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Update on the epidemiology of Canine Leishmaniosis in Southern Europe.
Guadalupe Miró

6

Immunology of Canine Leishmaniosis: What do I have to know?
Fernando Farinhas

9

Diagnosis of Canine Leishmaniosis: learning from difficult cases.
Lluis Ferrer

10

CaniLeish®: Development of a new preventive tool.
How do you produce a vaccine which really works?
David McGahie

13

Results of safety trials with CaniLeish®. What can be expected?
David McGahie

15

Results of efficacy testing with CaniLeish®.
How does it perform against high level natural challenge?
Christophe Rême

17

Practical use of CaniLeish®. What do I need to know to get the best from this new tool?
David McGahie

19

How can I best treat clinical cases of Canine Leishmaniosis? Current guidelines.
Guadalupe Miró

20

Treatment was successful...What next?
Lluis Ferrer

22

CaniLeish® was developed by BVT (BioVéto Test), a wholly-owned subsidiary of Virbac, in partnership with IRD (Institut de Recherche pour le Développement) and Virbac’s R&D teams. This project is based on an IRD’s patented invention relating to Leishmania culture, on which IRD has granted an exclusive patent licence to BVT for animal health applications.
Canine Leishmaniosis prevention: the challenge of a vaccine for Europe

Maite Verde

Canine Leishmaniosis (CL) is a high prevalence disease in dogs of the Mediterranean Basin countries. Any dog is susceptible to the disease when it is bitten by infested Phlebotominae. An individual can present the disease, clinically, primarily depending on the immune response, concomitant parasitic diseases, possible re-infections and the pathogenicity of the Leishmania strain.

In the last thirty years significant research has been achieved resulting in a disease model and a wealth of information about the clinical forms, the most sensitive and specific laboratory tests, prognostic considerations based on the patient biopathological situation and the other concomitant diseases, which has made us to bet more confident, making better decisions on how to treat and obtain successful outcomes.

The therapeutic options are based on the combined use of glucantime or miltefosine, with allopurinol. But in any case, the application of preventive measures to avert disease development is essential. We are attending the presentation of the latest progress in preventive medicine against CL in Europe: CaniLeish® (the first vaccine against CL registered in the European Medicines Agency in 2011, by Virbac Laboratories).

At this symposium we are honored to attend a CL state-of-the-art discussion that may interest animal clinicians hosted by Drs. Guadalupe Miró, Fernando Fanfas and Lluís Ferrer. These experts combine expertise and experience of the highest level in the field of immunology, pathology, parasitology, diagnosis and treatment of CL. In addition, Mr. David McGahie and Mr. Christophe Rème will provide data on the safety and efficacy of CaniLeish® vaccine and on how to use it from a practical perspective.

We are looking forward to take this opportunity to the next level in our CL fund of knowledge and the most expected preventive option.

Update on the epidemiology of Canine Leishmaniosis in Southern Europe

Guadalupe Miró

Canine Leishmaniosis extends mainly in three geographical areas: Brazil, China and the Mediterranean basin. In the Mediterranean basin, the dog is the main reservoir of L. infantum infection domestic cycle.

Also exists a sylvan cycle of the L. infantum infection affecting wild canidae such as the fox, the wolf or the jackal. The prevalence found in foxes using the PCR technique in investigations performed in Spain and Southern Italy was 40-75%; therefore, the fox could be considered a secondary reservoir which is thought to be the link between both cycles due to its preference for living near domestic populations. Leishmania infantum has also been detected in wolves, rodents, horses and cats (although there are many issues to solve about the epidemiological role of these species).

In endemic areas there is a high percentage (50%) of infected but clinically healthy dogs. In contrast, the percentage of dogs with leishmaniosis is lower (3-5%) because Canine Leishmaniosis is a disease in which infection is not always equal to clinical disease.

Therefore, Canine Leishmaniosis prevalences registered in Spain and Southern Europe are highly variable depending on whether the data obtained by molecular techniques refers to seroprevalence or infection.

In the epidemiology of this important zoonosis the arthropod vector Phlebotomus spp plays an essential role, and the presence of L. infantum will be influenced activity and incidence rates.
Currently, we should be considering other non-vectorial transmission routes of this infection, which have been proven: vertical transmission (although the cases included in the literature are anecdotal), blood transfusion (of great importance in endemic areas where blood donors may often be sub-clinically infected), venereal transmission (the presence of semen in infected males has been detected, who infected healthy females after intercourse). Their importance is still barely known and we will have to take into account in the near future.

Other hypothesis considered with respect to potential transmission routes, but that have not been proven yet, are transmission through other vectors, such as ticks and fleas (though experimental transmission to dogs is still to be proven) and transmission through direct dog-to-dog contact (a hypothesis considered to explain the presence of native cases in non-endemic areas in which the presence of any effective arthropod vector could be proven, as in the case of the United States). To define the infectious ability of dogs with parasites is very important, which can only be experimentally evidenced by direct xenodiagnosis. In general, sick seropositive dogs show greater infectivity for Phlebotominae than sub-clinical seropositive dogs and remain 'sterile' for Phlebotominae between 4 and 6 months after treatment.

Finally, it has been proposed to consider humans as a reservoir of Leishmaniosis in L. infantum/HIV co-infection, either by sharing used syringes or by the bite of Phlebotominae with parasites after being fed by sick people, in both cases we would be facing a real anthroponosis as the dog, and no other vertebrate than the human, take part in the disease cycle.

As for the characteristics of the host, it is worth mentioning that it is thought that certain dog breeds are more prone to having the disease such as, Boxer, Cocker Spaniel, Rottweiler and German Shepherd dog and that the development of the disease has an age-related bimodal curve, showing more cases in animals less than 2-3 years old and in dogs more than 8 years old.

Recommended references:
• Coutinho MT, Linardi PM, 2007, Can fleas from dogs infected with canine visceral leishmaniasis transfer the infection to other mammals? Vet Parasitol 147, 320-325.
• da Silva SM, Ribeiro VM, Ribeiro RR, Tafuri WL, Melo MN, Michalick MS, 2009, First report of vertical transmission of Leishmania (Leishmania) infantum in a naturally infected bitch from Brazil. Vet Parasitol 166, 159-162.
Immunology of Canine Leishmaniosis.
What do I have to know?

