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Short communication

Decrease of the incidence of human and canine visceral leishmaniasis after dog vaccination with Leishmune® in Brazilian endemic areas

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ABSTRACT

Leishmune®, the first prophylactic vaccine licensed against canine visceral leishmaniasis (CVL), has been used in Brazil since 2004, where seropositive dogs are sacrificed in order to control human visceral leishmaniasis (VL). We demonstrate here that vaccination with Leishmune® does not interfere with the serological control campaign (110,000 dogs). Only 1.3% of positivity (76 among 5860) was detected among Leishmune® uninfected vaccinees. We also analyzed the possible additive effect of Leishmune® vaccination over dog culling, on the decrease of the incidence of CVL and VL in two Brazilian endemic areas, from 2004 to 2006. In Araçatuba, a 25% of decline was seen in CVL with a 61% decline in human cases, indicating the additive effect of Leishmune® vaccination of 5.7% of the healthy dogs (1419 dogs), on regular dog culling. In Belo Horizonte (BH), rising curves of canine and human incidence were observed in the districts of Barreiro, Venda Nova and Noroeste, while the canine and human incidence of Centro Sul, Leste, Nordeste, Norte, Pampulha and Oeste, started to decrease or maintained a stabilized plateau after Leishmune® vaccination. Among the districts showing a percent decrease of human incidence (−36.5%), Centro Sul and Pampulha showed the highest dog vaccination percents (63.27% and 27.27%, respectively) and the lowest dog incidence (−3.36% and 1.89%, respectively). They were followed by Oeste, that vaccinated 25.30% of the animals and experienced an increase of only 12.86% of dog incidence and by Leste and Nordeste, with lower proportions of vaccinees (11.72% and 10.76%, respectively) and probably because of that, slightly higher canine incidences (42.77% and 35.73%). The only exception was found in Norte district where the reduced human and canine incidence were not correlated to Leishmune® vaccination. Much lower proportions of dogs were vaccinated in Venda Nova (4.35%), Noroeste (10.27%) and Barreiro (0.09%) districts, which according to that exhibited very increased canine incidences (24.48%, 21.85% and 328.57%, respectively), and pronounced increases in human incidence (14%, 4% and 17%, respectively). The decrease of canine ($p = -0.008$) and human incidences ($p = -0.048$) is directly correlated to the increase of the number of vaccinated dogs, confirming the additive control effect of Leishmune® vaccination over dog culling, reducing the parasite reservoir, protecting dogs and, in this way, reducing the risk of transmission of VL to humans and becoming a new effective control tool.

1. Introduction

Human visceral leishmaniasis (VL) or kala-azar is, in the Mediterranean and in the New World, a re-emergent canid zoonoses [1]. The parasite is exposed on the skin of dogs and wild canids and transferred to human through the bite of the specific sand flies. The present epidemiological control, as recommended by the World Health Organization, involves the treatment of human cases, the insecticide vector control and the removal for sacrifice of the *Leishmania*-seropositive dogs [1]. In Brazil, the impact of the control campaign has been either supported [2–5] or contested [6,7], mainly for being too laborious and of doubtful efficacy, probably due to the low sensitivity of the diagnostic methods [4,5,8,9] and delay in the removal of infectious dogs [8]. A mathematical model for the transmission of zoonotic visceral leishmaniasis (ZVL) was developed by Dye [10] who initially proposed that dog removal

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would not have impact on the decrease of the canine infectious population. Further analysis of the same model proved, in contrast, that dog culling might however be efficacious if sensitivity of diagnostic methods were increased [5,11] and if time between diagnosis and dog removal would be shortened [11]. On the other hand, one widely accepted conclusion of Dye's model [10] is that human or dog prophylactic vaccination would be useful and potent tools for the reduction of the disease incidence.

First generation *Leishmania* vaccine using BCG [12] or muramyldipeptide [13] failed to prevent canine visceral leishmaniasis (CVL) in Brazil and France, respectively, while the use of Alum+ BCG resulted in 69.3% of vaccine efficacy against the disease in Iran [14]. A second-generation vaccine composed of *Leishmania infantum* antigen in combination with muramyldipeptide (LiESAp) induced 92% of vaccine efficacy, recorded with sensitive molecular diagnosis methods in an endemic area where no deaths or severe cases of CVL occurred indicating its low infective pressure [15].

