CANINE LEISHMANIASIS: GUIDELINES FOR DIAGNOSIS, STAGING, THERAPY, MONITORING AND PREVENTION.

Part I: Diagnostic approach and classification of the patient affected by leishmaniasis and management of dogs with proteinuria

By CANINE LEISHMANIASIS WORKING GROUP (CLWG)
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Summary
*The "Canine Leishmaniasis Working Group"(CLW*G) *has elaborated guidelines for the diagnosis of canine leishmaniasis, its classification and the treatment of affected dogs with concurrent proteinuria. The guidelines are based on existing references and/or the experience of the CLWG members. The paper aims to provide the most updated information about the treatment of dogs affected by leishmaniasis. The veterinary clinician should critically evaluate the potential applicability of the present guidelines when treating cases of canine leishmaniasis.*
A dog with clinical signs and/or laboratory findings compatible with leishmaniasis (skin or ocular lesion, lymphadenopathy, lameness, anemia, dysproteinemia, azotemia, proteinuria) is considered affected if *the parasite is identified within lesions on cytology and/or there is a fourfold increase of the antibody titer above what is considered the lower positive level of the reference laboratory. If the antibody titer is mildly to moderately increased, and cytological specimens are negative, it will be necessary to perform histology/immunohistology (on skin lesions) or PCR on bone marrow*

and/or lymph node samples. If one of the previous tests is positive the dog must be considered infected. *If a clear association can be made with such lesions the dog is likely affected by leishmaniasis. When PCR is negative this result indicates that the dog has been exposed to the parasite and should be serologically monitored.*
Proteinuria should be quantified by the urine protein/creatinine urinary ratio (UP/UC). As proteinuria is associated with the development of renal failure, it is important to do not treat exclusively the parasite but include measure against the renal protein loss in dogs affected by leishmaniasis. ACE-inhibitor drugs can be used for this purpose. In dogs with concurrent renal failure modifying the diet is necessary.

INTRODUCTION

Canine leishmaniasis (CanL) is caused by *Leishmania infantum (Kinetoplastida: Trypanosomatidae)*, a protozoan characterized by the presence of an evident mitochondrial organelle, called kinetoplast. Flagelled forms of parasite, known as promastigotes, multiply in the gut of the insect vectors, the female of phlebotomus *(Diptera: Psychodidae),* that intradermically inoculates them in the host during the blood-feeding. Macrophages in the connective tissue phagocytize promastigotes, which become round-shaped and unflagelled and are named amastigotes. Amastigotes replicate in macrophages, destroying them and infecting progressively more and more phagocytes. Parasite dissemination in the body and possible development of the disease depend on type and efficiency of immune response of the infected dog. Prevalence of infection ranges from 2% to 40% in Mediterranean outbreaks of CanL. Studies on presence of *Leishmania*-specific cell-mediated immunity showed higher prevalence, suggesting that exposition rate is probably very higher.[1-3] In endemic outbreaks of Mediterranean Basin, main phlebotomus vectors belong to species *Phlebotomus perniciosus, P. ariasi, P. perfiliewi, P. neglectus and P. tobbi,* [4-11] all with crepuscular and nocturnal activity extending from late spring to late autumn.

Epidemiologically, until 1980s all northern Italy Regions, but some areas of Bologna Province, were CanL-free.[12] Beginning from early 1990s, incidence of CanL increased in every endemic regions and stable-type micro-outbreaks were reported in traditionally non-endemic areas too, like Piedmont, Aosta Valley, Lombardy, Trentino-Alto Adige, Veneto and Friuli Venezia Giulia.[14] Therefore, CanL endemy could seem in Italy and is quickly expanding to northern latitudes, being in these areas an emerging problem of veterinary health.

Leishmania infections have three pathogenetic features: (i) macrophages are the target of parasite, that can reply itself inside them; (ii) appearance and evolution of the disease depend on inflammatory or immune response of the host; (iii) infection persists in tissues. *Leishmania* tends to localize itself in all tissues with the higher levels of monocytic-macrophagic cells, where it can be find with direct methods just some weeks post-infection. A proportion of infected dogs can become negative to some diagnostic test after a generally short period from first positive result and without any treatment. Actually, it isn't known if these animals became infection-free or restrained the infection to a not-detectable level with the used method, or the parasite had localized in tissue different from those examined during the first diagnosis.[15] In mammals, *L. infantum* causes an infection, generally chronic, that sometimes can be asymptomatic and sometimes can evolve in a obvious clinical disease: immune response play a very important role in this dichotomy (infection *versus* disease), due to T helper CD4+ lymphocytes that can direct the immune system towards an humoral (Th2) or cell-mediated (Th1) response. The two boundaries of clinical expression include: (i) infected and clinically sound dogs, characterized by mild or no Th2 response and presence of a Th1-specific response against *Leishmania*; (ii) infected and severely sick dogs, characterized by an exaggerated Th2 response and an absent or mild Th1 response.[16]

Resistance to disease seems to be associated to a Th1-Th2 mixed-type immune response in dogs and in human beings, with Th1 cytokines predominance, while disease susceptibility seems to be associated to reduced production of cytokines, mainly of Th2-type.[16-21] In these cases,

continuous antigenic stimulation and exaggerated antibody response cause hypergammaglobulinemia, immune complexes deposition that can cause glomerulonephritis, vasculitis, polyarthritis, uveitis and meningitis and production of auto-antibodies against platelets and erythrocytes.[19] In addition, sick or asymptomatic dogs with mainly humoral response are characterized by parasite dissemination in body, decreased count of T lymphocytes CD4+ and immunosuppression.[19,22] The count of T lymphocytes CD4+ is inversely related and seropositivity rate is directly related to phlebotomus infectivity.[23,24]

On November, 2005, the "Canine Leishmaniasis Working Group"(CLWG) has been constituted [see website: http://www.gruppoleishmania.org/] to obtain a rationale and homogeneous approach to management of canine leishmaniotic patient as regards:

1. diagnostic approach;
2. classification of leishmaniotic patient;
3. therapeutic approach;
4. management of proteinuric leishmaniotic patient;
5. management protocol for prevention.

