LABORATORY TESTS FOR DIAGNOSING AND MONITORING CANINE LEISHMANIASIS

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Abstract: Although several reviews on canine leishmaniasis have been published, none thoroughly described clinicopathologic abnormalities and their clinical usefulness. The aim of this review was to provide information concerning current diagnostic tests relevant for clinical pathologists and from a practical perspective. Specifically, in canine leishmaniasis, non-regenerative normocytic normochromic anemia, thrombocytopenia, or leukogram changes may be present. Clinical chemistry and urinalysis may indicate renal dysfunction (azotemia, decreased urine specific gravity, proteinuria) and an inflammatory/immune response (increased acute phase proteins [APP] or α2- and/or γ-globulins). Although a potential gammopathy is usually polyclonal, it may also appear oligo- or monoclonal, especially in dogs coinfected by other vector-borne pathogens. When lesions are accessible to fine-needle aspiration (lymphoadenomegaly, nodular lesions, joint swelling), cytology is strongly advised, as the presence of Leishmania amastigotes in a pattern of pyogranulomatous inflammation or lymphoplasmacytic hyperplasia is diagnostic. If the cytologic pattern is inconclusive, the parasite should be identified by histology/immunohistochemistry or PCR on surgical biopsies. Alternatively, cytology and PCR may be performed on bone marrow samples where amastigotes, along with erythroid hypoplasia, myeloid hyperplasia, plasmacytosis, or secondary dysmyelopoiesis can be observed. Dogs with overt leishmaniasis generally have high antibody titers, while low titers predominate in immunologically resistant infected dogs or in exposed dogs with no parasite confirmation. Quantitative serology is recommended in clinically suspect dogs as high-titer antibodies titers may confirm the clinical diagnosis. In confirmed and treated dogs, renal function and inflammatory/immune response variables should be periodically monitored.

Contents
Introduction
Etiology and pathogenesis of canine leishmaniasis
Clinical signs of canine leishmaniasis
Laboratory abnormalities that may support or confirm leishmaniasis
Hematologic abnormalities
Immunophenotyping of lymphocytes

(continued)
**Introduction**

Leishmaniasis is a frequent infectious disease of dogs living in endemic areas, associated with important morbidity and, despite appropriate treatment, potential lethal outcome. Although several reviews have been published so far, none has fully described the diagnostic role of available laboratory tests that may be diagnostic or of value for monitoring dogs with leishmaniasis. Therefore, the aim of the present review was to provide information concerning typical laboratory abnormalities and current diagnostic tests that may be relevant for clinical pathologists, from a practical perspective.

**Etiology and Pathogenesis of Canine Leishmaniasis**

Canine leishmaniasis is caused by the protozoan parasite *Leishmania infantum* or its New World synonym *Leishmania chagasi*. Although nonvectorial transmission has been reported (e.g., transplacental, transfusional, or venereal)\(^2\)\(^-\)\(^4\), the parasite is usually transmitted by infected phlebotomine sand flies. Therefore, the geographic distribution and prevalence of the disease depend on the presence and abundance of competent vectors.\(^5\) Blood-sucking females ingest the nonflagellated form (amastigote) during the blood-meal on infected hosts. After multiplication, flagellated forms (promastigotes) transform into infectious metacyclic promastigotes that are inoculated into the host at the next blood meal. Parasites are phagocytosed by macrophages\(^6\); however, the amastigotes are resistant to phagolysosomal digestion due to interference with the oxidative activity of these cells\(^7\)\(^,\)\(^8\) and survive and replicate in macrophages. As a result, more and more macrophages become progressively infected and destroyed.

In longitudinal field studies on naïve dogs, *Leishmania* can be detected by PCR in bone marrow starting about 6 months from natural exposure to vectors.\(^9\) Once bone marrow has been colonized, it is generally accepted that the dog is persistently infected. However, a fraction of dogs with positive bone marrow PCR may become negative in the following months without any treatment; it is unknown whether in these dogs the parasite density falls below the threshold limit of the PCR test, amastigotes persist in other organs, or the infection is actually eradicated by host defense mechanisms.\(^7\) While most dogs develop an antibody response shortly after the first contact with the leishmania parasite, resistance or susceptibility to progressive infection depends on the balance between T helper 1 (Th1) (cell-mediated) and T helper 2 (Th2) (humoral) immune responses. Specifically, dogs with a predominant Th2 response are likely prone to parasite dissemination to multiple tissues and overt clinical signs, while dogs with a predominant Th1 response may keep the parasite in check and be clinically healthy.\(^10\)\(^-\)\(^13\) Hence, the presence of circulating antibodies does not necessarily imply that the dog is suffering clinical leishmaniasis, and the presence of amastigotes in tissue can be noted in clinically healthy dogs. Therefore, the guidelines for diagnosis and staging of canine leishmaniasis, released by the Canine Leishmaniasis Working Group (CLWG)\(^14\), suggest a combination of clinical and laboratory criteria for the classification of dogs into exposed, infected, or actually sick animals:

1) Exposed dogs: dogs that are clinically unremarkable, have a low-titer positive serology, and are negative by PCR and/or cytology.

2) Infected dogs: dogs that are clinically unremarkable, with normal hematology and clinical chemistry variables, but positive PCR and/or cytology in bone marrow, lymph node, spleen, skin, or peripheral blood.

3) Sick dogs: infected dogs with typical clinical or clinicopathologic changes.

4) Severely sick dogs: sick dogs with a severe clinical condition such as proteinuric nephropathy, chronic renal failure, and with concurrent problems that may or may not be related to leishmaniasis, such as ocular disease causing blindness and severe joint
disease impairing motility, and which require immunosuppressive treatment. These dogs include also animals with potential concomitant conditions such as coinfections or neoplastic, endocrine, or metabolic diseases, or those that are unresponsive to repeated courses of antileishmanial treatments.

Alternative guidelines released by the expert group known as Leishvet classify sick dogs in 4 different stages according to the severity of clinical signs, clinicopathologic findings, and serologic status.15

**Clinical Signs of Canine Leishmaniasis**

The interpretation of clinicopathologic, serologic, and molecular tests should take into account the history (eg, exposure to phlebotomine vectors), signalment (male dogs > 2 years are at high risk), and clinical presentation. The latter is characterized by a wide spectrum of clinical presentations, ranging from infections characterized by the absence of overt clinical findings in the presence of obvious laboratory abnormalities, to more or less marked clinical and laboratory abnormalities that may require hospitalization, especially in cases with severe life-threatening complications.14–26

**Laboratory Abnormalities that may Support or Confirm Leishmaniasis**

In addition to some typical clinical findings, laboratory abnormalities uncovered by routine hematology, clinical chemistry, or urinalysis may support the clinical suspicion of canine leishmaniasis. Moreover, especially in the early phases of leishmaniasis, some laboratory changes may arise suspicion in the absence of obvious abnormal findings at physical examination. The basic mandatory panel of tests and their significance for the classification of clinically suspect dogs or dogs with positive PCR and/or cytology indicating presence of amastigotes are summarized in Table 1.

