

# Development and Clinical Trial of a Novel DNA Vaccine as Immunotherapy during Canine Leishmaniasis

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## Abstract

**Background:** Visceral Leishmaniasis (VL) is a zoonotic disease caused by *Leishmania infantum* in the Mediterranean area. Dogs are the main reservoir of *Leishmania infantum* parasites. Disease management represents a serious problem, since anti-*Leishmania* drugs have limited efficacy in both symptomatic and asymptomatic dogs, which are infective to phlebotomine vectors. In many tropical and sub-tropical countries the development of a safe and easily-available vaccine has high priority. DNA vaccines represent one of the most recent innovations in the field of immunization.

**Findings:** This study aimed to evaluate the effect of a DNA vaccine based on two *Leishmania* antigens (Cpb1, PO) in leishmaniotic dogs. Twelve leishmaniotic dogs from a Leishmaniasis-endemic area (Naples, Italy) received three consecutive injections of DNA vaccine at 15-days intervals. Another group of five leishmaniotic dogs received the same amount of pVAX-1 without the coding sequences of *Leishmania* antigens. *Leishmania* DNA load, INF $\gamma$ , IL-4 mRNA expression levels and clinical parameters were tested before and after the therapy, every 3 months for a period of 12 months.

Analysis of the data in the vaccinated dogs showed: i) a decrease *Leishmania* DNA load in lymph node samples, ii) an increase of INF $\gamma$  and IL-4 mRNA expression levels in PBMC samples. All vaccinated dogs also showed an improvement in the clinical symptoms.

**Conclusion:** Our results show that the vaccine developed in this study may represent a useful tool in the treatment of leishmaniotic dogs. However, since the duration of positive effects is limited in time, further trials are needed to evaluate the effectiveness of immunotherapy alone or in association with conventional therapy.

**Keywords:** DNA vaccine; Real-time PCR; *Leishmania infantum*; Cytokine; Dog

## Introduction

Visceral Leishmaniasis (VL) or *kala-azar* is a zoonotic disease caused by *Leishmania infantum*, in the Mediterranean area, and by *Leishmania chagasi* in Latin America and China [1]. It is endemic in less developed countries; however, incidences are increasing in non-endemic areas due to changing patterns of international travel and to population migration [2]. VL currently affects 12 million individuals and it is an opportunistic infection in immunocompromised patients [3].

Dogs are the main reservoir of *Leishmania infantum* parasites, and they play a central role in the transmission to humans through phlebotomine sandflies [4,5]. A large amount of evidence has demonstrated that the prevalence and incidence of canine Leishmaniasis (CanL) has, until now, been underestimated [6,7]. In addition to dogs, wild canids, such as jackals (*Canis aureus*) and red fox (*Vulpes vulpes*), are potential feral reservoirs for *Leishmania infantum* [8].

After infection, some dogs can control the parasite and don't develop the disease in the short term, sometimes remaining subclinical for years or for their entire lifetime, whereas others may present progressive disease [9]. The presence of latent infection in dogs is typical and contributes to maintaining the long-term presence of the parasite in endemic regions. Disease management represents a serious problem since both symptomatic and asymptomatic dogs are infective to phlebotomine vectors [10], and the available antileishmanial drugs have limited efficacy in dogs [11,12].

The data currently available for canine VL show that the clinical

appearance and evolution of Leishmaniasis is a consequence of complex interactions between the parasite and host immune response. The outcome of infection depends on the ability of host macrophages to effectively destroy the parasite. This activity is determined by the balance between a heterogeneous set of cytokines [13]. Previous studies on the immune responses in experimentally infected dogs, have revealed that T lymphocytes and the cytokines play a crucial role in determining whether infection evolves toward a protective immunity status or a progressive and manifested disease [14].

Two different T helper (Th) cell subsets, termed Th1 and Th2, which differ from each other in the profile of secreted cytokines, have been described [15]. In the murine model for Leishmaniasis, the Th1 response confers protection, whereas the Th2 response renders the host susceptible to infection [16-18]. Studies on the immune response in dogs experimentally infected with *Leishmania infantum* have shown that the protective immunity is associated with the production of IL-2, IFN- $\gamma$  and TNF- $\alpha$  by peripheral blood mononuclear cells (PBMC)

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in addition to the induction of parasite-specific cytotoxic T cells [9]. Furthermore, the activation of macrophages by IFN- $\gamma$  has been shown to result in nitric oxide production which mediates the killing of intracellular parasites [19, 20]. On the other hand development of the disease is associated with the production of other interleukins such as IL 10, IL 12, IL 18 etc.