Fernando Fariñas

Introduction to the immune response

Infectious diseases in any individual are not only caused by the presence of a pathogen but by its interaction with the host immune system. The immune response can be classified in two types: the initial response that tries to contain rapidly the pathogen, in a non-specific, stereotyped way and unable to create any memory (innate immune response), and that able to set up a number of particular and highly specific strategies in order to eliminate the invading agent and to create a memory (adaptive immune response). To simplify, this adaptive response may consist of a predominant Th1 cellular response, producing high levels of cytokines IFN-γ, IL-12 and TNF-α and the production of IgG2a, a predominant Th2 humoral response with the production of cytokines IL-4, IL-10 and IL-5 and immunoglobulins A, E and IgG1, or a mixed Th1/Th2 response.

The immunity or innate response is an immediate response activated via receptors codified in a germline and known as pattern recognition receptors (PRRs) that can recognize molecular patterns that have been conserved through evolution in many pathogens. These molecules, known as pathogen-associated molecular patterns (PAMPs), stimulate cell signalling, gene expression and, therefore, the activation of inflammatory and antimicrobial functions. The innate response, apart from being a rapid line of defence against infection, initiates the process that enables the eventual development of the adaptive immune response and sets the immune memory.

Mammals can be infected by a huge number of microorganisms (viral, bacterial, protozoal, etc.) that fundamentally have different structures, biology, and spread mechanisms. However, the innate immune system has a limited number of available PRRs that enable the recognition of some common parts in very different infectious agents.

PAMPs are microbial molecular structures that have been conserved through evolution and, therefore, are shared by different microbial species. Moreover, PAMPs are essential for the growth of microbes and rarely modified by the organisms, enabling the recognition by the innate immune system. The second principle in the innate immune recognition is the detection of molecules with aberrant localization, mainly nucleotide structures.

Microbial infections are associated with the insertion of nucleotides (DNA and RNA) both in the endosome and cytoplasm, localizations considered as abnormal for these structures.

The innate immune system detects nucleotides in these aberrant localizations thanks to the expression of PRRs in the cellular endosome and cytoplasm in such a way similar to microbial recognition.

All viruses and a subset of bacteria and parasites spread in the infected host cell. Among parasites, some protozoa enter the cells and create a single membrane surrounding the cytoplasmic compartment, the parasitophorous vacuole, where replication takes place. The membrane glycoporphatidyl-inositol (GPI) is the main PAMP for the recognition of protozoa in the extracellular space, whereas in the endosome, receptors for DNA and dsRNA have been reported to contribute to the innate immune response against them.

Immunology of Canine Leishmaniosis

The resistance of dogs to Leishmania infection depends on the development of a strong cellular immune response, in which active CD4+ Th1 lymphocytes take part by means of the synthesis and release of cytokines such as interleukin-2, interleukin-12 and interferon-gamma (IFN-γ), each of them required for macrophage activation, the effector response of CD8+ cytotoxic T-cells and the cytotoxic activity of natural killer cells (NK), that lead to the destruction of the parasite. The leishmanicidal activity is due to the increased capacity to produce toxic oxygen and nitrogen radicals (NO) by macrophages in response to IFN-γ. However, if the response is leaded by CD4+ Th2 cells that produce interleukins 4 and 10, it may predispose to a more serious clinical picture.

Some published studies suggest that canine leishmaniasis could be a very useful model in the investigation of human visceral leishmaniasis. Certainly, one could argue that there are some differences between canine and human leishmaniasis clinical and pathological aspects, such as the greater human resistance to infection and the presence of clinical manifestations in dogs (e.g. ulcerative dermatitis associated with infection) that have never been reported in humans.
Despite this, both species have many immune similarities. Thus, as already mentioned, the resistance to infection is related to the type and quality of the immune response both in human and canine leishmaniasis and whether it is cutaneous, mucocutaneous or visceral leishmaniasis. In human cutaneous leishmaniasis, the local production of IFN-γ mediating Th1 responses plays a critical role in the resistance by means of the activation of infected macrophages. Likewise, during the active clinical phase of visceral leishmaniasis, there is a marked depression of both the Leishmania-specific lymphoproliferative responses and the production of IFN-γ and the delayed hypersensitivity responses against different antigens of the parasite. This anergy seems to be mediated, at least partially, by a suppressive effect of IL-10 and low levels of IL-12. All these mechanisms are essentially identical to those described in dogs except for the role of IL-10 in dogs, where it wouldn’t seem to play such an important or decisive role in the pathology.

Paying attention to humoral immunity against leishmaniasis in sick humans and dogs, there have been polyclonal, and sometimes monoclonal, increases in immunoglobulin production by hyperactivation of B cells. These antibodies are non-protective and their level is even positively correlated to the presence and severity of the disease. Thus, resistant humans and dogs tend to produce all kind of antibodies in a low frequency and concentration whereas the critically-ill ones do the contrary. Amongst these immunoglobulins, IgG predominate whereas IgM, IgE and IgA are produced in much lower amounts. Even, it’s been found that some of these antibodies created during infection have characteristics of auto-antibodies against erythrocytes, myocytes and nuclear components, mainly in the dog, which could explain in part the number of clinical manifestations of the disease in them.

On the other hand, Leishmania parasites are not inert beings but have a number of complex strategies to attack, infect and survive within macrophages. The host may fail to control the disease due to some strains that have the ability to resist the microbicidal action of the activated macrophages and to produce a state of severe immunosuppression.

As we said, Leishmania has several surface molecules that act as virulence factors as well as to evade the host immune system. These mechanisms include a dense glycocalyx formed by lipophosphoglycans and glycoprophosphatidylinositol as well as proteins secreted by the parasite, including an arsenal of glycoconjugates, sulfated proteoglycans, acid phosphatases and metalloproteinase gp63, that abounds in the surface of promastigotes.

There is no doubt that the knowledge of these immune mechanisms has contributed and will further contribute to the development of immunoprophylactic and immunotherapeutic strategies that will allow a better control and treatment of the disease.

Diagnosis of Canine Leishmaniosis: learning from difficult cases

Lluís Ferrer

Introduction

The basis for establishing a correct diagnosis is the understanding the difference between infection and disease. Studies show that the percentage of infected dogs in areas where the disease is endemic is very high (probably over 50%), but only some are seropositive and an even smaller percentage are developing the disease. It is known that once the promastigote is inoculated into the dog's skin, the disease progression follows different paths. Maybe (not shown in a reliable way), in a small percentage of animals, innate immune mechanisms abort the infection locally. In most cases, however, the infection spreads locally and triggers a specific immune response. Depending on the type of response, the infection progresses to clinical disease or remains under control. In those animals that develop a Th1-dominated cell-mediated immune response (probably most), there is activation of macrophages and destruction of parasites by mechanisms mediated by NO. In contrast, in those animals with a Th2-dominated immune response, with predominance of humoral antibody production (IgG1, IgG2), infection cannot be controlled and progresses to severe clinical disease.