In the present guidelines, diagnostic approach, classification and management of proteinuric leishmaniotic patient are considered. These guidelines are based on international scientific references and, where these are inadequate or not complete, on experiences of CLWG members. These guidelines, directed to veterinary practitioners, describe the ideal and most complete approach to disease and must be interpreted like recommendations to follow to improve diagnostic, therapeutic, managing and preventing capability without taking the place of the evaluation of the clinician, that, especially in a complex disease like leishmaniasis, has to evaluate their applicability on a case by case basis and/or modulate them according to different epidemiological and clinical situations.

1. DIAGNOSTIC APPROACH

[By Paltrinieri S. (coordinator), Castagnaro M., Crotti A., Fondati A., Gradoni L., Lubas G., Solano Gallego L., Zatelli A.]

Diagnosis of CanL should be based on a integrated approach considering signalment, history, clinical findings, clinicopathologic changes and results of direct and indirect etiologic diagnostic tests.

1.1. Signalment and history

Leishmaniasis can affect every canine breed, but some, like German Shepherd dog and boxer, seem to be predisposed.[25,26] Furthermore, sexual predilection could exist in males, that present an higher risk of developing the disease,[27,28] as reported in human beings[29] and hamster.[30] Also, the disease shows a bimodal distribution, with a peak in dogs < 3 years old and a second one from 8 to 10 years of age[19] and it is essential to know if the dog lives or traveled in endemic areas and/or if he is exposed to vectors, if has received preventive treatments potentially effective against phlebotomus or therapy capable of interfering with the efficiency of his immune system. History collection is completed by information about clinical signs noted by the owner and consistent with CanL, including weight loss, asthenia, dermatological changes, polyuria-polydipsia (PU/PD) and epistaxis.

1.2. Physical examination and diagnostic imaging

Most common clinical signs in cases of CanL are cutaneous ones and lymphadenopathy. However, it is possible to detect a wide and heterogeneous range of clinical signs and lesions[31-34] [see Box 1], some of which (e.g., hepatosplenomegaly, renal lesions) can be noted by diagnostic imaging too. Considering signalment and history, the practitioner will decide where insert the

disease, in order of probability, in his list of differential diagnosis. At any rate, if clinical signs allow to include leishmaniasis in differential diagnosis, it is advisable to perform laboratory tests [see 1.3] to rule-out/in its presence.

1.3. Laboratory tests and findings consistent with leishmaniasis

Minimum data base includes complete blood count (CBC), serum biochemical panel, serum protein electrophoresis and urinalysis. In CanL, these tests can allow to find one or more changes listed in Box 2.[31-34] Basing on results of minimum data base, further tests can be done, also listed in Box 2.[35-37]

BOX 1 **General and specific clinical findings of particular body areas in cases of CanL**	
General	Poor nutrition state up to cachexia Muscular hypotrophy Lethargy Pale mucous membranes Epistaxis Light to moderate volume increase of palpable lymph nodes Epistaxis Hepatosplenomegaly Lamenesses and joint swellings Fever
Cutaneous and mucocutaneous	Desquamative dermatitis (localized/generalized) Ulcerative dermatitis with varying appearance and distribution Mucocutaneous junctions Skin of paws Traumatized sites Papular dermatitis Nodular dermatitis Lupus/pemphigous-like nasal lesions Onychopathy Nasodigital hyperkeratosis
Ocular	Palpebral lesions: see cutaneous and mucocutaneous findings Diffuse and/or nodular conjunctival lesions Corneal lesions, mainly associated to conjunctiva (keratoconjunctivitis). Nodular keratitis and keratoconjunctivitis sicca are also possible. Scleral lesions: diffuse and/or nodular scleritis and episcleritis Diffuse and/or granulomatous lesions of anterior uvea and lesions of posterior uvea (chorioretinitis, hemorrhages and retinal detachments) Possible complications of uveal diseases are glaucoma and panophthalmitis Granulomatous orbital lesions, myositis of extrinsic muscles
Others	Gastrointestinal, neurological, etc.