In the last decade, the polymerase chain reaction (PCR) has been successfully introduced and proven to be a sensitive and powerful tool to detect *Leishmania* in clinical samples as well as for parasite characterization [21-23]. Very recently, PCR has also been used to analyse the immune response profile either in humans or in infected dogs [24]. However, the multiple steps of post-PCR manipulation require time and pose the risk of DNA contamination. A recent approach to estimating the parasitic load of mouse livers experimentally infected with *Leishmania* infantum has been based on the use of a double real-time quantitative PCR test targeting the parasite DNA polymerase gene and the mouse brain-derived neutrophilic factor gene [25,26]. Furthermore, a real-time PCR that simultaneously detects, quantitates and differentiates *Leishmania* organisms has been developed [27,28].

Control of Leishmaniasis currently includes the use of combined chemotherapeutic treatments and vector control by the use of insect repellent. Stamping out is a widely used practice in countries such as Brazil, however attempts to remove seropositive dogs has proven to be insufficient for eradicating visceral Leishmaniasis in dogs [29].

It is generally accepted that an effective vaccine against canine Leishmaniasis is the best strategy for disease control. Among vaccine type, the DNA vaccine seems to be the most promising due to their ability to efficiently stimulate both humoral and cellular immune immunity in many infectious disease models [30].

A DNA vaccine typically consists of a foreign gene, encoding a protein antigen of interest, cloned into a bacterial plasmid under the control of an appropriate promoter that can be injected into the skin or muscle of the the host. After uptake of the plasmid, the protein is produced endogenously and intracellularly and the antigenic peptides can be presented on the cell surface in the context of both the MHC class I and MHC class II pathway [31].

In this study, a new DNA vaccine, which expresses both Cysteine proteinases b1 (CPb1) and acidic ribosomal protein PO (LiPO) of *Leishmania infantum* was constructed.

The immunotherapeutic potential of this vaccine was assessed in 12 naturally infected dogs.

The quantitative real-time PCR (qPCR) assay was used for measuring: a) *Leishmania* DNA load in lymph node aspirates; b) the expression levels of cytokines relative to either Th1 or Th2 patterns.

Both parasite DNA load and cytokine expression levels were also evaluated in a group of healthy uninfected dogs used as the control group.

## Materials and Methods

### Plasmid construction and purification

The coding sequences of two *Leishmania* antigens Cpb1 and PO were inserted into the pVAX-1 vector to obtain the pVAX/Cpb1/Po plasmid. pVax CPB1/PO encodes the full-length sequences of the Cpb1 and PO genes placed downstream of the cytomegalovirus (CMV) promoter and upstream of the bovine growth hormone (BGH) polyadenylation sequence in the pVax1™ vector (Invitrogen). The

sequences of the Cpb1 and PO genes were derived from GenBank (accession number AJ628942.1 and X72714.1 respectively) and were synthesized by GenScript, USA Inc. in a concatameric formation with a linking sequence of repeats coding for a serine/glycine rich peptide flexible bridge rotating on a threonine hinge, as described previously (Robinson and Sauer, 1998). The pVax CPB1/PO plasmid was used for the invitro experiments and clinical vaccine trials described below. The plasmid pUC CPB1/PO encodes the Cpb1/PO fused genes cloned in the multiple cloning sites (MCS) of the pUC19 cloning vector and was used as negative control for *in vitro* experiments. Plasmid DNA for vaccine clinical trials was purified with the Endo Free Plasmid Giga Kit (Qiagen) using endotoxin-free material and eluted in endotoxin-free deionised water.

### Expression of Cpb1/PO genes in transfected cells

The human embryonic kidney cell line HEK 293 was transfected with pVax CPB1/PO and with the negative controls pUC/Cpb1/PO, using the CalPhos Mammalian Transfection Kit (Clontech), according to the manufacturer's instructions. Transfected cells were harvested 48 h later and washed with phosphate-buffered saline three times. Total RNA was extracted with Trizol reagent (Invitrogen) and treated with DNaseI (Invitrogen). Complementary DNA (cDNA) was obtained from RNA with the SuperScript™ First-Strand Synthesis System (Invitrogen). CPB1/PO cDNA was amplified by PCR. The amplified products were analyzed with a 1% agarose gel. Robustness of plasmids expressing CPB1/PO was evaluated by replicating the expression experiments three times.

