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Occurrence of *Lutzomyia longipalpis* (Phlebotominae) and canine visceral leishmaniasis in a rural area of Ilha Solteira, SP, Brazil

Ocorrência de *Lutzomyia longipalpis* (Phlebotominae) e leishmaniose visceral canina em uma área rural de Ilha Solteira, SP, Brasil

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Abstract

This study aimed to investigate the occurrence of *Lutzomyia longipalpis* and also the canine visceral leishmaniasis (CVL) in a rural area of Ilha Solteira, state of São Paulo. Blood samples were collected from 32 dogs from different rural properties (small farms) and were analyzed by ELISA and the indirect immunofluorescence antibody test (IFAT) in order to diagnose CVL. From these serological tests, 31.25% of the dogs were positive for CVL and these were distributed in 66.7% (8/12) of the rural properties, which were positive for *L. longipalpis*. CDC (Center for Disease Control and Prevention) light traps were installed in 12 properties (one per property) and insects were caught on three consecutive days per month for one year. *L. longipalpis* was present on 100% of the rural properties visited, at least once during the twelve-month interval, totaling 64 males and 25 females. The insects were more numerous after the peak of the rain, but the association between prevalence of peridomestic vectors and the climatic data (precipitation, relative air humidity and temperature) and the occurrences of CVL among dogs on each rural property were not statistical significant ($p < 0.05$). However, the occurrence of CVL cases in dogs and the presence of *L. longipalpis* indicate that more attention is necessairy for the control of this disease in the rural area studied.

Keywords: CDC, ELISA, IFAT, *Leishmania infantum*, *Lutzomyia longipalpis.*

Resumo

O objetivo desse trabalho foi o estudo da prevalência de *Lutzomyia longipalpis* e da leishmaniose visceral canina (LVC) em uma área rural do município de Ilha Solteira do estado de São Paulo. Amostras de sangue foram coletadas de 32 cães provenientes de pequenas propriedades rurais e analisadas por meio dos métodos sorológicos ELISA (imunoensaio enzimático indireto) e RIFI (reação de imunofluorescência indireta) para o diagnóstico da LVC. Pelos exames sorológicos, dos 32 cães avaliados, 31,25% foram diagnosticados positivos para LVC, os quais estavam diostribuídos em 66,67% (8/12) das propriedades positivas para *Lutzomyia longipalpis*. Armadilhas luminosas do tipo CDC (*Center for Disease Control and Prevention*) foram instaladas em 12 propriedades, sendo uma por propriedade, e as coletas dos insetos foram realizadas três dias consecutivos a cada mês, durante um ano. O inseto *L. longipalpis* foi encontrado em 100% das propriedades visitadas, pelo menos uma vez no ano, totalizando 65 machos e 25 fêmeas. A maior quantidade de insetos foi observada principalmente após a ocorrência dos maiores picos de precipitação pluvial, mas a associação entre a prevalência dos vetores peridomiciliares e os dados climáticos (precipitação, umidade

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relativa do ar e temperatura) assim como a ocorrência da CVL em cães em cada propriedade não foi estatisticamente significante (p< 0.05). No entanto, alerta-se que pela presença dos casos de LVC nos cães amostrados e também de *L. longipalpis*, maior atenção deve ser dada durante as investigações epidemiológicas para o controle dessa doença nessa área rural estudada.

Palavras-chave: CDC, ELISA, RIFI, *Leishmania infantum*, *Lutzomyia longipalpis.*

Introduction

Canine visceral leishmaniasis (CVL) is considered a parasitic zoonosis and is one of the most important public diseases in about 80 countries in Asia, Africa and Latin America (ASHFORD et al., 1992), distributed particularly in many tropical and subtropical regions of the world (ASHFORD, 2000; DESJEUX, 2004).

