

## Canines vaccinated against visceral leishmaniasis develop a serological response to the *Leishmania braziliensis* antigen

### Cães vacinados para leishmaniose visceral respondem sorologicamente aos antígenos de *Leishmania braziliensis*

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#### Abstract

American cutaneous leishmaniasis (ACL) is a zoonosis caused by *Leishmania*, a protozoan. Common antigens occur in the strains found in America, which allow antigenic cross-reactivity. Therefore, multivalent vaccines can be used for this pathogen. In this study, we investigated the efficacy of two different commercial vaccines for visceral leishmaniasis to induce an immune response to the soluble *L. (Viannia) braziliensis* antigens. In 2014, 70 seronegative dogs from the municipality of Iúna (Espírito Santo State, Brazil) were vaccinated and serologically evaluated by ELISA and immunoblotting by using the soluble antigen of *L. braziliensis*. Of the 121 dogs initially selected, only 70 received vaccination because 51 dogs tested positive by ELISA, yielding a positive frequency of 42.14% in the asymptomatic group. These 70 dogs were divided into two equal groups and administered three doses of each vaccine, according to the manufacturers' instructions. We found that the sera of dogs immunized with three doses of both vaccines A and B had antibodies against the soluble antigens of *L. (V.) braziliensis*, as determined by ELISA and immunoblotting 120 days post vaccination. Antibodies produced in response to vaccines A and B were found in 22/35 and 18/35 serum samples, respectively, at T1 (120 days), while 7/35 and 4/35 serum samples tested positive at T2 (240 days). Furthermore, immunoblotting allowed us to differentiate between vaccinated and asymptomatic dogs.

**Key words:** American cutaneous leishmaniasis. *Leishmania* vaccines. Diagnosis. ELISA. Immunoblotting.

#### Resumo

A Leishmaniose Tegumentar Americana (LTA) é uma zoonose causada por protozoários do gênero *Leishmania*. Existem antígenos comuns entre as várias espécies de *Leishmania* da América determinando reações antigênicas cruzadas o que possibilita vacinas multivalentes. Este estudo avaliou soros de cães vacinados com duas vacinas comerciais diferentes para leishmaniose visceral na indução de resposta imunitária cruzada para antígenos solúveis de *Leishmania (Viannia) braziliensis*. Durante o ano de 2014, 70 cães soronegativos para *Leishmania* spp. do município de Iúna (Espírito Santo, Brasil) foram vacinados e examinados sorologicamente por ELISA e imunoblotting utilizando o antígeno solúvel de *L. braziliensis*. Para 121 cães inicialmente selecionados, apenas 70 foram submetidos a vacinação porque

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51 dos animais foram positivos no teste sorológico ELISA para LTA, indicando uma frequência positiva de 42,14% para os animais LTA assintomáticos. Estes setenta animais foram divididos em dois grupos de tamanhos iguais e foram aplicadas três doses de cada vacina segundo recomendações dos fabricantes. Como resultado inédito verificamos que os soros de cães imunizados com as três doses de vacinas A e B apresentaram anticorpos contra antígenos solúveis de *L. (V.) braziliensis* quando avaliados por ELISA e immunoblotting com 120 dias pós-vacinação. As vacinas A e B apresentaram resultados positivos com presença de anticorpos nos testes serológicos, respectivamente, 22/35 e 18/35 positivos em T1 (120 dias), enquanto que em T2 (240 dias), 7/35 e 4/35 soros positivos. Além disso, o immunoblotting permitiu diferenciar o soro de cães de diferentes vacinas e cães assintomáticos.

**Palavras-chave:** Leishmaniose tegumentar. Vacinas *Leishmania*. Diagnóstico. ELISA. Immunoblotting.

## Introduction

Leishmaniasis is a disease caused by the protozoans of the genus *Leishmania*. Depending on the virulence factors of the parasite and the immune response developed by the host, this disease is characterized by skin lesions or general visceral symptoms. This protozoan is traditionally transmitted by the bite of an infected phlebotomine sandfly, which causes various forms of clinical leishmaniasis. The visceral form of leishmaniasis is mainly caused by *Leishmania chagasi/infantum* (VL - visceral leishmaniasis). In Brazil, the disease exists countrywide, in both cutaneous and visceral forms. In subtropical America, the cutaneous form is known as ACL (REITHINGER; DAVIES 1999).