The factors that make a certain animal progress towards the immune control of the disease or to the clinical disease are unknown. Genetics is probably the most important. There are dog breeds in which clinical disease is rare (Ibizan hound) and others in which clinical disease is very common (Rottweiler, German Shepherd, etc.). A recent study in two breeds (Boxer, German Shepherd) attributed to genetics a 60% of importance in the progression of the infection. An important aspect is that the situation of 'resistant' (non-effective response) or 'susceptible' (ineffective humoral response) is not definitive. An immunosuppressive disease, drug treatment or other factors may make that an animal that has kept the infection under control for years develops clinical signs.
of leishmaniasis. Similarly, the current therapy (antimonials, miltefosine, allopurinol) gets the immune response directed towards the effective cell-mediated response and controls the disease.

In animals where the infection progresses, several pathogenic mechanisms are triggered. First, the infection spreads to many organs and systems (spleen, lymph nodes, skin and mucous membranes, liver, pancreas, testes, bowel...), in which granulomatous inflammatory processes occur. Furthermore, circulating immune complexes (ICs) are produced and deposited in renal glomeruli, uvea, blood vessels and joint synovium. Deposition of ICs is a major cause of clinical sign. Moreover, other pathogenic mechanisms such as the formation of autoantibodies or chronic anaemia are produced in the course of the disease.

**Diagnosis of the disease**

The diagnosis of leishmaniosis can be very easy or very difficult. The difficulties arise from:

1. The different clinical presentations of disease
2. The confusion between infection and disease
3. The presence of secondary infections / parasitic diseases

It is well known that the clinical leishmaniasis is a very pleomorphic disease. It is expressed differently in each animal because of the genetic background and associated infections, amongst other factors. Briefly, the main clinical signs and clinical presentations of the disease are:

1. Skin lesions: exfoliative dermatitis, skin ulcerations and mucocutaneous junction ulceration, skin nodules, etc.
2. Lymphadenopathy (reactive lymphatic hyperplasia)
3. Weakness, anorexia, weight loss, muscle atrophy, mild hyperthermia
4. Renal failure (proteinuria, azotemia)
5. Ocular lesions (keratitis, uveitis, panophthalmitis, glaucoma)
6. Lameness (arthritis, myositis)
7. Epistaxis
8. Large bowel chronic diarrhoea (colitis)

The disease has an insidious onset and a chronic course and develops over weeks or months. Sometimes, it is useful to know that the process has not responded to antibiotics or steroids.

Clinical tests are also highly variable, reflecting the variety of pathogenic mechanisms as well as the development and severity of the disease. Hyperproteinemia due to hyper-gamma-globulinemia, moderate non-regenerative anaemia and proteinuria of varying severity are common findings, but there are very different presentations.

In summary, we could say that the diagnosis is established in a patient with consistent clinical signs or clinicopathological alterations and a positive quantitative serology (IFAT, ELISA).

It must be remembered that the diagnosis is a clinical opinion, issued by the surgeon after evaluating a series of findings and results (history, exams, analysis...). There are no ‘DIAGNOSTIC’ tests. According to the so-called evidence-based medicine, a good diagnostic test is that one that increases the probability of a specific diagnosis.

Quantitative serology is the best way to confirm a diagnosis of leishmaniosis in clinically suspected cases because:

i) It is known that a high antibody titre is correlated with clinical disease (i.e., it does not only indicate infection)

ii) It's simple, fast and inexpensive and is readily available

iii) It can be used to assess patient response to therapy

Much of the confusion stems from the use of PCR techniques, more adapted to the identification of the infection than to the regular diagnosis. The diagnosis of infection is only interesting in specific epidemiological or research works. It can be done by means of:

- PCR (different techniques)
- Serology
- Culture (low sensitivity and high cost)
- Cytology and histopathology (low sensitivity)
- Intradermal Test
The problem is that the use of PCR techniques (simple, nested or quantitative) has been extended on the conviction that it allowed a highly sensitive diagnosis of the disease. However, one must bear in mind that these techniques only report the presence of the parasite's DNA in the sample. Therefore, a negative result is compatible with:

1. A dog that is not infected, that does not suffer leishmaniosis
2. A dog infected with the parasite in other parts of the body but not in the sample (common situation)
3. A dog infected and with leishmaniosis, with the parasite in other parts of the body but not in the sample (rare but common situation)

Likewise, a positive result is consistent with an infected animal that has no leishmaniosis (a situation more common in endemic areas) and with a sick animal. The problem is that PCR is a qualitative technique (positive/negative) and does not help to distinguish an infected-healthy animal from an infected-sick one. In endemic areas, this is a serious problem. Quantitative PCR partially resolves this problem. However, the correlation between clinical picture and test results is much greater with serology, as shown by various studies.

The figure shows a possible protocol for a definitive diagnosis [Solano-Gallego et al, 2011]:

**Figure 1** Diagnostic approach to dogs with suspected clinical signs and/or clinicopathological abnormalities consistent with Canine Leishmaniosis

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Dog with clinical signs and/or clinicopathological abnormalities compatible with CanL

  POSITIVE  | Quantitative serology  | NEGATIVE
  HIGH  | LOW  | Cytological/histological evaluation
  YES  | Leishmania amastigotes
  YES  | PCR  | NEGATIVE

Confirmed CanL
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**References**

History of the development

The history of CaniLeish® research dates back to the early 1980s. At this time a category of proteins, the ESPs, standing for Excreted Secreted Proteins, had been identified in numerous species of unicellular parasites such as Leishmania, Babesia and some others. This category of proteins was thought to be involved in macrophage infection and therefore held potential for use as antigens, either in diagnostic kits or in immunotherapeutic or immunoprophylactic vaccine applications. However, these ESPs were produced only in very limited quantity and purification from the other components of the culture medium was difficult as Leishmania required very rich culture media and inoculation in animal hosts to complete its life cycle.

The first breakthrough was an invention from the team of Jean-Loup Lemesre in the Institut de Recherche pour le Développement (IRD) of a completely defined in vitro medium containing only precise quantities of small molecules such as sugars, amino acids and vitamins that was able to sustain the development and multiplication of the parasite. This medium is also serum free and cell free, which is important because the only proteins that will be found in the culture medium supernatant are of Leishmania origin – the Leishmania infantum ESP (LiESP). The IRD team then began a collaboration with BioVéto Test (BVT), a diagnostics company that is now a wholly-owned subsidiary of Virbac, to explore the potential of the LiESP antigens, initially in the context of some diagnostic tests they were developing and then later in the context of some therapeutic and prophylactic canine vaccination. The IRD granted an exclusive licence to BVT for the use of the invented method of producing LiESP for animal health applications.

