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Short communication

Immunogenicity assay of the Leishmune® vaccine against canine visceral leishmaniasis in Brazil

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Leishmune® is the industrialized version of the FML-saponin vaccine which has been shown to develop 92–95% protection in vaccinated dogs and 76–80% vaccine efficacy against field canine visceral leishmaniasis (CVL) in Brazil. Leishmune® has been proven to be safe and tolerable and a transmission-blocking vaccine which renders vaccinated dogs non-infectious to sand fly vectors. In the present investigation, 550 healthy seronegative dogs of endemic and epidemic areas of Brazil were monitored for Leishmune®-induced immunogenicity during a 2-year trial. Another group of 588 untreated exposed dogs was also studied in parallel. Both groups were seronegative on day 0. The strong immunogenicity induced by Leishmune® vaccine was demonstrated by the 98% of FML-seroconversion, increase in absorbencies, the 82.7% DTH positive reactions and increase in skin test size diameters, the average increase in CD8+ total lymphocytes population in blood (27.1%), expected for QS21 saponin-containing vaccine, the sustained proportions of CD4+ T cells, and the average increased proportions of CD21+ B lymphocytes (42.3%). The Leishmune®-induced protection against CVL is demonstrated by the results: 98.8% asymptomatic dogs (at the end of first year) and 99% healthy survivors (at the end of the second year) among vaccinated dogs, compared to the 79.4% asymptomatic and 61% survivor dogs (p < 0.001) monitored in the untreated exposed cohort. In spite of the low vaccine coverage, it was possible to detect a 66.1% (< 0.005) reduction in Aracatuaba of the incidence of CVL among vaccinated dogs, when compared to the global incidence of CVL of each town, respectively. Our preliminary results support the potential use of Leishmune® to prevent CVL epidemics.

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1. Introduction

Visceral leishmaniasis (VL), a chronic and severe protozoa infection, is fatal if untreated after the beginning of symptoms. The disease is a canid zoonosis (ZVL) caused by Leishmania chagasi in America and by Leishmania infantum in the Mediterranean basin and Middle East, and an anthropoosis caused by Leishmania donovani in Africa, India and Asia. Nowadays there are 500,000 new human cases registered annually worldwide [12]. The drug resistance and toxicity of chemotherapy, the increase of the disease incidence of immunocompromised subjects, and the difficulties of the epidemiological control, which is based upon sacrificing of seropositive dogs, emphasizes the need for safe prophylactic vaccines for both humans and dogs [3]. Mathematical modelling analysis indicates the need for human and canine vaccines as tools to reduce the incidence of the disease in endemic regions [4].

The most studied first-generation vaccine, composed of total Leishmania lysate and BCG, protected against VL in Sudan [5] and against canine visceral leishmaniasis (CVL) in Iran [6] but not in Brazil [7]. Lemesre et al. [8], using a second-generation vaccine with the culture media of L. infantum containing a 54-kDa excreted protein in formulation with MDP (LiESap) obtained protection in beagles in a kennel assay [8]. Regarding the recombinant vaccines, the multicomponent Leish-111f fusion protein in combination with MPL-SE or AdjuPrime was only immunogenic in dogs challenged with L. chagasi [9] and L. infantum (MML) [10] and failed to prevent L. infantum natural infection or the progression of disease in dogs in an open kennel trial [11]. A few third-generation DNA vaccines have been tested in dogs against experimentally induced CVL.
achieving different levels of protection, but no data from field trials of any DNA formulation is so far available [16].