Also, production of Cpb1/PO protein was detected by immunoblotting (B) using an antiserum leishmaniotic dog Vaccine clinical trial.

This study included 12 dogs, hospitalized at the Department of Clinical Veterinary Sciences of the University of Naples, naturally infected by *Leishmania infantum*. These dogs were living in the endemic area of Campania Region in South Italy and included different breeds, ranged in age from 1 to 10 years. All dogs were diagnosed as having CanL on the basis of clinical manifestations, immunofluorescence antibodies IFAT, and qPCR tests.

**Criteria for inclusion in this study were:** Clinical findings compatible with Leishmaniasis (weight loss, peripheral lymphadenopathy, skin lesions, ocular lesions, epistaxis, lameness, ulcers, diarrhea and moderate glomerulopathy), alterations of ematobiochemical parameters compatible with Leishmaniasis (thrombocytopenia, anaemia, leukopenia; total proteins and beta globulins higher than normal value), presence of anti-*Leishmania* antibody with titres above 1:80, and a *Leishmania* DNA load in lymph node aspirate ranging from 100 parasites/ml.

Animals were scored for clinical and laboratory parameters on a scale from 0 to 9 and the values were added up to give a clinical score [32].

### Vaccination protocol

At the time of diagnosis, 12 dogs which were treated contemporaneously with three intramuscular doses and three subcutaneous doses of 500  $\mu$ g of plasmid, Pvac Po\CPB1 plasmid on days 0, 15, 30.

Another group of five leishmaniotic dogs received the same amount of pVAX-1 without the coding sequences of *Leishmania* antigens (control group). These dogs were kept without treatment until there has

been a worsening of clinical symptoms, then, 6 months after the start of the study, they were subjected to specific treatment.

The efficacy of the therapy was evaluated at 0, 15 and 30 days from the beginning of the therapy, and 3, 6 and 12 months from the beginning of the therapy.

All dogs were given a clinical score on the basis of the presence and severity of clinical manifestations, the alterations of laboratory parameters, and the IFAT titre. In addition the *Leishmania* DNA load in the lymph node aspirates and mRNA expression levels of INF  $\gamma$  and IL4 in the blood were also measured at these times.

The Ethics Committee gave a positive opinion for treatment for compassionate use of dogs studied.

### Sampling

Whole blood samples were collected from the dogs in EDTA-containing tubes by cephalic venipuncture. Lymph node aspirates were obtained by using a thin biopsy needle. Samples were stored at  $-80^{\circ}\text{C}$ .

DNA extraction from tissue samples and from parasite cultures and RNA isolation and production of cDNAs were carried out as previously described [33].

