antibody titer will confirm the disease is present. On the contrary, a low antibody titer generally does not suggest the dog is diseased because of leishmaniasis; hence, the dog could be affected by a different disorder that shares similar signs. In dogs with a low antibody titer, other diagnostic procedures, chosen on the basis of the clinical signs, should be followed.

For skin lesions in dogs with suspected leishmaniasis, particularly those in which the cytologic pattern is consistent with leishmaniasis, collection of a cutaneous biopsy specimen is recommended to histologically determine whether *Leishmania* spp are present. Immunohistochemical analysis is also suggested when the histologic findings are consistent with leishmaniasis but routine stain does not reveal parasites. When this test also yields a negative result, a PCR assay for detection of *Leishmania* DNA in a skin biopsy specimen should be performed as a confirmatory test.

When noncutaneous lesions (eg, systemic signs) exist that are highly suggestive of leishmaniasis, a PCR assay must be performed on tissues such as bone marrow or lymph nodes because the PCR test provides a good chance to detect the DNA of the invading parasite. When molecular assays yield negative results, dogs with negative cytologic findings and low antibody titers against *Leishmania* spp should be considered as having had previous exposure to the parasite and being affected by a different disease. If molecular assays yield positive results, these dogs should be considered infected but with clinical signs attributable to another disease.

**Approach to Clinical Classification**

*Leishmania infantum* infection may develop over a period of a few weeks to several months toward clinical conditions that can be highly variable, and therefore, it is not always easy to classify dogs within specific categories. Nevertheless, when leishmaniasis is diagnosed, veterinarians should be able to ascribe the infection or disease to a parasitological and clinical stage to determine adequate treatment or to predict progression toward more serious and irreversible stages. Here, the CLWG proposes to classify dogs with positive results of serologic tests or in which the parasite has been identified via direct diagnostic methods in 4 clinical stages: A, exposed dogs; B, infected dogs; C, sick dogs (dogs with clinically evident leishmaniasis); and D, severely sick dogs. The proposed classification system should not be considered as a rigid and schematic outline for such a complex disease but as a useful tool for management of affected dogs.

**Stage A, exposed dogs**—Dogs with negative cytologic, histologic, parasitological, and molecular diagnostic findings as well as low-titer anti-*Leishmania* antibodies are included in this category. Dogs are clinically normal or have signs associated with other diseases. Usually, dogs exposed to *L. infantum* infection are those living or that have lived during 1 or more transmission seasons in a geographic region in which the presence of *Leishmania* vectors has been confirmed.

**Stage B, infected dogs**—This category includes dogs in which the presence of parasites was confirmed through direct (eg, positive results of microscopic analysis, organism culture, or PCR assay) methods and which have low-titer anti-*Leishmania* antibodies. Such dogs can be healthy or can have clinical or pathological signs associated with other diseases. In endemic areas, a positive finding of PCR assays of skin or peripheral obtained blood in the absence of lesions and obtained during the infection transmission period may not be sufficient to consider a dog infected.
Stage C, sick dogs (dogs with clinically evident leishmaniasis)—This category comprises dogs with positive cytologic results regardless of serologic tests, dogs with high-titer anti-Leishmania antibodies, and rarely, infected dogs. One or more clinical signs common to leishmaniasis are present (Table 1). Given the multifaceted manifestations of the disease, the signs indicative of disease can be different from those listed, as long as they can be clearly associated with the ongoing infection. In the absence of detectable signs at physical examination, such a dog should still be considered sick when it has hematologic, biochemical, and urinary alterations suggestive of leishmaniasis (Table 2). As indicated previously, laboratory changes not listed in this report can be indicative of disease, provided they are clearly associated with the infection.

Stage D, severely sick dogs—Sick dogs with a severe clinical condition are included in this category, as indicated by 1 of the following: evidence of proteinuric nephropathy or chronic renal failure; concurrent problems, such as ocular disease causing functional loss or severe joint disease impairing mobility, which are related or unrelated to leishmaniasis and that require immunosuppressive treatment; presence of concomitant conditions including various coinfections or neoplastic, endocrine, or metabolic diseases; and clinical unresponsiveness to repeated courses of anti-Leishmania drugs.

References

Correction: Multicenter veterinary practice assessment of the effects of omega-3 fatty acids on osteoarthritis in dogs

In the article “Multicenter veterinary practice assessment of the effects of omega-3 fatty acids on osteoarthritis in dogs” (J Am Vet Med Assoc 2010;236:59–66), the units for serum concentrations of omega-6 and omega-3 fatty acids in Table 3 are incorrect. Concentrations were measured in units of mg/dL, not mg/mL.