BOX 2 **Results consistent with CanL in basic and special laboratory tests**		
Basic tests	**Findings consistent with leishmaniasis**	**Further tests**

Complete Blood Count (CBC)	Poorly regenerative or non-regenerative anemia Possible regenerative anemia (due to immunomediate processes) Neutrophilic and monocytic leukocytosis with lymphopenia and eosinopenia (stress leukogram/inflammation) Leukopenia Possible thrombocytopenia	Cytofluorimetry to detect anti-erythrocytes antibodies Bone marrow cytology Complete coagulation profile (e.g., increased FDP* and decreased AT*) Research of co-infection (e.g. with *Ehrlichia canis*) Cytofluorimetry to detect anti-platelets antibodies
Basic coagulation profile	Hyperfibrinogenemia, possible increase of PT and aPTT	Complete coagulation profile (as above)
Serum biochemical panel	Hyperproteinemia, hypoalbuminemia, hyperglobulinemia, altered Albumin:Globulin ratio Azotemia (high serum values of urea [BUN} and creatinine) Increased hepatic enzymes concentrations	Acute phase protein: CRP*, Hp*, SAA (useful for monitoring) Lipid parameters (hypercholesterolemia) Electrolytes (Hypokalemia) Minerals, Ca/P, Mg, (hyperphosphatemia, hypermagnesemia) Blood gas analysis (metabolic acidosis) Liver function tests
Serum protein electrophoresis	Hypoalbuminemia, increased α_2-globulins and poly/oligoclonal gammopathy	Acute phase protein: CRP*, Hp*, SAA (useful for monitoring)
Urinalysis	Isosthenuric urine (SG *: 1008-1012) or poorly concentrated urine (< 1030) Proteinuria (determined by dipstick and UP/UC*)	SDS-AGE urine (consistent with leishmaniasis: mixed or glomerular proteinuria)

** FDP = Fibrin/fibrinogen Degradation Products; AT = antithrombin III; CRP = C Reactive Protein; Hp = haptoglobin; SAA = serum amyloid A; SG = Specific Gravity; UP/UC = urinary protein:creatinine ratio; SDS-AGE = sodium dodecyl sulfate-agarose gel electrophoresis.*

1.4. Etiologic diagnosis

To identify the parasite or the body response against it, the integration of different methods of etiological diagnosis is necessary. Indeed, positive results of bone marrow or lymph nodes aren't always an index of persistent infection, neither permit to assign to *Leishmania* any detected clinical sign. On the contrary, identifying parasite in organs presenting lesions consistent with leishmaniasis allow to ascertain with good probability a cause-effect relationship between parasite and lesions.

Diagnostic methods actually available [cyto-histologic, parasitologic, molecular, serologic and evaluating cell response] are described. Remember that it is better ask to the laboratory which are the most appropriate rules for collecting, to storage and shipping samples.

1.4.1 Cyto-histologic methods

Cytologic examination. This method permits to show the presence of amastigotes inside intralesional macrophages or extracellularly. Collecting the sample in injured organs, there are cytologic changes (lymphoplasmocytic and/or granulomatous-pyogranulomatous inflammations, lymphonodal reactive hyperplasia, myeloid hyperplasia and/or erythroid hypoplasia in bone marrow, etc.) consistent with leishmaniasis. Thus, cytologic study should be done on following samples:

(i) papular, nodular and ulcerative skin lesions: collect by needle-infixion (or needle-aspirate) and/or apposition; however, ulcerative lesions of ischemic origin may result negative.

(ii) Bone marrow and lymph nodes in presence of clinical signs or clinicopathologic changes related to their involvement (anemia, lymphoadenomegaly, etc.);

(iii) Other sites: biologic fluids collectible from sites with lesions (e.g., synovial fluid in case of arthritis/polyarthritis, cerebrospinal fluid in presence of neurological signs, etc.)

In the absence of lesions that could be sampled, organs and tissues where the parasite is most easy to find include bone marrow, lymph node and spleen and blood, in descending order of diagnostic sensitivity.[38,39] Material sampled for cytology could be stored too and, in case of negative cytologic result, send to laboratory for PCR search for genoma of *Leishmania* (see below).

Histologic examination. The parasite could be shown in sections obtained from lesions and prepared with routine hematoxylin-eosin stain. In addition to parasite, changes consistent with CanL could be find too, including lymphoplasmocytic or granulomatous-pyogranulomatous inflammations and/or vasculitis in several organs, ischemic skin diseases, lymphoplasmocytic dermatitis of dermoepithelial junctions, lymphoid hyperplasia of spleen and lymph nodes. When a strong suspect of CanL remains despite negative cytology, histologic examination is always advisable, mainly in presence of dermatitis and in skin diseases characterized by focal lesions.

Should histologic changes like those described above are present, but identifying the parasite in hematoxylin-eosin stained sections is impossible, it will be appropriate to perform immunohistochemical stains using antibodies against *Leishmania* antigens. If this approach results negative too, bioptic sample could be used for PCR search for *Leishmania* genome (see below).[40,41]

1.4.2 Parasitological methods

Culture test. This is the most specific test because the development in culture of vital promastigotes can be attribute exclusively to genus *Leishmania* if the samples were collected in endemic areas of the Old World. However, it has the disadvantage of requiring long labour times and being performed *only* at specialized laboratory of some Italian *Istituti Zooprofilattici Sperimentali*.

Xenodiagnosis. The method consists in feeding on the suspected dog some phlebotomus breeded in laboratory and then examine them some days later searching for the presence of promastigotes in the gut. The method is very sensitive, but – of course – poorly applicable in practice.