### Selection of primers and probes

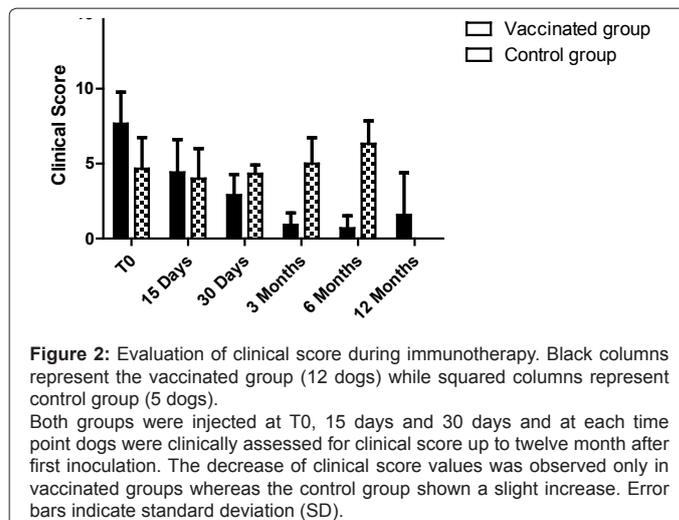
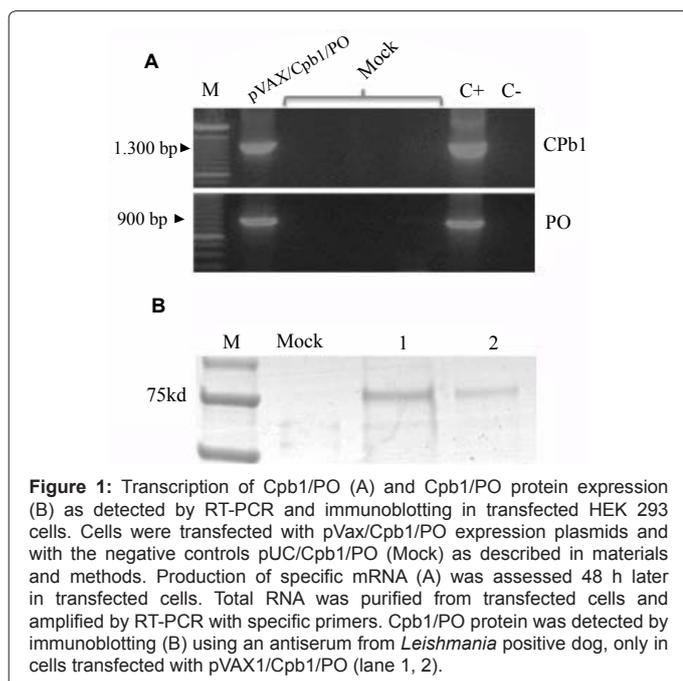
All TaqMan primers and probes were selected as previously reported [33]. All primers and FAM probes were provided from Applied Biosystems.

#### Real-time PCR analysis

Real-time PCR was performed as previously described [34,35].

#### Statistical analysis

Statistical analysis was carried out with GraphPad InStat software 3.0 application. The significance of differences between the two groups was evaluated by using the Anova tests. All conclusions were based on significance levels of  $p < 0.05$ . Boxplot of results were generated by using the same software.



### Results

Plasmid P<sub>vax</sub>/Cpb1/PO was designed in order to investigate the immunotherapeutic potential of DNA vaccines against canine Leishmaniasis. To provide an enhanced immunological response, the proteins were linked together with a sequence encoding a glycine bridge. DNA sequencing confirmed the correct synthesis of Cpb1/PO in P<sub>vax</sub>1. To ensure that Cpb1/PO fusion proteins could indeed be transcribed in mammalian cells, the presence of specific mRNA was established by RT-PCR from total RNA isolated from HEK 293 cells transfected with P<sub>vax</sub>/Cpb1/PO plasmid. As shown in Figure 1, an amplicon of approximately 1330 bp and 900 bp corresponding to Cpb1 and PO respectively was obtained from transfected cells (Figure 1A). Immunoblotting performed on HEK 293 cell lysates confirmed the presence of correct transcription of fusion protein Cpb1/PO (Figure 1B).

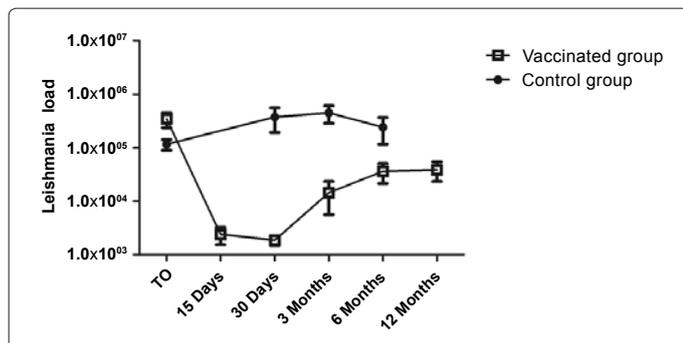
Before immunotherapy began, vaccinated dogs (Group1) had a mean clinical score of 7, while control dogs had a mean value of 5. Three months after the start of therapy, vaccinated dogs showed a progressive improvement in their clinical manifestations, as demonstrated by the decrease in their clinical scores (Figure 2). In addition the average clinical score was lower than before starting therapy. This decrease continued until 6 months after the beginning of therapy. At this point the average of clinical score of the vaccinated dogs started to progressively increase, and finally three dogs had a relapse.

The average of value of clinical score for the control group progressively increased from the beginning of the therapy to the end (Figure 2).