**Hematologic abnormalities**

Hematologic changes in canine leishmaniasis are non-specific.27 Neutrophilia, due to a systemic inflammatory response, may be present and particularly prominent in cases with ulcerative cutaneous lesions and secondary bacterial infection.27,28 Conversely, quantitative or qualitative morphologic changes in the other leukocyte populations are less common, although lymphopenia, lymphocytosis, or eosinophilia are occasionally described28–30 Amastigotes are rarely documented in circulating neutrophils, lymphocytes, and monocytes of infected dogs (< 0.5% of cases).29,31 As the percentage of infected cells is so low, a microscopic search in peripheral blood smears is generally not rewarding. Rather, in cases of systemic disease and suspected peripheral blood dissemination, the test of choice is PCR/quantitative PCR as it is more sensitive (see below).

The most common hematologic change in canine leishmaniasis is a mild to moderate normocytic normochromic anemia, an anemia of chronic disease.27,28,32,33 However, the pathogenesis of anemia in leishmaniotic dogs includes additional mechanisms such as reduced erythropoietin synthesis due to renal failure. Moreover, it is very likely that the anemia also has a hemolytic component as suggested by a positive Coombs test in a minority of cases.34 The latter may be associated with a “lupus-like” reaction along with other clinical or laboratory changes, such as a positive anti-nuclear antibody (ANA) test35 or the presence of perinuclear anti-neutrophil cytoplasmic autoantibodies.36

Mild to moderate thrombocytopenia is fairly frequent in leishmaniotic dogs without concurrent infections. In cases with marked thrombocytopenia, co-infection with other vector-borne pathogens (eg, *Ehrlichia canis*, *Anaplasma phagocytophilum*, or *A platys*) or other possible causes of reduced platelet concentration should be suspected. The most likely mechanism responsible for thrombocytopenia in leishmaniasis is an immune-mediated peripheral destruction of circulating platelets, as anti-platelet antibodies have been demonstrated in leishmaniotic dogs.33,37,38

In addition, platelet loss may be associated with hypercoagulability caused by a decreased concentration of antithrombin III due to protein-losing nephropathy39 (see below). Disseminated intravascular coagulation (DIC) has been occasionally reported in leishmaniotic dogs.40 However, thrombocytopenia in leishmaniotic dogs can also be due to suppressed platelet production in the bone marrow. Finally, reduced platelet function has been described in dogs with normal platelet concentrations in canine leishmaniasis41, although this reduced function is rarely responsible for hemostatic abnormalities.

**Immunophenotyping of lymphocytes**

Flow-cytometric determination of the CD4/CD8 lymphocyte ratio in peripheral blood of dogs infected with *Leishmania* sp. has been evaluated with the rationale that a decreasing Th1 response and consequent
increased susceptibility to overt clinical leishmaniasis is accompanied by a lower CD4/CD8 ratio due to decreased CD4+ lymphocyte numbers. Therefore, a seropositive or PCR-positive dog with a low CD4/CD8 ratio is expected to be more predisposed to develop clinical signs than a similar dog with a CD4/CD8 ratio in the reference range. However, due to the high individual variability, the definition of a cutoff for proper clinical staging is difficult. Hence, the CD4/CD8 ratio may be more suitable for monitoring the post-treatment follow-up rather than initial staging of dogs suspected to have leishmaniasis.

**Bone marrow evaluation**

The hematologic profile of leishmaniotic dogs may be complemented by bone marrow cytology, which may be highly diagnostic just by the microscopic identification of amastigotes in macrophages. In addition, the quantitative assessment of the frequency of infected macrophages in a bone marrow smear may allow differentiation between an infected and a sick dog, as the parasite load and the magnitude of cytologic alterations are generally more prominent in dogs with clinical signs, although some histologic studies demonstrated that parasite density can be high despite few clinical signs. Therefore, rare infected macrophages may occasionally be seen in the absence of other pathologic or clinical findings in infected dogs, whereas sick dogs are expected to have higher numbers of parasites and abnormal bone marrow findings. The latter would include erythroid hypoplasia with normal proportions of proliferating and maturing pools, as well as occasional dysgranulopoiesis.

**Table 1. Summary of typical, frequent, and occasional laboratory findings in canine leishmaniasis.**

<table>
<thead>
<tr>
<th>Typical Abnormalities</th>
<th>Frequent Abnormalities</th>
<th>Occasional Abnormalities</th>
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<tbody>
<tr>
<td><strong>Routine CBC—leukogram</strong></td>
<td>Neutrophilia27,28</td>
<td>Lymphopenia; Lymphocytosis; Eosinophilia28–30</td>
</tr>
<tr>
<td><strong>Routine CBC—erythrogram</strong></td>
<td>Normocytic normochromic nonregenerative anemia</td>
<td>Megakaryocyte hyperplasia; Secondary dysmyelopoiesis (dyserthropoiesis or dysmegakaryopoiesis, occasionally dysgranulopoiesis)29,48</td>
</tr>
<tr>
<td><strong>Routine CBC—thrombogram</strong></td>
<td>Erythroid hypoplasia; Myeloid hyperplasia; Macrophage proliferation-hyperplasia; Presence of intracytoplasmic amastigotes; Plasmacytosis17,28,30,32,44–48</td>
<td>Decreased Antithrombin III</td>
</tr>
<tr>
<td><strong>Bone marrow cytology</strong></td>
<td>Erythroid hypoplasia; Myeloid hyperplasia; Macrophage proliferation-hyperplasia; Presence of intracytoplasmic amastigotes; Plasmacytosis17,28,30,32,44–48</td>
<td>Megakaryocyte hyperplasia; Secondary dysmyelopoiesis (dyserthropoiesis or dysmegakaryopoiesis, occasionally dysgranulopoiesis)29,48</td>
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<tr>
<td><strong>Hemostasis</strong></td>
<td></td>
<td>Decreased Antithrombin III</td>
</tr>
<tr>
<td><strong>Routine clinical chemistry</strong></td>
<td>Renal Azotemia14,16,34,65,67–70,78,80</td>
<td>Prolonged PT and APTT40</td>
</tr>
<tr>
<td></td>
<td>Hyperproteinemia with hypoalbuminemia and inverted A:G ratio14,15,65,81,103–105</td>
<td>Hypercoagulability detected by thromboelastography or thromboelastometry49</td>
</tr>
<tr>
<td><strong>Serum protein electrophoresis</strong></td>
<td>Polyclonal gammopathy14,15,65,81,103–105</td>
<td>Oligoclonal gammopathy108</td>
</tr>
<tr>
<td><strong>Acute phase proteins and other markers of inflammation</strong></td>
<td>Increased C-reactive protein, serum amyloid A, haptoglobin, ceruloplasmin, Ferritin; decreases of total iron-binding capacity111–116</td>
<td>Decreased PON-1 and high-density lipoprotein117–119</td>
</tr>
<tr>
<td><strong>Urinalysis</strong></td>
<td>Proteinuria; decreased urine specific gravity14–16,34,81; mixed proteinuria in SDS-electrophoresis94–97</td>
<td>Increased markers of renal tubular damage (γ-glutamyl transferase and N-acetyl-β-N-glucosaminidase)96,100</td>
</tr>
</tbody>
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neutrophils, and a moderate to marked plasmacytosis characterized by a higher number of plasma cells, mott cells, and lymphocytes. Megakaryocyte hyperplasia can also be present, especially when peripheral consumption of platelets occurs.