It is a widespread zoonotic disease and is considered an emerging and reemerging health problem in more than 15 countries (GRIMALDI; TESH 1993). Currently, the southern region of the State of Espírito Santo in Brazil is endemic for the ACL caused by *L. (Viannia) braziliensis*, with many cases occurring in both humans and dogs in the last decade. Several studies have suggested that the domestic dog (*Canis familiaris*) might play the role of a reservoir in the domestic transmission of ACL caused by *L. braziliensis* to humans (FALQUETO et al., 2003; MARZOCHI; MARZOCHI. 1994; MADEIRA et al., 2003). Many questions are still unanswered, regarding the role of dogs in the cycle of disease transmission, with the dogs being considered an accidental host (REITHINGER; DAVIES 1999). Polymerase chain reaction (PCR)-based amplification of a prepronociceptin (PNOC)

gene fragment present in the blood meal sources of the sand flies occurring in the leishmaniasis-endemic areas and subsequent DNA sequencing showed that 1 (3.7%) of these flies had fed on a horse (*Equus caballus*), 16 (59.3%) on pigs (*Sus scrofa*), and 10 (37%) on dogs (*Canis lupus familiaris*). This indicated that the transmission cycle of leishmaniasis depends on the movement of the potential animal reservoirs from the forest to the domestic households as well as from humans and domestic animals to the forest (BAUM et al., 2015). Using mathematical models, which were fitted to the data on the proportion of positive results as a function of dog age was estimated a basic reproductive number ( $R_0 \pm S.E.$ ;  $1.22 \pm 0.09$ ), which indicated that the disease is endemic in dogs. Nevertheless, this information by itself is insufficient to incriminate dogs as ACL reservoirs, given the inability to detect parasites (or their DNA) in seropositive dogs and the previously reported failures to experimentally infect vectors feeding on dogs carrying ACL parasites (CALZADA et al., 2015).

Studies on leishmaniasis vaccines have been undertaken in the last few years because the cell-mediated immunological mechanisms that can control the infection were understood. This species present a genetic homology of antigens; therefore, the use of pan-*Leishmania* vaccines have been suggested (HANDMAN, 2001; CARVALHO et al., 2002; MENDONÇA et al., 2016). It is mandatory to screen serological tests before vaccinating dogs against leishmaniasis to avoid vaccinating sick or

asymptomatic animals, as a thorough evaluation of the infection in these animals is necessary to decide the course of action to be followed according to the recommendations of the sanitary surveillance system and the instructions of the manufacturers.

Previous findings indicate that indirect ELISA and immunoblotting have high sensitivity for detecting *L. (V.) braziliensis* infections and are considered a valuable tool for screening and diagnosing this disease, given that the association of two or more indirect methods helps increase the sensitivity of diagnosis (RIBEIRO et al., 2007; SZARGIKI et al., 2009; ZANINI et al., 2010). Conventionally, diagnostic serological tests such as enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence assay (IFA) have a sensitivity of 91.8% and 90.8% and a specificity of 83.4% and 53.4%, respectively (ARRUDA et al., 2016).

Currently, vaccine antigens have been studied in dogs, because the development of vaccines against canine leishmaniasis can form a prophylactic barrier, although the antibody response detected by standard diagnostic techniques cannot distinguish between vaccinated and naturally infected dogs (MARCONDES et al., 2013). In 2009, the Ministry of Agriculture, Livestock, and Food Supply of Brazil (MAPA) (Clarification Note 03/05/2009) issued a provisional license allowing two private laboratories to produce and trade vaccines against visceral leishmaniasis in dogs. According to the manufacturers, 21 days after the third dose, the animals should be protected. These vaccines have been administered across countries to protect against VL, independent of ACL. The protection measures related to ACL are not specified in the technical reports and documents issued by the manufacturers, although they do acknowledge the cross-protection observed between different species of *Leishmania* (FALQUETO et al., 2003; REIS et al., 2009).

