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Nucleoside hydrolase DNA vaccine against canine visceral leishmaniasis

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Abstract

The Nucleoside Hydrolase (NH36) is the main marker of the FML complex of *Leishmania donovani*, antigen of the licensed Leishmune® vaccine for prophylaxis of canine visceral leishmaniasis. As a DNA vaccine in mice, it induces a TH1 immune response. We vaccinated mongrel dogs with the VR1012NH36 vaccine for prophylaxis and immunotherapy against a high dose *Leishmania chagasi* infection (7 x 10^8 infective amastigotes). The untreated controls developed more symptoms, higher parasite/lymphocyte ratio, smaller DTH reactions, lower proportions of NH36-specific CD4+ cells and sustained NH36-specific CD8+ cell counts than dogs of the prophylaxis group. In the immunotherapy treated group, enlarged DTH reactions, enhanced CD4+ and sustained CD8+ lymphocyte proportions were also detected, however, without reduction of symptoms or parasite/lymphocyte ratio, indicating that the vaccine was sufficiently potent to prevent but not to control the disease. Both treatments determined higher survival rates. Anti-FML antibodies increased in vaccinated and control dogs while anti-NH36 antibodies were only increased in vaccinees (p= 0.000). The parasite load of an untreated survivor control dog (638.05 parasites) felt outside the IC95% of that of vaccinated dogs (32.02, IC95% 9.45-64.59) suggesting that both vaccination treatments succeeded in reducing the *Leishmania* infective burden. Accordingly, an untreated control dog showed lower levels of IFN γ-β, IL-2, IL4 but not IL-10 β actin-relative quantification. We conclude that the VR1012-NH36 vaccine induces strong prophylactic protection and a milder immunotherapeutic effect against a high dose canine experimental infection with *Leishmania chagasi*

Keywords: Leishmanioses; Nucleoside hydrolase; DNA vaccines; canine visceral leishmaniasis; prophylaxis; immunotherapy

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Vaccination with the FML-saponin Leishmune® vaccine is efficient in prevention [1,2], immunotherapy [3,4] and in blocking the transmission of canine visceral leishmaniasis (CVL) between dogs and to humans [5]. NH36, a nucleoside hydrolase of 34.3 kDA and 314 amino acids, is the main antigenic marker of the FML complex [6]. It cleaves nucleosides from imported DNA to release free purine or pyrimidic bases for *Leishmania* replication and according to this very relevant function it has been recently recognized as an important and conserved phylogenetic marker of the *Leishmania* genus [7]. As a DNA vaccine (VR1012-NH36) in mice it induced 88% of protection, 91% of curative potential against visceral and 65% of protection against coetaneous leishmaniasis, developing a TH1 immune response [8].

In this work we used 19 SRD dogs (4 months old), of 4 different broods, that were vaccinated against rabies, canine distemper, Type 2 Adenovirus, Coronavirus, Parainfluenza, Parvovirus and Leptospira and treated with anti-helminthic drugs. All dogs were healthy and seronegative for *Leishmania* antibodies in the FML-ELISA assay [9]. Aiming to avoid any bias based on genetically determined susceptibility [10], each brood was randomized by draft into 3 experimental groups (saline, prophylaxis and immunotherapy). For prophylaxis, 6 dogs were vaccinated with 3 doses of 750 μg of VR1012-NH36 plasmid, through the im route on days 0, 21 and 42, while the other 13 dogs received only saline. On day 67, all the animals were challenged with 7 x 10^8 amastigotes from a *L chagasi* strain maintained for 3 passages in hamsters and originally isolated from an infected dog. On day 160, all the 13 untreated dogs were already *Leishmania*-seropositive and symptomatic. Six of them were treated for immunotherapy with 3 doses of 750 μg of theVR1012NH36 vaccine, while the other 7 remained as untreated controls. The sera of all animals was assayed for the presence of anti-NH36 and anti-FML antibodies [9] and the cellular immune response was evaluated by the assessment of the DTH response against leishmanial lysate and of the proportion of CD4+ and CD8+ specific lymphocytes after *in vitro* proliferation of PBMC with 0.5 μg NH36. Dogs were also monitored for their clinical symptoms scores and for their parasite/lymphocyte ratio in Giemsa stained smears obtained after fine needle lymph-node punctures. All cohorts were monitored until day 517. At the end of the experiment, the parasite load of the survivor dogs was evaluated in PBMC and the cytokine expression in whole blood *ex-vivo* was assayed by Real Time PCR as modified from Manna et al., (2006) using the primers and probes represented in Table 1 and the (Taq man system®) [11].

