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Prevalence and incidence of canine visceral leishmaniasis and its clinical–immunological features in an endemic area of the Brazilian Amazon

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Abstract

Background: A cohort study for 2 years period analysed the prevalence, incidence and clinical–immunological features of canine *Leishmania (L.) chagasi*-infection in 316 mongrel dogs in a visceral leishmaniasis-endemic area in Pará State, Brazil.

Objective/Methods: Diagnosis of infection was performed by the indirect fluorescent antibody test (IFAT-IgG), the leishmanin skin test (LST) and a parasite search (from the popliteal lymph node aspiration) at the beginning of the study and at 6, 12 and 24 months intervals.

Results: IFAT/LST revealed three immune profiles of infection: (I) $IFAT^{(+)}/LST^{(-)}$ (81), (II) $IFAT^{(-)}/LST^{(+)}$ (17) and (III) $IFAT^{(+)}/LST^{(+)}$ (13). Prevalence of profiles I, II and III were 25.6, 5.4 and 4.1%, and an overall prevalence 35.1%. Incidence of profiles I, II and III were 5.4, 0.3 and 0.0%, and an overall incidence 5.7% dogs per month. Incidence at the age ranges <1 year, ≥1 year, <7 years and ≥7 years evidenced a highest rate in the age range <1 year (6.6% dogs per month). Parasitological diagnosis was positive in 19% dogs at the prevalence (85.7% profile I), and in 11% at the incidence (100% profile I). The clinical picture of 179 infected dogs showed 145 (81%) of profile I (82% subclinical); 21 (11.7%) of profile II (100% subclinical); and 13 (7.3%) of profile III (84.6% subclinical). Conversion from subclinical to sick dogs was higher ($p < 0.05$) in profile I (40.2%) than in profiles II (5.8%) and III (9%). Immunological conversion showed that only 3.2% of profile I dogs (prevalence) converted to $LST^{(+)}$ (two at the end of the first 6 months and 1 after 24 months), while 82.3% of profile II dogs converted to $IFAT^{(+)}$ (11 in the first 6 months, whereas three after 12 months). A 100% death rate was observed in dogs from profile I alone.

Conclusion: These results reinforce the need of adopting preventive strategies against CVL as early as in the first semester of the dog's life.

KEYWORDS
Brazilian Amazon, canine visceral leishmaniasis, clinical–immunological features, incidence, prevalence

1 | INTRODUCTION

Visceral leishmaniasis (VL) is an anthropozoonotic disease that affects populations across five continents. It is estimated that approximately 50,000–90,000 new cases of VL occur annually (WHO, 2020). In Latin America, this disease is known as American visceral leishmaniasis (AVL) or 'neotropical Kala azar' (Lainson & Shaw, 2010; Silveira et al., 2013), with *Leishmania (Leishmania) chagasi* Cunha & Chagas 1937 as its aetiologic agent (Lainson, 2010; Silveira & Corbett, 2010; Marcili et al., 2014; Silveira et al., 2021, 2023).

From an epidemiological point of view, it is not possible today to dissociate human AVL from canine visceral leishmaniasis (CVL) by the same *Leishmania* agent given that both go hand in hand, with CVL being considered even more important than AVL due to its higher prevalence (Silveira et al., 2012). Moreover, not only subclinical dogs but also diseased ones may act as the source of infection for the major phlebotomine vector, *Lutzomyia longipalpis* (Psychodidae: Phlebotominae) in Latin America (Barata et al., 2005; Lainson & Rangel, 2005; Laurenti et al., 2013).

As regards the clinical spectrum of CVL, it is well known that natural canine infection can evolve from a subclinical or an apparently healthy state to a systemic, clinical state that culminates in the dog's death. These clinical signs include lymphadenopathy, periorbital and nasal dermatitis, onychogryphosis, fever, apathy, diarrhoea, intestinal haemorrhaging, loss of weight, splenomegaly, hepatomegaly and ulceration of the nose, ears and tail (Genaro et al., 1998; Ferrer, 1999; Ferreira et al., 2007).

In Brazil, the AVL control program prioritizes three types of action: (i) diagnosing and eliminating the infected (either apparently healthy or sick) dogs to reduce sources of infection, (ii) diagnosing and providing precocious treatment for human AVL (iii) and coming at against the vector in areas where human cases occur (Brasil, 2006). However, some studies have questioned the effectiveness of these actions, as their impact on human transmission has been both limited and expensive (Tesh, 1995; Paranhos-Silva et al., 1998; Courtenay et al., 2002; Miró et al., 2017). Therefore, there is a consensus among researchers regarding the need for producing an efficient vaccine against CVL to control AVL (Moreno & Alvar, 2002; Reithinger & Davies, 2002; Solano-Gallego et al., 2017). Alternatively, there is at present a treatment regimen for CVL in Brazil that combines Miltefosine with Allopurinol, the results of which have demonstrated a significant reduction in the clinical score and parasite load in 76% of the dogs that received this treatment regimen, thereby improving the quality of animal life (Lisboa et al., 2018).

As for the canine immune response against the *L. (L.) chagasi* infection, it was demonstrated for the first time in Brazil that the combined use of the indirect fluorescent antibody test (IFAT-IgG) and the delayed-type hypersensitivity test (DTH) is capable of recognizing three different immune response profiles: (I) $IFAT^{(+)}/DTH^{(-)}$, (II) $IFAT^{(-)}/DTH^{(+)}$ and (III) $IFAT^{(+)}/DTH^{(+)}$. Moreover, after comparing these profiles among 138 naturally infected dogs, it was found that the frequency of profile I (77.5%) was higher than the frequencies of profile II (13.0%) and profile III (9.5%), suggesting a higher expression of profile I in both the clinical stages of infected dogs, that is, subclinical (apparently healthy) or sick (Silveira et al., 2012). Thus, it has been demonstrated by our research group that the IgG1-antibody response, but not IgG2, is strongly associated with canine susceptibility to CVL (Lima et al., 2017).

Considering all this, there can be no doubt about the significance of identifying the resistance and/or susceptibility markers of CVL based on the canine immune response profiles against infection. Therefore, the present study sought to amplify the understanding of CVL by focusing not only on its prevalence and incidence but also on the clinical–immunological features of the infection to address specific questions about the interactions of the parasite with the canine immune responses to contribute to new control strategies, therapies, or candidates for vaccines against CVL in Latin America.