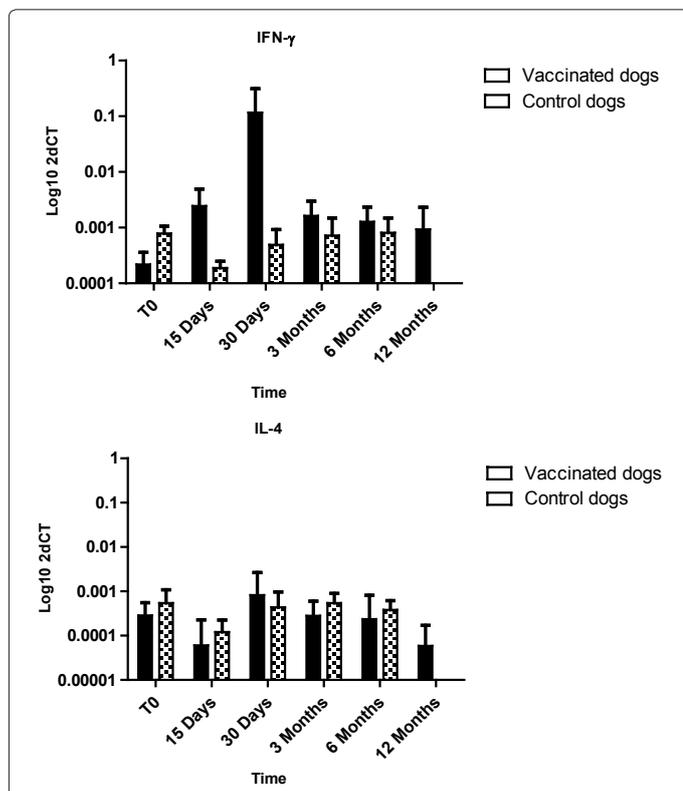
30 days after the start of the immunotherapy, vaccinated dogs showed a relevant decrease of *Leishmania* DNA that continues until 3 months after the beginning of therapy. From this time point the *Leishmania* DNA load started to progressively increase without reaching original load. On the contrary the control group shows a progressive increase of DNA *Leishmania* load (Figure 3).

The IFN- $\gamma$  and IL-4 mRNA expression levels in group 1 and in the control group were monitored by real time PCR (Figure 4).

IFN- $\gamma$  mRNA levels of vaccinated dogs show a significant increase at days 15 and 30 post inoculation, whereas no difference



**Figure 3:** Evaluation of parasite load during immunotherapy. Squares represent vaccinated group (12 dogs) while circles indicates control group (5 dogs). Fine-needle biopsy were taken and *Leishmania* DNA copies was estimated by qPCR at each time point. The axis indicates the number of *Leishmania*/ml expressed in logarithmic values. In contrast to control group the vaccinated group exhibits a reduction of parasite load since 15 days after first inoculation.



**Figure 4:** Transcriptional analysis of PBMC cytokines mRNA expression levels during immunotherapy. Cytokine mRNA levels in PBMC shows no relevant differences between the vaccinated and control group with the exception of the 15 days and the 30 days time point of vaccinated group that shows a significant increase of IFN-γ. Error bars indicates standard deviation (SD).

in IL-4 mRNA levels where observed between the vaccinated and the control group.

## Discussion and Conclusions

DNA vaccination has been demonstrated to induce both humoral and cellular immune responses. Th1 cells can activate macrophages to destroy intracellular microorganisms and also activate B cells to produce strongly opsonizing antibodies such as IgG2a and IgG2b [31].

In this study, a pVAX based plasmid expressing the Cpb1 and PO proteins of *Leishmania infantum* was designed and evaluated in dogs diagnosed with symptomatic Leishmaniasis. As shown in Figure 1, Cpb1/PO mRNA was observed in transfected HEK 293 cells, and the Cpb1/PO fusion protein was detected by Western blotting analysis.

Cpb1 and PO may represent an ideal candidate for the development of a DNA vaccine against Leishmaniasis. Cysteine proteinases (CPs) are enzymes that belong to the papain superfamily, which are found in a number of organisms from prokaryotes to mammals. Previous data indicates that the Cysteine proteinases (CPA and CPB) inoculated as recombinant protein or as DNA vaccines is able to elicit a protective immune response against *L. major* in the murine model [36-38]. Analysis of the immune response showed that vaccinated animals developed a specific Th1 immune response, which was associated with an increase of IFN-γ production. PO is an acidic ribosomal protein. Administration of the PO antigen from *Leishmania infantum*, either as soluble recombinant or as a plasmid DNA formulation confers protective immunity to *Leishmania major* infection in BALB/c mice [39].