Albeit less frequent, secondary dysmyelopoiesis can be found and is characterized by peripheral cytopenia (eg, anemia and thrombocytopenia) associated with hypercellular bone marrow displaying signs of asynchrony and dysplastic features (Figure 2). In canine leishmaniasis, these mostly include dyserythropoiesis with abnormal mitoses, asynchronous nucleo-cytoplasmic maturation, nuclear fragmentation, and/or late-stage maturation arrest and dysmegakaryopoiesis with dwarf megakaryocytes and emperipolesis, while dysgranulopoiesis with abnormal

Figure 1. Canine bone marrow smear summarizing the main findings in canine leishmaniasis. May–Grünwald–Giemsa. (A–C) Bar = 15 μm. (D and F) Bar = 20 μm. (E) Bar = 70 μm. (A) Several Leishmania sp. amastigotes in the cytoplasm of infected macrophages. (B, D, F) Free amastigotes in the background (arrows). (C) Infected macrophage with erythrophagia. (D) Infected macrophage with cytophagia. (E) Myeloid hyperplasia, erythroid hypoplasia, and infected macrophages. (F) Marked plasmacytosis, myeloid hyperplasia, and a Mott cell.
maturation of granulocytes and ring forms is rather rare. Therefore, the presence of secondary dysmyelopoiesis is not diagnostic for leishmaniasis except in the presence of amastigotes, and primary dysmyelodysplastic syndromes should be carefully ruled out based on serology and PCR.

**Hemostatic abnormalities**

Hemostatic abnormalities are uncommon in leishmaniotic dogs. Activated partial thromboplastin time and prothrombin time may be prolonged; however in most cases, this is likely due to preanalytic factors such as an increased concentration of total globulins, which is frequent in dogs with leishmaniasis. Alternatively, prolonged coagulation times may result from DIC, although this complication is uncommon in leishmaniotic dogs. Conversely, hypercoagulability may be common in leishmaniotic dogs if affected by severe protein-losing nephropathy. This is mostly due to glomerular loss of ATIII, a protease inhibitor involved in the regulation of blood coagulation that prevents the conversion of fibrinogen into fibrin. The lack of this physiologic anticoagulant may induce hypercoagulability that in turn promotes thrombosis and subsequent consumption coagulopathy. Antithrombin III concentration can be measured by immunoturbidimetic methods, and is particularly recommended in dogs with protein-losing nephropathy.

The hyperviscosity syndrome due to the increased circulating globulins also favors hypercoagulability. Hypercoagulability in leishmaniotic dogs was also demonstrated through a shorter clot formation time and increased global clot strength using

![Figure 2](image_url)

*Figure 2. Canine bone marrow smear with secondary dysmyelopoiesis associated with leishmaniasis, May–Grünwald–Giemsa. (A and B) Bar = 20 μm. (C and D) Bar = 60 μm. (A) Atypical mitotic figure and infected macrophage. (B) Myeloid hyperplasia, plasmacytosis, and atypical mitosis and asynchrony in an erythroid precursor (arrowhead). (C) Dwarf megakaryocytes; (D) Emperipolesis in a megakaryocyte. (A and C) Free amastigotes in the background (arrows).*
thromboelastography (TEG).49 In contrast, the clot formation of leishmaniotic dogs assessed by thromboelastometry (TEM, a technique similar to TEG) was within normal limits in another report.50 It is worth noting that TEM and TEG are affected by the RBC mass51,52, which possibly explains the different results obtained by TEM and TEG.

Biochemical abnormalities

The clinical presentation of dogs with leishmaniasis is quite variable, and the type of biochemical abnormalities varies accordingly. Renal dysfunction and inflammation and/or immune reactions are relatively frequent; therefore, the respective analytes should be evaluated in each dog with suspected or confirmed leishmaniasis. Likewise, biomarkers of hepatobiliary or pancreatic dysfunction may be altered in cases with pyogranulomatous infiltrates in these organs.14,16 Activities of enzymes released from affected skeletal muscle such as CK and LDH can also increase.53 The activity of the brain isoenzyme of CK can be increased in the presence of neurologic signs54 due to Leishmania-related cerebrovascular alterations55,56, while increases in the myocardial isoenzyme of CK can occur with cardiomyopathy. In such latter cases, increased troponin I has also been reported.27,57 Abnormal endocrine variables are rare, although amastigotes and inflammatory lesions have been found in the adrenal cortex of leishmaniotic dogs.58,59

Assessment of renal function

The deposition of circulating immune complexes at the glomerular level induces inflammatory changes detectable histologically and ultramicroscopically26,60–62 leading to a proteinuric nephropathy.52 The resulting chronic kidney disease (CKD) is characterized by glomerulosclerosis, renal hypertension, and tubulointerstitial nephritis.61,62 Advanced stages of CKD are characterized by azotemia and may be associated with systemic hypertension, both factors contributing to comorbidity in dogs with leishmaniasis.62,63 Therefore, the clinical and laboratory approach to leishmaniotic dogs with proteinuric nephropathy is the same as the one recommended by the International Renal Interest Society (IRIS)64 for any type of CKD. This approach is based on a thorough clinical evaluation including the measurement of arterial pressure, and on the quantification of urinary proteins and of markers of renal function such as the urine specific gravity and the serum concentration of creatinine.64 This latter increases frequently in leishmaniotic dogs.14–16,34,65 However, creatinine is not sensitive enough to detect the earliest stages of renal insufficiency,66 and research is currently underway to identify early markers for decreased glomerular filtration rate (GFR), either in leishmaniotic dogs or in dogs affected by other types of CKD. The direct measurement of GFR through clearance tests would be the best method to assess real-time functionality of the kidneys.67 The serum concentration of Cystatin C (Cys C) has been assessed in dogs with leishmaniasis68, but in contrast to other types of early CKD69, Cys C was not a good marker in dogs with leishmaniasis.70 Recently, symmetric dymethilarginine (SDMA) has been proposed as an early biomarker for early diagnosis of CKD.71,72 Currently, there are no studies on the diagnostic power of SDMA in early CKD in leishmania-affected dogs that have proteinuria in the presence of normal creatinine concentrations.

Other serum markers may provide additional information in leishmaniotic patients with CKD. For example, in people, the increased serum concentration of homocysteine (Hcy), endothelin-1 (ET-1), or C-reactive protein (CRP) may predict hypertension and/or inflammation associated with CKD.73–76 Increases of Hcy and ET-1 also have been reported in dogs with CKD, some of which were affected by leishmaniasis.77,78 However, further studies are needed before these markers can be recommended as ancillary tests for the management of leishmaniotic dogs with CKD. Conversely, inflammatory markers such as CRP, ferritin, and adiponectin may increase in the urine of leishmaniotic dogs, sometimes in the absence of elevated serum creatinine.79,80 However, their increase is usually due to a systemic inflammatory state rather than to CKD. Finally, tubulointerstitial dysfunction may develop secondarily to proteinuria caused by glomerular damage in leishmaniotic dogs. Markers of tubular injury in urine are described in the section on urinalysis.