A prophylactic vaccine against canine visceral leishmaniasis was recently licensed in Brazil under the trade name of Leishmune® [16]. It is the first in the world licensed second-generation vaccine against leishmaniaisis [17,18], consisting of an industrial formulation of FML-saponin vaccine which was immunoprophylactic and immunotherapeutic in mice, hamsters and dogs [19–25] and showed safety [26], 92–95% of protective effect in vaccinated dogs and 76–80% of vaccine efficacy (VE) in previous field assays in Brazil [19,20]. Dogs vaccinated with Leishmune® showed reduced exposure of parasites to sand flies [16] and Leishmune® behaves as a transmission blocking vaccine, raising antibodies in protected dogs which impede the binding of *Leishmania* to the sand flies midguts curtailing the transmission of the infection in nature [27]. Preliminary results of xenodiagnosis also support these findings [18]. A recent assay with the Leishmune® commercial preparation on 550 seronegative dogs of endemic areas disclosed 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 [28]. In spite of the low vaccine coverage in Brazil, it was possible to recently detect a 66.1% ($p < 0.005$) and a 80.2% ($p < 0.005$) reduction of CVL incidence among vaccinated dogs of Belo Horizonte and Araçatuba, respectively, when compared to the global incidence of each town [28].

In the present work, we describe the effect of the use of Leishmune® for canine prophylaxis on the decrease of the incidence of canine and human disease, after three years of vaccination (2004–2006), in two Brazilian endemic areas: Araçatuba, São Paulo state and Belo Horizonte, Minas Gerais state. We also analyzed the influence of vaccination on the reduction of the proportion of dogs required to be sacrificed by the control campaign, in order to interrupt the epidemics. We also report that dogs vaccinated with Leishmune®, while partially seropositive in the FML ELISA assay [19,28,29], show negative results in the anti-*Leishmania* ELISA test recommended by the Brazilian Ministry of Health as the official control test, meaning that the Leishmune® vaccination does not interfere with the serological control campaign.

2. Material and methods

2.1. Effect of the use of Leishmune® prophylactic vaccine in dog and human incidence of visceral leishmaniasis in endemic areas

In the epidemiological cycle of VL in the New World, only dogs are capable of infecting sand flies. The contribution of humans to transmission is considered negligible [10]. Canine serological screenings for the presence of anti-*Leishmania chagasi* antibodies are regularly performed by Public Heath organs, in Brazilian regions were CVL and VL are endemic. We here reproduced the official numbers of human cases registered in the towns of Araçatuba (from 2002 to 2006), Sao Paulo state (SP), and Belo Horizonte (BH), Minas Gerais state (MG) (from 1999 to 2006) [30], by the respective Zoonoses Control Centers. In these two towns, the insecticide vector control is applied regularly, not eradicating however the disease while other control tools such as insecticide impregnated dog's collars or dog chemotherapy are not systematically but only occasionally used. These regions are considered new epidemic areas of VL and CL in Brazil.

As in other Brazilian endemic areas [31] and as recommended by WHO [1], the epidemiological control in Araçatuba and Belo Horizonte is based upon the treatment of human patients, insecticide treatment of residences and removal and sacrifice of dogs found seropositive to leishmanial total antigen of *Leishmania major*-like or *Leishmania braziliensis*, in the ELISA assay of eluates of blood according to the Ministry of Health policy [31], using the Biomanguinhos test (Fundação Oswaldo Cruz, Rio de Janeiro, Brazil) and confirmed by the Indirect Immunofluorescency test [9,32]. Reactions are considered positive if fluorescent at a 1:40 dilution. This is the usual limit value for the diagnosis of leishmaniasis in immunofluorescence assays in field control in Brazil considered as referenced by the Brazilian National Foundation for Health (FUNASA). The methods used for serological control were identical then in Araçatuba and Belo Horizonte, turning the results useful for comparison. In Belo Horizonte, dog's serological screening was performed by Center for Zoonoses Control BH, using the ELISA assay on blood eluates, since 1997. The samples from Araçatuba were diagnosed by the Adolfo Lutz Institute, SP, using the same recommended methods since 2002. Data of Araçatuba and Belo Horizonte [30] correspond to the screening of the whole town dog population. No changes in removal practices occurred during the studied period.

Data about the total number of healthy seronegative dogs vaccinated with Leishmune ® were obtained from the vaccine manufacturer, Fort Dodge Animal Health, Campinas, SP, Brazil. Briefly, dog vaccination was done with 3 doses of Leishmune ® (Fort Dodge Animal Health, Campinas, SP, Brazil), in a 21-day interval, through the subcutaneous (*sc*) route [26,28] and one a booster on month 12. Before vaccination, all dogs were seronegative to the FML antigen, asymptomatic and show good physical condition. Leishmune® is only applied by Fort Dodge registered veterinarians, who did all the manipulations, keeping the animal suffering the minimal as possible. We performed an initial comparison between the number of dogs that underwent complete vaccination (including annual booster) estimated either by the number of doses distributed by the manufacturer to the veterinarians (50,917) or by the actual number of dogs vaccinated as reported by 26/207 veterinary clinics form BH and found that both variables were highly correlated ($p = 0.000$) as did also their cumulative percent counts ($p = 0.001$) (results not shown). In order to facilitate the analysis of the results we only show the total numbers of vaccinated dogs according to the manufacturer information which are more representative.