Despite the recent intensification in research for a canine vaccine only two second-generation vaccines with native antigens have progressed to Phase III field trials: the FML-saponin [17,18] and the LiESap vaccines [19]. The FML (fucose–mannose ligand) glycoprotein complex [20], antigenic for both humans [21] and dogs [22], was formulated, as a second-generation vaccine, with Quillaja saponaria saponin and underwent Phase I–III trials becoming the Leishmune® licensed vaccine in Brazil [23]. The FML was immunogenic, immunoprophylactic and immunotherapeutic, in mice and hamsters and canine field trials [17,18,24–27]. In the first Phase III dog assay [17], 4 deaths and 6 symptomatic cases among 30 placebo-treated dogs (33%) were detected and confirmed by parasite analysis and PCR. No deaths were detected among vaccinated and infected was confirmed in 3/36 oligosymptomatic dogs (8.33%), resulting in 92% protection and 76% vaccine efficacy [17]. In the second field assay [18], the infective pressure was higher and 2 years after vaccination, deaths were detected in 8/33 (25%) placebo-treated and 1/20 (5%) vaccinated dogs, resulting in 95% of protection and 80% vaccine efficacy. This protection lasted for at least 3.5 years and was concomitant with the reduction of the human incidence of the disease in the area [18]. The FML-vaccine also produced an immunotherapeutic effect when administered to L. donovani- or L. chagasi-infected dogs while they were still asymptomatic [28]. The decrease in the canine and human incidence of visceral leishmaniasis in the vaccinated area [18] and the maintenance of normal proportions of CD4 and CD21 lymphocyte levels in the blood of vaccinated dogs [28] indicate that dog vaccination with the FML-vaccine reduces dog infectivity to sand flies [23]. LiESap + MDP vaccine, the other second-generation vaccine with native antigens, was recently used in a field assay with naturally exposed dogs of South France [19]. In this trial any dog showing clinical and/or serological evidence, infection was confirmed (or not) by the presence of parasites in bone marrow cultures and by PCR analysis. After 2 years, the incidence of infection was 0.61% (1/165) in vaccines versus 6.86% (12/175) in placebo-treated dogs (92% vaccine efficacy) [19]. The authors claimed a vaccine efficacy of 92% based on the confirmation of infection by very sensitive methods such as PCR or culture, instead of CVL deaths [19] which did not occur in this area of lower incidence. The FML-vaccine unlike the LiESap vaccine revealed protection not only against infection, but also against severe disease and deaths due to CVL [17,18] reducing morbidity and mortality [17,18] which are much stronger criteria of protection [29].

The FML-vaccine was licensed in Brazil, for dog prophylaxis against ZVL, under the brand Leishmune® [23]. Dogs vaccinated with Leishmune® (FML-licensed vaccine) are not infectious for sand flies [23], as indicated by a complete absence of clinical signs and of parasites in the skin, lymph node and blood PCR-amplified samples. Exposed untreated controls on the other hand, were symptomatic (25%) and showed parasites in their lymph nodes (56.7%), Leishmania DNA detected by PCR in blood (15.7%) and immunohistochemical reactions in skin (25%) [23]. Leishmune® is a transmission-blocking vaccine [30] and when used with increased adjuvant concentration was also effective in immunotherapy on experimental CVL [31].

Recently we described the safety analysis of Leishmune® vaccine performed in a cohort of 600 dogs from Brazilian endemic and epidemic areas of canine and human visceral leishmaniasis. The vaccine proved to be tolerable and safe [32]. In the present investigation we report the immunogenicity assay of Leishmune®, monitored in the same dog cohort, confirming the immunoprotective potential previously described for the FML-saponin vaccine [17,18] and disclosing the potential use of Leishmune® vaccine to interrupt epidemics.

2. Materials and methods

2.1. Animals and study design

Six hundred healthy dogs from the canine visceral leishmaniasis endemic towns of Aracatuba, Andradina, Valparaíso, Guaraparé, Bauru (São Paulo state) and Belo Horizonte, Nova Lima, Sete Lagoas (Minas Gerais state), Brazil, showing previous negative results in Leishmania-serology by the immunofluorescent assay [33] were selected for vaccination with three doses of Leishmune® (Fort Dodge Animal Health, Campinas, SP, Brazil), in a 21-day interval, through the subcutaneous (sc) route [32] and a booster in month 12. On day 0, before vaccination, 50 from the 600 dogs were excluded due to their positive reaction to the more sensitive FMLELISA assay [22]. The remaining 550 dogs, seronegative to the FML antigen, asymptomatic and showing good physical condition, became the trial group of this investigation. Each of the 30 veterinarians participating in this trial vaccinated 20 dogs with three doses of Leishmune®, making a total of 1800 doses. The animals were monitored for their anti-FML IgG serum antibody titers by the FML-ELISA assay [22] at days 0 and 70 and in months 7 and 12, and by their intradermal response to the L. donovani promastigote lysate [17,18] antigen in months 7 and 12. The serum was collected and intradermal test was carried out before injection of the vaccine booster in month 12. Also, clinical evaluations were performed every 3 months, during the 2-year period (2003–2005). Alopecia, onychogryphosis, cachexia, anorexia, apathy, disseminated ulcers, skin lesions, keratitis, renal failure, loss of weight, lymph node enlargement or diarrhoea were recorded as visceral leishmaniasis symptoms. In vaccinated symptomatic animals, Leishmania infection was confirmed either by PCR analysis of lymph node aspirates and/or blood samples [23] or by direct microscopic observation of Leishmania amastigotes in Giemsa stained lymph node smears [31]. The Leishmune®-vaccinated dog cohort included 511 animals (85%) from 61 different breeds and of 89 (15%) mongrel dogs [32]. All animals were previously vaccinated against distemper, parvovirus, parainfluenza virus, leptospirosis, coronaviruses, type 2 adenoviruses and rabies.