2 | MATERIAL AND METHODS

2.1 | Study area

The present study was undertaken in an AVL endemic area named Santana do Cafezal, located approximately 7 km from the administrative center of Barcarena (01°30′S×48°37′W), Pará State, Brazil (Figure 1). This area was chosen as it demonstrates ecological and social economic conditions favourable to AVL transmission (Silveira et al., 2009). These aspects were described in earlier studies, which recorded an 85% prevalence of the major phlebotomine vector, *Lu. longipalpis* (Souza et al., 2005), as well as a 43% prevalence of canine infection (based on the IFAT-IgG and DTH) (Silveira et al., 2012) in and around the human habitations in this region.

2.2 | Canine population examined

The study population consisted of 316 mongrel dogs residing in Santana do Cafezal, out of which 172 (54.4%) were males and 144 (45.6%) were females. Their ages varied from 6 months to 15 years (mean = 2 years and 3 months, suggesting a young population).

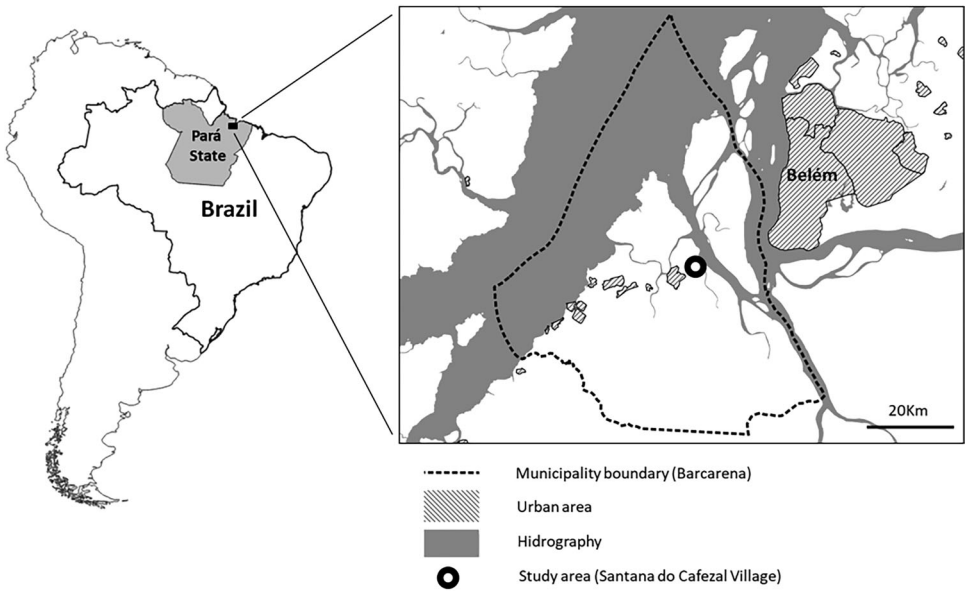

FIGURE 1 Endemic area of American visceral leishmaniasis in Santana do Cafezal village, located nearly 7 km from the administrative center of Barcarena municipality (01°30′S×48°37′W), and geographicaly close to Belém, the capital of Pará State, Brazil.

2.3 | Study design

The present study was designed to analyse the prevalence and incidence of the canine *L. (L.) chagasi*-infection and the dynamics of its clinical–immunological features. For this, it was necessary to conduct a prospective study to follow up the cohort of 316 mongrel dogs for a period of 2 years, from August 2012 to July 2014. The laboratory diagnosis of the infection was processed using the following: the leishmanin skin test (LST) to access the delayed-type hypersensitivity (DTH), the IFAT-IgG to access the IgG-antibody response (IFAT-IgG) and the parasite search (from popliteal lymph node aspiration) to evaluate the prevalence and the incidence at three time intervals (at 6, 12 and 24 months). As such, these laboratory procedures were performed on dogs who initially tested negative for prevalence, and then again at the aforementioned three time intervals. Dogs with positive LST, which is related to immune-genetic resistance against infection (Cardoso et al., 1998; Dos-Santos et al., 2008; Maia & Campino, 2008), were not subjected to a repeat LST as it was assumed that this character was then definitive in their lives. Among the dogs that showed reactivity to both tests, only IFAT-IgG was performed again as this test does not require a new antigen injection, unlike LST. Finally, among the dogs that showed reactivity only against IFAT-IgG, which, in contrast to LST, represents an immune-genetic state of susceptibility to infection (Barbiéri, 2006; Pinelli et al., 1994), it was necessary to perform both tests in subsequent surveys to analyse the evolution of both immune responses, that is, humoral (IFAT-IgG) and cellular (LST). As for the parasite search, it was only repeated when the prior result was negative.

To gain a better understanding regarding the dynamics of the clinical–immunological features of infected (apparently healthy or ill) dogs, their population was stratified into three age classes, that is, (i) less than (<) 1 year, (ii) greater than or equal to (≥) 1 year but less than (<) 7 years and (iii) greater than or equal to (≥) 7 years, which comprised 70 (22,2%), 234 (74%) and 12 (3,8%) dogs, respectively. Thus, considering the need to carry out this analysis on the clinical–immunological evolution of the infection, only dogs with a parasitological diagnosis were excluded (euthanized) from the analysis, while those with reactivity to IFAT, or LST, or both, were kept in their domiciles until the end of the study.

2.4 | Criteria for the identification of canine infection

As IFAT-IgG indicates the humoral response (i.e., susceptibility) and LST (DTH) indicates the cellular response (i.e., resistance), the definition of canine infection was considered to be marked by reactivity to either one or both of these immunological tests, whether associated with a positive parasite search or not. As such, it was assumed that serological reactions (IFAT) with a titre ≥80 (IgG) and skin reactions (LST) forming papules or nodules ≥5 mm in diameter were considered positive ('cut-off') for IFAT-IgG and LST (Silveira et al., 2012).

2.5 | Clinical evaluation of canine infection

Before performing the procedures (i.e., LST and IFAT-IgG) for the laboratory diagnosis of infection, all the dogs were submitted to clinical evaluations during all stages of the present study – that is, at the prevalence and during the incidence surveys at the intervals of 6, 12 and 24 months – to search for signs that could be related to active CVL. As such, dogs who were considered subclinical or apparently healthy did not show any clinical signs suggesting CVL, while sick or ill dogs manifested clinical signs of CVL (Manciati et al., 1988). Therefore, the designation 'subclinical' throughout this study refers to apparently

healthy dogs, while an ill dog refers to a dog that was sick (Baneth et al., 2008). The main clinical signs that were considered in these evaluations included alopecia and skin ulcerations, conjunctivitis, onychogryphosis, loss of weight, apathy, anorexia, weakness and lymphadenopathy. The clinical evaluations were recorded in specific clinical files and performed by a professional veterinarian. This evaluation has been widely used in clinical and immunological surveys to access the clinical spectrum of canine infection due to *L. (L.) chagasi* (Cardoso et al., 2007; Moreira et al., 2007; Rondon et al., 2008; Alves et al., 2009; Reis et al., 2009).