In order to evaluate the possible reactivity of sera of Leishmune® vaccinated dogs in the ELISA test used by the official serological control campaign for visceral leishmaniasis, data of the serological control campaign of 2007 of Campo Grande, Mato Grosso state, Brazil were also included. These are official data from the Center for Zoonoses Control (CCZ) of Campo Grande. Sera samples were tested for antibodies in the ELISA and IF test of Biomanguinhos (Fundação Oswaldo Cruz, Rio de Janeiro, Brazil) and in the *Leishmania chagasi* HSP70 recombinant ELISA test (s7 fragment of the heat shock protein HSP70-Biogene, Recife, Brazil). To assay the infection, the presence of amastigotes of *Leishmania* was assayed on Giemsa stained smears of lymph node or bone marrow puncture samples.

2.2. Statistical analysis

For correlation coefficient analysis we used the Pearson's bivariate test, two-tailed test of significance (SPSS). To test the significance of the differences between groups we used the 95% confidence interval of the averages.

3. Results

3.1. Impact of Leishmune® vaccination on the decrease of canine and human incidence of visceral leishmaniasis in the field

We investigated if the preventive vaccination with Leishmune® has an additive effect to the dog culling on the decline of the epidemics. We reproduce here the official number of canine and human cases of ZVL of Araçatuba (2002–2006) (Fig. 1) and Belo Horizonte (1999–2006) (Fig. 2).

In Araçatuba (Fig. 1A and B), dog removal based on seropositivity began in 2002, attained a maximum in 2003 (10,527 dogs, 30.01% of the total population) and started a significant decline during 2004. This is evident either if expressed as the total number of removed seropositive dogs (Fig. 1A) or as the percentage of seropositive sacrificed dogs among total dog population (Fig. 2B). The number of human cases of visceral leishmaniasis displayed a similar curve with a peak in 2002 (52 cases) and a mild decline towards the end of 2003 (Fig. 1C), probably as a result of the initiation of infectious dog removal the year before (Fig. 1A and B). However, while only a 25% of decline in the number of removed dogs was noticed from 2004 to 2005 (9268–6909, respectively) (Fig. 1B), a 61% severe decline was seen in the human cases during the same period (36 to 14 cases). Since no significant change in total dog population (30,000 in 2004 to 28,000 dogs in 2005), or leishmaniasis control tools occurred during this period, we attribute the pronounced decrease of human cases to the beginning of vaccination of the healthy and seronegative dogs, with the preventive Leishmune® vaccine, after its license,

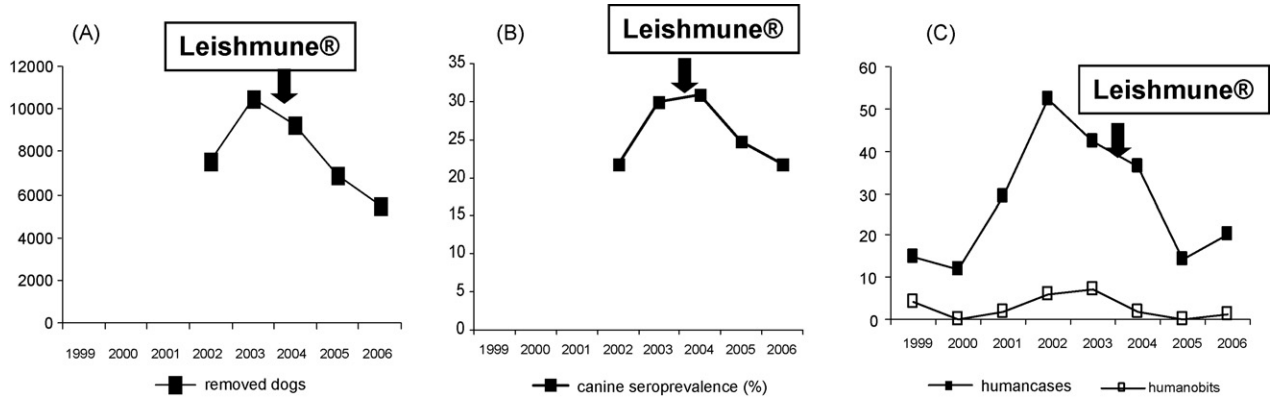

Fig. 1. Additive effect of the Leishmune® canine vaccination on the removal of infected dogs in the decrease of human visceral leishmaniasis incidence in Araçatuba. (A) Evolution of the number of *Leishmania*-seropositive dogs removed to sacrifice by the control campaign from 2002 to 2006; (B) evolution of the canine seroprevalence (percent of number of *Leishmania*-seropositive dogs among the total dog population) removed to sacrifice by the control campaign from 2002 to 2006; and (C) evolution of the number of human cases of VL and human obits from 1999 to 2006.