For ethical reasons, veterinarians were not able to keep an untreated and exposed control dog population. For the purpose of comparison, 588 asymptomatic FML-seronegative dogs from another endemic area (Jardim Progresso, Natal, RN, Brazil), with similar canine incidence [34] were included in this study as the exposed untreated group. In this investigation, all manipulations performed on the animals were conducted to ensure minimal animal suffering, as recommended by the NIH regulation.

2.2. Vaccine preparation

Each Leishmune® prophylactic vaccine dose [23] was composed of lyophilized FML antigen adjuvanted with saponin, reconstituted in 1 ml NaCl 0.9% sterile saline solution at the moment of vaccination and administered subcutaneously. The FML-vaccine, Leishmune®, is patented: INPI number: PI1100173-9 (18 March 1997) assigned to Universidade Federal do Rio de Janeiro, Brazil and is the first second-generation vaccine licensed against leishmaniasis, since 11th June 2003 [23].

2.3. Delayed-type hypersensitivity (intradermal reaction to promastigote lysate)

This was determined by injecting dogs intradermally, in the inner aspect of the right hind leg, with 0.1 ml of L. donovani freeze-thawed antigen containing 200 µg protein in NaCl0.9% sterile saline solution (10⁸ stationary phase promastigotes/ml). The left hind leg
received only 0.1 ml saline. Measure of the increase of intradermal reaction was performed 48 h after antigen injection. Indurate areas were marked and each time the values of the saline control were subtracted from the reaction due to the *Leishmania* antigen. Reactions showing diameters  \( \geq 5 \) mm were considered positive [17,18].

### 2.4. Flow cytometry analysis of PBMC

In month 18 after vaccination, PBMC of 15 randomly chosen *Leishmune*-vaccinated dogs from Aracatuba and Andradina were analysed by Flow cytometry. Three milliliters of blood from the cephalic vein was collected from each dog in Heparin-tubes, transported at room temperature and processed 48 h after collection [31]. For the *ex vivo* analysis, 30 \( \mu l \) of blood was incubated for 30 min at room temperature, with 30 \( \mu l \) of each one of the following monoclonal antibodies diluted in Facs dil solution (10% FCS-supplemented PBS buffer): anti-Thy-1 (Rat-lgG2b-clone YKIX337.217) (1:800), anti-CD5 (Rat-lgG2a-clone YKIX322.3) (1:800), anti-CD4 (Rat-lgG2a-clone YKIX302.9) (1:12500), and anti-CD8 (Rat-lgG1-clone YCATE55.9) (1:100). Facs dil solution was used as negative control. After this period, 2 ml of PBS-W (PBS buffer with 0.5% bovine serum albumin and 0.1% sodium azide) were added to each tube and the mixture was homogenised, and centrifuged at 1300 rpm, at room temperature, for 7 min. The supernatants were aspirated and pellets homogenised and added to 60 \( \mu l \) of anti-rat FITC conjugate (1:200) (Serotec, UK) except for the Facs dil cell control. At this time, 4 \( \mu l \) of the FITC-labelled mouse anti-human-CD21 (Mouse-lgG1-clone I0B1a) monoclonal antibody (Immunotech Co., Marseille, France) was used in a direct immunofluorescence procedure. All suspensions were homogenised, incubated for 30 min at room temperature in the dark and treated with 2 ml of the 1/10 diluted lysis solution during vortex homogenisation (Becton & Dickinson, USA). The mixtures were further incubated for 10 min at room temperature in the dark and further centrifuged at 1300 rpm for 7 min. Supernatants were discarded and the pellet-containing tubes were inverted on to absorbent paper. All these procedures were repeated twice after the addition of 2 ml PBS. The pellets were homogenised carefully and finally fixed with 300 \( \mu l \) of 2.8% formaldehyde–PBS. The relative immunofluorescence of cells was counted in a total of 10,000 events measured in a Becton Dickinson Facscalibur apparatus and further analysed using Windows Multiple Document Interface Flow Cytometry Application (WinMDI) Version 2.8 software [31]. As control, FACS analysis of PBMC of nine normal healthy untreated dogs was also performed.