2.6 | Collection of tissue samples

Before collecting the tissue samples as well as venous blood and performing popliteal lymph node aspiration, the dogs were physically restrained. The cephalic vein was punctured to obtain venous blood (2.0 mL) with posterior extraction of the serum, which was stored at −20°C until IFAT-IgG was performed. Aspiration of the popliteal lymph node was performed to confirm the parasitological nature of the infection by seeding in the Difco B45 culture media (Walton et al., 1977).

2.7 | Criteria for defining death due to canine *L. (L.) chagasi*-infection, other damages and loss

To define mortality due to canine *L. (L.) chagasi* infection, we considered dogs, preferentially sick, who demonstrated clinical signs suggestive of CVL and had a positive serological result (IFAT-IgG) and a negative LST. Additionally, in cases of sick dogs, death would have to have occurred at least 4 months after a positive diagnosis (Carneiro, personal observation). A total of 38 dogs died during the course of the present study. It is important to note that the bodies of 31 (81.5%) of these dogs were recovered by community health agents within the family health program of the municipality and submitted to the necropsies and posterior immunohistochemical analysis of their viscera, that is (spleen and liver) with polyclonal antibodies against *Leishmania* sp. This analysis confirmed the leishmanial aetiology in 28 cases (90.3%), as has been published earlier (Lima et al., 2010). The three dogs with negative results in the immunohistochemical analysis using IFAT-IgG and LST were assumed to have died in response to other conditions. Additionally, the cases where the dogs could not be located later (due to changes of residence of the owners or other reasons) were considered definitive losses.

2.8 | Immunological test procedures

The procedures for IFAT-IgG and LST testing were the same as those described earlier (Silveira et al., 2012). The immunological assays (IFAT-IgG and LST) were performed with species-specific *L. (L.) chagasi*-antigens, that is, amastigote from experimental infection in 'hamster' for IFAT-IgG, and promastigote from culture medium (Difco B45) for LST, both produced in the 'Ralph Lainson' leishmaniasis laboratory, at Evandro Chagas Institute, Ministry of Health, Brazil.

2.9 | Parasite search

To determine the aetiologic agent of canine infection, we performed popliteal lymph node aspiration on each dog before seeding the contents into the Dfico B45 culture media (Walton et al., 1977), as previously described (Silveira et al., 2012).

2.10 | Data analysis

The data were statistically analysed using simple percentages to express the prevalence and the incidence of canine infection at 6, 12 and 24 months. The results were examined using the chi-square test (χ^2) by considering a level of significance (α) of 0.05 for the rejection of the null hypothesis ($p \leq \alpha$). For this, the BioEstat 5.0 software was used (Ayres et al., 2007). As for values lower than five, the *G*-test was used. In both tests, when the contingency data demonstrated only one degree of freedom, the Yates and *G*-test (Williams) corrections were applied respectively.

The data of clinical evolution were also analysed by using the actuarial test, which consists of observing the set of dogs divided into constant time periods, which eventually furnishes a number that corresponds to the probable time needed for a subclinical dog to develop and demonstrate signs of infection. To that end, we used three data columns, where the number of sick dogs corresponded to the number of serum-positive animals, the column of occurrences corresponded to the number of dogs that converted from subclinical to sick, and the censored animals corresponded to those that were excluded from the study or died. This test was implemented using the BioEstat 5.0 software (Ayres et al., 2007).

The relative risk (RR) factor was applied to the variables of the evolution to death to obtain the proportions of the incidence of death in the population of dogs that showed different immunological response profiles, with a statistically significant p value of <0.05 and a confidence interval of 95% (CI 95%). For this, the BioEstat 5.0 software was used (Ayres et al., 2007).

The graphs were elaborated using the GraphPad Prism 6.0 software for Windows (GraphPad software) and Microsoft Office Excel (Ayres et al., 2007).

3 | RESULTS

3.1 | Prevalence of canine *L. (L.) chagasi*-infection

The overall prevalence of canine *L. (L.) chagasi*-infection was 35.1% (111 out of 316). Moreover, the prevalence of profile I was 25.6% (81 out of 316), which was higher ($p < 0.05$) than the prevalence of profiles II (5.4%; 17 out of 316) and III (4.1%; three out of 316) (Figure 2).

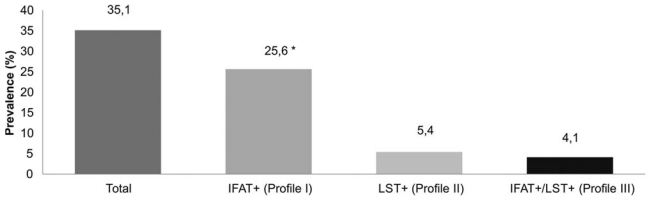

FIGURE 2 Prevalence of canine *L. (L.) chagasi*-infection in Santana do Cafezal, Barcarena, Pará State, Brazil; $^{*}p \leq 0.0001$.

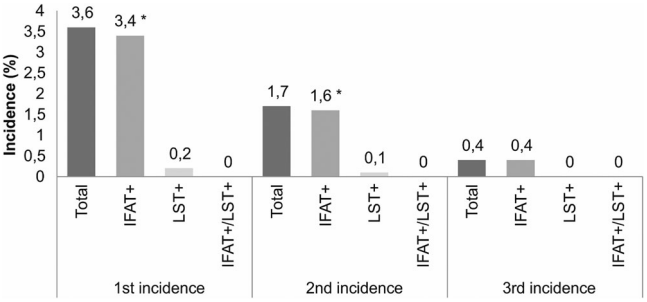

FIGURE 3 Incidence of canine *L. (L.) chagasi*-infection in Santana do Cafezal, Barcarena, Pará State, Brazil; $^{*}p \leq 0.0001$. 1st incidence = at 6 month; 2nd incidence = at 12 month; and 3rd incidence = at 24 month.

3.2 | Prevalence of canine *L. (L.) chagasi*-infection according to age groups

The prevalence in the age group of ≥1 year <7 years was 27.5% (87 out of 316), which was higher ($p < 0.05$) than the prevalence in the age groups of <1 year (5.3%; seven out of 316) and ≥7 years (2.2%; seven out of 316).