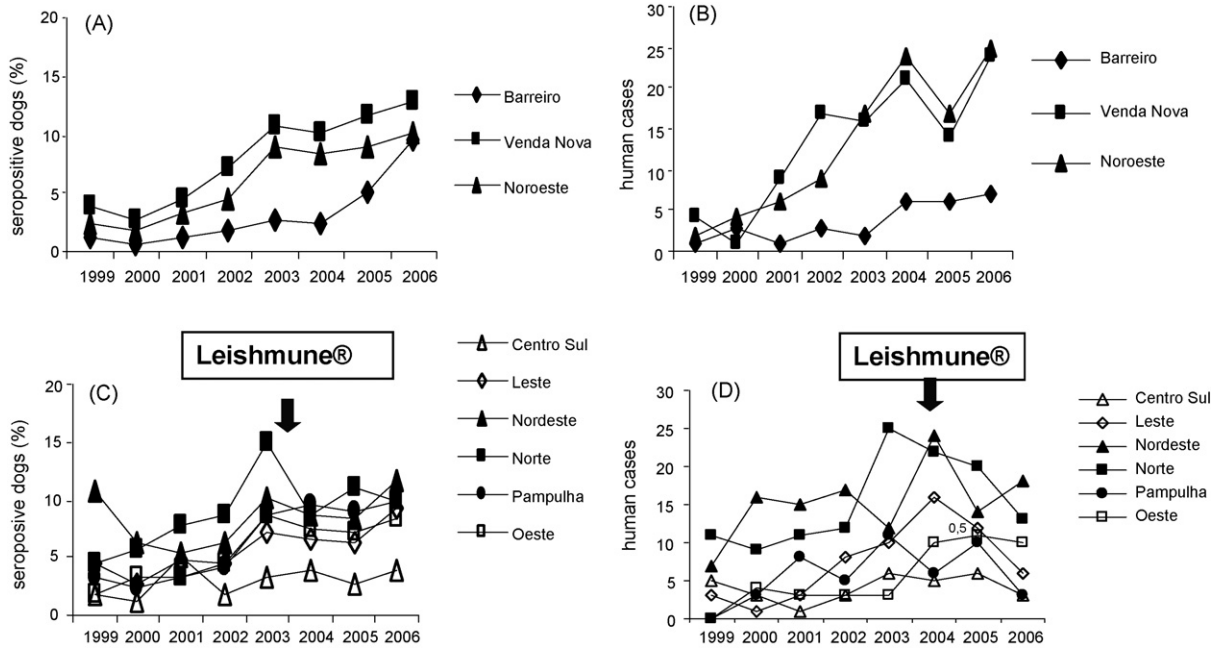

Fig. 2. Additive effect of the Leishmune® canine vaccination on the removal of infected dogs in the decrease of human visceral leishmaniasis incidence in Belo Horizonte. Evolution of the percent of seropositive dogs removed by the control campaign along the time (from 1999 to 2006) in the districts with increased (A) and sustained or decreased human incidence after the beginning of vaccination with Leishmune® (C). Increased of the number of human cases of VL along the time (from 1999 to 2006) (B) and decreased or sustained number of human cases incidence in districts with higher Leishmune® vaccine coverage (D).

in August 2004. The detected decline in the human and canine incidence of the disease would be the result of the additive effect of Leishmune® vaccination of 5.7% of the healthy dogs (1419 dogs) on the regular dog culling, which exhibit itself a 29% decrease, from 2004 (30.89%) to 2006 (21.77%).

A different picture is obtained by the analysis of data of another endemic area: the town of Belo Horizonte, which is subdivided into 9 districts. In Fig. 2, we represent the distribution of CVL cases (Fig. 2A and C) and human VL cases (Fig. 2B and D), during the period 1999–2006. The terms dog seroprevalence or canine seropositivity indicate the percent of total dogs seropositive in the official ELISA test. These are the dogs with CVL, further removed to sacrifice, therefore we also refer to these counts, as cases of CVL or canine incidence of the disease. Of note, the increase of canine and human incidences is significantly correlated ($p = 0.000$), confirming the importance of the dog as the infectious reservoir of the disease. The data are very impressive, since the total number of dogs in town is much higher than that of Araçatuba (149,470 in 2005) and differences are observed in the co-evolution of human and canine cases, starting from August 2004, when the vaccine started to be used. In Fig. 2 we observed the rising curves of canine (Fig. 2A) and human incidence (Fig. 2B) of the districts of Barreiro, Venda Nova and Noroeste, and the curves of canine (Fig. 2C) and human incidence (Fig. 2D) of the districts of Centro Sul, Leste, Nordeste, Norte, Pampulha and Oeste, that started to decrease or maintained a stabilized plateau after Leishmune® vaccination, starting on 2004. In Table 1, we therefore separated the districts according to the percentual variation of the human incidence from 2004 to 2006. While Oeste district showed no variation and Centro Sul, Leste, Nordeste, Norte and Pampulha showed decreased percent values of human incidence (Table 1 column 10), Barreiro, Venda Nova and Noroeste showed an increase in the human incidence for the same period.