### 2.5. Statistical analysis

Comparison of proportions was carried out using the \( \chi^2 \)-test. To test the significance of the differences between groups we used the 95% confidence interval of the averages.

### 3. Results

Five hundred and fifty dogs previously assayed for *Leishmune*-safety analysis [32] were also monitored for the vaccine-induced immunogenicity during a 2-year trial (2003–2005). Another group of 588 untreated exposed dogs were also studied. The differences between the two groups after vaccination were highly significant in all variables (\( p < 0.005 \)) (Table 1). Both the vaccinated and the untreated groups were seronegative at day 0, but a strong FML-seroconversion (98%) was detected after complete vaccination on day 70. By this time, 15% of the untreated controls developed anti-FML antibodies due to their exposure to natural infection. The vaccine increased, not only the number of dogs with positive

| Table 1  | Two-year evolution of immunogenicity and incidence of ZVL in cohorts of *Leishmune*-vaccinated and untreated dogs |
|----------|--------------------------------------------------------------------------------------------------------------|
| Time     | Symptoms of ZVL/alive (%) | DTH positive (%) | FML ELISA positive (%) | ZVL confirmed deaths (%) |
| D0       | 86.0 (423/492)             | 0 (0/550)        | 0.0 (0/550)            | 58.9 (129/219)           |
| D70      | 98.0 (423/432)             | 0.0 (0/550)      | 0.0 (0/550)           | 92.7 (205/225)           |
| D9       | 13.5 (65/477)              | 0.0 (0/550)      | 0.0 (0/550)           | 9.7 (26/279)             |

Data obtained from *Leishmune*-vaccinated dogs of Aracatuba and Andradina, SP, and from untreated exposed dogs of Jardim Progresso, RN, 2 years after vaccination. D, day; M, month; ND, not determined.
antibody response (cutoff Abs. 492 nm = 450) but the absorbency values in the FMLELISA assay as well. The mean average ± S.D. of sera absorbencies was 0.925 ± 0.201 (n = 423), on day 70 and 0.875 ± 0.277 (n = 195), in month 12 (p < 0.05), indicating a sustained humoral response along time, probably related to the natural booster of L. chagasi-infected sand flies in the endemic area.

The delayed type of hypersensitivity response (DTH) against the leishmanial lysate was positive in 59% of the vaccinated dogs, in month 7, and increased along time (Table 1) disclosing that, as desired for a protective vaccine against CVL, Leishmune® prophylactic vaccine enhances not only the humoral response but also triggers the cellular immune response against the parasite as well. The size of skin test reactions can be used as a measure of potency of a vaccine. The mean ± S.D. diameter of the skin tests at month 7 was 7.39 ± 2.33 mm (n = 129). The skin tests diameters significantly increased until month 12 to 8.24 ± 2.62 (n = 105, p < 0.005), when DTH reactions were positive in 82.7% of the vaccines. These results confirm that the natural booster in an endemic area, while sustaining the humoral response of Leishmune®, contributes to enhance the specific anti-L. chagasi cellular immune response which is known to be responsible for protection against CVL.

While both vaccinated and untreated dogs were both healthy at the beginning of the study, the untreated dog group developed a greater number of ZVL symptoms along time reaching 20.6% of the cohort by the end of the first year. Meanwhile, clinical signs were detected in only 1.2% of the vaccines during the same period (Table 1). Accordingly, while the cumulative proportions of deaths due to confirmed ZVL in the untreated dogs reached 39% of the cohort, only 1% of the vaccinated dogs died of ZVL during the trial. Our results indicate the strong protective prophylactic effect of Leishmune® in seronegative dogs of endemic areas.