Considering the above-detailed factors, we investigated the serological response to the two VL vaccines with respect to the production of antibodies

against the *L. braziliensis* antigen, by using ELISA and immunoblotting during the recommended period of protection extended by the vaccines.

## Materials and Methods

### *Characterization of the study population*

This study was carried out in Iúna Municipality of the State of Espírito Santo, Brazil, an ACL-endemic region. This state has a population of approximately 26,000 and an average elevation of 661 m. It is about occupies 460 km<sup>2</sup> and has an average temperature of 7–30°C, with a tropical climate, and Atlantic Forest as its characteristic vegetation. It is situated in the southern region of the state (Caparaó Region) and is bordered by the municipalities Muniz Freire in the east, Ibitirama in the south, Irupi and Ibatiba in the north, and the State of Minas Gerais in the west according to the data of Prefeitura Municipal de Iúna, 2011.

To select dogs for this study, five ACL-endemic districts from the municipality, which had reported dog and human infections, were considered. The animals were characterized as mongrels (*Canis lupus familiaris*), over one year of age, and living within 1000 meters from a site where there were registered cases of ACL in humans. With consent from the guardian of the dogs, 121 dogs clinically negative for ACL were selected and subjected to serological testing. All animals were electronically identified through numerical chips. Serum samples were used to determine the presence of antibodies against *Leishmania* species by using ELISA and immunoblotting.

Calculation of *n* sampling was based on the canine population figures provided by the Health Surveillance for the municipality of Iúna-ES by using the formula for finite populations. The 70 vaccinated dogs were divided into two groups: 35 animals (24 males, 11 females) formed group 1 (G1) that were vaccinated with vaccine A [a purified glycoprotein fraction of *L. donovani* called fucose mannose-ligand (FML)] and 35 animals (21 males,

14 females) formed group 2 (G2) for vaccination with vaccine B (A2 recombinant protein of *L. donovani* and saponin).

The vaccination protocol followed the manufacturers' guidelines, which recommend that the first dose should be followed by two more doses at 21-day intervals. Serum samples of the dogs were evaluated in three stages to detect antibodies only against the *L. braziliensis* antigen:  $T_0$  - before the first dose;  $T_1$  - 120 days after the second dose; and  $T_2$  - 240 days after the third dose. These blood samples were collected by venipuncture, transferred to test tubes without anticoagulant, kept at room temperature (25°C) until clot retraction, and then centrifuged at 1500 rpm for 10 min to separate the serum; this was maintained at -80°C until further analysis.

#### *Enzyme linked immunosorbent assay (ELISA)*

The soluble fractions obtained from the promastigote forms of *L. (V.) braziliensis* (MHOM/BR/75/M2903) and *L. (L.) infantum* (MHOM/BR/1974/PP75) were used to screen the serum samples of 121 dogs by ELISA to identify any reaction to *Leishmania* antigens. Ribeiro et al. (2007) had reported the detection of IgG antibodies against *Leishmania* species by a modified indirect ELISA method. Briefly, ELISA, performed in triplicate, involved fixing the antigen on the wells of 96-well polystyrene plates (concentration, 1.1 mg mL<sup>-1</sup>; diluted in carbonate-bicarbonate buffer, pH 9.6) for 12 h at 4°C. Then, the wells were washed with the washing solution [sodium chloride (NaCl; 9 g), Tween 20 (0.5 mL), and distilled water (1000 mL)]. After the fixing step, a blocking step was performed to avoid non-specific reactions, by adding 100 µL of the blocking solution to each well. The blocking solution contained 616 µL of fetal bovine serum (FBS), 10 mL of phosphate-buffered saline [PBS-1X; sodium chloride (NaCl; 8 g), potassium chloride (KCl; 0.2 g), dibasic sodium phosphate (Na<sub>2</sub>HPO<sub>4</sub>; 1.44 g), monobasic potassium phosphate (KH<sub>2</sub>PO<sub>4</sub>;