The protective immunity against *Leishmania* is believed to be due to the action of the subpopulation of lymphocytes T helper type 1 [40]. The T lymphocyte activation determines proliferation, differentiation and production of cytokines such as IL-12, IFN-γ and TNF-α [41]. In presence of TNF activated macrophages produce NO which is considered the main mechanisms for *Leishmania* elimination [42].

Results in this study demonstrate that vaccination with pVAX/Cpb1/PO determines a clinical improvement since 15 day after the first inoculation along with a decrease of DNA parasite load in the lymphnode. Improvement of clinical score and decrease of parasite burden progresses until 30 days post inoculation and stabilizes until 3 months time point.

The clinical improvement is probably due to a decrease of organ parasitism. The remarkable decrease in parasite burden is a consequence of activation of effective immunity response. The outcome of infection by *Leishmania* depends on the balance between host ability to induce immune mechanisms against *Leishmania* and the parasite strategies to interfere in the process of antigen presentation and thus modulate the immune response mediated by T lymphocytes [43]. Expansion in the number of CD4 + T lymphocytes subset has been correlated to a reduction in organs parasitism in dogs undergoing immunotherapy and in vaccinated animals [44,45].

Previous studies analyzing peripheral blood lymphocyte correlates protective immune response against *Leishmania infantum* with CD4+ Th1 cells expressing IFN-γ, IL-2 e TNF-α. Cytokine patterns (IL-4) of CD4+ Th2 subset has been correlated to symptomatic state of the disease [46].

In our study, at 15 days and 30 days time points there is a significant increase of IFN-γ mRNA levels, coinciding with the lowest DNA parasite load and lowest clinical score in vaccinated dogs. IL-4 mRNA levels selected as Th2 response marker do not show significant variations along the clinical trial. Results suggest that pVAX/Cpb1/PO trigger an effective Th1 immune response. These effects are transient and more or less around the thirty months after the first inoculation we assist a progressive decline of the clinical status.

In conclusion, our results show that the vaccine developed in this study may represent a useful tool in the treatment of leishmaniotic dogs. However, further trials are needed to evaluate the effectiveness of immunotherapy alone or in association with conventional therapy.