Table 2 summarizes the results of the immunophenotype analysis of PBMC of a randomly selected sample of 15 dogs, collected among Leishmune® vaccines, 18 months after vaccination, and of 9 normal and healthy untreated control dogs. We observed that the mean average of CD4+ lymphocytes of normal Leishmune®-vaccinated dogs (44.61%) fell inside the 95% confidence interval of the normal dogs (44.07%, CI 95% 21.4–51.28). The proportions of CD8+ (26.69%) and of CD21+ (10.53%) lymphocytes in Leishmune® vaccines are, on the other hand, increased, falling outside the respective confidence intervals (19.45%, CI 95% 11.27–22.50 and 6.07%, CI 95% 3.01–7.98) of the normal dogs, indicating the modulation of the cellular immune response due to Leishmune® treatment. The average increase in proportions was 27.1% for the CD8+ T cells and 42.3% for the CD21+ B lymphocytes.

The great difference in the incidence of ZVL disclosed in vaccines and controls (Table 1) could be partially due to the different infective pressure of the towns where Leishmune®-vaccinated dogs and untreated controls were located. However, when we compared the incidence of ZVL in Leishmune®-vaccinated dogs to the incidence of the total dog population of the same town, the vaccine-induced protection was also evident. Table 3 shows the official data mean values of canine incidence of ZVL during the period 2003–2005. We show that the ZVL incidence in Belo Horizonte, decreased from 7.97% in the total population [35] to 2.70% (p < 0.005) in the Leishmune®-vaccinated dogs, in the first 2 years of the vaccine use. The more striking effect of the prophylactic vaccination was seen in Aracaju, where the incidence of ZVL decreased from 29.67% of the total population to 5.88% in vaccines (p < 0.005), leading to an 80.2% reduction in the ZVL incidence and thus confirming the strong protective effect of Leishmune®.

4. Discussion

The cohort of vaccinated dogs analysed in this investigation was the same previously used for the safety analysis of the Leishmune® vaccine [32], which confirmed that the formulation was tolerable and safe. In this investigation, we confirmed the strong immunogenicity of Leishmune® vaccine, previously shown for the FML-saponin vaccine in the field [17,18] indicating that the commercial formulation maintains the characteristics of the laboratory-prepared vaccine. Soon after complete vaccination, the percent of seropositivity to FML in dogs treated with the FML-saponin vaccine was 62% [17], while it reached 98% in Leishmune® vaccines. This indicates the earlier achievement of humoral response induced by the commercial formulation, which is an important indicator of the vaccine potency. The humoral response was also sustained at high levels for 12 months after vaccination, probably due to the natural booster effect of L. chagasi-infected sand flies of the endemic area. Conversely, a previous kennel experiment in a non-endemic area, showed in Leishmune®-vaccinated unexposed dogs, a decrease in absorbencies from day
70 (1.017 ± 0.250) to month 12 after vaccination (0.673 ± 0.160) (unpublished results).

Twelve months after vaccination, a positive DTH response to leishmanial antigen was present in 91% of the FML-saponin-vaccinated dogs (7.79 mm average skin test diameter) [17], and in 82.7% of Leishmune®-vaccinated dogs which showed slightly larger diameters of skin tests (8.2 mm), indicating that beside the antibody response, the induction of the cellular immune response against Leishmania lysate was also preserved in the commercial formulation.

Leishmune® vaccination induced an increase in CD8+ total lymphocytes population in blood, also observed after dog immunotherapy with the FML-saponin vaccine [28]. This was expected for a vaccine containing the QS21 saponin adjuvant of Q. saponaria Molina and it was related to its hydrophobic normonoterpene moiety [36,37] but also present in the deacylated saponins of Q. saponaria Molina [36] and the decacylated saponins of Calliandra pulcherrima [38] which lack the hydrophobic moieties.

Evidence of the involvement of CD8+ lymphocytes in protection against intracellular parasitic infection has increased recently [39]. CD8-defficient mice failed to control Leishmania parasite growth [40]. Also, CD8 specific cells are primed during natural infection or human vaccination and secrete IFN-γ, when re-stimulated in vitro with Leishmania antigens [41–43]. In naturally infected asymptomatc dogs, increased levels of CD8+ lymphocytes appeared as the major phenotypic feature, as well as in dogs bearing a lower parasite load [44], indicating a correlation with natural protection. While no effect was observed after L. infantum lysate vaccination [45], an increase in CD8+ T cells was observed after vaccination with the Q. saponaria saponin-containing vaccines: FML-QuilA (56.02%), FML-saponin (38.18%) [28], Leishmune® (6.00%) [46] and the L. brasiliensis lysate-saponin vaccine (4.00%) [47]. The intense cell proliferation and increased nitric oxide production during in vitro stimulation by L. chagasi soluble antigens, suggests the induction of a potential resistant profile [47].