3.3 | Incidence of canine *L. (L.) chagasi*-infection

The incidence of canine infection at 6 months was 3.6% dogs per month (i.e., 45 new cases out of 205 noninfected dogs from the prevalence), out of which an incidence rate of 3.4% dogs per month (42 out of 205) was diagnosed by IFAT-IgG (profile I) alone, while a lower ($p < 0.05$) rate of 0.2% dogs per month (three out of 205) was diagnosed by LST (profile II) alone. Notably, not a single animal was diagnosed by both tests (profile III).

The incidence at 12 months was 1.7% dogs per month (i.e., 16 new cases out of 158 noninfected dogs from the 6-month incidence), with the incidence of 1.6% dogs per month (15 out of 158) being diagnosed by IFAT-IgG (profile I) alone, which was higher ($p < 0.05$) than the rate of 0.1% dogs per month (one out of 158) diagnosed by LST (profile II) alone. Notably, no cases were diagnosed by both tests (profile III).

The incidence at 24 months was 0.4% dogs per month (seven new cases out of 136 noninfected dogs from the 12-month incidence), which was diagnosed by IFAT-IgG (profile I) alone. Notably, no cases were diagnosed by either LST (profile II) alone or both tests (profile III) (Figure 3).

As such, the overall incidence of canine infection was 5.7% dogs per month (5.4% of profile I; 0.3% of profile II; and 0.0% of profile III). A progressive decline ($p < 0.05$) in the incidence surveys of the infection can be observed at the time intervals of six (3.6% dogs per month), 12 (1.7% dogs per month) and 24 months (0.4% dogs per month).

3.4 | Incidence of canine *L. (L.) chagasi*-infection according to age groups

The incidence of canine infection at 6 months in the age class of <1 year was 4.8% dogs per month (i.e., 13 new cases out of 45 noninfected dogs), which was higher ($p < 0.05$) than the incidence in the age class of ≥7 years (3.3% dogs per month; i.e., one new case out of five noninfected dogs) as well as in the age class of ≥1 year and <7 years (3.1% dogs per month, i.e., 26 new cases out of 141 noninfected dogs).

The incidence of infection at 12 months in the age group of ≥1 year and <7 years was 1.9% dogs per month (i.e., 13 new cases out of 115 uninfected dogs), which was higher than the incidence in the age group of <1 year (1% dogs per month; i.e., two new cases out of 32 noninfected dogs) and in the age group of ≥7 years, which showed no new cases.

The incidence of infection at 24 months in the age group of <1 year was 0.8% dogs per month (i.e., three new cases out of 30 noninfected dogs), which was higher ($p < 0.05$) than the incidence in the age group of ≥1 year and <7 years (0.3% dogs per month, i.e., four new cases out of 102 noninfected dogs). There were no recorded cases of infection in the age group of ≥7 years.

As such, the overall incidence of canine infection in the age group of <1 year was 6.6% dogs per month, which was higher ($p < 0.05$) than the incidence in the age group of ≥1 year and <7 years (5.3% dogs per month) and the age group of ≥7 years (3.3% dogs per month).

3.5 | Parasitological diagnosis of canine *L. (L.) chagasi*-infection

The parasitological diagnosis of canine infection was confirmed in 19% (21 out of 111) of the dogs at the prevalence, most of whom (85.7%) were from profile I, out of which 61.1% (11 out of 18) were sick ($p < 0.05$) and 38.9% (seven out of 18) were subclinical. As for the remaining 14.3%, all were from profile III, with 66.6% (two out of three) being subclinical and 33.3% (one out of three) being sick. In terms of the incidence, the parasitological diagnosis was confirmed in 11% (five out of 45) of the new cases of canine infection, with four new cases diagnosed at 6 months and one at 12 months. In profile I, 60% (three out of five) of the dogs were subclinical ($p < 0.05$) and 40% (two out of five) were sick.

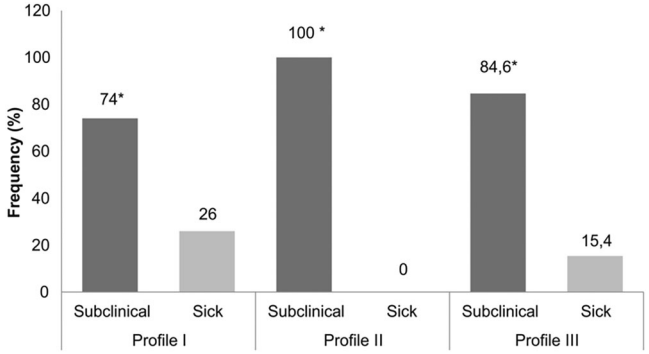

FIGURE 4 Canine *L. (L.) chagasi*-infection in Santana do Cafezal, Barcarena, Pará State, Brazil. Clinical evaluation according to the immunological profiles of infection at the prevalence; $^{*}p \leq 0.0001$.

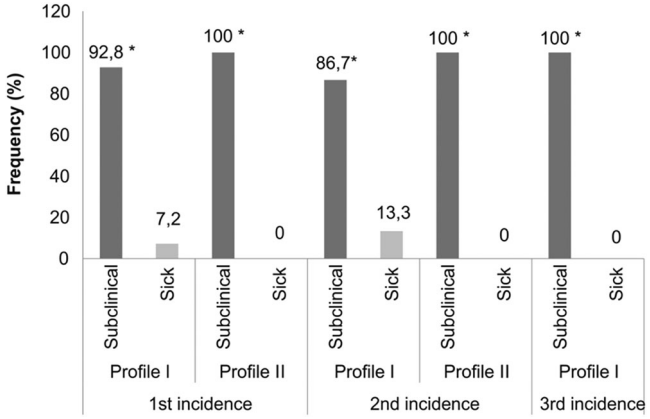

FIGURE 5 Canine *L. (L.) chagasi*-infection in Santana do Cafezal, Barcarena, Pará State, Brazil. Clinical evaluation according to the immunological profiles of infection at the incidence; $^{*}p \leq 0.0001$. 1st incidence = at 6 month; 2nd incidence = at 12 month; and 3rd incidence = at 24 month.