Between 1997 and 2003, in conjunction with the French National Veterinary School of Lyon, trials were performed with a prototype LiESP vaccine adjuvanted with muramyl dipeptide (MDP) which produced encouraging results when used as immunotherapy and also for disease prevention in both challenge model and field conditions. In the field trial the infection rate found in the control group was low (less than 7%), but the potential for use of ESP in a prophylactic vaccine was confirmed. However the MDP adjuvant was only used in experimental settings and it is known that its derivatives can also induce a Th2 response in aqueous conditions. Furthermore ESP production at industrial levels had not been defined. Therefore significant further work was required to develop the concept of ESP use to a vaccine which could be produced commercially. The Virbac R&D team joined the collaboration to add their expertise in vaccine formulation and development in order to bring the vaccine to its current form.

Developmental steps

Various developmental steps were required. It was necessary to define the parameters required for the industrial scale-up of the ESP production from the small volumes of the pilot batches to batch sizes which would enable the product to be made available widely to general practitioners. It was also necessary to further define the character of the ESP. As part of this process the presence of the major antigens in the vaccine – parasite surface antigen (PSA) was confirmed. These proteins are present in a membrane-bound form (on both promastigotes and amastigotes) and also excreted/secreted in free form and are involved in macrophage-parasite interactions making them suitable vaccine targets. The unique production system used for CaniLeish®, using the parasites themselves to produce the proteins, means that the proteins retain their native conformation. The characterization of the ESP also allowed the further development of specific quality-control assays which provide assurance of the consistency of the protein profile from batch to batch.

In addition to characterizing and defining production methods for the ESP it was also necessary to select a suitable adjuvant for the vaccine. The main requirement is the safe induction of a Th1 profile response. Various options were considered and finally QA-21 was selected as it has an optimum benefit-risk profile and is an excellent inducer of the interferon-gamma (IFN-γ) mediated Th1 response.

Proving the concept – the action on the immune system

Choosing an antigen and adjuvant based on the literature evidence and pilot studies is not sufficient to provide confidence in the ability of the vaccine to perform well in full efficacy testing. It is also necessary to follow the impact the vaccine has on the immune system of the dog. This can also allow some calibration of the correct levels of antigen and adjuvant.
During the development of CaniLeish®, several assays were used to assess the way in which it modulated the immune response. In conjunction with the laboratory of Dr J. Moreno in the WHO Collaborating Centre for Leishmaniasis, Instituto de Salud Carlos III in Madrid, the ability of the vaccine to induce memory cells which would then proliferate specifically in response to exposure to Leishmania infantum was confirmed. In the same laboratory this was then taken a step further and by means of the ELISpot test, which measures the proportion of the replicating cells which are producing IFN-γ, the Th1 polarity of these memory cells was confirmed. The combined use of these assays demonstrated that when experimental dogs were vaccinated with CaniLeish®, they were able to produce the expected Th1 memory cells to L. infantum.

However, it is also possible to take this one step further. In conjunction with the laboratory of Professor I. Vouldoukis at INSERM, UPMC-UMRS 945, Immunité et Infections, Paris, it was possible to utilize the Canine Macrophage Leishmanicidal Assay (CMLA) which confirmed that not only did the vaccine induce memory cells of a Th1 polarity, but that these memory T cells were capable of stimulating autologous macrophages to kill L. infantum promastigotes even 1 year after the vaccination. Further assays also confirmed that the level of parasite killing was correlated with induction in the macrophages of inducible nitric oxide synthase and production of a nitric oxide-based oxidative burst. This is consistent with the known mechanism of action of the desired Th1-dominated cell-mediated immune response.

Further use of these assays during an experimental challenge trial confirmed that even after a vaccinated dog is challenged with massive levels of living L. infantum parasites the correct Th1-dominated cell-mediated immune profile is retained. Combining this knowledge of the mode of action of the vaccine with the positive results of an experimental challenge study provides the confidence to progress to a natural challenge study which is the only suitable way to provide a final assessment of the efficacy of any vaccine against Canine Leishmaniosi.

Conclusion
The successful production of the first vaccine in Europe against Canine Leishmaniosis began with the selection of an appropriate antigen and adjuvant combination with each component being known for their potential in this regard. This was followed by significant investment to ensure that consistent production of the antigens was possible and by further work to understand the mechanism of action of the vaccine in the immune response of dogs. This culminated in the decision to progress to a natural challenge study over two years (which will be discussed in another session of this symposium) and the production of the relevant data required for the successful registration through the EMEA in the first half of 2011.

References
Results of safety trials with CaniLeish®. What can be expected?
David McGahie

Introduction

The safety profile of any vaccine is a very important factor for practitioners considering its use. Vaccines are normally used to prevent disease and therefore are used in previously healthy animals where administering the vaccine is a choice for the owner. This means that adverse reactions are perceived as being less acceptable than with medications used to treat serious illnesses. Additionally, the population which is vaccinated is likely to be much larger than the population which would receive a therapeutic medication and so even rare adverse responses may be more apparent.

Adverse reactions to vaccines are generally considered using two different approaches:
1) Reactions are classified as either 'normal' or 'inappropriate'. Transient fever, malaise, local inflammation and mild local pain have been classified as 'normal' reactions to vaccines because they are the result of the normal immune response to vaccination. Reactions persisting over a long duration or of greater intensity would be classified as 'inappropriate'.
2) Reactions are divided into 'local' and 'general'. Local reactions occurring at the site of injection soon after the vaccination is administered are relatively easy to link with the act of vaccination. General reactions such as gastrointestinal upsets are less easy to link to the administration of the vaccine with confidence, as they can also be caused by other unrelated events.

Significant work has been done throughout the development of CaniLeish® to ensure that the high level of protection it provides is also associated with a good safety profile and to assess the expected reactions that may be encountered during its use.

There are three main sets of data available to be discussed during this session.
A) Specific laboratory studies to assess the safety of extra doses and overdoses
B) Field trials to assess the safety of the vaccine under more normal conditions of use in a wide range of breeds and ages
C) Post-launching pharmacovigilance and informal feedback from Portugal where the vaccine is currently marketed

Safety data

A) Laboratory studies assessing the safety of an extra dose or an overdose

Two studies were performed. Firstly the safety of four doses - the normal protocol is three doses at three week intervals, and secondly the safety of an overdose where a double dose is given.