Abnormalities of urinalysis

As for any suspected proteinuric nephropathy, it is necessary to confirm CKD, proteinuria, and tubular damage not only by serum chemistry but also by urinalysis.14–16,34,81

Physico-chemical analysis. Urine specific gravity (USG) tends to decrease in dogs with tubulointerstitial damage due to loss of concentrating function and should be assessed by a refractometer on urine that has previously been centrifuged.66 The supernatant should be tested with a urine dipstick to provide the pH and the concentration of proteins, keeping in mind that an overestimation of proteinuria can occur with alkaline
urine (pH 8). The presence of glucose is a potential indicator of tubular damage.

Sediment analysis is another important component in the evaluation of leishmaniotic dogs, as an active sediment (e.g., presence of high numbers of leukocytes, erythrocytes, or bacteria) may indicate a lower urinary tract infection superimposed on the primary disease (leishmaniasis) and may also result in overestimation of proteinuria. The presence of granular or cellular casts may be consistent with tubular damage.

**Evaluation of proteinuria.** The evaluation of proteinuria is mandatory, as proteinuria is a risk factor for the progression of nephropathy. According to the guidelines by the American College for Veterinary Internal Medicine (ACVIM), proteinuria should be assessed in any dog suffering from a predisposing disease, such as leishmaniasis. This assessment includes certain recommended standards such as collection by cystocentesis to avoid contamination from the lower urinary tract. However, a first screening may be done on voided samples, as results recorded with the 2 methods of collection overlap when the sediment is inactive. Proteinuria may be first investigated using a dipstick, and if the result is negative, the dog is likely nonproteinuric according to the IRIS classification and no further evaluation of proteinuria is necessary. In contrast, if the dipstick is weakly positive in dogs with low USG, or strongly positive regardless of USG, the dog is likely proteinuric. In this latter case, in order to classify the level of proteinuria, the urinary protein-to-creatinine (UPC) ratio must be determined. Nonproteinuric dogs have a UPC ratio < 0.2, borderline proteinuria ranges from 0.2 to 0.5, and proteinuric dogs have a UPC ratio > 0.5 according to the IRIS classification (recently revised for the diagnosis of glomerular disease). In the interpretation of borderline results, particular attention should be paid to potential analytic factors that can influence the UPC ratio such as type of reagent, methods, or instruments.

Quantification of proteinuria must be repeatedly assessed (3 times in 2 weeks or once on pooled urine) because additional investigations or treatments should only be pursued if proteinuria is persistent. Finally, the origin of urinary protein should be assessed by a histologic examination of a renal biopsy. However, according to the recent IRIS guidelines, renal biopsy is recommended only in the case of rapid progression of CKD or in dogs not responding to conventional treatments. Alternatively, the origin of proteinuria can be argued on the basis of surrogate methods such as qualitative analysis of urinary proteins (see below).

**Markers of tubular injury.** In order to stage dogs with a tubular component of proteinuria and advanced renal disease, urinary markers may be used. Some conventional markers such as granular or cellular casts and glycosuria in normoglycemic dogs are very specific indicators of tubular damage, but are not sensitive enough to detect dogs with early tubular damage and are rarely observed in leishmaniotic dogs. Sodium dodecylsulphate (SDS) electrophoresis of urinary proteins or measurement of more recently defined urinary markers may provide an earlier indication of tubular damage.

The SDS-mediated denaturation and negative charge of urinary proteins allows mass-dependent migration during polyacrylamide gel (SDS-PAGE) or agarose gel (SDS-AGE) electrophoresis. This differentiates large proteins present in urine due to glomerular damage, from small proteins of tubular origin. Results of SDS-PAGE or SDS-AGE correlate well with histopathology of renal biopsies, especially for the differentiation between glomerular and severe tubulointerstitial damage. Using SDS-AGE, it has been shown that leishmaniotic dogs have a mixed glomerular and tubular pattern. Only a minority of dogs, likely those with early CKD, have a pure glomerular proteinuria. However, SDS-AGE may not be accurate with very concentrated or diluted urine. Occasionally, low molecular weight proteinuria with no signs of glomerular disease may be seen, possibly due to free light chain proteinuria (prerenal proteinuria associated with highly activated antibody production) rather than to tubular damage.

Enzymuria is considered a good marker of tubular damage. Specifically, the enzymes of interest are located in the cytoplasm of tubular epithelial cells and may be found in urine when integrity of tubular cells has been disturbed. The 2 most popular urinary enzymes are GGT and N-acetyl-β-N-glucosaminidase. They are both unstable in untreated urine and their activity must be measured immediately after sampling. Increases of these and other enzyme activities (e.g, ALP or β-glucuronidase) have been reported in dogs with leishmaniasis, and the increase of GGT correlates with the presence of tubular bands in SDS electrophoresis of urine. In contrast, no information is available on the utility in leishmaniotic dogs of the measurement of other urinary analytes used to detect
tubular damage in dogs with CKD not associated with leishmaniasis.\textsuperscript{92,101,102}

Assessment of inflammatory/immune reactions

Leishmaniotic dogs with overt disease mount an intense inflammatory response and produce significant amounts of various molecules involved in the immune response, including antibodies. There are several tests including serum protein electrophoresis or measurement of APP to assess and monitor these mechanisms.

Serum protein analysis including electrophoresis may reveal abnormalities very early during the course of the disease.\textsuperscript{17} For instance, total proteins and total globulins are frequently increased\textsuperscript{14,15,65,81,103}, and the increase of total proteins has been shown to correlate with the severity of the clinical score.\textsuperscript{104} The only exception is albumin, which decreases because it is a negative APP (see below) but can also be lost due to proteinuric nephropathy, resulting in a decreased albumin-to-globulin (A/G) ratio.\textsuperscript{65,103} The decrease of the A/G ratio is so frequent that it has been considered by some authors to be one of the most sensitive tests for canine leishmaniasis\textsuperscript{103}, and hypoalbuminemia is considered a negative prognostic factor in leishmaniotic dogs.\textsuperscript{105} The typical electrophoretogram of leishmaniotic dogs with overt clinical signs (Figure 3) displays hypoalbuminemia, a moderate increase of $\alpha_2$-globulins, which include most of the positive APP, and a marked increase of $\gamma$-globulins, due to the high titers of circulating antibodies, immune complexes, and other molecules with $\gamma$-globulin-like mass and charge. Occasionally, peaks of circulating antibodies are found in the $\beta$ region, where IgM and some APP migrate. The gammopathy in leishmaniasis is typically polyclonal but sometimes the peak may appear narrower (ie, oligoclonal), biclonal\textsuperscript{106}, or definitely monoclonal\textsuperscript{107}, especially using capillary zone electrophoresis (Figure 4).\textsuperscript{108} However, although monoclonal peaks associated exclusively with leishmaniasis have been described, the presence of monoclonal peaks should also include consideration of concurrent disease such as other vector-borne diseases or multiple myeloma.\textsuperscript{109,110}