3.6 | Clinical evaluation of canine *L. (L.) chagasi*-infection according to their immunological profiles

When the infection prevalence was analysed, it was observed that out of the 81 dogs from profile I, 74% (60 out of 81) of the dogs were subclinical and 26% (21 out of 81) were sick. Moreover, while 100% (17 out of 17) of the dogs from profile II appeared to be subclinical, among the 13 dogs from profile III, 84.6% (11 out of 13) were subclinical and 15.4% (two out of 13) were sick (Figure 4). As such, the three immunological profiles of infection identified a higher prevalence of subclinical dogs (79.3%; 88 out of 111; $p < 0.05$) than that of sick dogs (20.7%; 23 out of 111).

When the incidence was analysed at 6 months, it was observed that among the 42 dogs from profile I, 92.8% (39 out of 42) were subclinical ($p < 0.05$) and 7.2% (three out of 42) were sick. Moreover, among the three dogs from profile II, 100% (three out of three) were subclinical, whereas no cases of profile III were observed.

When the incidence was analysed at 12 months, it was found that among the 15 dogs from profile I, 86.7% (13 out of 15) were subclinical ($p < 0.05$) and 13.3% were sick (two out of 15). Moreover, 100% (one out of one) of the dogs from profile II were subclinical, whereas no cases of profile III were observed.

Finally, when the incidence was analysed at 24 months, it was observed that all of the seven dogs from profile I (100%; seven out of seven) were subclinical, whereas no cases of profiles II and III were observed (Figure 5).

3.7 | Clinical evolution of canine *L. (L.) chagasi*-infection according to their immunological profiles

This parameter considered the time that an apparently subclinical infected dog took to become ill over 2 years of the study. It is important to note that the number of dogs in this analysis varied due to the necessity of eliminating the dogs with positive parasitological diagnoses, regardless of their clinical status.

Out of the 60 apparently subclinical dogs from profile I diagnosed at the prevalence, seven were eliminated due to positive parasitological diagnoses, leaving 53 dogs for this evaluation. Out of these 52 dogs, 5.7% (three out of 53) became sick during the first 6 months, 11.3% (six out of 53) became sick after 12 months and 3.8% (two out of 53) became sick after 24 months, which amounted to a clinical conversion rate of 20.8%. Out of the 17 subclinical dogs that expressed profile II, 5.8% (one out of 17) became sick after 24 months, whereas out of the 11 subclinical dogs that expressed profile III, 9% (one out of 11) became sick after 12 months. Therefore, it was possible to record a higher conversion rate ($p < 0.05$) in dogs from profile I (20.8%) in those from profiles II (5.8%) and III (9%) during the 2-year duration of the study. Moreover, it was noted that compared with the dogs from profile I, the few subclinical dogs from profiles II and III required greater durations (i.e., 24 and 12 months, respectively) to convert to sick.

Out of the 39, apparently subclinical dogs from profile I diagnosed at the 6-month incidence, three were eliminated due to positive parasitological diagnoses, which left 36 dogs for evaluation. Out of these 36 dogs, 11.1 % (four out of 36) became sick after 18 months. All three dogs that expressed profile II (100%) remained subclinical until the end of the study.

While examining the 13 apparently subclinical dogs from profile I diagnosed at the 12-month incidence, one was eliminated due to a parasitological diagnosis, which left 12 dogs for this analysis. Out of these 12 dogs, 8.3% (one out of 12) became sick by the end of the study. The single subclinical dog from profile II retained that clinical status until the end of the study.

Thus, after comparing these rates of clinical conversion from subclinical to sick pertaining to dogs from profiles I, II, and III, it was observed that the conversion rate of profile I (40.2%) was higher ($p < 0.05$) than the conversion rates of profiles II (5.8%) and III (9%), which revealed that a subclinical dog from profile I had a chance of becoming ill within 8.4 months, whereas a subclinical dog from profile II would only become ill after 27 months (Figure 6).

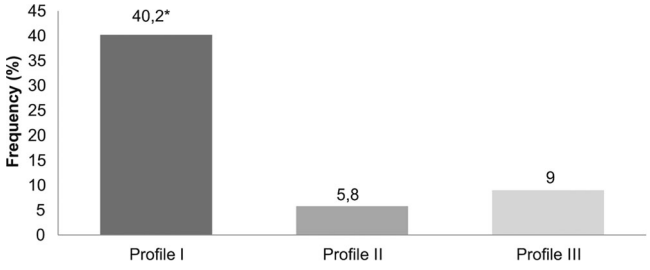

FIGURE 6 Canine *L. (L.) chagasi*-infection in Santana do Cafezal, Barcarena, Pará State, Brazil. Clinical conversion according to the immunological profiles; $^{*}p \leq 0.0001$.

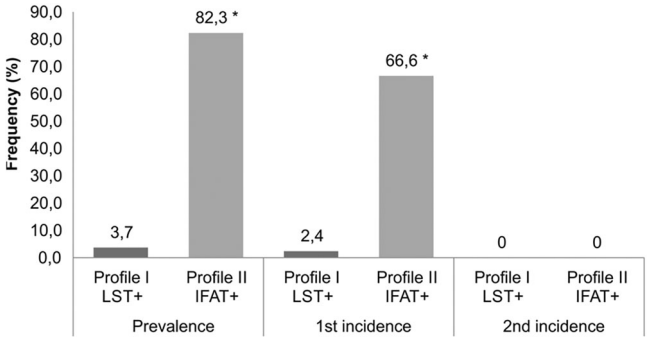

FIGURE 7 Canine *L. (L.) chagasi*-infection in Santana do Cafezal, Barcarena, Pará State, Brazil. Serological [IFAT-IgG] and cellular [LST] immune responses conversions according to the immunological profiles; $^{*}p \leq 0.0001$. 1st incidence = at 6 month; 2nd incidence = at 12 month.

3.8 | Conversion of the serology (IFAT-IgG) and the delayed-type hypersensitivity (LST) in the canine *L. (L.) chagasi*-infection

While analysing the 81 (73%) infected dogs that expressed profile I ($IFAT^{(+)}/LST^{(-)}$) at the prevalence, it was found that only 3.7% (three out of 81) converted to $LST^{(+)}$ (two at the end of the first 6 months and one after 24 months). All of these dogs were in the age group of ≥1 year and <7 years. Moreover, before the LST conversion, two of these dogs showed IFAT-IgG titres of 320−640 and one showed IFAT-IgG titres of ≥1280.