Among the districts showing decrease of human incidence, Centro Sul, which showed the highest vaccination percent (63.27% of the dogs) exhibited also a decrease of 3.36% in dog incidence and Pampulha, which vaccinated 27.27% of their dogs, showed only 1.89% increase in dog incidence (Table 1, columns 8 and 6). They were followed by Oeste, that vaccinated 25.30% of the animals and experienced an increase of only 12.86% of dog incidence and by Leste and Nordeste, with lower proportions of vaccinees (11.72% and 10.76%, respectively) and probably because of that, slightly higher canine incidences (42.77% and 35.73%), but still showing diminished numbers of human cases. Much lower proportions of dogs were vaccinated in Venda Nova (4.35%) and Noroeste (10.27%) districts, which according to that exhibited increased canine incidences (24.48% and 21.85%, respectively), and pronouncedly increase of human incidence (14% and 4% of human cases) (Table 1, columns 8, 6 and 10). Finally, the lowest proportion of vaccinated dogs (0.09%) was found in Barreiro which displayed, according to that, the most pronounced increase in canine (328.57%) and human incidence (17%) (Table 1). A total of 12,113 dogs were then vaccinated in Belo Horizonte from 2004 to 2006 making an 8.1% of the total dog population (149,470 dogs). These results indicate that, as the proportion of Leishmune® vaccinated dogs increases, the incidence of canine disease, the proportion of sacrificed dogs and the human incidence decrease concomitantly, indicating the additive effect of vaccination over dog culling in the control of CVL in the field, and its possible impact on the decline of human incidence of VL. Supporting our conclusions, the decrease of canine ($p = -0.008$) and human incidences ($p = -0.048$) are directly correlated to the increase of the number of vaccinated dogs, confirming the impact of the use of Leishmune® vaccination on the protection of dogs, reduction of the reservoir of the disease and, in this way, reduction of the risk of transmission of the visceral leishmaniasis to humans.

Still supporting our interpretation, the average values of the groups of districts, the numbers of total and of seropositive dogs (Table 1, columns 3 and 4), as well as the canine incidence (Table 1, column 5) of the most vaccinated districts were not different from those of the less vaccinated or untreated districts, since their means fell within the respective CI 95% of the untreated districts (Table 1). This indicates that the infectious power or endemicity of the two groups of districts is heterogeneous and compatible. On the other hand, more dogs were vaccinated in Centro Sul, Leste, Nordeste, Norte, Pampulha and Oeste districts (average 1271.89 dogs/year) (Table 1, column 7) than in Barreiro, Venda Nova and Noroeste (388.11 dogs/year; CI 95% 82.35–693.87). Indeed, the percent of total doses used in the high vaccinated districts was 85.7% (43,643 out of 50,917) while the low vaccinated areas only used 14.3% of the doses (7274 out of 50,917), and the cumulative percent of vaccinated dogs was higher in Centro Sul, Leste, Nordeste, Norte, Pampulha and Oeste (23.12%), than in Barreiro, Venda Nova and Noroeste districts (4.90; CI 95% −2.18 to 11.98) (Table 1, column 8). Simultaneously and of note, in the six districts showing higher vaccination, the number of human cases showed an average decline of 36.5%, from 2004 to 2006, falling outside of the CI 95% of the less vaccinated districts (CI 95% 2.23–21.11) which instead showed an average increase of 11.67% (Table 1, column 10).

The distribution of VL in Norte district constituted the only exception showing a reduced human incidence (−41%) (Table 1) and low canine incidence increase (12.41%), however not related to Leishmune® vaccination which was very low (0.4% of the dogs) (Table 1). These results might be related to other factors (insecticide treatment of the residences, socio-economical status), which were not analyzed in this investigation, since no comparative quantitative data of other variables was available to us. Although all these variables existed before the vaccination treatment began, the complex epidemiological dynamic of CVL and VL in BH, including all tools used for control deserves further integrated study.

Up to the end of 2007, 7.513% (1713/22,800) of the Araçatuba dogs and of 11.03% (17,165/155,643) of the Belo Horizonte dogs were vaccinated with Leishmune®. Even at this low vaccine coverage, the number of infectious dogs needing to be sacrificed by the control campaign decreased in Araçatuba and in the most vaccinated districts of Belo Horizonte, and the number of human cases also significantly decreased in both towns indicating that the prophylactic vaccination with Leishmune® has begun to be a control tool.

Since the FML-saponin-Leishmune® vaccine is known for its strong capacity of generating anti-FML antibodies, there was a major concern about the possible interference of the vaccinial antibodies in the serological control campaign for VL. Our data obtained in 2007 exclude this possibility (Table 2). In the town of Campo Grande-Mato Grosso, an important endemic and epidemic area for both human and visceral leishmaniasis, blood samples of 110,000 dogs (the whole dog population) were collected in 2007. A total of 5680 dogs vaccinated with Leishmune® were included in this screening. Sera samples of all the 110,000 dogs were obtained and tested by the official ELISA assay. Only 76 among the 5860 vaccinated dogs showed seropositivity in the ELISA assay which was further confirmed by the results of IF assay, constituting a 1.3% of the whole vaccinated population. The same dogs showed no antibodies for the heat shock protein recombinant antigen and no parasites in either lymph node or bone marrow samples, confirming they carry no *Leishmania* infection (Table 2). Our results prove that in spite of previous concerns, the Leishmune® vaccination does not interfere with the serological control of CVL.