1.4.3 Molecular methods

***Polymerase chain reaction* (PCR).** This technique allows to amplify specific sequences of *Leishmania* genome. The method is very sensitive, mainly if amplify "multicopy" genomic sequences, i.e. present in high numbers in every single parasite, like DNA of minicircles of kinetoplast.[42] Thus, it is possible identifying very low amount of DNA of protozoans present in the examined biological material. The three most common used techniques are:

(i) Conventional or traditional PCR: *Leishmania* DNA is amplified using a couple of primers (base sequences complementary to target sequence contained in *Leishmania* DNA);[41-45]

(ii) "Nested" PCR: this is a modification of traditional PCR, more sensitive but less specific, because increasing the numbers of passages tends to rise the risk of contamination by foreign DNA and, thus, of finding false positive results;[43,46]
(iii) Quantitative PCR ("real time"): using fluorescent probes, it is possible quantifying the number of DNA copies present in the biologic sample. This test has a sensitivity similar to PCR "nested", but if it is performed with "closed systems" is more specific because the sample is undergoing to lesser number of handlings and therefore is less exposed to contaminations. According to recent preliminary data,[47] this test can also give information (e.g., the number of present parasites) useful during monitoring, so it could be worthwhile using immediately this approach, if reference laboratory offers it.

These techniques could be done on several biologic samples. Besides injured tissues, in case of generalized disease other tissues having the highest probability to identify by PCR the DNA of any present parasites include, in decreasing order of sensitivity: bone marrow/lymph nodes, skin, conjunctive, buffy coat and peripheral blood. However, it must be remembered that in resistant dogs *Leishmania* inoculation may not be followed by parasite dissemination: so, any skin positivity in absence of skin lesions in endemic zone doesn't mean necessarily that the dog is infected and will develop infection; [44] in the same way , any marrow positivity may be followed by negativization.[15]

As a general rule, the use fresh or frozen material is always advisable, or fixed in 95% ethyl alcohol. Formalin-fixed and parafinized samples yield lower diagnostic performances, but anyway they can be used. It is therefore advisable to request this test to the laboratory in cases when cytologic and histologic studies are negative despite a strong diagnostic suspect (see above).

1.4.4 Serological methods

Seroconversion occurs in the period of some months from infection, on average 5 months (range: 1-22) for natural infections and 3 months (range: 1-6) for experimental ones.[48] *Only* in dogs with parasitic dissemination antibody titers tend to be high or increasing. Several diagnostic techniques are available. Someone, like Western Blotting, shows very good diagnostic performances, but aren't widely employed because labour times and costs. Those most commonly available include rapid immunomigration test, ELISA techniques and indirect immunofluorescent assay test (IFAT), schematically described below.

Rapid immunomigration test. It is easy to do and can be performed in-house too, but its diagnostic efficiency is lower than ELISA and IFAT: while specificity is mean-high, sensitivity is low (30-70%)[44,49,50] and can cause false negative results. In these cases, if a strong diagnostic suspect persists, serologic study must be extended using one of the other two tests. In the case of positive result, the limit is that the test doesn't allow to evaluate antibody titer, which instead can be useful to identify animals with parasite dissemination and to monitor therapeutic response.

ELISA test. Serum to evaluate is placed in *Leishmania* antigens-coated microplates. In positive cases, colorimetric reaction appears that can be quantified by spectrophotometry and therefore isn't dependent on operator-related variables. It is a specific test with a mean-high sensitivity (70-100%). Sensitivity is very high when used tests are based on association of several antigens of promastigotes, to increase the numbers of epitopes that can fix any occurring antibody.[44,49,51-53] Furthermore, the test allows to quantify specific antibodies.

IFAT. IFAT is performed placing the serum to evaluate on slides with *Leishmania* promastigotes. Any present antibody bind to promastigotes and positivity is shown using fluorescent anti-antibodies. In this case it is also possible to determine antibody titer using serial dilution of serum to evaluate. Because IFAT sensitivity and specificity are near 100%,[44,50,51] the test is considered by World Organization for Animal Health (OIE – *Office International des Epizooties*) as reference serologic method.[54]

As for ELISA and IFAT, it is advisable to make sure that reference laboratory always performs "end point" titrations, i.e. until the last positive dilution and not simply until a

predetermined threshold value for positivity. Although antibody titer isn't always related to seriousness of clinical signs (especially for mean-low values), as a general rule antibody titer determination allows to distinguish infected but non sick dogs, that will have a tendency to low titers, from sick ones with parasite dissemination, that will have a tendency to high titers. Definitions of "low" and "high" titer must always refer to positivity threshold indicated by reference laboratory. Most of them consider negative dogs with IFAT titres lower than 1:40, positive those with titers equal or higher than 1:80 and doubt dogs with a titer from 1:40 to 1:80. However, because some laboratories use different threshold titers, referring always to the same laboratory is advisable, especially in cases when any increase or decrease of antibody titers has to be verified. At any rate, considering the high variation of coefficient inter- and intra-assay that characterize serological studies, like in many other infectious disease it is adequate to consider as "high" only titers showing at least a fourfold difference with threshold value of positivity for the reference laboratory (e.g. if laboratory considers "positive" a titer equal or higher than 1:80, a titer higher than 1:640 has to be considered "high").

1.4.5 Methods to evaluate cellular immune response

Evaluation of cellular response is used to perform research on immune response in clinically obvious disease or on resistance to it. However, many techniques that can be used for scientific research are still unavailable in clinical practice. Nevertheless, indirect information on the state of cell-mediated response can be get from tests feasible *in vivo*, like intradermic test with leishmanin and determination of T lymphocytes CD4/CD8 ratio in peripheral blood by cytofluorimetry.[37]

1.5. **How to integrate the collected data to reach the diagnosis**

To reach the diagnosis of leishmaniasis, the integratation of signalment, history, any clinical sign and laboratory test results is necessary.