For both of these studies we have a similar study design:
- The vaccine doses were formulated to contain 10% more antigen than normal commercial doses.
- Ten dogs were vaccinated, and five dogs were kept as controls.
- The dogs were all four months old beagles (two months younger than the recommendation on the license, younger dogs being more likely to suffer adverse reactions to vaccination).
- The injection site was shaved to allow easy and accurate assessment of any reactions occurring in the local area.
- After each vaccination a detailed examination was performed of the clinical status and the local injection site daily for 14 days.
- Weekly haematological analysis was performed.

In the first study, ten out of ten puppies had local swellings on at least one occasion. The maximum size was ten centimeters, but most of the swellings were only noticeable by palpation, despite the site being shaved. All resolved spontaneously within 12 days at the most. A single puppy had mild hyperthermia but only after the first injection. Overall the four injections were well tolerated.

In the second study, where a double dose was administered, there was a slightly higher incidence of mild hyperthermia (three of the ten puppies, each for only 1 day). Seven of the ten demonstrated a local swelling; maximum size seven centimeters, maximum duration five days. Again the vaccine was very well tolerated.

B) Field trials:

Again two studies were performed. Both involved client-owned animals of various breeds and ages given the normal primary course of 3 injections at 3 week intervals. The injection sites were not shaved for these studies.
The first study involved 151 dogs. 61 were puppies aged 4 or 5 months, and 90 were 6 months of age or older (the licensed age range). All animals received veterinary examinations pre-vaccination, 4 hours after vaccination and days 2, 7, 14 and 21 post-vaccination as well as daily checks by the owners to specifically include examination of the injection site. The puppies received additional veterinary examinations on days 1, 3 and 4. Around 25% of the animals aged 6 months or older had detectable local reactions at the injection site when assessed by the veterinary surgeons. The maximum duration was 15 days in a single animal after the first injection. The maximum duration after the second and third injections was 1 week. In this age group the incidence of animals showing at least one general sign such as lethargy, hyperthermia, gastrointestinal upsets etc was in the region of 15 to 20% after each injection. However this includes events such as apathy and diarrhoea beginning as late as 14 days after the administration of the vaccine which could therefore be unrelated. The incidence of local reactions and general signs was, unsurprisingly, higher in the underage puppies. With regards to the general signs, in most cases it was not the same animals which reacted after each vaccination. For example, with the animals displaying hyperthermia, one puppy had hyperthermia after the first and third injections but not the second. One adult had hyperthermia after each of the three injections, but this animal also recorded episodes of hyperthermia unrelated to vaccination. All other cases of hyperthermia were isolated and not repeated. The second field study obtained the perception of the owners, who were aware that this was a vaccine in development and therefore had to be assessed for safety. It involved 231 dogs aged 6 months to 12 years. Around one quarter of these were young dogs close to 6 months old. The dogs were assessed by a veterinarian on the days of the vaccinations, and 2 weeks after the third vaccine. The owners were asked to check their animal each day and specifically to check the injection site for swelling or pain. Any abnormality was to be reported to the veterinarian. In this study, 8 dogs (3.5%) had local reactions reported, and in only one of these dogs was it reported after each of the injections. In this dog it was of shorter duration and milder intensity at each subsequent occasion. The most common general sign noted was lethargy, noted in 5 dogs (2.2%) lasting for 1 day in 4 of the dogs, and 3 days in the other. Therefore when used in a study under normal field conditions we saw a slightly higher rate of reported reactions than is usual for a conventional vaccine such as DHPPiL, but still found the vaccine to be very well tolerated in the majority of animals.

C) Post-launching pharmacovigilance and informal feedback

Clearly this is a dynamic situation, and at the time of writing insufficient data are available to draw any meaningful conclusions as the vaccine has been marketed for only a very short time. In the coming months and years this will be an important source of more detailed information.

Conclusion:

Veterinarians can expect some dogs to show short-term lethargy, moderate hyperthermia and local reactions. In most cases these should be self-limiting or respond rapidly to symptomatic treatment if required. Very occasionally this may be prolonged and rare cases of allergic reactions have been reported.

Notifying the owner that some short-term lethargy or local swelling may be expected in a minority of animals is sufficient in most cases to ensure that their expectations are met and to reduce any disappointment if this occurs. Only in very rare cases is it deemed necessary to not continue with the remainder of the primary vaccination schedule due to an adverse response after assessment of the risk benefit in the individual situation by the veterinarian. Indeed there appears to be a trend for local reactions to be of a smaller size and reduced intensity at subsequent vaccinations.

Overall this vaccine is well tolerated and the data confirms that CaniLeish® has a good safety profile for an effective anti-Leishmania vaccine.

Reference:

Results of efficacy testing with CaniLeish®. How does it perform against high level natural challenge?

Christophe Rème

Tools and classification
To understand how the efficacy of CaniLeish® was assessed, it is necessary to first appreciate the different tools used to monitor infection with Leishmania and how they were used to classify the progression of the disease.

The first set of tools looks at the effects of the parasite on the dog. This includes, for example, monitoring clinical signs such as apathy, anorexia and dermatological signs, and measurement of laboratory abnormalities such as reversed albumin-globulin ratio or increased total proteins. The measurement of the level of antibodies produced by the dog in response to the parasite is also done using IFAT.

The second set of techniques is for detection of the parasites themselves in the dog. Live Leishmania may be directly observed in aspirates from the bone marrow or the lymph nodes, and these aspirates can also be cultured by inoculation in specific medium. Culture is the more sensitive method of the two.

An even more sensitive method is the use of PCR to detect Leishmania DNA. This technique allows us to detect even traces of the parasites, and quantitative PCR has the additional advantage of allowing us to measure the parasite load.

These tools can be combined to evaluate the status of the dog towards infection and to classify this into several categories:

- **Stage I:** Dogs that have never met the parasite are Leishmania free. They will be negative in all tests.
- **Stage 2:** Dogs that harbor the parasite, but in which the parasite is not actively multiplying or is present at only very low levels will normally be PCR positive but negative on all other tests. This is termed subpatent infection.

In these first two categories, there is no evidence of active infection.