Table 1. Primers and Probes

	Primer forward	**Primer reverse**	**Probe (FAM-MGB)**
β-act.	5'CTGGCACCACACCTTCTACAA 3'	5'GCCTCGGTCAGCAGCA3'	5'GCCTCGGTCAGCAGCA3'
INFg	5'GCGGAAAAGGAGTCAGAATCT GT3'	5'GCGGAAAAGGAGTCAGA ATCTGTT3´	5'GCGGAAAAGGAGTCAGAATCTG TT3'
IL-2	5'GAAGTGCTAGGTTTACCTCAA AGC3'	5'CAGATCCCTTTAGTTTCA GAAGTGTTACA3'	5'ACACCAAGGAATTAATCAGC3'
IL-4	5'GCTCCAAAGAACACAAGCGAT AAG3'	5'CTGCCGCAGTACAGTAGC A3'	5'CTCTGCAGAAGATTTC3'
IL-10	5'CCTGGGAGAGAAGCTCAAGAC 3'	5'CACAGGGAAGAAATCGG TGACA3'	5'CTGAGACTGAGGCTGCGAC3'
L. inf.	5'GGCGTTCTGCGAAAACCG3'	5'AAAATGGCATTTTCGGGC C3'	5'TGGGTGCAGAAATCCCGTTCA3'

Furthermore, the expression of IFN gamma, IL-2, IL-4 and IL-10 were also studied by Real Time PCR after *in vitro* proliferation of PBMC of one dog of the untreated control and one dog of the immunoprophylaxis group, at day 667 and parasite load was also obtained by absolute quantification-Real Time PCR [11].

For statistical analysis means were compared by ANOVA analysis simple factorial test and by one way ANOVA- Tukey´s honestly significant difference method (SPSS for windows). To test the significance of the differences between groups we also used the 95% confidence interval of the averages. All the animals included in this investigation were treated following the guidelines for animal experimentation of the USA National Institute of Health, and experiments were done in accordance with the institutional guidelines in order to minimize animal suffering.

The results of the serological survey are summarized in **Figure 1**

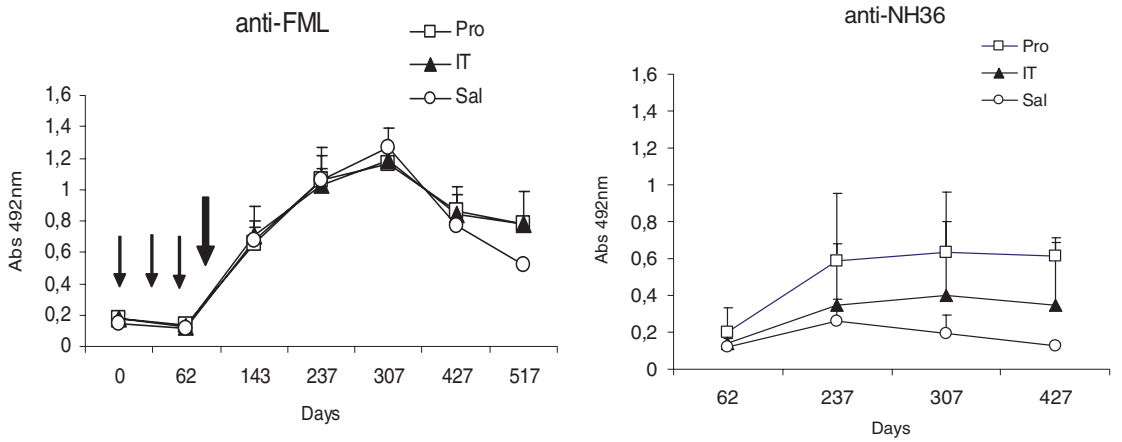

Figure 1. Evolution of the anti-FML and anti-NH36 serological response in dogs treated for prophylaxis or immunotherapy with the VR1012-NH36 DNA vaccine.

The serological immune response against FML and NH36 only started after the infective challenge, meaning that seropositivity is not a consequence of NH36 DNA vaccination. This is a very beneficial effect of the vaccine that can be potentially used in the field for the control of CVL. As a canid zoonoses, the epidemiological campaign removes for sacrifice dogs seropositive to *Leishmania* for they are considered infected and reservoir of disease [12]. The ANOVA analysis of the anti-FML response showed significant differences between times (ANOVA p= 0.000) but not by treatments (p> 0.05) since anti-FML antibodies increased in both vaccinated (prophylaxis and immunotherapy) and control dogs simultaneously. The serological response against the NH36 recombinant protein was, conversely, specifically increased in both prophylaxis and immunotherapy treated dogs (ANOVA p= 0.000) while remained low in infected animals. This is another beneficial effect of the DNA vaccine that discriminates the infected from the vaccinated animals.

The results of clinical, parasitological and cell immune responses of dogs vaccinated with the NH36 DNA vaccine and untreated controls are summarized in **Table 2**.