4. Discussion

The described prophylactic [19,28], immunotherapeutic [24,25] and transmission blocking effect of the Leishmune® vaccine [16,18,27] pointed out its potential use for the reduction of infec-

Table 1
Distribution of canine and human incidence of visceral leishmaniasis and number of Leishmune® vaccinated dogs in Belo Horizonte in the period 2004–2006.

Districts	Year	Total dogs	Positive dogs	Dog incidence (%)	Δ dog incidence	Cumulative L® vaccinees	Cumulative % vaccinated dogs	Human cases	Δ human cases (%)
Centro Sul	2004	5,190	201	3.87		1900		5	−40
	2005	10,298	274	2.66		3686		6	
	2006	6,823	255	3.74	−3.36	4317	63.27	3	
Leste	2004	12,337	811	6.57		389		16	−63
	2005	16,925	1039	6.14		768		12	
	2006	8,103	760	9.38	42.77	950	11.72	6	
Nordeste	2004	12,151	1044	8.59		374		24	−25
	2005	18,684	1544	8.26		851		14	
	2006	10,792	1258	11.66	35.73	1161	10.76	18	
Norte	2004	11,551	1014	8.78		27		22	−41
	2005	20,817	2295	11.02		46		20	
	2006	12,190	1203	9.87	12.41	49	0.40	13	
Pampulha	2004	3,899	372	9.54		824		6	−50
	2005	11,998	1082	9.02		1741		10	
	2006	7,993	777	9.72	1.89	2180	27.27	3	
Oeste	2004	6,116	456	7.46		652		10	0
	2005	11,799	850	7.20		1338		11	
	2006	6,484	546	8.42	12.86	1641	25.30	10	
Average/year		11027.89	882.83	7.88	17.05	1271.89	23.12	11.94	−36.5
Barreiro	2004	8,783	197	2.24		2		6	17
	2005	7,609	375	4.93		4		6	
	2006	6,434	617	9.6	328.57	6	0.09	7	
Venda	2004	11,434	1177	10.29		134		21	14
Nova	2005	19,378	2263	11.68		259		14	
	2006	12,072	1547	12.81	24.48	525	4.35	24	
Noroeste	2004	9,021	760	8.42		375		24	4
	2005	17,127	1516	8.85		1000		17	
	2006	11,572	1187	10.26	21.85	1188	10.27	25	
Average/year		11926.33	1113.56	8.94	124.97	388.11	4.90	16	11.67
CI 95%		5698.73–18153.93	215.74–2011.37	6.55–11.33	−119.45–369.39	82.35–693.87	−2.18–11.98	4.82–27.18	2.23–21.11

Dog incidence (%): percent of seropositive dogs among the total dogs; Δ dog incidence: variation on percentual of dog incidence in 2006 in relation to 2004; cumulative L® vaccinees = cumulative number of Leishmune® vaccinated dogs that receive complete vaccination (3 doses in the first year and one annual booster in the following years) distributed to the veterinarians; cumulative % of vaccinated dogs: percentual variation of the number of vaccinated dogs in 2006 relative to 2004; Δ human cases: percentual variation of the number of human cases in 2006 in relation to 2004. IC 95% = 95% confidence interval.

Table 2
Reactivity of sera of Leishmune® vaccinated dogs as detected by the official serological control campaign for visceral leishmaniasis.

Total dogs in 2007	Total Leishmune® vaccinated dogs	Leishmune® vaccinated dogs positivity				
		ELISA	IF	%	Anti-HSP Biogen ELISA	Parasites in lymph node or bone arrow
110,000	5,860	76	76	1.3	0	0

Results of the serological control campaign of Campo Grande, Mato Grosso state, Brazil, in 2007. The official campaign obtained sera samples of the whole town dog population and analyzed them through the ELISA assay confirming seropositivity by immunofluorescence (IF) tests. Both tests used by the Brazilian Ministry of Health, contain crude antigen of total promastigotes of *Leishmania major*-like. The samples were also tested for their antibodies against a recombinant Heat Shock protein of *Leishmania chagasi* (HSP Biogene test). The potential presence of infection was analyzed in Giemsa stained smears of lymph node and/or bone marrow samples obtained by puncture of the seropositive dogs.