As a general rule, in dogs with clinical signs and modification of results of minimum data base of laboratory tests highly consistent with CanL, PCR, serology and (only in some tissues) cytology all have an high sensitivity.[44,55,56] In endemic areas, the problem could be ascertaining a cause-effect relationship between the occurrence of parasite and detected anomalies, because there is the risk of overestimating CanL. Therefore, in dogs with consistent clinical signs the approach that allows to optimize diagnostic efficacy of different methods and minimize operations on patient speeding up diagnostic times consists in performing immediately serologic examination and cytological analysis of explorable lesions, if any, and going on with more specific tests according to results of these initial assays. Possible combinations of results and their interpretations are reported in Box 3, showing that the patient has to be considered definitely affected by CanL when cytologic examination performed in tissues with consistent lesions (including bone marrow in case of anemia) is positive, independently of serologic results, that however in these cases should be most probably positive (except very rare cases of very localized lesions or in very early phases of infection). On the other hand, if cytology results negative serology become vital to consider a patient as sick or simply "suspected sick". While any high antibody titer denotes that the

BOX 3
Schematic draw of different combination of test results that can be obtained in dogs with clinical signs and clinicopathological changes consistent with leishmaniasis

Analysis	Result				
Serology	Positive	Negative	Positive†	Positive§	Positive§
Cytology	Positive	Positive	Negative	↓	↓
Other tests	↓ ↓ ↓ ↓	↓ ↓ ↓ ↓	↓ ↓ ↓ ↓	Presence of cutaneous clinical signs ↓ Histology,	No cutaneous clinical signs ↓ PCR on biopsy of bone

	↓ ↓ ↓	↓ ↓ ↓	↓ ↓ ↓	immunohistochemistry, PCR on skin biopsy Positive ↓ Negative ↓	marrow and/or lymph node Positive ↓ Negative ↓
Diagnosis	Sick‡	Sick‡	Sick‡	Infected/sick‡ Exposed[a;b]	Infected/sick[a;b] Exposed[a]
‡ sick with leishmaniasis; † high titer = 4-fold threshold of positivity for the reference laboratory; § low-intermediate titer; [a] monitor by serology; [b] rule-out other possible causes of present clinical signs.					

patient is sick, a low antibody titer doesn't allows to rule-out *Leishmania* infection, with the animal affected by different disease. In this case further information could be collected deepening diagnostic surveys in different ways according to present lesions:

- if cytologically negative lesions are cutaneous and cytologic pattern is highly consistent with leishmaniasis, the use of both cutaneous biopsy and histologic examination to detect the presence of *Leishmania* is necessary. Immunohistochemistry is suggested in presence of histological pattern consistent with CanL but routine stain don't show any parasites; if this approach is also negative, a PCR on cutaneous biopsy has to be performed. If this survey is negative too, the dog must be considered as "not sick" (with antibody titer meaning previous exposure to organism) or as "suspected infect", with a disease different from CanL. In this case, monitoring serial antibody titers is necessary, because they tend to rise with infection reactivation.
- If lesions aren't cutaneous and pattern is highly consistent with leishmaniasis, PCR must be performed in site with the highest probability to find the parasite, mainly including bone marrow and/or lymph nodes. If these surveys are negative the dog must be considered as exposed to infection and monitored during time. Instead, if they are positive, the dog must be considered infected or sick if it is possible to relate clearly the lesion to the infection. At any rate, possible progression towards disease must be monitored by serial serologic surveys.

2. CLASSIFICATION

[by Oliva G. (coordinator), Grotti A., Maroli M., Roura X., Solano Gallego L., Zatelli A.]

L. infantum infection can evolve, in few weeks or many months, to disease patterns extremely variable and polymorphous, not always easy to classify. Nevertheless, at the time of diagnosis the practitioner should always try to set the infection-disease in its evolutive stage, both to allow adequate therapy and to anticipate possible progressions towards more serious or irreversible phases. Therefore, the following classification isn't an attempt to "entrap" in a scheme this complex disease, but only a way to offer a useful tool for management of affected patients.

2.1. Definition of "exposed dog"

Dogs clinically healthy, with negative cyto-histologic, parasitologic and molecular diagnostic tests, and with specific antibody titres, but not higher than fourfold of threshold value of the reference laboratory are defined as "exposed". Usually, dogs exposed to infection with *L. infantum* are those living or having lived, during one or more transmission seasons, in an area where presence of phlebotomus vector of parasite has been established.

2.2. Definition of "infected dog"

A dog is defined as "infected" with *L. infantum*, when shows presence of parasite, demonstrable with direct (microscopy, culture, or PCR) and indirect (evidence of specific antibody)

methods. In endemic areas, the only positivity to PCR on cutaneous material in absence of lesions during transmission season (June-October) could not be sufficient to define a dog as infected.

2.3. Definition of "sick dog"

An infected dog can be defined "sick" when shows one or more clinical signs suggestive of leishmaniasis (Box 1). Given the extreme clinical polymorphism of canine leishmaniasis, an infected dog can be defined as "sick" even if showing one or more clinical signs from those reported in Box 1, as long as they could clearly be related to ongoing infection.

A dog infected with *L. infantum* can be defined as sick even if, in absence of detectable clinical signs, shows hematological, hematochemical and urinary alterations that can be related to leishmaniasis or presents one or more laboratory changes (Box 2), provided that they can be surely related with ongoing infection.