0.24 g), and distilled water (1000 mL)], and required an incubation period of 45 min at 37°C. Then, 100 µL of the serum sample was added to each well, diluted 1:40 in PBS-T [solution of PBS 1X (1000 mL) and Tween 20 (0.5 mL)]. After 45 min of incubation at 37°C, the plate was washed four times with the washing solution, and 100 µL of conjugated dog anti-IgG peroxidase (sheep anti-dog total IgG HRP-conjugated/A40-121P; Bethyl Laboratory Inc., Montgomery, TX) diluted to 1:5000 in PBS-T was added to each well. Again, after incubating for 45 min at 37°C, the plate was washed four times. Approximately 100 µL of a solution containing 2 µL of 30% dihydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and 1 mg of ortho-phenylenediamine dihydrochloride (OPD/Sigma®) in 10 mL of phosphate citrate buffer [citric acid (C<sub>6</sub>H<sub>8</sub>O<sub>7</sub>; 5.19 g), dibasic sodium phosphate (Na<sub>2</sub>HPO<sub>4</sub>; 7.19 g), and distilled water (1000 mL)], pH 5.0, was added to each well, and the plate was further incubated for 10 min at 37°C. The reaction was interrupted after 10 min, by adding 32 µL of 2.5M H<sub>2</sub>SO<sub>4</sub> to each well; the plate was read immediately at 490 nm by using a spectrophotometer (Multiskan Go; Thermo Scientific, Vantaa, Finland).

Determination of reactivity was based on the readings above the cut-off point (cut-off), which was calculated as the average optical density of the serum samples obtained from five negative and five positive control dogs plus twice the standard deviation of the optical density of these sera.

#### *Immunoblotting analysis*

For immunoblotting, a peroxidase-conjugated secondary anti-total IgG was used to differentiate the antibody panel of vaccinated and positive control dogs in the presence of the antigens of *L. (V.) braziliensis*. Immunoblotting analysis was performed according to the method described by Zanini et al. (2010) with minor modifications. Briefly, 10 µg of the soluble antigens of *L. braziliensis* (one in each lane) and SDS-PAGE Molecular Weight Standards (Biorad Laboratories;



Catalog number: 161-0304) were separated by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis using a mini gel apparatus. Polypeptides from the gels were electroblotted onto 0.45- $\mu$ m-thick nitrocellulose membranes by using a semidry blotter at 200 mA for 1.5 h (Biorad, USA), following the supplier's instructions.

For immunodetection, strips were cut from previously blotted membranes and blocked for 90 min with 5% skimmed milk in PBS. Strips were then washed with PBS-T (three times, 10 min each), followed by incubation with the serum sample diluted 1:100 in PBS-T for 12 h at 4°C. After incubation with the primary antibodies, the strips were washed four times, as described, and incubated for 1 h with peroxidase-conjugated secondary anti-total IgG canine immunoglobulins (Bethyl Laboratories, Inc., Montgomery, TX, USA) diluted in PBS-T at concentrations of 1:1000, 1:500, and 1:1000, respectively, for 1 h at room temperature. After the strips were washed three times with PBS-T, color development was allowed by adding 0.6 mg mL<sup>-1</sup> 4-chloro-1-naphthol (Sigma®) prepared in 0.15M PBS, pH 7.3, along with 0.02% H<sub>2</sub>O<sub>2</sub>. The final reaction was stopped by washing the strips with distilled H<sub>2</sub>O.

*Statistical analysis*

Data were statistically analyzed by using the  $\chi$ -squared test of a variable or a 2 × 2 contingency

table. All analyses were performed using SPSS® (version 13.0 for Windows) at a level of significance of 0.05.

*Ethics*

All procedures involving experimental animals were conducted according to the Brazilian regulations and the guidelines issued by the Colégio Brasileiro de Experimentação Animal (COBEA). The project was approved by the Ethical Committee for Animal Research, Universidade Federal do Espírito Santo (CEUA-UFES), Protocol 006/2009.

**Results**

Only 70 of the originally selected 121 dogs were chosen for vaccination because they tested positive by ELISA.