Acute phase proteins are powerful indicators of inflammation. Pro-inflammatory cytokines elicited during an acute phase response stimulate the release of neutrophils from storage pools, activate myelopoiesis, and modulate protein synthesis in the liver.\textsuperscript{75} This latter phenomenon leads to a decreased serum concentration of negative APP and an increased concentration of the positive APP that

\begin{figure}[h]
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\caption{Examples of electrophoretograms from a normal dog (A) or dogs with clinical leishmaniasis (B–F) using agarose gel electrophoresis. (A) Normal canine electrophoretogram for comparison ($\alpha =$ albumin; $\alpha_1$, $\alpha_2$, $\beta_1$, $\beta_2$, $\gamma =$ globulin fractions); (B) Marked increase of $\alpha_2$ and $\gamma$-globulins, with polyclonal gammopathy. (C) Mild increase of $\alpha_2$-globulins (detectable only in the early phase of the disease). (D) Severe hypoalbuminemia and polyclonal gammopathy. Also $\beta_2$-globulins are likely increased in this case. (E) Marked increase of $\alpha_2$-globulins and polyclonal gammopathy, with a prominent peak in the $\beta_2$-region and a less evident polyclonal peak in the $\gamma$-region. (F) Marked hypoalbuminemia and oligoclonal gammopathy. This dog was co-infected with \textit{Ehrlichia canis}.}
\end{figure}
includes a series of immunomodulators, scavenger or transport proteins, antiproteases, and other proteins involved in host defenses. Therefore, it is not surprising that the serum concentration of positive APP is high in dogs with overt canine leishmaniasis. The list of APP whose concentration increases in serum of leishmaniotic dogs is long and includes CRP, haptoglobin (Hp), ceruloplasmin (Cp), serum amyloid A (SAA), and ferritin. Similarly, a decrease of negative APP other than albumin has also been reported. They include transferrin (total iron-binding capacity or TIBC) that results in a reduction of iron, and a decreased activity of the enzyme paraoxonase 1 (PON-1). Paraoxonase 1 is a negative APP that is bound to high-density lipoproteins (HDL) and represents a link between inflammation and oxidative stress. Decreased PON-1 activity is not always seen in leishmaniotic dogs, but it may become evident when oxidative stress is particularly severe. In these cases, the concentration of HDL, which is converted into low-density lipoprotein (LDL) after detachment from PON-1, also decreases and may prove an inexpensive marker of inflammation and oxidative stress associated with leishmaniasis. Recently, reduced serum activity of adenosine deaminase and butyrylcholinesterase, 2 enzymes involved in modulating immune responses, have also been reported in dogs with leishmaniasis.

The APP changes summarized above are not diagnostic per se as mild increases of positive APP have been reported also in infected dogs without clinical signs, and markedly increased levels may occur in diseases other than leishmaniasis. In a dog with confirmed leishmaniasis, however, the magnitude of APP changes may reflect the extent of the systemic inflammatory response and thus provide prognostic information. In particular, decreased PON-1 activity may be a negative prognostic indicator.

Tests for Etiological Diagnosis that may Support or Confirm the Diagnosis of Leishmaniasis

Tests for an etiologic diagnosis are used to confirm the presence of the parasite or its components (direct tests) or of the host’s response to the parasite (indirect tests). As previously mentioned, positive indirect tests (ie, serology) may or may not indicate a current infection. Conversely, positive direct tests (cytology, histology, immunohistochemistry, PCR, culture, and xenodiagnosis) demonstrate that the dog is actually infected with Leishmania sp. The diagnosis of an actual presence of disease has to rely on clinical findings and clinicopathologic tests. The most common tests for etiologic diagnosis are discussed below.

Serology

Methods

There are techniques such as Western blotting that is highly accurate but not available in routine practice, while others have been proposed but are not extensively used, such as the latex agglutination test or detection of antibodies through immunosensors or flow cytometry. The most common techniques used to detect antileishmanial antibodies are based on 3 analytic principles: immunofluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), and immunochromatographic test (ICT). The latter method is the basis of all rapid in-clinic assays, which only provide a qualitative result (ie, presence/absence of specific reactive bands or spots). Several commercial ICT kits are available, which are based on single or multiple recombinant Leishmania sp. antigens to be incubated with serum, plasma, whole blood, or blood spots dried onto filter paper. The
specificity of these tests is quite acceptable, but sensitivity is usually low (in the approximate range of 30–70%) and largely dependent on leishmaniasis stage.\(^{126}\) Lowest test sensitivity is associated with infected dogs without clinical signs, the highest ones with dogs with overt disease.\(^{127}\) Therefore, ICT may be used as a first in-clinic test to complete the laboratory evaluation of clinically suspect dogs. In case of a positive result, a quantitative serology (ELISA or IFAT) should be performed to obtain a numeric result. Also, due to its low sensitivity, a negative ICT in light of strong clinical signs should be questioned, and followed up by IFAT or ELISA. Recently, an ICT kit claiming detection of antibodies developed after natural infection but not those elicited by vaccination with the LiESP-based vaccine has been proposed as a tool to differentiate vaccinated from infected dogs.\(^{128}\) The principle of the test is sound, and a preliminary study reported a high sensitivity of this ICT format\(^{129}\), while a further study reported a low sensitivity.\(^{130}\)

The IFAT is considered the reference method for anti-Leishmania serology in dogs\(^ {126-131}\) based on the high sensitivity and specificity (near 100% for both) except in areas endemic for the New World parasite Trypanosoma cruzi that may give false-positive results. The ELISA is also very sensitive and specific when a combination of immunodominant recombinant proteins is used as antigen, whereas it has slightly lower specificity when crude parasite lysates are employed instead.\(^ {124,130-132}\) In comparison with the IFAT that is based on the operator-dependent evaluation of promastigote fluorescence by UV microscopy, the ELISA is easier to standardize as results are read by an automated spectrophotometer. Both IFAT and ELISA have the advantage of providing quantitative results reflecting the final antibody titer (the last 2-fold serial dilution of sample providing a positive result) or, for ELISA only, optical density values converted based on a reference titered sample. Owing to the unavoidable variability due to operator-dependent or analytic factors (antigen stability, antiserum, or equipment performances), reference sera with standardized anti-Leishmania antibody titers are not universally available. At this point, a titer is considered high if it is 4-fold higher than the threshold value of the laboratory.\(^ {14}\) Similarly, 4-fold titer variations in sequential samples of the same dog should be expected with seroconversions, or with a positive therapy outcome. Hence, sequential samples must always be analyzed by the same method and in the same laboratory.

**Interpretation**

Serologic tests detect and quantify antibodies present in serum or plasma. As not every dog will seroconvert after infection, it is difficult to precisely determine seroconversion in naturally infected dogs. Antibodies can be found in blood as early as one month after exposure to infected phlebotomines; the median time for seroconversion was estimated to be about 5 months in natural conditions and 3 months in experimental studies using artificial infection.\(^ {133}\) Therefore, dogs living in highly endemic regions may seroconvert during the active period of the sand fly (from late spring to early autumn in temperate zones, all year in tropical areas).\(^9\) If the vector-transmitted parasites are efficiently controlled by the host’s immune response, the antibody titers, if present, tend to remain low and therefore these clinically healthy dogs can be classified as exposed (when the infection is not confirmed by direct tests) or infected.\(^ {14}\) Conversely, uncontrolled parasite dissemination is associated with an exaggerated humoral response. Antibody titers will be high when the disease is evident in dogs classified as sick or very sick by CLWG classification\(^ {14}\) or stage II, III, or IV (mild, severe, or very severe disease) by the Leishvet classification.\(^ {15}\) While a direct relationship between the clinical score and antibody titers exists\(^ {104,134}\), low-to-medium antibody titers may also be detected in dogs with clinical signs. These have been classified as stage I or II (mild or moderate disease) according to the Leishvet classification.\(^ {15}\)