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## Results

- Guerin PJ, Olliaro P, Sundar S, Boelaert M, Croft SL, et al. (2002) Visceral leishmaniasis: current status of control, diagnosis, and treatment, and a proposed research and development agenda. *Lancet Infectious Disease* 2: 494-501.
- World Health Organization (2001) WHO information by topics or disease.
- Desjeux P, Alvar J (2003) Leishmania/HIV co-infections: epidemiology in Europe. *Ann Trop Med Parasitol* 97: 3-15.
- Courtenay O, Quinnell RJ, Garcez LM, Shaw JJ, Dye C (2002) Infectiousness in a cohort of Brazilian dogs: why culling fails to control visceral leishmaniasis in areas of high transmission. *J Infect Dis* 186: 1314-1320.
- Chamizo C, Moreno J, Alvar J (2005) Semi-quantitative analysis of cytokine expression in asymptomatic canine leishmaniasis. *Vet Immunol Immunopathol* 103: 67-75.
- Solano-Gallego L, Morell P, Arboix M, Alberola J, Ferrer L (2001) Prevalence of *Leishmania infantum* infection in dogs living in an area of canine leishmaniasis endemicity using PCR on several tissues and serology. *J Clin Microbiol* 39: 560-563.
- Alvar J, Cañavate C, Molina R, Moreno J, Nieto J (2004) Canine leishmaniasis. *Adv Parasitol* 57: 1-88.
- Dipineto L, Manna L, Baiano A, Gala M, Fioretti A, et al. (2007) Presence of *Leishmania infantum* in Red Foxes (*Vulpes vulpes*) in Southern Italy. *J Wildl Dis* 43: 518-520.
- Moreno J, Alvar J (2002) Canine leishmaniasis: epidemiological risk and the experimental model. *Trends Parasitol* 18: 399-405.
- Molina R, Amela C, Nieto J, San-Andres M, Gonzalez F, et al. (1994) Infectivity of dogs naturally infected with *Leishmania infantum* to colonized *Phlebotomus perniciosus*. *Trans R Soc Trop Med Hyg* 88: 491-493.
- Herwaldt BL (1999) Leishmaniasis. *Lancet* 354: 1191-1199.
- Gradoni L (2001) An update on antileishmanial vaccine candidates and prospects for a canine *Leishmania* vaccine. *Vet Parasitol* 100: 87-103.
- Rhalem A, Sahibi H, Lasri S, Jaffe C (1999) Analysis of immune responses in dogs with canine visceral leishmaniasis before, and after, drug treatment. *Vet Immunol Immunopathol* 71: 69-76.
- Pinelli E, Gonzalo RM, Boog CJ, Rutten VP, Gebhard D, et al. (1995) *Leishmania infantum*-specific T cell lines derived from asymptomatic dogs that lyse infected macrophages in a major histocompatibility complex-restricted manner. *Eur J Immunol* 25:1594-1600.
- Mosmann TR, Moore KW (1991) The role of IL-10 in cross-regulation of TH1 and TH2 responses. *Immunol Today* 12: 49-53.
- Rogers KA, DeKrey GK, Mbow ML, Gillespie RD, Brodsky CI, et al. (2002) Type 1 and type 2 responses to *Leishmania major*. *FEMS Microbiol Lett* 209: 1-7.
- Sacks D, Noben-Trauth N (2002) The immunology of susceptibility and resistance to *Leishmania major* in mice. *Nat Rev Immunol* 2: 845-858.
- Gumy A, Louis JA, Launois P (2004) The murine model of infection with *Leishmania major* and its importance for the deciphering of mechanisms underlying differences in Th cell differentiation in mice from different genetic backgrounds. *Int J Parasitol* 34: 433-444.
- Pinelli E, van der Kaaij SY, Slappendel R, Fraggio C, Ruitenber EJ, et al. (1999) Detection of canine cytokine gene expression by reverse transcription-polymerase chain reaction. *Vet Immunol Immunopathol* 69: 121-126.
- Gradoni L, Ascenzi P (2004) [Nitric oxide and anti-protozoan chemotherapy]. *Parassitologia* 46: 101-103.
- Belkaid Y, Mendez S, Lira R, Kadambi N, Milon G, et al. (2000) A natural model of *Leishmania major* infection reveals a prolonged "silent" phase of parasite amplification in the skin before the onset of lesion formation and immunity. *J Immunol* 165: 969-977.
- Reithinger R, Espinoza JC, Courtenay O, Davies CR (2003) Evaluation of PCR as a diagnostic mass-screening tool to detect *Leishmania* (*Viannia*) spp. in domestic dogs (*Canis familiaris*). *J Clin Microbiol* 41: 1486-1493.
- Manna L, Vitale F, Reale S, Caracappa S, Pavone LM, et al. (2004) Comparison of different tissue sampling for PCR-based diagnosis and follow-up of canine visceral leishmaniasis. *Vet Parasitol* 125: 251-262.
- Babaloo Z, Kaye PM, Eslami MB (2001) IL-13 in Iranian patients with visceral leishmaniasis: relationship to other Th2 and to Th1 cytokines. *Trans R Soc Trop Med Hyg* 95: 85-88.
- Bretagne S, Durand R, Olivi M, Garin JF, Sulahian A, et al. (2001) Real-time PCR as a new tool for quantifying *Leishmania infantum* in liver in infected mice. *Clin Diagn Lab Immunol* 8: 828-831.