*ELISA*

At zero time, the vaccinated dogs tested seronegative on ELISA, while 51 asymptomatic dogs and serum-positive controls tested ELISA positive for ACL (Table 1). Serum samples of some asymptomatic dogs showed borderline results (ELISA) but they were not chosen for vaccination.

**Table 1.** Number of dogs that tested positive for *Leishmania infantum* and *L. braziliensis* from the groups vaccinated with vaccines A and B.

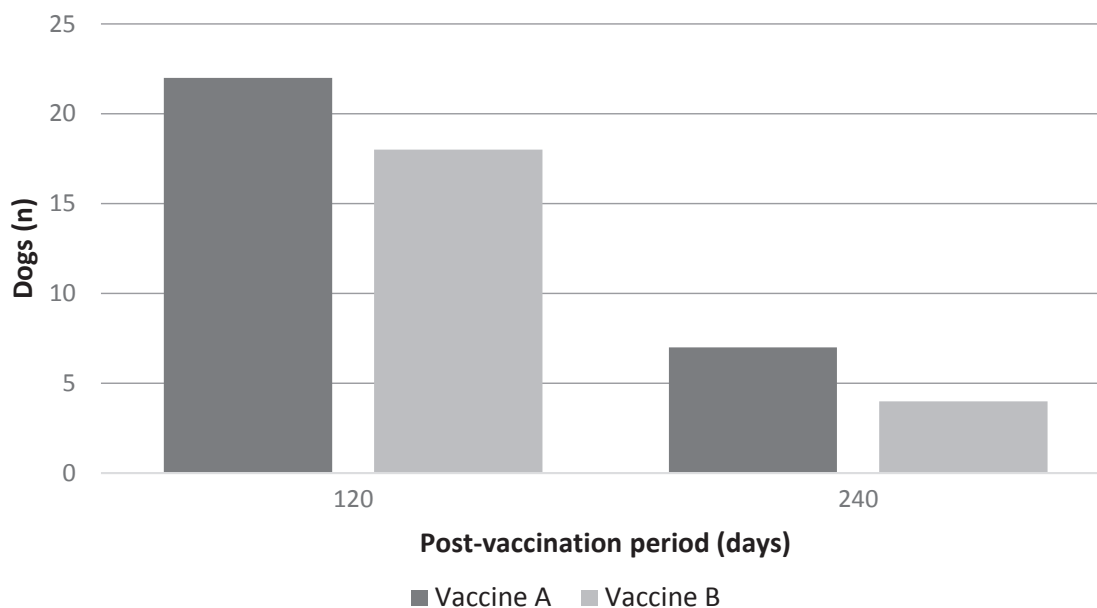
districts of Íuna-ES	Selection		Vaccine A			Vaccine B			Total Vaccinated
	TS	T <sub>0</sub>	TV	T <sub>1</sub>	T <sub>2</sub>	TV	T <sub>1</sub>	T <sub>2</sub>	
São João do Príncipe	23	8	7	5	1	7	6	1	14
Serrinha	25	12	7	6	2	7	3	1	14
Figueira	20	6	7	4	1	7	4	-	14
Tinguaciba	23	8	7	3	2	7	2	1	14
Trindade	30	16	7	4	1	7	3	1	14
Total	121	51	35	22	7	35	18	4	70

TS, Total selected dogs; T<sub>0</sub>, Positive animals excluded from vaccination; TV, Total vaccinated dogs; T<sub>1</sub>, Positive animals 120 days post-vaccination; T<sub>2</sub>, Positive animals 240 days post-vaccination.

During the post-vaccination period, after three doses of vaccines A and B, respectively, 22 and 18 were positive at  $T_1$  (120 days - 62.85% and 51.42%), while at  $T_2$  (240 days - 20% and 11.42%), seven and four samples tested on ELISA and were confirmed by immunoblotting using the soluble antigen of *L. braziliensis*. It was observed that for the trademark

A ( $\chi^2 = 7.76$ ;  $p = 0.005$ ) as well as for the trademark B ( $\chi^2 = 8.91$ ;  $p = 0.003$ ), there was a statistically significant difference (Figure 1) in the number of reactive animals showing a decrease between the two evaluated time points.