Out of the 17 (15.3%) dogs that expressed profile II ($IFAT^{(-)}/LST^{(+)}$) at the prevalence, most (82.3%−14) converted to $IFAT^{(+)}$, 11 at the end of the first 6 months, whereas three converted in the subsequent 6 months (12 months). These dogs showed different IFAT-IgG titres, that is, ≥1280 for six (42.8%), 320−640 for five (35.7%) and 80−160 for three (21.5%). In this case, 64.3% of the dogs were in the age group of ≥1 year and <7 years. Moreover, 28.5% of the dogs were in the age group of <1 year, whereas 7.2% were in the age group of ≥7 years.

Out of the 42 (93.3%) dogs that expressed profile I ($IFAT^{(+)}/LST^{(-)}$) at the 6-month incidence, only 2.4% (one out of 42) converted to $LST^{(+)}$ after the first 6 months, which amounted to a single dog in the age group of ≥1 year and <7 years with an IFAT-IgG titre ≥ 1280.

Out of the three (6.6%) dogs that expressed profile II ($IFAT^{(-)}/LST^{(+)}$) at the 6-month incidence (45 cases), 66.6% (two out of three) converted to $IFAT^{(+)}$ after the first 6 months with IFAT-IgG titres of 80−160. These two dogs were in the age group of ≥1 year and <7 years.

Out of the 15 (93.8%) dogs that expressed profile I ($IFAT^{(+)}/LST^{(-)}$) at the 12-month incidence, none converted to $LST^{(+)}$ until the end of the study. Likewise, the only dog (6.2%) that expressed profile II ($IFAT^{(-)}/LST^{(+)}$) did not convert to $IFAT^{(+)}$ before the end of the study (Figure 7).

3.9 | Evolution to death due to canine *L. (L.) chagasi*-infection, other damages, and loss

While analysing the 60 subclinical dogs that expressed profile I at the prevalence, it was observed that seven were eliminated due to positive parasitological diagnoses, which left 53 for this analysis. Out of these 53 dogs, 15.1% (eight out of 53) died in the first 6 months, and an additional 5.1% (eight out of 53) died at the end of 12 months; however, no deaths occurred during the last 12 months. Thus, 69.8% (37 out of 53) of the subclinical dogs were left at the end of 2 years.

Out of the 21 sick dogs that expressed profile I at the prevalence, 11 were eliminated due to positive parasitological diagnoses, which left 10 dogs for this evaluation. Out of these 10 dogs, 50% (five out of 10) died during the first 6 months, and an additional 30% (three out of 10) died by the end of 12 months. Moreover, no dogs died during the last 12 months. This left only 20% (two out of 10) of the sick dogs.

As such, the death rate of the sick group (80%) was higher ($p < 0.05$) than that of the subclinical group (30.2%); consequently, the survival rate was higher ($p < 0.05$) in the subclinical group (69.8%) than in the sick group (20%).

While analysing the 17 subclinical dogs that expressed profile II at the prevalence, no death associated with CVL was observed, although 23.5% of them eventually died due to unknown causes, which left 76.5% at the end of the study. Therefore, excluding the unknown causes of death (four), all 13 of the subclinical dogs that expressed profile II (100%) remained alive until the end of the study.

Out of the 13 dogs that expressed profile III at the prevalence, 11 (84.6%) were subclinical and two (15.4%) were sick. Out of the 11 subclinical dogs, two were eliminated due to positive parasitological diagnoses and one died due to unknown causes—all during the first 12 months of the study. Thus, eight (72.7%) dogs were left after 24 months. Finally, among the two sick dogs, one was eliminated for having a positive parasitological diagnosis and the other died of unknown causes, which made a more conclusive analysis of this group impossible (Figure 8).

Therefore, canine deaths due to *L. (L.) chagasi*-infection at the prevalence added 38% (24 out of 63) of the dogs that expressed profile I, with mortality being more frequent ($p < 0.05$) among the sick dogs (80%) than the subclinical ones (30.2%) and survival rate being higher among the subclinical dogs (69.8%; $p < 0.05$) than the sick ones (20%).

Out of the 42 dogs that expressed profile I at the 6-month incidence, 92.8% (39 out of 42) were subclinical and 7.2% (three out of 42) were

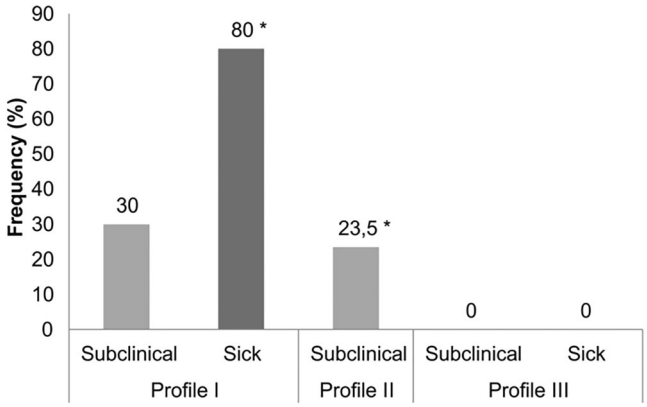

FIGURE 8 Canine *L. (L.) infantum chagasi*-infection in Santana do Cafezal, Barcarena, Pará State, Brazil. Evolution to death, other damages and loss regarding dogs diagnosed at the prevalence; $^{*}p \leq 0.0001$; × = death by other damages.

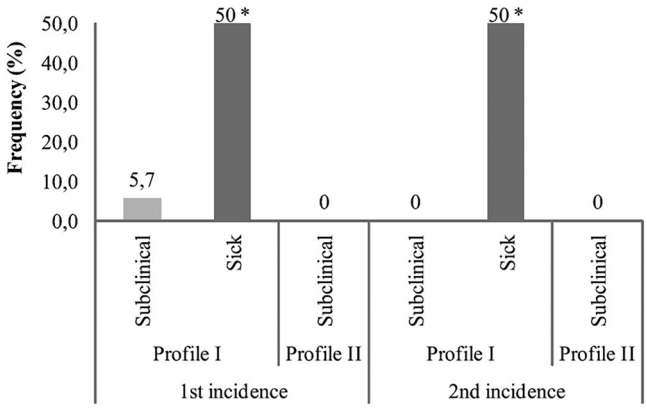

FIGURE 9 Canine *L. (L.) chagasi*-infection in Santana do Cafezal, Barcarena, Pará State, Brazil. Evolution to death, other damages and loss regarding dogs diagnosed at the incidence; $^{*}p \leq 0.0001$. 1st incidence = at 6 month; 2nd incidence = at 12 month.

sick. Out of the 39 subclinical dogs, three were eliminated due to positive parasitological diagnoses and one died due to unknown causes, which left 35 dogs for this analysis. Out of these, 5.7% (two out of 35) died after 6 months, which left 94.3% of the dogs. Moreover, out of the three sick dogs, one was eliminated due to a positive parasitological diagnosis, which left two-one of which died after 6 months, while the other remained alive until the end of the study. All of the three dogs that expressed profile I (100%) were subclinical and none of them died during the study. No cases of profile III were documented.