2.3.1 Definition of sick dog with severe clinical picture

A dog infected with *L. infantum* can be defined as sick with severe clinical picture if:

a) it has already been treated with one or more anti-*Leishmania* drugs and doesn't show any remission of clinical signs;
b) it is affected by proteinuric nephropathy;
c) it is affected by chronic renal failure;
d) it is affected by severe ocular disease that can cause functional loss and/or require immunosuppressive treatments;
e) it is affected by severe joint disease that can impair motility and/or require immunosuppressive treatments;
f) it is concurrently affected by other severe infectious, parasitic, neoplastic, endocrine or dysmetabolic disease.

3. MANAGEMENT OF THE LEISHMANIOTIC PATIENT WITH PROTEINURIA

[by Zini E. (coordinator), Castagnaro M., Lubas G., Zatelli A.]

3.1. Pathogenesis of renal injury

CanL is a common cause of glomerulonephritis in Mediterranean Basin. Renal injury caused by leishmaniasis can be primarily attributed to intraglomerular deposition of circulating immune complexes, formed by organism antigens and antibodies produced against it. Immune complexes deposition is mainly glomerular.[57,58] The deposition of immune complexes induce complement activation by classical way. The main useful functions of this way include immune complexes solubilization and their phagocytosis by macrophages and neutrophilic granulocytes. C3a and C5a components of complement have a recruiting action on natural immune cells, promoting the development of a local inflammatory condition that can be, however, harmful for the whole nephron.[59] In addition, some interleukines produced by macrophages have a chemotactic action on lymphocytes, with consequent increase of immune response. The activation of immune system causes mainly glomerular inflammation. By contiguity, tubulointerstitial compartment is involved too. Renal lesions cause proteinuria and promote renal failure development.[60]

3.2. Pathogenesis of proteinuria

In physiological conditions, the glomerule allows free filtration of proteins with molecular weight (MW) lower than 69 kD (e.g., lysozyme, β2-microglobulin) and a little amount of albumins (MW = 69 kD). On the other hand, proteins with higher molecular weight are retained in blood stream (e.g., transferrin, immunoglobulins). In addition, on surface of glomerular basal membrane there are several negative charges that are opposing filtration of protein with negative net charge (e.g., albumins). Proximal convoluted tube has the role of reabsorbing proteins filtered by glomerule. Normally, filtered amount of proteins with MW equal or lower than 69 kD is nearly

entirely reabsorbed and in definitive urine their concentrations are only minimal.[61] In case of glomerular injury, overall amount of proteins in the filtrate increases, especially in favor of proteins with MW equal or higher than 69 kD.[62] If tubular integrity is maintained, proximal tubular cells increase their activity to reabsorb the whole protein amount in excess. However, if tubular protein receptors are saturated, the amount of proteins detectable in definitive urine increases (proteinuria). There is a competition for tubular reabsorption in case of glomerular proteinuria, because tubular receptors for urinary proteins have normally a low specificity. Proteins with high MW compete within them, but also with low MW protein and vice versa.[61] Therefore, in definitive urine finding of proteins with both high and low MW is possible, although in case of isolate glomerular injury high-MW proteins can be find in higher concentrations.[62] In leishmaniasis, however, inflammatory infiltrate induced by immune complex deposed in glomerules involves both glomerular and tubular pools.[57,58] Because renal injury is mixed-type, thus, detectable proteinuria has both glomerular and tubular origin (mixed proteinuria).[60,62,63]

3.3. Clinical evaluation of proteinuria

Quantitative measurement of proteinuria by urinary protein:creatinine ratio (UP/UC) and qualitative tests by sodium dodecyl sulfate-agarose gel electrophoresis (SDS-AGE), or SDS-polyacrylamide gel electrophoresis (PAGE) are non-invasive ways to identify the presence of a renal injury in several glomerulonephritis, including those leishmaniasis-induced.[60,62,63] In dogs, quantitative evaluation by UP/UC involves some interpretative problems, because authors don't agree on univocal threshold value to define presence/absence of proteinuria and standardized protocol for protein measurement in urine doesn't exist. In general opinion, UP/UC values lower than 0.5 show unsignificant proteinuria, from 0.5 to 0.7 are doubt and require further evaluations and over 0.7 are indicative of proteinuria.[64] In addition, in dogs with renal proteinuria UP/UC doesn't allows to differentiate the type of current injury. However, in dogs with leishmaniasis quantitative measurement of proteinuria plays a role of primary importance, mainly in follow-up during therapy. In fact, periodic UP/UC evaluation offers to the practitioner a useful parameter to evaluate therapy efficacy.[65]

In contrast to quantitative investigation, the qualitative urinalysis in dogs with nephropathies of different etiology seems could characterize renal lesions.[62] Although qualitative examination doesn't give information about the type of glomerular injury, it is able to define the extent of tubulointerstitial damage. Especially, the occurrence of protein bands with MW from 10 to 20 kD is particularly sensitive and specific for identification of dogs with severe tubulointerstitial damage. Generally, however, except a promising contribute to characterization of dogs with most serious tubulointerstitial damages, in veterinary medicine the number of reported studies on qualitative method isn't still sufficient to allow to define diagnostic and prognostic value, unequivocally.