tious dog and human populations and for the interruption of epidemics. Leishmune® vaccinated exposed dogs show no evidence of parasites in blood, lymph node and skin [16]. The antibodies generated by the vaccine impede the parasite development in the sand flies, as shown by an *in vivo* membrane assay [18,27] and by preliminary xenodiagnosis results [18], leading to the interruption of the transmission of the parasite in nature. Before the vaccine license, a safety and immunogenicity trial showed that 98.8% of the first 550 vaccinated dogs were protected and healthy after two years of vaccination [28]. Recently, veterinary clinics of different states of Brazil reported that among 8393 exposed dogs, vaccinated from August 2004 to August 2008, only 229 developed the disease, representing 97.3% of achieved protection (complete absence of symptoms or signs of CVL). This data was gathered from 67 out of 228 clinics from different towns of the West of Sao Paulo state reporting protection in 4626/4752 vaccinated dogs, 15 among 207 clinics of Belo Horizonte, in 1817/1864 dogs and by 7 among 59 clinics of Campo Grande (Mato Grosso do Sul state) in 1721/1777 vaccinated dogs, making also 97% of protection in each respective area. Furthermore, a total of 65,000 healthy dogs were vaccinated with Leishmune® in Brazil up to October 2008.

Being a vaccine against a canid zoonoses, the generation of dog protection is expected to result in a decrease of parasite reservoir, in the number of dogs infectious to sand flies and subsequently, in a decrease in human cases [10]. The human cases of VL indeed declined from 15 to 0 in the area where the pre-commercial formulation of Leishmune® (the FML saponin vaccine) was tested, in field Phase III trial, showing on dogs, 92–95% of protection and 76–80% of vaccine efficacy [19,20].

The mathematical model for the control of leishmaniasis developed by Dye [10], already pointed out that a canine vaccine would be an excellent and potent tool for decreasing both the human and canine incidence of the disease. The development of anti-zoonotic vaccines for animal reservoirs, aiming the subsequent decline of human cases of the disease is strongly encouraged [1,10,33]. Although a few other CVL vaccines were studied in Phase III field assays [12–15,34], and two of them showed efficacies [14,15], no data about the influence of these assays on the decline of human disease was ever reported.

We chose for our study the towns of Araçatuba and Belo Horizonte, because, besides Leishmune® vaccination, no other official control tool was used there, except for dog culling and insecticide treatment of the residences. Being aware of the possible interference of the sensitivity of different serological assays in the number of dogs detected as seropositive [5,6,9,19,35,36] we chose for our study, the towns of Araçatuba and BH that shared the same methods and procedures for the epidemiological control campaign. Therefore, sensitivity was not a variable. Detailed information about the regular insecticide treatment of residences in the different districts of Belo Horizonte was not available to us [30]. Therefore, it was not possible to perform a correlation analysis in order to exclude this factor as a possible variable affecting incidence. The same happened with the number of dogs treated with chemotherapy, which is still not recommended by WHO and the Brazilian Ministry of Health, and with the Social Vulnerabilty index [37], which represent the different socio-economical status of the different districts [30]. An integrated study with numerical data of all these possible variables should be done to evaluate the possible contribution of each treatment. Since however, there were no changes in serological diagnostic tools, government policies or insecticide treatment in Araçatuba and BH during the studied period and before (since 2002 in Araçatuba and since 1997 in BH), when the incidences were increasing, and the decrease in human and dog cases only started after the beginning of Leishmune® vaccination, the results suggest that the only variable affecting the incidence was the vaccine. During the same period, a decrease in canine cases was also noted (Dr. Aziz Abdelnour, Centro de Controle de Zoonoses of Andradina, personal communication) in Andrandina, another town of São Paulo state that used Leishmune® vaccination and where insecticide dog collar necklaces were introduced as an additional official control tool for the disease. For this reason we did not include in our study the data of Andradina, in spite of our previously obtained significant results indicating that untreated dogs remained infectious while Leishmune® vaccinated did not, whether or not they used the delta-methrin insecticide collar necklace [16].

While the decline of human and canine disease of Araçatuba was very clear as a whole, since the total dog and human population is small, the distribution of the disease in Belo Horizonte was more complex. We initially separated the districts according to their human incidence variation along the time. We detected that the canine incidence variation followed the variation of human incidence of their respective districts, and finally observed that the decline of human cases and of canine incidence was highly correlated to the use of Leishmune® vaccine. Indeed, the vaccine had an additive effect on dog culling in the control of VL. Districts that showed intense vaccination needed to sacrifice less dogs in order to keep reduced the number of human cases. Districts that had lower proportions of vaccinated dogs, needed to sacrifice more infected dogs. Vaccination with Leishmune® acted then as a control tool with additive effect on dog culling and our results indicate that the future increase in the proportion of vaccinated dogs might substitute dog culling in the epidemiological control of VL.