Transmission of the parasite to humans and animals occurs through the bite of phlebotomine sandflies (Diptera; Psychodidae) of the genera *Phlebotomus* and *Lutzomyia*. *Lutzomyia* is the most common genus in Latin America, with more than 400 known species (YOUNG & DUNCAN, 1994). The species *Lutzomyia longipalpis* is considered the main vector of the parasite and it feeds on a large numbers of hosts, including birds, domestic and wild animals and even human beings (BRASIL, 2006).

Dogs are considered very important reservoirs in several visceral leishmaniasis foci in both rural and periurban areas (MARZOCHI & MARZOCHI, 1994; SILVA et al., 2001). Occurrences of this disease have been correlated with the presence of sand fly vectors (VIEIRA & COELHO, 1998).

The importance of this vector in the epidemiology of leishmaniasis is the factor that has stimulated the search for knowledge about the phlebotomine fauna responsible for CVL transmission to the susceptible reservoirs (SARAIVA et al., 2006). Camargo-Neves et al. (2001) considered that there was a need for studies on the density of phlebotomines correlated with environmental factors such as the presence of natural vegetation and organic matter in the soil (leaves, fruits, domestic animal feces, food and plant waste), which could represent possible sites for vector breeding, development or survival. Moreover, presence of human homes in inappropriate locations, with remnant forested areas close to the peridomestic environment and a lack of basic sanitation, are considered very common in rural or periurban areas (MUNIZ et al., 2006), thus favoring presence or maintenance of vector breeding sites. In addition, temperature, humidity and rain are important factors related to the phlebotomine population in certain areas in Brazil. Macedo et al. (2008) observed that the phlebotomine fauna presented seasonal distribution associated with the rainfall rate and relative humidity, such that greater vector density occurring during the rainy season.

The epidemiological surveys on CVL that have been undertaken in urban areas of Ilha Solteira, state of São Paulo, have shown that *L. longipalpis* is very well adapted to survival in peridomestic areas even without the presence of abundant vegetation. This adaptation can contribute towards allowing it to become established under different environmental conditions, thereby increasing the sand fly population and dispersing it to many varied habitats (PAULAN et al., 2012).

The municipality of Ilha Solteira is classified as a suitable local for human and canine leishmaniasis transmission (SÃO PAULO, 2006). Therefore, it is recommended that canine serological investigations should be conducted to diagnose leishmaniasis, in addition to phlebotomine surveys, in order to control the disease. However, rural areas are not covered by local programs for CVL control. Thus, we decided to carry out an epidemiological survey in order to investigate the presence of the vector *L. longipalpis* in relation to positive findings of CVL among dogs and in relation to climatic data, in a rural area called "Cinturão Verde" (greenbelt), which is very close to the urban area of Ilha Solteira, state of São Paulo, Brazil.

Materials and Methods

Area under study

This study was carried out in a rural area called "Cinturão verde" (greenbelt), which is close to the urban areas of Ilha Solteira, state of São Paulo, Brazil. Its area is 880.46 hectares (ha), divided into 91 small rural properties (small farms or settlements) for agriculture (crops or vegetables) or animal production (poultry, pigs, sheep) on which around 200 families live.

Twelve rural properties in the "Cinturão Verde" were chosen for phlebotomine surveys as prescribed by Macedo et al. (2008), who recommended that the areas to be surveyed should have characteristics favoring the maintenance of vector breeding sites. This means that the homes involved should have large peridomestic areas with abundant organic matter in the soil, presence of domestic animals (dogs, cats, birds, pigs, sheep or horses), small bushes, remnant forest, fruit trees and vegetable gardens.

Capture and identification of sand flies

Sand fly collections were carried out from September 2012 to August 2013, for three consecutive days per month, using CDC (Center for Disease Control and Prevention) light traps that were installed in the peridomestic areas of 12 rural properties (one light trap per property). The distribution of these collection sites is presented in Figure 1.