- Nicolas L, Prina E, Lang T, Milon G (2002) Real-time PCR for detection and quantitation of *Leishmania* in mouse tissues. *J Clin Microbiol* 40: 1666-1669.
- Schulz A, Mellenthin K, Schonian G, Fleischer B, Drosten C (2003) Detection, differentiation and quantitation of pathogenic *Leishmania* organisms by a fluorescence resonance energy transfer-based real-time PCR assay. *J Clin Microbiol* 41: 1529-1535.
- Vitale F, Reale S, Vitale M, Petrotta E, Torina A, et al. (2004) TaqMan-based detection of *Leishmania infantum* DNA using canine samples. *Ann N Y Acad Sci* 1026: 139-143.
- Courtenay O, Quinnell RJ, Garcez LM, Shaw JJ, Dye C (2002) Infectiousness in a cohort of Brazilian dogs: why culling fails to control visceral leishmaniasis in areas of high transmission. *J Infect Dis* 186: 1314-1320.
- Lowe DB, Shearer MH, Kennedy RC (2006) DNA vaccines: Successes and limitations in cancer and infectious disease. *J Cell Biochem* 98: 235-242.
- Gurunathan S, Wu CY, Freidag BL, Seder RA (2000) DNA vaccines: a key for inducing long-term cellular immunity. *Curr Opin Immunol* 12: 442-447.
- Manna L, Reale S, Vitale F, Picillo E, Pavone LM, et al. (2008) Real time PCR assay in *Leishmania*-infected dogs treated with meglumine antimoniate and allopurinol. *Vet J* 177: 279-282.
- Manna L, Reale S, Viola E, Vitale F, Manzillo VF, et al. (2006) *Leishmania* DNA load and cytokine expression levels in asymptomatic naturally infected dogs. *Vet Parasitol* 142: 271-280.
- Manna L, Reale S, Picillo E, Vitale F, Gravino AE (2008) Interferon-gamma (INF $\gamma$ ), IL4 expression levels and *Leishmania* DNA load as prognostic markers for monitoring response to treatment of leishmaniotic dogs with miltefosine and allopurinol. *Cytokine* 44: 288-292.
- Manna L, Vitale F, Reale S, Picillo E, Neglia G, et al. (2009) Study of efficacy of miltefosine and allopurinol in dogs with leishmaniasis. *Vet J* 182: 441-445.
- Rafati S, Salmanian AH, Taheri T, Vafa M, Fasel N (2001) A protective cocktail vaccine against murine cutaneous leishmaniasis with DNA encoding cysteine proteinases of *Leishmania major*. *Vaccine* 19: 3369-3375
- Zadeh-Vakili A, Taheri T, Taslimi Y, Doustdari F, Salmanian AH, et al. (2004) Immunization with the hybrid protein vaccine, consisting of *Leishmania major* cysteine proteinases Type I (CPB) and Type II (CPA), partially protects against leishmaniasis. *Vaccine* 22: 1930-1940.
- Rafati S, Ghaemimanes F, Zahedifard F (2006) Comparison of potential protection induced by three vaccination strategies (DNA/DNA, Protein/Protein and DNA/Protein) against *Leishmania major* infection using Signal Peptidase type I in BALB/c mice. *Vaccine* 12: 3290-3297.
- Iborra A, Mayorga M, Llobet N, Martinez P (2003) Expression of complement regulatory proteins [membrane cofactor protein (CD46), decay accelerating factor (CD55), and protectin (CD59)] in endometrial stressed cells. *Cell Immunol* 223: 46-51.
- Strauss-Ayali D, Baneth G, Jaffe CL (2007) Splenic immune responses during canine visceral leishmaniasis. *Vet Res* 38: 547-64.
- Sperling AI, Bluestone JA (1996) The complexities of T-cell co-stimulation: CD28 and beyond. *Immunol Rev* 153: 155-182.
- Pinelli E, Gebhard D, Mommaas AM, van Hoesj M, Langermans JA, et al. (2000) Infection of a canine macrophage cell line with *leishmania infantum*: determination of nitric oxide production and anti-leishmanial activity. *Vet Parasitol* 92: 181-189.
- Panaro MA, Lofrumento DD, Saponaro C, De Nuccio F, Cianciulli A, et al. (2008) Expression of TLR4 and CD14 in the central nervous system (CNS) in a MPTP

- 
- mouse model of Parkinson's-like disease. *Immunopharmacol Immunotoxicol* 3: 729-740.
44. Guarga JL, Moreno J, Lucientes J, Gracia MJ, Peribáñez MA, et al. (2002) Evaluation of a specific immunochemotherapy for the treatment of canine visceral leishmaniasis. *Vet Immunol Immunopathol* 88: 13-20.
45. Borja-Cabrera GP, Cruz Mendes A, Paraguai de Souza E, Hashimoto Okada LY, de A Trivellato FA, et al. (2004) Effective immunotherapy against canine visceral leishmaniasis with the FML-vaccine. *Vaccine* 22: 2234-2243.
46. Heidarpour M, Soltani S, Mohri M, Khoshnegah J ( 2012) Canine visceral leishmaniasis: relationships between oxidative stress, liver and kidney variables, trace elements, and clinical status. *Parasitol Res* 111: 1491-1496.