**Figure 1.** Post-vaccination period, after three doses of vaccines A and B Positive results on ELISA were confirmed by immunoblotting using the soluble antigen of *Leishmania braziliensis*.



To verify the association between the findings of ELISA and the vaccine trademark, a chi-square test using a  $2 \times 2$  contingency table was performed. It was observed that there was no association between a positive ELISA test and the different trademark vaccines at 120 days ( $\chi^2 = 0.40$ ;  $p = 0.527$ ) as well as 240 days ( $\chi^2 = 0.818$ ;  $p = 0.366$ ). This result was corroborated by a representative chi-square test, which showed no significant difference in the number of positive ELISA tests for the two trademark vaccines at 120 days ( $\chi^2 = 0.61$ ;  $p = 0.435$ ) or 240 days ( $\chi^2 = 0.82$ ;  $p = 0.37$ ). Thus, the two vaccines behave in the same way at different

evaluation time points, with respect to ELISA test findings.

#### *Immunoblotting analysis*

Immunoblotting showed differences in the reactivity of the serum samples of symptomatic and vaccinated animals toward *L. (V.) braziliensis* total IgG antibodies. However, a similar number of proteins were detected using antigenic panels. The five positive control sera showed many soluble protein profiles on SDS-PAGE; bands immunoreactive to *L. (V.) braziliensis*, with a relative

MW value ranging from 17 to 150 kDa (17, 21, 27, 32, 45, 50, 54, 58, 66, 80, 97, 110, and 150 kDa), were observed after incubation with secondary anti-total IgG canine immunoglobulins. The serum samples from asymptomatic dogs predominantly contained proteins over 54 kDa (54, 66, and 97 kDa). On the other hand, the serum samples of animals immunized with vaccine A contained proteins ranging from 27 to 97 kDa (27, 32, 50, 66, and 97 kDa), while those of animals immunized with vaccine B contained proteins of approximately 50 kDa (45, 50, and 54 kDa).

## Discussion

Of the initially selected 121 dogs, only 70 received vaccination because 51 of these animals tested positive for the serological ACL test based on ELISA, yielding a positive frequency of 42.14% for the asymptomatic ACL animals (Table 1). It is important to direct attention to the high number of dogs that were serologically positive, but asymptomatic. According to Arraes et al. (2008), Zanini et al. (2010), and Lakhali et al. (2012), asymptomatic animals are often observed in endemic areas and can be detected by serological methods such as ELISA, immunoblotting, and IFI. Sera from asymptomatic dogs (subclinical and self-healing forms) often test positive for >54-kDa protein (ZANINI et al. 2010). These results clearly indicate the need for a rigorous screening protocol to select suitable animals for vaccination in endemic regions. It is worth noting that the official test for LV, DPP® (Dual Path Platform - CVL rapid test, Bio-Manguinhos/Fiocruz, Rio de Janeiro, Brazil) does not detect animals positive for ACL (GRIMALDI et al., 2012). The importance of differential diagnosis in leishmaniasis and association of different tests to enhance detection sensitivity has been demonstrated previously (RIBEIRO et al., 2007; SZARGIKI et al., 2009; ZANINI et al., 2010).

This study is different from previous reports in that it evaluated the serological response to the