Each property was identified using the numbers 1 to 12. The locations were georeferenced using the global positioning system (GPS) (Garmin Model II-12). These data were imported into a geographical information system (GIS) using SPRING/INPE (CAMARA et al., 1996) in order to visualize their spatial distribution. By means of the SCARTA module, a spatial map

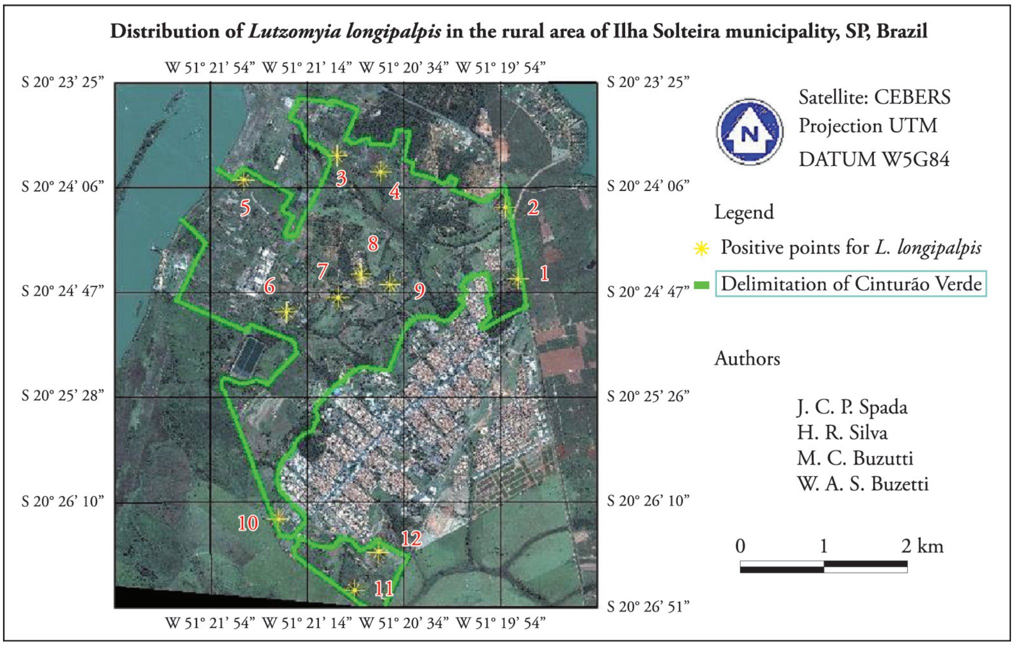

Figure 1. Sites where CDC light traps were placed for catching *Lutzomyia longipalpis* sand flies in the rural area named "Cinturão Verde" in the municipality of Ilha Solteira, state of São Paulo, Brazil. The number of each rural property is represented in red color.

was constructed and edited with the points at which each light trap was placed.

The phlebotomines that were caught were sent to the Superintendency for Endemic Disease Control (SUCEN) in Araçatuba, state of São Paulo, and were identified in accordance with the taxonomic key of Galati (2003), in order to confirm the species.

Serological tests on CVL

Serum samples were collected from 32 dogs that were distributed on the 12 properties on which the light traps were kept for phlebotomine surveys. The serum samples were used for CVL diagnosis by means of ELISA (indirect enzyme immunoassay) and by means of an indirect fluorescence antibody test (IFAT).

ELISA and IFAT

An indirect ELISA method was used in accordance with Machado et al. (1997) and adapted by Oliveira et al. (2008). Crude soluble antigen of *L. infantum* was used at the concentration of 5 μg/ml diluted in 0.05 M carbonate/bicarbonate buffer at pH 9.6. Alkaline phosphatase conjugate rabbit anti-dog IgG whole molecule (Sigma, cat. no. A-0793, USA) was used at a dilution of 1:8,000 plus 0.5% normal rabbit. The substrate of the reaction was P-nitrophenyl phosphate (Sigma, cat. no. N-9389, USA) at 1 mg/ml in diethanolamine buffer (pH 9.8). The plates were read at the wavelength 406 nm using a microplate reader (Dynex Technologies, USA). To control inter-plate variation, positive and negative control were included in each plate. The cutoff point was determined to be an ELISA level ≥ 3 (EL ≥ 3).