Out of the 15 dogs that expressed profile I at the 12-month incidence, 86.6% (13 out of 15) were subclinical and 13.4% (two out of 15) were sick. Out of the 13 subclinical dogs, one was eliminated due to positive parasitological diagnoses, which left 12 for this analysis. None of these dogs died after 12 months. However, out of the two sick dogs, one died after 12 months of disease evolution. Conversely, the only subclinical dog that expressed profile II remained alive until the end of the study. There was no case of profile III at the 12-month incidence (Figure 9).

As such, canine death was observed in the incidence survey, but only among the dogs that expressed profile I and mainly in the sick group at a rate of 50%, which was higher ($p < 0.05$) than that of the subclinical group (4.3%). Therefore, based on an analysis of the RR quotient, it was possible to conclude that a dog that expressed profile I was 34.2 times more likely to die than a dog that expressed profile II.

4 | DISCUSSION

This is the first prospective study to follow a cohort of 316 mongrel dogs subjected to natural *L. (L.) chagasi* infection in an AVL-endemic area in Pará, Brazilian Amazon, over 2 years. The prevalence and incidence of canine infection, as well as the dynamics of the clinical–immunological features of CVL were analysed to better understand the interactions of the parasite with the canine immune responses.

The present diagnostic approach revealed an overall prevalence of canine infection (35.1%) that was higher than those found through the use of only one serological diagnostic assay (i.e., either ELISA or IFAT-IgG) in other AVL-endemic areas in northeastern Brazil, such as Bahia (23.5%), Ceará (24%) and Pernambuco (16%) (Paranhos-Silva et al., 1996; Rondon et al., 2008; Santos et al., 2010). This confirms the usefulness of this diagnostic approach for determining subclinical or symptomatic canine *L. (L.) chagasi*-infection.

Another advantage of the present diagnostic approach is the possibility of analysing the specific prevalence of infection based on the three immunological profiles identified against infection. It was apparent that the prevalence of profile I (25.6%) was higher than the prevalence of profile II (5.4%) or III (4.1%), indicating that profile I is the most prevalent expression of the canine immune response against infection. As such, the low prevalence of profiles II (5.4%) and III (4.1%) together (9.5%), characterized by $LST^{(+)}$, reflects a dog's poor ability to develop a cellular immune response against infection. Therefore, although there is no clear evidence of a dichotomy between the cellular and humoral immune responses against canine *L. (L.) chagasi* infection (Barbiéri, 2006), the IgG-antibody response is undoubtedly more prevalent than the cellular one.

Based on this assumption, it is possible to project that a significant number of dogs expressing profile I will convert from subclinical to ill within a period of 1 or 2 years. Along that line, it was observed that the rate of conversion from subclinical to sick among the dogs that expressed profile I at the prevalence (20.8%) was higher ($p < 0.05$) than those that expressed profile II (5.8%) or III (9%) during the 24 months of the study, which confirmed that the risk of clinical conversion was higher among the dogs that expressed profile I.

As regards the age groups of dogs with canine infection, it was observed that the prevalence in the age group of ≥1 year and <7 years (27.5%) was higher ($p < 0.05$) than the prevalence in either the age group of <1 year (5.3%) or the age group of ≥7 years (2.2%), which suggests that most infections occurring in this period of life are a result of the cumulative effects on the dogs who were infected before completing their first year as well as those who were infected ≥1 year and <7 years. Conversely, it is important to note that the low prevalence

(2.2%) in the age group of ≥7 years might be an indication that most of the dogs infected in the age group of ≥1 year and <7 years die of CVL before reaching 7 years of age, although there is evidence that subclinical dogs infected with *L. (L.) infantum* in the Mediterranean region in Europe can later convert to a clinical state between 3 months and 7 years of age (Oliva et al., 2006; Solano-Gallego et al., 2001).

As regards the incidence of canine infection, the present results demonstrate a clear progressive decline in the infection incidence, going from a rate of 3.6% dogs per month at 6 months to 1.7% dogs per month at 12 months and then 0.4% dogs per month at 24 months, which suggests that the source of infection reduced over that period, possibly because of the elimination of 26 dogs through positive parasitological diagnosis as well as the elimination of 28 dogs that expressed profile I due to natural mortality resulting from CVL. Thus, 54 dogs in total doubtlessly ceased to be the sources of infection in the study area and, therefore, contributed to the progressive reduction of the infection incidence.

It is difficult to draw comparisons between the aforementioned incidence of canine infection and the data available in the existing literature, not only because of methodological divergences of the infection diagnosis but also because of the need of following a cohort of dogs for a period of up to 2 years. For instance, Paranhos-Silva et al. (1998) used serological diagnosis (ELISA) to track canine infection during an 18-month period to find an overall incidence of 0.5% dogs per month in an AVL-endemic area in northeastern Brazil (Jequié, Bahia), which was lower than that reported in the present study (5.7% dogs per month) based on the combined use of immunological (IFAT-IgG/LST) and parasitological parameters. Similarly, Coura-Vital et al. (2013), used molecular (PCR) and serological (ELISA) diagnostic tools to determine canine infection during a 26-month study to report an overall incidence of 5.8% dogs per month. Although these authors employed a more sensitive method (PCR/ELISA) than the ones used in the present work (IFAT-IgG/LST/parasite search), no significant difference was found between the results, which indicated that the serological diagnostic assay (IFAT-IgG) used in the present study provides high specificity and sensitivity for the diagnosis of CVL (Silveira et al., 2012).

Another interesting point regarding the incidence of canine infection according to the age groups of the dogs is that most of the new cases of infection occurred in the age group of <1 year (6.6% dogs per month), followed by the age group of ≥1 year and <7 years (5.3% dogs per month) and then the age group of ≥7 years (3.3% dogs per month). This suggests that, as opposed to the prevalence (which demonstrated a greater accumulation of cases in the age group of ≥1 year and <7 years), the infection transmission before the first year of life represents an important event in the lives of those dogs – a fact that should be given priority by adopting preventive strategies in the form of vaccination programs, repellent collar, responsible ownership and environmental management against CVL as early as the first semester of a dog's life.