Table 2. Clinical, parasitological and cell immune response results in dogs vaccinated with the NH36 DNA vaccine.

Treatment	Symptom scores	Parasite/lymphocyte ratio	DTH	CD4+*	CD8+*	Average time of survival (days)
Prophylaxis	39.4 (57.7-21.10)	0.64 (1.93-0.65)	7.18 (8.30-6.07)	42.00 (46.31-37.69)	22.98 (29.63-16.34)	343.25 (211.42-473.08)
Immunotherapy	44.4 (69.62-19.19)	1.18 (2.96-0.60)	5.76 (8.44-3.08)	43.27 (47.31-39.24)	24.99 (34.64-15.30)	367.33 (238.40-496.26)
Saline control	58.4 (80.4-36.40)	2.05 (4.01-0.01)	3.18 (5.55-0.80)	32.10 (38.97-25.23)	26.52 (34.12-18.92)	278.60 (166.56-390.64)

For all variables, the results are shown as the average and CI95% values recorded during the 17 months period of the experiment (**Table 2**). Only the results of parasite/lymphocyte ratio at lymph nodes were recorded at day 307 after the initial vaccine dose. Animals were monthly clinically evaluated. A score of the clinical signs was prepared. We considered the diameters of small (up to 1cm); medium (1-1.5cm) and large lymph nodes (≥2cm) and loss of weight was considered mild (0-2kg), medium (3-5kg), severe (6-8kg) and extremely severe (≥9kg). While asymptomatic cases receive value=0, to the detection of alopecia, onycogryphosis, skin lesions or conjunctivitis was

attributed value = 1; to anaemia or oedema was attributed value=2; to ictericia, cachexia, cough, asthenia, cataract or nasal purulent secretion was attributed value = 4 and to death caused by CVL was attributed value =10. The scores for normal undetectable, small, medium and enlarged lymph nodes were= 0,1,2,3, respectively, and a score of = 2,3,4 or 5 was attributed, respectively, to mild, medium, severe and extremely severe loss of weight. The CD4+ and CD8+ lymphocyte proportions correspond to the counts obtained after *in vitro* proliferation with NH36.

As expected for the development of CVL, the analysis of the CI95% showed that, the untreated controls developed more symptoms, higher parasite/lymphocyte ratio, smaller DTH reactions, lower proportions of NH36-specific CD4+ cells, and sustained NH36-specific CD8+ cell counts, when compared to dogs of the prophylaxis group disclosing the protective potential of preventive vaccination with the VR1012-NH36 DNA vaccine.

In the immunotherapy treated group, enlarged DTH reactions, enhanced CD4+ and sustained CD8+ lymphocyte proportions were also detected, however, without reduction of symptoms or parasite/lymphocyte ratio, when compared to the untreated controls indicating the NH36 DNA vaccine, as 3 doses of 750ug/each was not sufficiently potent to control the disease provoked by 7 x 10^8 amastigotes obtained from a dog infection. Future experiments should be done with smaller infective doses such as those used in other investigations [13] (5 x 10^6 *in vitro* cultured promastigotes) in order to better define the immunotherapeutic potential of the vaccine. The high infective challenge used in this investigation was also responsible for the detected high mortality (**Table 2**). In spite of that, it was possible to see a delay in mortality rates generated by the vaccine. Indeed, the average time of survival of dogs treated for prophylaxis and immunotherapy was 64.65 and 88.73 days higher, respectively, than that of untreated controls (**Table 2**), confirming the preventive and therapeutic effect of the NH36 DNA vaccine.

At the end of the experiment (day 490), the *Leishmania* parasite load (**Figure 2A**) and the cytokine expression (**Figure 2B**) were assayed in PBMC and whole blood, respectively, from one dog of the prophylaxis and one dog of the control group, by PCR Real Time techniques. Also, the cytokine expression was measured after *in vitro* proliferation of PBMC of one control and one dog of the prophylaxis group, at day 667 (**Figure 2C**).