According to the mathematical model for the control of leishmaniasis developed by Dye [10], the expected efficacy for the vaccine in bringing down the canine incidence of the disease is disclosed by plotting in the *y* axis the dog incidence after vaccination divided by the dog incidence before vaccination and expressed as percentage, and in the *x* axis the fraction of the susceptible dogs converted to resistant by the vaccine treatment. In Araçatuba, the dog incidence after vaccination declined from 30.01% (2003) to 21.77% (2006) giving a percent ratio of 72.54% which corresponds to a transformation of 25% of the dog population from susceptible to resistant or protected. This was achieved vaccinating only 7.531% of the dog population. More extensive vaccine coverage would certainly help in the interruption of the epidemics. In Belo Horizonte, on the other hand, the decline in human incidence was observed after dog vaccination in several districts. In Nordeste district, human cases declined from 24 to 18 giving a percent ratio of 72% which

corresponded to 28% of the human population moving to a resistant class. In Leste district, the human cases declined from 16 to 6, giving a percent ratio of 37.5% which corresponded to 62% of the human population becoming resistant or indirectly protected. This was achieved with vaccination of only 11.03% of the whole dog population. An increase in vaccine coverage would certainly approach to the interruption of epidemics.

There was a concern that Leishmune® vaccination could lead to seroconversion detectable by the diagnostic methods used by the official control survey campaign [29]. It was argued that the vaccinated dogs would be confused with the infected dogs. If seropositivity induced by the vaccine would be so significant, one would expect to see an increase in dog seroprevalence in the more vaccinated areas. The findings disclosed by this investigation support the opposite effect, since the more vaccinated districts of BH showed the lowest seroprevalences. Therefore, this concern was not justified. Indeed, only 1.3% of the Leishmune® vaccinees were seropositive in the official test and since they showed no parasites and no antibodies against the heat shock protein of *L. chagasi*, they were considered non-infected and then were not sacrificed. These results are very significant considering that the analysis was done on sera samples, instead of blood eluates, turning the sensitivity much higher [5]. The Leishmune® vaccinated dogs showed 50–98% of FML-seropositivity after complete vaccination [19,28]. In these investigations, the FML, a purified highly glycosilated glycoprotein specific antigen [38] of *Leishmania donovani*, was both the vaccine antigen and the antigen of the diagnostic test. Therefore, it can recognize with high sensitivity and specificity the anti-FML antibodies generated by the vaccine. On the other hand, the total promastigote lysate of *Leishmania major* and *Leishmania brasiliensis*, which are the antigens of the official ELISA test used for screening, can react with all the antibodies generated during the multiplication of parasites in the infected host. While the purified FML antigen interacts with a defined fraction of sera antibodies, the total lysate interacts with the whole plethora of antibodies directed against the total parasite, masking or diluting the response against the purified FML antigen, which is not the major *Leishmania* antigen. The difference of results is then due to the differential affinity of the antigens. Our results demonstrate that, the seropositivity induced by Leishmune® vaccine does not interfere with the control campaign. A similar discrepancy was informed in relation to the predominant IgG subtype of antibody [17]. In dogs vaccinated with the CPa and CPb cystein proteinases, the work of Rafati et al. (2005) [39] shows that higher IgG2 than IgG1 titers are detected against the recombinant vaccinial antigens but not against the *Leishmania infantum* lysate [17,39]. Supporting our results, recent comparative study developed by Grecco F and Allegretti (personal communication) in Leishmune® vaccinated dogs from a non-endemic area, disclosed 73% of positivity in the FML ELISA assay (19 sample among a total of 26), 0% of reactivity in the official anti-*Leishmania* test (24 negative and 3 undefined samples) and 0% of reactivity (28 samples, all negative) in a laboratory prepared anti-*Leishmania chagasi* ELISA test.

The epidemiological data analyzed in this investigation are the best data gathered from the field regarding the impact of an anti-*Leishmania* vaccine. A Phase IV trial, is recommended by WHO for a vaccine that already demonstrated 50% of efficacy in Phase III double-blind random control test [40]. In a Phase IV test, vaccination is done on a large population (10,000 to 100,000 individuals) and the impact of the vaccine is measured by the difference of the incidence before and after the vaccine treatment. WHO recommends a Phase IV trail for it is un-ethical to use untreated controls when the efficacy of the vaccine was already proven in a Phase III assay and the vaccine is industrialized [40]. Since no such test was run with Leishmune® by the Brazilian government yet, the data showed here is the best approximation of the impact of the use of Leishmune®, mainly considering that in Belo Horizonte, the existence of some less vaccinated districts serves as potential untreated controls.

Among the many formulations tested for anti-*Leishmania* vaccination, Leishmune® is one of the only three vaccines licensed for commercialization all over the World, the only one of second-generation kind and the only one against the canine disease [17,18]. To our knowledge, Leishmune® is the first anti-*Leishmania* vaccine to be used in large scale vaccination in the field and to induce a decline of human and canine cases of the disease.

In this work, and although the Leishmune® vaccine coverage is so far considered low, we show that the vaccination of 11.3% of the dogs (17,165/155,643) of Belo Horizonte and of 7.513% of the dogs of Araçatuba (1713/22,800) has begun to be a control tool, protecting vaccinees, reducing the parasite reservoir in infectious dog and human populations and by doing so, decreasing the number of dogs sacrificed. The larger use of Leishmune® would certainly help in the eradication of the disease.

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