In conclusion, quantitative serology should be always be performed when, despite strong clinical suspicion of leishmaniasis, lesions approachable by fine-needle aspiration are not present or when cytologic analysis of lesions, lymphoid organs, and bone marrow fail to reveal the typical pattern associated with leishmaniasis, despite a possible PCR-positivity. In this case, a high antibody titer is often consistent with leishmaniasis, while, if the antibody titer is low, leishmaniasis should be considered only if other diseases potentially responsible of the clinical presentation have been ruled out.\(^ {14,15}\)

The increasing use in southern Europe of the LiESP vaccination, known to elicit long-standing low-to-medium titers of antileishmanial antibodies, may further complicate the interpretation of serology in vaccinated dogs. Practical laboratory protocols allowing discrimination between the humoral response in Leishmania-infected and LiESP-vaccinated dogs are not yet available.
PCR

Methods

Several methods have been proposed to detect the presence of parasite DNA in various biologic samples. Some of these methods are not commonly used or were not recently validated, such as those based on the use of probes labeled with gold nanoparticles or the loop-mediated isothermal amplification (LAMP). Conversely, conventional PCR, nested PCR, and quantitative (real-time) PCR are widely used in routine practice. Sensitivity and specificity vary according to the method and the target DNA sequence. Most of the PCR tests currently used are targeting multicopy DNA sequences, such as the small subunit ribosomal RNA genes or the kinetoplast DNA minicircles, thus increasing the sensitivity of the test. Compared with conventional and nested PCR, the quantitative PCR techniques offer 2 main advantages: they may be run in closed systems and are therefore less prone to contamination, and they provide information about the copy number of DNA present in the sample. This latter aspect may be relevant during the follow-up to monitor the efficacy of leishmanicidal treatments, and therefore it may be advisable to use quantitative PCR at first diagnosis (before any treatment) for the establishment of a baseline value for comparison of future results during the follow-up. Overall, it does not seem that quantitative PCR techniques are more sensitive than conventional or nested PCR to diagnose canine leishmaniasis. One additional limitation of quantitative PCR is that standardized methods to accurately quantify the DNA copies may not be offered by some laboratories.

Samples

Polymerase chain reaction techniques may be applied virtually on any tissue or biologic fluid. Theoretically, it may be superfluous to use molecular tests in affected tissues in which Leishmania amastigotes have been visualized by cytology or histology. However, these latter methods are less sensitive than PCR and therefore, a negative cyto- logic result does not exclude that a low number of amastigotes is indeed present. Hence, when a fine-needle aspirate or a tissue biopsy is performed, it may be advisable to prepare cytologic or histologic specimens and to store the remaining material in the preservatives recommended by the laboratory to run PCR. If needed, PCR may also be performed on cytologic material already fixed on glass slides or on formalin-fixed and paraffin-embedded material.

In routine practice, PCR is rarely run on biopsies from lesions for which cytology and histology are preferred, but rather if cytology and histology are not diagnostic. When there are no lesions eligible for fine-needle aspiration or biopsy (eg, when the only prevalent clinical symptoms include anemia or proteinuric nephropathy), bone marrow and/or lymph nodes and spleen are the tissues with the highest potential prevalence for detection by PCR, especially in sick dogs, provided that the quality of the sample is adequate. Recent studies demonstrated that conjunctival and, to a lesser extent, oral and nasal swabs are very sensitive for the detection of Leishmania DNA and, in addition, can provide positive results earlier than other tissues. Whole blood or buffy coats may also be used for conventional or quantitative PCR analysis. The sensitivity is lower than in the above tissues, but blood collection is less invasive, and when positive, provides a rapid and inexpensive diagnosis.

Interpretation

When interpreting PCR results, the difference between infected and sick dogs must be kept in mind. Ultimately, the detection of the parasite’s DNA indicates that the dog is infected. The correlation between infection and disease must then be based on the presence of clinical and laboratory abnormalities. From this perspective, the detection of Leishmania DNA in lesions with cytologic or histologic patterns highly consistent with leishmaniasis, or in blood or bone marrow of a dog with systemic signs of leishmaniasis supports the diagnosis of disease. Conversely, positive PCR results in dogs without signs clearly referable to leishmaniasis do not support the hypothesis that the infected dog is also affected by clinical leishmaniasis, unless any other possible disease is excluded. For example, a transient PCR-positivity in bone marrow may be found a few months after the natural exposure to sand fly bites, without necessarily meaning that the dog is definitively infected or even sick. Similarly, PCR-positivity in intact skin of dogs frequently exposed to vectors does not necessarily mean that dermal “contamination” by infectious bites will be followed by systemic parasite dissemination. Positive skin PCR results may in fact depend on the presence of recently inoculated promastigotes, or of amastigotes phagocytosed by resident macrophages that, in resistant dogs, may efficiently control (or even eliminate) the parasite on a local level.
Cytology

Samples and methods

Fine-needle aspiration should be performed in all cases with cutaneous papular or nodular lesions and/or lymph node enlargement.14 Ulcerative cutaneous lesions can be sampled by scraping the lesion or using less invasive methods such as imprint smears. Additionally, reports describing the presence of amastigotes and associated lesions in nodular masses with atypical localization such as the tongue19,23, the testis154,155, and oral or nasal masses156 have been reported. Therefore, any nodular lesion in dogs with clinical or laboratory signs potentially consistent with leishmaniasis (eg, anemia, CKD, alterations of the electrophoreograms, positive serology) should be sampled by fine-needle aspiration. Nasal lesions may also be sampled using brush cytology.157 Similarly, when clinical or clinicopathologic patterns are consistent with leishmaniasis, the possible presence of *Leishmania* should be investigated also in pathologic body fluids such as joint fluids158,159, effusions29, or cerebrospinal fluid (CSF), although in this latter sample, cellularity is usually so low that PCR may detect the parasite better than cytology.55 When cutaneous lesions, nodular lesions in other organs, lymph node enlargement, or abnormal accumulation of fluids are absent but the clinical suspicion of leishmaniasis is high, the presence of parasites should be investigated in tissues that contain many cells of the monocyte-macrophage series, such as bone marrow, lymph nodes, or spleen.14,15,44

Interpretation

Cytology aims to demonstrate the presence of *Leishmania* amastigotes within the macrophages, or when the parasite burden is high and cell lysis occurs, also in the background (Figure 5). The detection of amastigotes

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**Figure 5.** Examples of cytology specimens from dogs with *Leishmania* sp. infection. May–Grünwald–Giemsa. (A, C, D) Bar = 20 µm. (B) Bar = 15 µm. (A) Imprint of an ulcerated skin lesion with pyogranulomatous inflammation (degenerate and nondegenerate neutrophils, macrophages, lymphocytes, and plasma cells). Varying sized pigmented material likely resulting from cytophagia in the macrophage and in the background could be confused with amastigotes. (B) Cytocentrifuged synovial fluid from a swollen joint. There are two amastigotes inside a large mononuclear cell with signs of nuclear degeneration. Neutrophils and lymphocytes, indicating inflammation, are also visible. (C) Fine-needle aspirate of spleen with intracytoplasmic amastigotes, plasma cells, and neutrophils. (D) Fine-needle aspirate of a lymph node. No amastigotes are visible but in this case the diagnosis is supported by the presence of reactive hyperplasia, characterized by variably sized lymphocytes, neutrophils, and plasma cells.
may be difficult in ulcerative cutaneous lesions, where necrosis and cellular debris or contaminating bacteria may mask the presence of amastigotes. Attention should be paid to not misinterpret cellular or granular debris as amastigotes in such lesions.