- **Stage 3:** When the parasite starts to multiply the dog is not only PCR positive but will also become culture positive. Initially, there are still no clinical signs, and so this active infection is termed asymptomatic active infection. However, it is only a matter of time before the disease progresses and becomes clinically apparent.
- **Stage 4:** In this final stage the IgG titre rises and clinical signs start to develop. This is therefore symptomatic active infection.

In these two final stages, there is confirmation of active infection, and already the dog is losing the battle with the parasite. The goal of any vaccine against leishmaniosis is to stimulate and direct the immune response of the dog such that it responds appropriately to parasite infection before it progresses to this active stage, preventing dogs from switching to the disease state.

Experimental challenge model
CaniLeish® was first evaluated using an experimental challenge model. 10⁵ Leishmania infantum taken from an infected dog’s spleen were injected by IV route to 20 dogs. Half of these dogs had been vaccinated one year before with CaniLeish®, with three injections as a primary course, while the other ten dogs were kept unvaccinated. The dogs were observed over a forty-seven week period after the challenge with regular blood sampling to assess haematology, biochemistry and IFAT parameters, and regular bone marrow aspirates to detect the parasite using both culture and quantitative PCR methods.

Seven of the ten control dogs developed a persistent infection throughout the study with positive PCR and positive culture results confirming active infection. This extremely high number emphasizes the severity of the challenge as it is normally expected that much less than 70% of dogs are naturally susceptible to developing the disease. One control dog became positive with PCR by the end of the study but remained negative on culture and so this was classified as a subpatent infection. In the other two dogs there was a transient positive PCR result, but these dogs returned to the negative state by the end of the study period. They blocked the infection and these dogs are naturally highly resistant to the disease.
By contrast seven of the ten vaccinated dogs blocked the infection. Four of these were positive for PCR and/or culture at some point but they reverted to the *Leishmania* free state by the end of the study and the other three were *Leishmania* free at all test points.

In three dogs, however, persistent active infection developed during the study period. This is a small number considering the severity of challenge and the results in the control group, and the fact that the challenge took place one year after the vaccination course and no booster injections were given.

This study confirmed that CaniLeish® induces a long-lasting immune response that was proven to be effective over one year after the last vaccination.

**High level natural challenge – the pivotal efficacy assessment.**

In order to truly assess the efficacy of a vaccine against Canine Leishmaniosis, it is necessary to see how it performs against high level natural challenge where there is a constant high infection pressure provided by wild-living sandfly vectors. The pivotal efficacy study with CaniLeish® used such a natural challenge under field conditions. This study was conducted in two parts. In the first part, 80 *Leishmania* free beagles of approximately 6 months age were housed in protected laboratory conditions and randomized into two groups. One group was given a primary course with CaniLeish® (three injections at three weeks intervals), while the other half of the dogs remained unvaccinated. The dogs were followed for nearly one month after vaccination to monitor the serological and cellular response in vaccinated dogs.

The dogs were then moved to open kennels in two very contaminated areas; one in Spain (near Barcelona) and the other in Italy (near Naples) with equal numbers of vaccinated and control dogs at each site. The dogs were transferred during the sand fly season to ensure rapid strong exposure and they were exposed to this natural challenge over two consecutive transmission seasons in 2007 and 2008. No repellent was allowed for these dogs, or any treatment against leishmaniosis. The vaccinated dogs received an annual booster with CaniLeish®.

The dogs were assessed every three months using clinical and paraclinical examinations, as well as culture of bone marrow or lymph node aspirates and quantitative and qualitative PCR to detect parasites. These parameters were used as explained previously to classify the dogs into the four infection categories and specifically to evaluate whether an active infection was developing in these dogs or not.

The severity of the natural challenge in these conditions was confirmed because 72% of the control dogs became positive by PCR at least once over the two year period of the study, clearly indicating a very strong parasitic pressure. This is further emphasized by the fact that 23% of control dogs developed clinical symptoms of the disease within the two years of the study, and in a further 10% an asymptomatic active infection was observed. Meaning that in total about one-third of the control dogs developed active infection.

In the vaccinated group only 7% developed symptomatic infection and 5% developed an asymptomatic active infection, meaning that nearly nine out of 10 vaccinated dogs did not develop active infection over the study despite the intense challenge received. This reduction in the number of dogs progressing to active infection by the end of the trial is statistically significant (p=0.025). Examining the results over time by means of a survival analysis gives a similarly significant result (p=0.027).

When only the development of symptomatic disease is compared between groups the differences are also significant. At the end of the trial, 93% of dogs in the vaccinated group remained free of symptoms (p=0.046) and survival analysis of the difference over time also confirms that the vaccine lowers the probability of developing symptomatic disease (p=0.047).

The impact of a vaccination at population level is interesting, but of course it is the individual benefit of vaccination for the individual patient which is of greatest interest in small animal medicine. In order to establish this, risk calculations are used and this can provide a measure of efficacy applicable to an individual dog. The odds are a classical measure of relative risk used in clinical studies. They are calculated from the probability of an event happening divided by the probability of that event not happening and groups are compared by calculating odds ratios. In this study the odds ratio for active infection is 1 to 3.6, and for symptomatic disease the odds ratio is 1 to 3.8, which in practical terms means that the vaccine reduces the risk of disease approximately four-fold. This four-fold reduction of risk is therefore the official claim on the approved SPC of the product.

**Conclusion**

Under severe challenge conditions, in highly endemic areas, CaniLeish® has demonstrated a significant ability to reduce the risk of a dog developing active infection or symptomatic disease four-fold. The results obtained with the vaccine are consistent with the mode of action. In common with the vast majority of vaccines it does not prevent the pathogen from entering the body, but rather stimulates and directs the immune response in order to cope with the challenge, thus preventing progression to active infection.
Practical use of CaniLeish®. What do I need to know to get the best from this new tool?

David McGahie

Introduction
The purpose of this session is to consider some of the data sheet recommendations and requirements as well as some practical suggestions to enable practitioners to understand the optimal use of the vaccine when it becomes available in their countries.

The basic protocol
With CaniLeish®, the basic initial starting protocol is three injections (subcutaneously) at three week intervals. This is slightly different to a conventional parvovirus or distemper vaccination and is because the objective is to induce a Th1-dominated cell-mediated immunity. Simply inducing antibodies is not sufficient for protection against Leishmania. Studies performed during development have confirmed that this is the optimal schedule for the primary course. The declared onset of immunity is four weeks after the third injection.

The starting age from which puppies can be vaccinated is six months of age and older. The efficacy testing in all of the developmental studies was done from the age of six months and it is believed that the maturation of the cell-mediated immune response capability in young dogs is slower than that of the humoral response.