As regards the parasitological diagnosis of infection, it is important to note that 19% of the dogs tested positive at the prevalence, most of whom (85.7%) expressed profile I (out of which, 61.1% were sick), which once again points to the high susceptibility of profile I to infection. This high susceptibility can be corroborated by the poor ability of these dogs (3.7%) to convert to $LST^{(+)}$ during the study. Furthermore, the parasitological diagnosis was confirmed in the dogs that expressed profile III in lower proportions (14.3%) than what was observed in relation to profile I (85.7%), which suggests that humoral immunity exercises dominance over the cellular immunity, a fact that reflects a high expression of profile I in all parameters of canine infection addressed in this study.

At the time of the incidence surveys, the parasitological diagnosis of infection was confirmed in 11% of all of the new cases, with four at 6 months, one at 12 months and all of them (100%) with profile I (60% subclinical and 40% sick). However, contrary to the prevalence, most of the dogs (60%) appeared to be subclinical, which suggests that although clinical conversion from the status of subclinical to sick requires an indeterminate amount of time, susceptibility to infection is strongly associated with profile I. We recently demonstrated that IgG1 response, but not IgG2, is associated with canine susceptibility to CVL, which reinforces this hypothesis (Lima et al., 2017).

Concerning the clinical spectrum of canine infection, it was noted that, at the time of the prevalence, 79.3% of dogs were subclinical and only 20.7% were sick, which was compatible with the results of previous studies conducted in Brazil (Madeira et al., 2004; Dantas-Torres et al., 2006). This includes the recent results obtained by our research group in the same region (76% subclinical and 24% sick) (Silveira et al., 2012) as well as in the Old World where the causal agent of infection is *L. (L.) infantum* (Cabral et al., 1998; Baneth et al., 2008; Solano-Gallego et al., 2001). Thus, the health status of the infected dogs initially appeared benign and was then contradicted when it was revealed that most of these dogs (68.2%) had an immune response that was consistent with profile I [$IFAT^{(+)}/LST^{(-)}$], which is known to have no resistance against the infection (Paranhos-Silva et al., 1998; Reis et al., 2009; Reithinger & Davies, 2002; Rondon et al., 2008).

When the clinical spectrum of infection was considered at the incidence, it was observed that 18.2% of the subclinical dogs that expressed profile I became sick, which did not differ from the 20.8% observed at the prevalence, which once again points to the pathogenic potential of profile I at the course of canine infection. In contrast, 100% of the dogs of profile II remained subclinical until the end of the study, confirming that the cellular immunity [$LST^{(+)}$] observed in those dogs appears to develop a protective role against infection. Thus, after comparing the cumulative prevalence and incidence rates of clinical conversion from subclinical to sick within profiles I, II and III, it was found that the conversion rate of profile I (39%) was higher ($p < 0.05$) than that of profile II (5.8%) or III (9%), indicating that dogs of profile I have 33.2 and 30% more chances of becoming sick than dogs of profiles II and III, respectively. Moreover, it was concluded that subclinical dogs that expressed profile I would most likely develop clinical signs of CVL within 8.4 months, while subclinical dogs that expressed profile II would most likely develop clinical signs within 27 months.

As for the serological [$IFAT^{(+)}$] and cellular [$LST^{(+)}$] conversions of the immune profiles of the infected dogs, it is important to note that, at the prevalence, only 3.7% of the dogs that expressed profile I con-

verted to LST(+), and all of them were in the age group of ≥1 year and <7 years, which demonstrates the poor ability of the dogs to develop an efficient cellular immune response against the infection. However, somewhat unexpectedly, 82.3% of the dogs that expressed profile II evolved to IFAT(+), although none of them died due to CVL. Therefore, it can be concluded that although the dogs of profile II have converted to IFAT(+) and assumed profile III, only 5.8% of these dogs converted to the sick status.

Similarly, when we consider the new cases of infection at the incidence, it was found that only 1.7% of the dogs that expressed profile I converted to LST(+) at the 6-month incidence, which reaffirms the inexpressive ability of the dogs of profile I to convert to a cellular immune response against infection. Conversely, it was observed that 50% of the dogs that expressed profile II converted to IFAT(+), with two subclinical animals in the age group of ≥1 year and <7 years that maintained their subclinical status until the end of the study, which ratifies the protective role played by DTH [LST(+)] against infection.

Finally, when CVL was analysed in terms of the deterioration of canine health and the consequent death of the animals, it was noted that among the 31 dogs that evolved to death, 28 (90.3%) were due to CVL and all expressed profile I, out of which 24 (85.7%) were counted at the prevalence (80% sick) and only four (14.3%) at the incidence (50% sick), confirming the critical association of profile I with a dog's susceptibility to CVL. In other words, the RR quotient demonstrated that the dogs that expressed profile I had 34.2 times more chances of dying due to CVL than the dogs that expressed profile II.

5 | CONCLUSION

Although some important features of the pathophysiology of CVL have been discussed here, the most notable are the findings concerning the incidence of infection, which have shed light on one of the most striking aspects of the dynamics of infection transmission to dogs. Through this, it was possible to demonstrate that the largest share of new cases of infection (6.6% dogs per month) occur among those animals that have not yet completed the first year of life (i.e., age group of <1 year), which represents a high-risk factor for the development of CLV, especially if these animals are exposed to repeated sections of infection transmission in that period of life. This highlights the need of adopting preventive strategies such as vaccination programs, repellent collars, responsible ownership and environmental management against CVL. These measures should be undertaken as early as the first semester of a dog's life.

AUTHOR CONTRIBUTIONS

Conception/design of the study: F. T. S. and M. D. L. *Data collection*: L. A. C., L. V. L., M. B. C., T. V. S., P. K. R., M. D. L. and F .T .S.; *data analysis*: L. A. C., L. V. L., M. B. C., T. V. S., P. K. R., M. D. L. and F. T. S.; *manuscript writing – original draft*: L. A. C., M. D. L. and F. T. S.; *manuscript writing – critical revision*: L. A. C., L. V. L., M. B. C., T. V. S., P. K. R., M. D. L. and F. T. S.; *financial support*: F. T. S. All authors read and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest concerning this work.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

This study was approved by the Ethical Committee of Animal Use 'CEUA' of the Evandro Chagas Institute, Surveillance Secretary of Health, Ministry of Health, Brazil, under protocol number 0018/2011/CEUA/IEC/SVS/MS/Brazil.

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PEER REVIEW

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