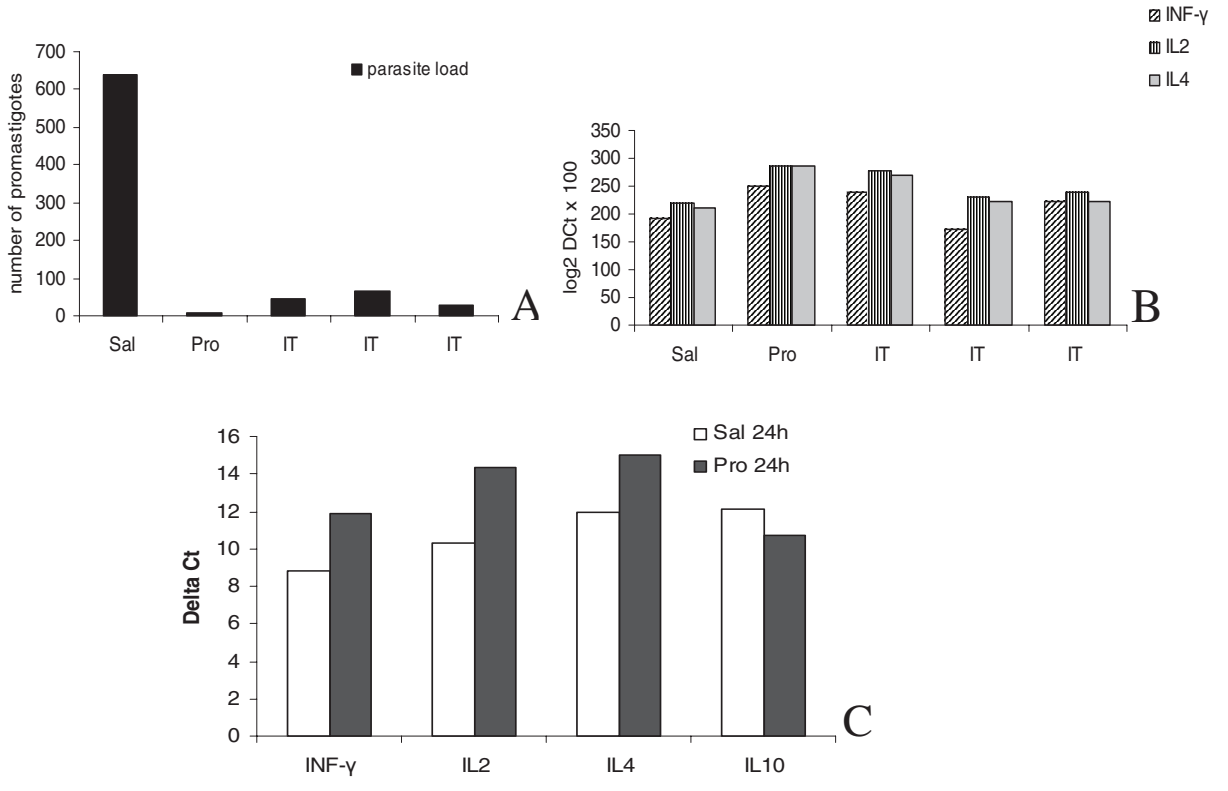

Figure 2. Parasite load obtained by absolute quantification-Real Time PCR in whole blood *ex-vivo* (A) and IFN gamma, IL-2, IL-4 and IL-10 expression in PBMC *ex vivo* (B), and after *in vitro* proliferation with 0.5 μg NH36 (C),

of individual dogs. In (A) the bars represent the number of promastigotes in 2 x 10^6 PBMC, according to a *Leishmania infantum* promastigote standard-curve ranging from 10^1 to 10^6, while in (B) the results of cytokine expression are expressed as relative quantification = $\log_2$ Delta (Ct of each cytokine–Ct of β actin) x 100 and in (C), to delta Ct (Ct of each cytokine–Ct of β actin) [manna 2006].

At day 490, the parasite load of an untreated survivor control dog (638.05 parasites) felt outside the IC95% of that of vaccinated dogs (32.02, IC95% 9.45-64.59) (**Figure 2A**), either by prophylaxis or by immunotherapy suggesting that both vaccination treatments succeeded in reducing the *Leishmania* infective burden provoked by an artificially high parasite challenge of 7 x 10^8 infective amastigotes. Accordingly, the untreated control dog showed a IFN γ-β actin relative quantification (191.72) lower than the average of vaccinated dogs (220.79) although did not felt outside their IC95% (182.34-259.25) **(Figure 2B)**. The IL-2 and IL4 relative quantifications for control dog were 219.91 and 210.80, respectively, and were higher for vaccines: 258 (IC95% 226.53-289.50) and 250.85 (IC95% 213.71-287.98) indicating the global enhancement of the secretion of IFN- γ, IL-2 and IL-4 due to NH36 DNA vaccination treatment. When measuring the cytokine expression of the NH36 specific-lymphocytes after *in vitro* incubation (**Figure 2C**) an increase in IFN-γ, IL-2 and IL-4 and a decrease in IL-10 expression were observed in the vaccinated dog (prophylaxis) relative to the untreated control dog, indicating the presence of a TH1 cell immune response. The decrease in IL-10 after vaccination is a promising result since this cytokine is related to progression to severe CVL [11].

From the analysis of our results we conclude that the VR1012-NH36 vaccine induces strong prophylactic protection and a milder immunotherapeutic effect against a high dose canine experimental infection with *Leishmania chagasi.*

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