Currently there is no data on the simultaneous administration of CaniLeish® with other vaccines such as DHPPiL. Therefore the current recommendation is to separate the administration of CaniLeish® from all other vaccinations by at least 2 weeks, which is a fairly common recommendation in situations where specific testing has not been performed to confirm compatibility. There is a requirement for an annual booster with a single dose of the vaccine to maintain immunity.

The vaccine
The vaccine itself is supplied in a very conventional manner: a lyophilized pellet (freeze dried pellet) and the solvent which is isotonic saline solution. It is interesting to note that the lyophilized pellet looks significantly larger than for other conventional dog vaccines. The solvent is a normal volume (1 ml), and the lyophilized pellet dissolves into the diluent to produce a normal 1 ml volume of a reddish solution for injection.

As the vaccine contains a high protein content, vigorous shaking during reconstitution will result in the formation of some foam, and it is recommended that the solution is simply mixed gently to ensure complete dissolution, which occurs very easily.

In common with most other vaccines it should be stored in the fridge, and it should be taken from the fridge and reconstituted immediately before use. As with any refrigerated vaccination it is always appropriate to warm it slightly in the hand to bring it closer to body temperature for the comfort of the animal when injected, and as normal it is recommended to lightly massage the injection site.

Pre-vaccination screening
The marketing authorization is for the active immunization of Leishmania negative dogs. The objective of vaccination is to prevent disease, not to treat disease, and therefore, a dog which is already well on the way to developing the disease is not a candidate for prevention but rather requires treatment. We know with Leishmania that active infection, when the dog has progressed to having a high parasite load of actively replicating parasites, rarely if ever regresses back to the negative state without assistance.

Various options are possible to detect infection:
- Serology is a conventional and routine method, and high titers in serology tests such as IFAT are closely associated with advanced and progressive disease. It is simple, rapid and inexpensive to perform.
- Direct visualization of amastigotes in smears from the lymph node or the bone marrow confirms that the dog is infected. However, this technique is not sensitive enough for routine pre-vaccination screening and dogs may still test negative despite having the disease.
Immunohistochemistry of such smears can increase the sensitivity, but is not routinely used by many practices and is not adapted to the screening situation.

Parasite culture is even more sensitive again, but is not available for routine use in general practice.

Finally, PCR is the most sensitive technique of all, but a positive PCR result gives no indication as to whether the parasite is actually still present in a living state. During the active transmission season, dogs may transiently test PCR positive despite not developing the disease. It is not adapted to the screening situation.

It is clear that serological testing is the most appropriate method of screening before vaccination in the general practice situation. The data sheet of CaniLeish® states 'the detection of Leishmania infection using a rapid serological diagnostic test is recommended prior to vaccination'. Rapid tests allow almost instantaneous real-time results, allowing vaccination to take place on the same day as testing where required which is more convenient for the owner. However, it is clear that other serological tests such as IFAT are also well adapted for pre-vaccination screening in cases where there is no urgency for the result, and in the event of a positive test have the additional advantage of providing a titre which is useful when assessing future courses of action.

When using a rapid test in the context of pre-vaccination screening, it is important that a test with excellent concordance with IFAT is used and ideally with a known threshold which is not excessively high to avoid situations where dogs which are already developing the disease are vaccinated. One such test is Speed Leish K™ where recently presented data showed an excellent concordance with IFAT and an appropriate cut-off threshold of between 1/80 and 1/100 on the IFAT scale. If a dog tests negative with an appropriate rapid test such as Speed Leish K™ or by IFAT, the dog can be vaccinated.

Other tips
As with any vaccination, it is recommended that animals should be dewormed prior to vaccination. It is well known that heavy worm burdens promote a TH2-dominated immune response, and the requirement for prevention of leishmaniosis is to stimulate a TH1-dominated response. Other parasites may do the same; there are published suggestions that Ehrlichia may well promote the establishment and development of leishmaniosis in dogs. Therefore good general parasite control, including internal parasites such as worms and external parasites such as ticks, is not only beneficial for the general health of the dog, but also reduces other factors which may act contrary to the beneficial effects of CaniLeish®.

Use of the vaccine should not prevent measures to reduce exposure to sandflies. The reason being that they are working in entirely different ways. Reduction of exposure to sandflies will minimise the challenge the dog receives. However CaniLeish® is the only option aimed at stimulating immune responses to the challenge that will still inevitably be encountered.

References

How can I best treat clinical cases of Canine Leishmaniosis? Current guidelines
Guadalupe Miró

Canine Leishmaniosis can present with a broad range of clinical manifestations. The basic principle of the treatment should include a good definition of clinical cases of leishmaniosis for the assessment and accurate diagnosis.

Currently, there are not 100% effective drugs against this disease; but in general, the clinical condition improves after treatment in most dogs. 'Parasitological cure' doesn't happen. In some cases, relapses may be possible that required reconsidering diagnosis and treatment.
Even though treatment protocols and clinical follow-up has changed considerably in the last years, the drugs used are unchanged. Furthermore, thanks to the improvement in veterinary assistance, the expectations of clinical—not parasitological—cure are much higher than in the past. Occasionally it is not possible to prevent relapse from immunosuppression cases (stress, debilitating diseases, etc.). Among all available drugs, the most used leishmanicides are pentavalent antimonials (Glucantime®), followed by miltefosine (Milteforan®) in combination with allopurinol (Zyloric®) the leishmaniostatic agent par excellence, due to innocuity and efficacy.

Currently, the trend is toward general consensus on antimonial administration protocols, recommending cycles of 75-100 mg/kg/day for 4-8 weeks (with total doses divided twice a day). Pharmacokinetic studies have shown that pentavalent antimonials subcutaneous administration is the most adequate form of administration. Antimonial toxicity may lead to stibointolerance that appears after the first doses, or to stibotoxicity that may occur at the end of treatment by cumulative effect. Both conditions present with asthenia, exercise intolerance, vomiting and diarrhea, appetite decrease and in some cases fever. Even though sometimes it is difficult to determine if the disorders that occurs in a sick person are side effects of chemotherapy or a consequence of the disease itself; the toxicity presents in patients with nephrotoxicity is greater in both dogs and humans. The combination of pentavalent antimonials and allopurinol is the most documented therapy. Both drugs have a synergistic effect that boosts efficacy, prolongs clinical remission and delays the relapse.

Another option that could be applied is an alkyl phospholipid, miltefosine (Milteforan®), at an oral dose of 2 mg/kg for 4 weeks, always administered with fodder. It is indicated in animals that have neither lost appetite nor have digestive problems. It is the first option in dogs with proteinuria and/or renal disease.