tested vaccines by using a soluble antigen of *L. braziliensis*. Nevertheless, other authors using different antigens found similar results by ELISA. In this study, ELISA showed that 62.85% dogs immunized with vaccine A tested positive for the soluble antigen of *L. braziliensis* at 120 days after the third dose. This finding is in agreement with the observations of Marcondes et al. (2013), who, after evaluating the same vaccine for 180 days after the third dose, reported that 88.8% tested positive on an “in house” ELISA, 33.3% on IFAT, 11.1% on standard ELISA, and 5.5% on DPP®. These values (62.85% and 88.8%) indicated a significant post-vaccination reaction after 180 days. Reinforcing these findings, Fernandes et al. (2014) observed positive serology for 32.5% (13/40) 11 months after the third vaccination. Meanwhile, Ribeiro et al. (2015) using *L. major*-like antigen to assess 71 dogs vaccinated with Vaccine A and found that only one presented with positive results in the ELISA/*L. major*-like and IFAT assays. For Vaccine B (A2, a recombinant protein of *L. donovani*), we found that 51.42% tested positive at T1 and 11.42% at T2, by ELISA using the soluble antigen of *L. braziliensis*; however, A2 gene sequences (*L. donovani*) were not detected in the genome of *L. braziliensis* (GHEDIN et al., 1997). However, anti-A2 antibodies were detected in 60% of the patients from the state of Minas Gerais (Brazil) with mucosal leishmaniasis, which is mainly caused by *L. braziliensis* (CARVALHO et al., 2002). On the other hand, Fernandes et al. (2014), who used an *L. infantum* antigen, found that 30.9% (13/42) tested positive on ELISA 280 days after the third vaccination. Our statistical results indicate that both vaccines have similar outcomes when tested by ELISA at different time points, and that from 120 to 240 days post vaccination, a significant drop was noted in the number of positive tests.

Studies on leishmaniasis vaccines have been undertaken in the last few years because the cell-mediated immunological mechanisms that can control the infection were understood.

*Leishmania* species presents a genetic homology ranging from 69% to 90%. Therefore, the use of heterologous antigens for the immunodiagnosis of VL and vaccination using pan-*Leishmania* vaccines have been recommended (HANDMAN, 2001; CARVALHO et al., 2002). Recently, this was confirmed using the LBSap vaccine (MENDONÇA et al., 2016), a vaccine containing 60 µg of *L. braziliensis* antigen and 50 µg saponin/dose displayed immunological and parasitological profiles similar to other commercially available anti-CVL vaccines, namely, Leish-Tec® (Hertape S.A., Juatuba, Brazil), and Leishmune® (Zoetis, Campinas, Brazil). An efficacy analysis using real-time PCR showed a reduction in the extent of parasitism in the spleen (Leishmune®: 64%; LBSap: 42%; and Leish-Tec®: 36%) and liver (Leishmune®: 71%; LBSap: 62%; and Leish-Tec®: 48%).

Few studies have reported immunoblotting analysis (PALATNIK-DE-SOUSA et al., 2008; TESTASICCA et al., 2014) of the vaccines. SDS-PAGE analysis of FML, a species antigen of *L. donovani* (Vaccine A), showed major glycoprotein components with approximate molecular sizes corresponding to 9, 28, 39, 43 to 45, 58 to 64, 68, and 92 to 95 kDa (PALATNIK-DE-SOUSA et al., 2008). Some cross-reactions between these glycoproteins of *L. donovani* and the antigenic components of *L. braziliensis* justify the identical nature of the profiles obtained by immunoblotting and ELISA. Evaluation of vaccine B was performed by immunoblotting with the recombinant A2 protein; Testasicca et al. (2014) found a recombinant 52-kDa protein in the sera of vaccinated animals, but not *L. braziliensis* antigens. Positive serological reactions for ELISA were observed by Fernandes et al. (2014) for an antigen of *L. infantum* when the dogs remained 30.9% reactive within six months of the first dose of Vaccine B, whereas Porrozzi et al. (2007) detected specific anti-rA2 antibodies in only one of nine *L. braziliensis*-infected dogs.

Thus, our findings as well as previous findings indicate the existence of some cross-reactivity between the antigens of *L. infantum*, *L. braziliensis*, and the components of Vaccine B.

It must also be considered that the antibody titer detected by ELISA at 240 days after vaccination might actually represent the extent of protection exerted against *L. braziliensis* infection.

Dogs vaccinated with the two different commercial vaccines were found to be serologically positive according to ACL ELISA, primarily after 120 and 240 days of vaccination. This study disagreed with the information supplied by the two vaccine manufacturers that vaccinated dogs are immunized, but do not develop serological response after 21 days of the third dose (by routine ELISA). Thus, it is essential that dogs vaccinated with the tested vaccines be strictly registered to ensure that they are not identified as asymptomatic, yet positive for leishmaniasis, in future.

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