3.4. Therapeutic approach to proteinuria

3.4.1 General aspects

In some experimental models of proteinuria it has been shown that excessive protein reabsorption by cells of proximal convoluted tube can perpetuate renal injury, leading to progressive reduction of the number of nephrons.[61] In fact, proximal convoluted tubular cells having excessive protein reabsorption can act as antigen-presenting cells with consequent lymphocytes recruitment and flogosis activation. Not only the tubules, but the glomeruli too can be damaged by an excessive protein filtration. Particularly, mesangial cells of proteinuric patient proliferate and synthesize higher amount of matrix. In human beings and in experimental animals, the level of proteinuria is recognized as an independent risk factor for development and progression of renal failure.[66] Recently, an association of level of proteinuria at the time of the diagnosis and likelihood of developing renal function worsening, uremic crisis and death has been shown in dogs with chronic renal failure.[67]

An up-to-date list of therapeutic tools is reported in following sections that can be used to treat protein-losing nephropathy in dogs, especially in leishmaniasis.

3.4.2 Allopurinol and pentavalent antimonium

Immune complexes play an essential role for the pathogenesis of renal injury and proteinuria development in cases of leishmaniasis.[57,58,60] Therefore, the correct use of anti-protozoan therapy (e.g., allopurinol, pentavalent antimonium and N-methyl glucamine) could decrease with time the number of circulating immune complexes. Reduction of immune complexes deposed into the glomerule could be advantageous to anatomic and functional integrity of the whole nephron. Concerning this, recently beneficial effects of allopurinol have been shown when the drug was used as single therapy in dogs with leishmaniasis.[65] Especially, it has been noted that, compared to placebo, after six months of allopurinol administration (10 mg/kg/q12h, PO): i) prevents proteinuria development in infected dogs, that show neither proteinuria nor increased creatininemia; ii) decreases the level of proteinuria in infected and proteinuric dogs without increasing the creatininemia. In infected and proteinuric dogs with increase of creatininemia, level of proteinuria doesn't show a significant worsening after six months. However, no dogs of this group received placebo; therefore, it is impossible to establish if allopurinol has caused a positive effect on the whole. In addition, compared to placebo allopurinol prevents deterioration of glomerular filtration rate (GFR) in infected and proteinuric dogs without any increase of creatininemia. In this group, beneficial effects on GFR are associated with improvement of tubulointerstitial lesions.

Similar studies on the use of pentavalent antimonium, alone or associated with allopurinol, have not been carried out in dogs with leishmaniasis.

Administration

Several therapeutic protocols have been published to treat leishmaniotic dogs with allopurinol and pentavalent antimonium.[68] While some of them use only one drug, others employ a combination. Which protocol is superior for treatment of proteinuria or renal injury hasn't been still verified. In this section, CLWG limits to report current knowledge about side effects of the two drugs on renal function.

In dogs with leishmaniasis, six months of therapy with allopurinol (10 mg/kg/q12h, PO) can cause xanthine cristalluria.[65] Cristalluria is associated neither with clinical signs nor with stones formation. In human beings, half-life of the drug increases if GFR is decreased. The dosage of allopurinol is sometimes reduced in dogs with increased creatininemia, although it is not known if half-life increase in this species too and if it is associated with side effects.

As for CLWG experience, allopurinol doesn't seem to have detectable adverse effects on kidneys. At the above dosage, allopurinol reduces proteinuria and nephropathy progression in dogs without any increase of creatininemia. Efficacy hasn't been still showed in dogs with increased creatininemia.[65]

Pentavalent antimonium can cause tubular toxicity in human beings, and patients with reduced GFR are more susceptible. The excretion of pentavalent antimonium in dogs is through renal for more than 80% of total. Clear side effects on renal function following the use of the drug have not been reported in dogs with leishmaniasis. However, dosages so far reported in veterinary literature have been somewhat variable (20.4-50 mg/kg, q12-24h, for 1-6 weeks, usually IM or SC), and most studies involved animals with normal creatininemia. In a recent study, pentavalent antimonium (75 mg/kg, q12h, for 21 days, IM) has been administered as single therapy in three dogs with increased creatininemia and, in one case, clinical signs of azotemia.[69] This therapy caused renal function improvement in two asymptomatic dogs. Symptomatic dog died three days after the beginning of treatment because of worsening of clinical signs. It has been supposed that pentavalent antimonium further impaired renal function.

On the basis of this information, the CLWG underlines the opportunity of verifying over and over again serum creatinine and BUN during treatment, especially in dogs with increased

creatininemia. If blood levels of one or both parameters are increased, it is necessary to reduce the dose or to discontinue administration of pentavalent antimonium.

3.4.3 ACE-inhibitors

ACE-inhibitor enalapril (0.5 mg/kg/q24h, PO) proved to be useful in dogs with idiopathic glomerulonephritis.[70] Enalapril reduced amount of protein lost in urine, probably because of reduction of glomerular endocapillar pressure and mesangial hypertrophy.

Concerning the use of ACE-inhibitors in dogs with chronic renal failure, in an experimental model (partial nephrectomy) enalapril (0.5 mg/kg/q12h, PO) showed its efficacy in decreasing glomerular endocapillar pressure and mesangial hypertrophy and slowing the development of tubulointerstitial lesions.[71]

Administration

The CLWG suggests to use routinely enalapril at the dose reported by Grauer et al.[70] (0.5 mg/kg/q24h, PO) in dogs affected by leishmaniasis with proteinuria (UP/UC ratio > 0.7) and /or increased creatininemia. Because ACE-inhibitors can reduce systemic pressure and possibly renal blood flow, verifying over and over again serum creatinine and BUN is vital. Particularly, according to experience of CLWG, the worsening of renal function can occurs especially during the first two weeks of treatment. In this case, reducing the dose of the drug or discontinuing the administration can be necessary.