The typical cytologic patterns associated with leishmaniasis are usually characterized by granulocytic-macrophagic or pyogranulomatous inflammation associated with a moderate to severe lymphoplasmocytic infiltration in skin or nodular lesions with atypical localization (Figure 5) and, in lymph nodes, by a reactive hyperplasia of variable degree, characterized by lymphoplasmocytic and macrophagic infiltration, usually associated with numerous neutrophils. Similarly, cytologic patterns typically associated with leishmaniasis may be found in the bone marrow, as described above. Neutrophils, lymphocytes and macrophages can also be found in body fluids of dogs affected by leishmaniasis.

The diagnosis of leishmaniasis is easy when amastigotes are detected in samples that show the cytologic patterns described above. However, when cytologic patterns consistent with leishmaniasis but no amastigotes are seen, leishmaniasis should not be ruled out, as it is known that the etiologic diagnostic sensitivity of cytology is low. In these cases, tests that have higher analytic and diagnostic sensitivity, such as PCR, must be run. Alternatively, affected tissues can be biopsied to perform histology and immunohistochemistry, as described below. Conversely, when amastigotes are seen in the absence of cytologic abnormalities, or cytology is done on bone marrow, lymph node, or spleen, positive results must be interpreted carefully, as systemic signs may be due to diseases other than leishmaniasis. Similarly, a diagnostic workup to differentiate sick from infected dogs should be done when Leishmania is incidentally found in lesions that clearly have a different origin. For example, several reports describe the association between the presence of amastigotes and neoplasms such as lymphoma, transmissible venereal tumors, and others. In such cases, it is important to determine whether the dog is affected by both diseases or affected by a neoplastic disease while simply being infected with Leishmania sp.

**Histology**

Histology can demonstrate the presence of Leishmania in routinely hematoxylin- and eosin-stained sections when cytology is negative on tissues with a cytologic pattern highly consistent with leishmaniasis. Compared with PCR and cytology, histology has 2 main disadvantages: it is more laborious and time-consuming, and the identification of amastigotes may be more difficult than in cytologic samples. However, amastigotes can be confirmed by immunohistochemistry (Figure 6), in situ hybridization, or PCR on formalin-fixed and paraffin-embedded specimens. On the other hand, histology has the advantage of providing additional information on the cytoarchitectural pattern of the lesions. This is a great advantage as it may allow to discriminate between dogs in which the parasite is associated with typical lesions and those in which the infection does not seem to be associated with the disease. Therefore, according to some guidelines, histology should always be performed. The interpretation of histologic results is well documented by numerous publications describing the typical lesions and the distribution of parasites associated with active disease, mostly characterized by lymphoplasmocytic or granulomatous-pyogranulomatous inflammation and/or by vasculitis either in organs usually affected by Leishmania (bone marrow, spleen, skin, lymph nodes, kidney, etc), and also in unusual tissues such as heart, lung, adrenal gland, genital tract, central nervous system, skeletal muscle, gastrointestinal tract, nails, lacrimal glands, and ocular muscles.

**Parasite culture and biologic test for infectiousness (xenodiagnosis)**

Conclusive diagnosis of active infection should be based on tissue cultures, which not only confirm
whether dogs harbor parasites but also demonstrate that the protozoa are viable. A diagnostic *Leishmania* sp. culture requires biphasic blood-agar media with fresh components. A conclusive test for infectiousness (xenodiagnosis) requires that naive (laboratory-reared) sand flies are allowed to feed on infected dogs and are examined thereafter for the presence of promastigotes in the gut. However, both tests are unpractical and restricted to specialized reference centers. Therefore, these tests are mainly intended for research and cannot be recommended for routine practice.

**Future Perspectives**

Several studies investigated the diagnostic potential of innovative markers in leishmaniotic dogs. For example, iron superoxide dismutase (Fe-SODe) secreted by the parasite has been evaluated as a possible marker of infection. Proteomic analysis has revealed a series of proteins that are over- or underexpressed in leishmaniotic dogs. The expression level of cytokines or molecules such as leptin or inducible nitric oxide synthetase in blood or tissues is different in leishmaniotic dogs compared to controls, and increased activities of matrix metalloproteinases have been reported in serum or CSF of leishmaniotic dogs. Recently, the scientific attention has been focused on markers of oxidative stress. Inflammation is characterized by the release of reactive oxygen metabolites from phagocytes recruited to inflammatory sites, eventually leading to a consumption of antioxidant compounds. Accordingly, increased concentrations of oxidants or oxidized molecules (eg, reactive oxygen metabolites, malonyldialdeide, lipoperoxides, thiobarbituric acid reactive substances) and decreased concentrations of antioxidant compounds (total antioxidant capacity, trace elements, paraoxonase) have been reported in leishmaniotic dogs.

However, none of the studies cited above provided, to date, exhaustive information on the possible practical diagnostic applicability of these markers. Nevertheless, preliminary results from these investigations are encouraging and useful in design of future research to explore their potential clinical application.

**Tests for monitoring the posttreatment follow-up**

Laboratory tests during the follow-up should be focused on the monitoring of possible toxic effects of treatment as well as the clinical and the parasitologic status of the patient following administration of drugs according to conventional treatments protocols. These mainly include the administration of antimonials or miltefosine, both in combination with allopurinol. Alternative drugs should be carefully considered only when conventional treatments are not effective.

**Monitoring the possible toxic effect of treatment**

Theoretically, the possible toxic effects of treatment should be monitored. However, despite some studies which reported possible nephrotoxicity of antimonials, others did not confirm this finding. Recent investigations demonstrated that no toxic effects on cardiac or pancreatic tissue are induced by these drug classes in dogs, differently from what is observed in human patients. Therefore, toxic effects should be monitored only in selected dogs, particularly when peculiar clinical findings are present or history might suggest any drug adversity.

The only possible adverse effect of allopurinol is the formation of xanthine crystals in urine and possibly urolithes. These crystals occur very frequently and may sometimes be abundant. However, associated clinical signs and urolith formation are not common and suspension of treatment is unusual. Therefore, the analysis of urine sediment should always be included in the laboratory database when allopurinol is administered for a long time or when urine appears macroscopically turbid or forms an evident pellet after centrifugation (Figure 7).

**Figure 7.** Urine sediment from a dog with leishmaniasis treated with allopurinol. Xanthine crystals appear as roundish brown-yellow crystals of different size, single or forming small to medium clusters. Unstained sediment, bar = 15 μm. (Courtesy of Dr. Tiziana Vitiello, DiMeVet, University of Milan).
Monitoring the clinical status

As the clinical presentation of leishmaniasis in dogs can be extremely variable, it is not possible to define, a priori, a common and standardized laboratory procedure to be used during the follow-up. However, 2 main aspects must always be monitored, namely the presence of renal disease and systemic inflammation.