Both leishmanicides are always used in combination with allopurinol at a dose of 10-20 mg/kg BID orally administered for long periods of time (mean 12-18 months) in the cases of classic clinical leishmaniasis. Prolonged treatment with allopurinol is effective to prevent remission of the clinical condition depending on the animal’s ability to control the disease; this will have a time variability. Sometimes, it is necessary to monitor the possible adverse reactions present in the long term in some patients, e.g. xanthine induced urolithiasis and/or nephrolithiasis. Clinical improvement of sick dogs will vary depending on their previous clinical-pathological condition, and it is normally seen between the first and third month of treatment. It is well known that dogs with severe renal damage will generally have a worse clinical recovery. Discontinuation of allopurinol is recommended when the patient is completely recovered from the clinical perspective (history, physical exam and laboratory tests) and when the antibody levels are negative or slightly positive.

### Recommended references

- Mateo M, 2007, *Estudios sobre la eficacia comparada y la tolerancia de la miltefusina y el antimoniatmico de n-metilglucamina, y la monitorización post-tratamiento con allopurinol en la infección natural por Leishmania infantum en el perro*. Tesis doctoral. Universidad Complutense de Madrid

Treatment was successful... What next?

Lluís Ferrer

Introduction
Canine Leishmaniosis, as stated before, is an extraordinarily complex and diverse disease. Thus, it cannot have a sole adequate therapeutic protocol for all cases and requires a clinical classification that allows the implementation of different therapeutic approaches that are more appropriate and a more accurate prognosis.

As we saw before, in the most common stages (II and III), the most effective and safe treatment is the combination of a parasiticide drug such as antimony salts (meglumine antimoniate - Glucantime®) or miltefosine, with allopurinol, a parasitostatic drug. Currently published papers report a similar efficacy for the combination of miltefosine with allopurinol and for the classical treatment (meglumine antimoniate + allopurinol) (Table I) [Miró et al, 2009].

Table I | Treatment of Canine Leishmaniosis

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose</th>
<th>Duration</th>
<th>Side effects</th>
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<tbody>
<tr>
<td>Meglumine antimoniate</td>
<td>80-100 mg/kg/day, SC</td>
<td>4 weeks</td>
<td>Weakness, anorexia, pain</td>
</tr>
<tr>
<td>Miltefosine</td>
<td>2 mg/kg/24 h, PO</td>
<td>4 weeks</td>
<td>Vomiting, diarrhoea, anorexia</td>
</tr>
<tr>
<td>Alopurinol</td>
<td>10 mg/kg/12 h, PO</td>
<td>1 year</td>
<td>Urolithiasis (xanthine)</td>
</tr>
</tbody>
</table>

This treatment produces very good outcomes in dogs without a severe renal disorder (stages II and III). More than 80% of animals exhibit an evident clinical improvement within the first two months, a 90% after 3 months. Generally, clinical and pathological parameters normalise within 3 months (including the protein electrophoresis). Antibodies titres are another matter. While the mean titre tends to fall slowly in treated animals, seronegativity cannot be expected within the first six months of treatment. In addition, antibody titre remains high for years in a significant percentage of cases [Torres et al, 2010].

Patient follow-up during and after the treatment is as important as the treatment itself. The goals of patient follow-up are:

1. Detect if the response is the expected one and if the patient shows an adequate clinical progress. Detect treatment failures.
2. Identify potential adverse effects of treatment and correct them.
3. Identify late-onset lesions associated with the disease (caused by immune complexes, associated to immunodeficiency, co-infections, etc.)
4. Detect leishmaniosis relapses as soon as possible.
Thus, the follow-up protocol must be aimed to achieve all these goals. Table 2 summarises a standard follow-up protocol once the treatment has begun. Generally, clinical examination, blood tests (particularly protein electrophoresis) and serology allow the assessment of the progression of the disease in a patient. The biggest difficulty might be the correct evaluation of an animal being treated for months that has responded to medication and presents clinical signs consistent with leishmaniosis (weight loss, weakness, lameness, skin or ocular lesions). Must these be simply attributed to the previously diagnosed leishmaniosis? How to make a decision?

Table 2 | Monitoring of patients with leishmaniosis from the treatment on

<table>
<thead>
<tr>
<th>I. Check-up at 1, 2, 3, 6 and 12 months</th>
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<tbody>
<tr>
<td>II. Then, twice a year</td>
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<tr>
<td>III. Every check-up must include:</td>
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<tr>
<td>- Full clinical examination.</td>
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<tr>
<td>- Total protein – protein electrophoresis.</td>
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<tr>
<td>- Serology (won’t be needed for 3 months).</td>
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<tr>
<td>- Chemistry and urinalysis.</td>
</tr>
<tr>
<td>- Blood tests.</td>
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<tr>
<td>IV. Occasionally, it can also include:</td>
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<tr>
<td>- CD3+, CD4+ and CD8+ count.</td>
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<tr>
<td>- Leishmanin skin test.</td>
</tr>
<tr>
<td>- Assessment of proteinuria.</td>
</tr>
<tr>
<td>- Quantitative blood and/or bone marrow PCR (identification of relapses).</td>
</tr>
<tr>
<td>- Acute Phase Protein (PCR, Haptoglobin)</td>
</tr>
</tbody>
</table>

Normally, if the clinical signs are associated with a change in protein electrophoresis and serology, we are in front of a relapse. If not (e.g., the dog has always maintained a high titre), it may be more difficult. In these cases, it is best to use C-reactive protein and, even better, quantitative PCR (Real Time) [Martinez et al., 2011].

Furthermore, it is very important that the animals that have undergone clinical leishmaniosis and have been treated will receive special cares, including:

1. Highest quality nutrition according to the kind of dog and its lifestyle. Consider using a diet for the prevention of urolithiasis.
2. Provide permanent access to fresh water (prevention of xanthine uroliths associated with allopurinol).
3. Regular parasiticide treatments (both for endo and ectoparasites). Annual faecal exams.
4. Use sandfly repellents (deltamethrin, permethrin) since sick dogs have the highest parasite burden.
5. Avoid, whenever possible, any immunosuppressive treatment (steroids).
6. Avoid drugs that could interact, particularly with allopurinol (azathioprine).

References

2. Miro G et al. Multicentric, controlled clinical study to evaluate effectiveness and safety of miltefosine and allopurinol for canine leishmaniosis. Veterinary Dermatology (2009); 20:397-404