The total levels of CD4+ and CD21+ lymphocytes are expected to decrease in advanced canine visceral leishmaniasis [31,45,48,49] with the specific decrease of CD4+ cells being correlated with dog infectivity to sand flies [45]. Indeed, the average percent value of CD4 lymphocytes in naturally infected dogs with visceral leishmaniasis was 20.47% [45]. On the other hand, vaccination against ZVL is expected to expand or sustain the CD4+ T cell levels. The expansion or sustentation of CD4+ T cell levels of dogs treated with L. infantum vaccine immunotherapy was 35% [50], with the FML-QuilA vaccine it was 43.30%, with FML-saponin R 44.94% [28] and with the Leishmune® vaccine was 35.00% [46]. Sustained CD4+ T cell were found also in this investigation in dogs vaccinated with Leishmune®, and sustained L. chagasi-specific CD4+ T cell proportions were found before in dogs treated with Leishmune® after infection [31] indicating that the commercial formulation maintains the immunogenity and potency demonstrated by the FML-saponin vaccine in the prophylaxis [17] and immunotherapy [28] against ZVL. Both the Leishmune® prophylactic vaccine, which contains 0.5 mg of [23] and the Leishmune® immunotherapeutic [31] or the L. brasiliensis [47] vaccines, that contain 1mg of the Riedel de Haen saponin, maintain the normal levels of CD4+ lymphocytes in vaccinated unexposed [47], exposed [28] or challenged [31] dogs.

Higher proportions of CD21 cells were detected in this investigation in the Leishmune®-vaccinated dogs. The levels of CD21+ cells were higher in vaccinees, with a mean average that fell outside the CI95% interval of the normal dogs. Increased CD21+ B cell levels were also found in dogs vaccinated with the L. brasiliensis-saponin vaccine that uses the same adjuvant included in Leishmune® [47] and in unexposed dogs vaccinated with Leishmune® [46]. The increase in total and in Leishmania-specific CD21+ B circulating lymphocytes was synchronous with the induction of an intense humoral response [47]. Also, a positive correlation was found between PBMC proliferation in response to L. chagasi antigen and CD21+ B cells. This was considered an indication of the major APC function of the CD21+ cells [47]. On the other hand, decreased CD21+ B cell proportions are expected to occur in untreated infected dogs with advanced ZVL [45,48], which also exhibit hypergammaglobulinemia, a hallmark of human and canine visceral leishmaniasis [51]. These facts suggest that the CD21+ B lymphocyte population, which increased after vaccination with Leishmune® is involved not merely in the expansion of the total humoral response but in the increase of the specific synthesis of the IgG2 anti-FML antibodies that are related to protection [52–56] and blockade of the transmission of VL in the field [30].

In this investigation, we have demonstrated the strong immunogenicity of Leishmune® in healthy exposed dogs in epidemic areas, which consequently exhibit a strong and sustained humoral and cellular immune response against the parasite. A classic Phase III trial, with random double-blind selected controls could not be performed because of the ethical restrictions of the veterinarians from these epidemic areas, who refused to include untreated healthy dogs as exposed controls. Leishmune®-induced protection against ZVL, is however suggested by the results of 98.8% of asymptomatic dogs (at the end of first year) and 95% healthy survivors (at the end of the second year) among vaccinated dogs, compared to the 79.4% of asymptomatic and 61% survivor dogs monitored in an untreated exposed cohort in another endemic area. Although a vaccine against ZVL is considered an efficient tool for eradication of human and canine visceral leishmaniasis [4] and Leishmune® is the first vaccine in the world to be licensed against ZVL [16], its vaccine coverage in Brazil is still very low. In spite of this the incidence of ZVL among vaccinated dogs in Belo Horizonte suffered a significant reduction of 66.1% (p < 0.005), and there was an 80.2% (p < 0.005) significant decline in Aracatuba, when compared to the global incidence of ZVL of both towns, respectively. Thus our preliminary results then support the potential use of Leishmune® for the prevention of ZVL epidemics.

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