According to CLWG, potential beneficial effects on proteinuria usually occur after 1-2 months of administration.

3.4.4 Acetylsalicylic acid

Acetylsalicylic acid is used to reduce platelets activation, because they can exacerbate the condition of hypercoagulability occurring in protein-losing nephropathies. In dogs, at low dosage (0.5 mg/kg/q24h, PO) the drug inhibits platelet cyclooxygenase, sparing endothelial one.[72] Although with unproved usefulness, acetylsalicylic acid is used for adjunctive therapy in proteinuric dogs to control hypercoagulability.[70]

Administration

The use of acetylsalicylic acid is recommended in dogs with leishmaniasis and proteinuria, irrespective of the presence or absence of increased creatininemia. With the above reported dosage, the CLWG noted neither alterations of coagulation profile nor worsening of renal function.

3.4.5 Diet

The level of proteinuria can be reduced using a low-protein diet.[73] However, it's worth noting that low protein diets may have difficult to maintain adequate body weight and muscular mass (Body Condition Score) and normal plasma albumin concentrations. For this reason and because dogs with leishmaniasis show often poor muscular mass, we think that the use of a low-protein diet has to be evaluated case by case, separately. However, low protein diet can reduce clinical signs if the dog affected with leishmaniasis has an increased creatininemia and shows clinical signs of azotemia. In addition, a decrease of phosphorus in diet can slow renal function decline and increase survival times in dogs with increased creatininemia.[74]

Administration

The role of a restricted protein diet hasn't still been clearly defined in dogs with leishmaniasis and proteinuria, in the absence of an increase of creatininemia. Instead, the use of a commercial low-protein and low-phosphorus diet is indicated in dogs with increased creatininemia, irrespective of the presence or absence of proteinuria. If body weight of the dogs decreases, it is possible that the animal doesn't eat adequately or the diet doesn't satisfy daily requirements. In the first case it is recommended to identify factors that can induce anorexia (e.g., nausea,

gastrointestinal ulcers, drugs overdose) and treat them adequately (e.g., antihemetics, H_2-antagonists). In the latter case, it could be indicated to increase the offered amount of feed or to use a different diet.

3.4.6 ω-3 essential fatty acids

Dietary supplementation with ω-3 fatty acids showed a nephroprotective effect in dogs partially nephrectomized.[75] Because of a beneficial effect on glomerular hemodynamics, ω-3 essential fatty acids prevented the deterioration in GFR and renal structure compared to saturated fatty acids or ω-6 fatty acids supplementation. Supplementation also maintained the level of proteinuria on slightly low values. However, this latter effect hasn't been significant. Essential ω-3 fatty acids could be useful as a therapeutic help in dogs with leishmaniasis and increased creatininemia.

Administration

The use of ω-3 essential fatty acids in dogs with leishmaniasis needs to be further studied, since studies on this subject are lacking.

3.4.7 Anti-hypertensive therapy

In leishmaniasis, 61.5% of dogs with proteinuria and increased creatininemia show systemic arterial hypertension. Systemic hypertension is also present in about 10% of proteinuric dogs without any increase of creatininemia.[76] Systemic hypertension has been proved as a risk factor for renal injury progression in dogs.[77]

Therefore, measuring systemic pressure is necessary in dogs with leishmaniasis, as like as treating hypertension, if any. At present good anti-hypertensive action is obtained using the calcium channel blocking agent such as amlodipine besylate (0.1-0.5 mg/kg/q12-24h, PO).[78] Association of an ACE-inhibitor drug is indicated to improve glomerular hemodynamics, because the drug could increase glomerular capillary pressure.[79]

Administration

Systemic anti-hypertensive treatment is administered when systolic arterial pressure is higher than 180 mmHg (Doppler method), or between 150 and 179 mmHg in association with clinical signs or laboratory changes consistent with systemic hypertension (e.g., retinal hemorrhages, hypertensive cardiopathy, cortical neurological signs, proteinuria, azotemia).[76] Anti-hypertensive therapy is indicated both in dogs with preserved renal function and in dogs with increased creatininemia. The incorrect use of anti-hypertensive therapy can highly reduce glomerular filtration pressure and GFR. Therefore, monitoring also creatininemia besides systemic pressure is recommended during therapy. An increase of creatininemia could indicate a treatment-induced worsening of renal hemodynamics.

Usually, systemic pressure reduction is obtained in few hours from the start of treatment.[79] Pressure monitoring should be performed several times during the first 2-3 days and afterwards regularly (e.g. every 1-2 weeks). As for the experience of CLWG, in some dogs the association of amlodipine and ACE-inhibitor isn't sufficient to reduce systemic pressure. In these cases, modifying amlodipine dosage (if used at low doses) or adding another drug (e.g., β-adrenergic blocking agents, aldosterone antagonists) could be indicated. Usually, CLWG suggests to add a selective β-adrenergic blocking agent (atenolol: 0.5-1.0 mg/kg/q12-24h, PO) as first choice drug. The goal of anti-hypertensive therapy is to reduce systolic arterial pressure to values lower than 150-160 mmHg (Doppler method). If this goal should be impossible to obtain, pressure should be however reduced of at least 50-60 mmHg in comparison with initial values.

Keywords

Leishmaniasis; dog; guidelines; diagnosis; classification; proteinuria.

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