Renal function should be evaluated through the analysis of serum concentrations of creatinine and, especially, through sequential quantification of proteinuria, due to its role as a risk factor for the progression of CKD. Proteinuria has been recently shown to be a negative prognostic factor in leishmaniotic dogs. After conventional leishmanicidal treatment, the degree of proteinuria decreases in 4–8 weeks. Thus, additional pharmacologic treatments for proteinuria should be decided thereafter. The possibility to restore normal renal function depends on the severity of renal damage at the time of first diagnosis. Therefore, serum creatinine and proteinuria should be repeatedly assessed during the follow-up. The frequency of testing depends on the severity of CKD. Dogs in IRIS stages 3 or 4 should be frequently tested also during the treatment period, while dogs in IRIS stages 1 or 2 should be tested at the end of the first treatment cycle. Posttreatment evaluation should be done after 12 months in stage 1 dogs, every 6 months in dogs in stage 2, every 3 months in dogs in stage 3, and every 6 weeks in dogs in stage 4.

The inflammatory status may be monitored through sequential analysis of electrophoretograms and of APP. The simple evaluation of total proteins, albumin, or albumin-to-globulin (A/G) ratio, may not be helpful because it is very likely that, despite decreases in globulin concentrations in response to treatment, albumin concentrations will remain low in dogs with persistent glomerular damage and proteinuria, in turn leading to only minor changes in the A/G ratio. Serum protein electrophoresis can be used to monitor a progressive decrease of α- and γ-globulins with successful treatment. These changes start to become evident after 2–3 weeks and 4–6 weeks, respectively, following treatment with antimonials. Therefore, the first useful electrophoretogram to monitor the efficiency of treatment should be run no earlier than one month after treatment initiation. The complete normalization of electrophoretograms, however, requires at least 90–120 days. If after 2–3 months the electrophoretograms still show abnormal profiles, the possible presence of concurrent diseases such as other vector-borne infections should be considered, especially if the gammopathy tends to be characterized by narrower peaks (Figure 3). Treatments with miltefosine or with other drugs may require more time to be beneficial (more than 2 months to observe a decrease in γ-globulins) and are also characterized by more frequent relapses after transient normalization of laboratory profiles.

Compared with serum protein electrophoresis, monitoring the concentration of APPs provides earlier information regarding the success of treatments with antimonials. C-reactive protein and SAA start to decrease within 2 weeks after treatment and may return to within the RI in about one month. The normalization of PON-1 and HDL is even more rapid: significant increases may be observed 3–7 days after treatment and values return to within the RIs in 2 weeks. Therefore, to assess the efficacy of treatment, it may be advisable to measure the serum activity of PON-1 or the concentration of HDLs or APPs one week after the first administration of drugs, when other clinical or clinicopathologic changes are likely still abnormal.

Monitoring the parasitologic status

As at first diagnosis, the parasitologic status can be monitored indirectly, through the assessment of antibody titers, or by direct evaluation of parasite presence. In case of successful treatment, a decrease in antibody titers may be expected over time; hence, serology should be repeated during the follow-up. Significant reduction in titers can be detected by 30 days post treatment in sick or severely sick dogs with good clinical response to therapy. However, most of the responders will show a noticeable decrease of titers about 6 months after initiation of treatment. It should be kept in mind that a complete disappearance of antileishmanial antibodies is unlikely, especially in dogs living in endemic areas that may be repeatedly exposed to the parasite, boosting the antibody response. Therefore, sequential serologic testing during the follow-up is intended to see a decreasing antibody titer reaching values consistent with simple exposure (ie, < 4-fold the threshold value of the laboratory). In order to assess whether treatment leads to complete elimination of the infection, ideally the presence of parasites should be assessed in tissues where Leishmania sp. typically establishes a latent infection and using very sensitive techniques such as repeated quantitative PCR analyses on bone marrow, spleen, or lymph nodes. However, this procedure is invasive.

and owner compliance may be challenging in cases where treatment appears successful and the dog looks clinically healthy. Therefore, in routine practice, the evaluation of treatment efficacy is usually assessed by serology or quantitative PCR analysis of blood. If treatment has been successful, quantitative PCR should show a clear reduction in *Leishmania* DNA copies after 3–6 months of therapy, with complete absence between 6 and 12 months.\(^{138}\)

### Concluding Remarks and Recommended Protocols

Diagnosing leishmaniasis in dogs may be difficult due to the complex pathogenesis and broad spectrum of clinical and clinicopathologic findings. Hence, tests that need to be included in the diagnostic protocol may vary according to case presentation or epidemiologic scenario (Figure 8).\(^{210}\)

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**Figure 8.** Flowchart illustrating the recommended diagnostic approach and laboratory tests for clinically healthy dogs living in or traveling to endemic regions, or for dogs showing clinical signs consistent with leishmaniasis.
In dogs with a strong clinical suspicion of leishmaniasis, the use of quantitative serology is advisable, as it can be conclusive for diagnosis when high titers of antibodies are present. In clinically healthy dogs living in or having traveled to an endemic area, again serology may be the test of choice to assess any possible exposure to parasites. Based on the median time required for seroconversion\(^{133}\), serology should be performed at least 6 months after exposure (eg, in February–March where transmission is seasonal, or every 6–12 months where transmission occurs all year long). If serology is positive, it is important to determine the antibody titer. A low antibody titer may be consistent with exposure or an early phase of infection, while a high antibody titer can be suggestive of infection or disease.\(^{14,15}\) Therefore, the subsequent diagnostic steps should confirm the suspected infection by cytologic and PCR analysis of target tissues, and/or by identification of typical clinical or laboratory alterations, especially in dogs with high antibody titers. If serology or PCR are positive and samplings have been performed during a nontransmission period, the laboratory workup should aim to identify the most common abnormalities in dogs with leishmaniasis in the absence of overt clinical signs (eg, anemia, abnormal serum protein electrophoresis, proteinuria). In the presence of typical changes, additional clinical or laboratory tests must be performed in order to stage the disease (eg, tests recommended by the IRIS guidelines for CKD\(^{64}\) and/or APP for inflammation).

If a dog is examined because of clinical abnormalities, the veterinarian should sample any accessible lesion to obtain cytologic smears or biopsies.\(^{15}\) If Leishmania sp. amastigotes are documented and the cytologic or histologic pattern is consistent with leishmaniasis, the dog should be considered sick. Thus, next diagnostic steps should clarify whether there is a systemic involvement (eg, hematologic disorders, inflammation, nephropathy) and the antileishmanial antibodies and/or the parasite burden should be quantified with quantitative PCR to obtain baseline values useful for monitoring. Conversely, if amastigotes are not observed but cytologic patterns are consistent with leishmaniasis, the lesion can be further analyzed by histology combined with immunohistochemistry, in situ hybridization, or PCR.\(^{14,15}\) A positive result with one of these additional tests should lead to further investigation of the general health status of the sick dog. Conversely, if these tests are negative, the presence of infection should be assessed in the bone marrow through cytology and/or PCR and, in case of positive results, further clinicopathologic tests should be performed as discussed above.\(^{14,15}\)

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Laboratory diagnosis of leishmaniasis

Paltrinieri et al


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