IFAT was performed in accordance with the procedure of Oliveira et al. (2008), which can be briefly described as follows. *Leishmania* promastigotes were isolated from the bone marrow of naturally infected dogs and were maintained in RPMI culture medium, which was used for antigen preparation. The serum dilutions were used in duplicate, starting at 1:40, and were placed over the antigen on the slides at 37 °C for 30 minutes. The slides were then incubated with anti-dog IgG serum conjugated for fluorescein isothiocyanate (KPL, cat. no. 02-19-02, USA). The slides were then examined under a fluorescence microscope (Olympus, BX-FLA, Japan). In all the experiments, reference serum samples were included as negative and positive controls. The cutoff point was serum samples at 1 ≥ 40.

Climatic data

Data on monthly mean temperature (ºC), relative air humidity and rainfall (mm), covering the period from September 2012 to August 2013, were obtained from the Experimental Weather Station of São Paulo State University (UNESP), Ilha Solteira, state of São Paulo, Brazil.

Statistical analysis

Two-dimensional statistical analysis making comparisons among groups was performed by means of chi-square tests. Variance analysis was used to make comparisons among the monthly *L. longipalpis* collections. Correlations regarding the presence of CVL-positive dogs and the presence of *L. longipalpis* on each rural property were made by means of univariate analysis using the chi-square test (PIMENTEL-GOMES, 2000). These analyses were performed using the R software, version 2.15.3

(TEAM, 2014). Analysis comparing the monthly numbers of specimens of *L. longipalpis* caught and also comparing the climatic characteristics (temperature, relative humidity and rainfall) were performed using the Tukey test (significance level of $p \leq 0.05$) and the Pearson correlation coefficient. Analysis of variance was performed using the Sisvar software, version 5.0 (FERREIRA, 2011).

Animal Ethics Committee

This study was approved by the Animal Ethics Committee of São Paulo State University (UNESP) in Ilha Solteira, state of São Paulo, under the Protocol No. 002/2011/CEUA.

Results and Discussion

Figure 1 shows the distribution and locations of the 12 CDC light traps that were placed in the rural area and it shows their proximity to the urban area of the municipality of Solteira.

The "Cinturão Verde" is considered to be a suitable rural area for phlebotomine breeding because it still presents remnant native forest and abundant organic matter on the ground (leaves, fruits, tree trunks, domestic animal feces, food and plant waste), which provide excellent biotic conditions for reproduction, proliferation and development of phlebotomine fauna. Furthermore, the presence of domestic or wild animals in peridomestic areas may contribute with blood sources for female sand flies. All of these factors are considered favorable for outbreaks of CVL disease, as reported by Camargo-Neves et al. (2001). According to Dias et al. (2007), sand flies particularly prefer primary and secondary forests or calcareous rocks for refuge and survival. However, the adaptation of many phlebotomine species has been undergoing changes, especially in environments modified by humans. These changes can cause these vectors to come closer to human homes, thereby favoring transmission of leishmaniasis. Most environmental factors affecting the epidemiology of leishmaniasis provide evidence of the adaptation of parasites and their vectors to ecological changes due to socioenvironmental processes such as deforestation and urbanization. Because these parasites and vectors have adapted to modified environmental conditions in order to survive, the epidemiological profile of the disease has also undergone modifications (MARZOCHI & MARZOCHI, 1994). In addition, *L. longipalpis* can resist and adapt to adverse conditions and can explore new environments (MICHALSKY et al., 2009).

According to Brasil (2006), *L. longipalpis* females feed on the blood of a great variety of vertebrate hosts, including birds, wild or domestic mammals and humans. Thus, the presence of other domestic animals (poultry, pigs, cats, cattle, horses, etc) in peridomestic areas may attract *L. longipalpis* to households, thereby increasing the risk of *Leishmania* spp. transmission. Because of the proximity of the "Cinturão Verde" to the periurban area of Ilha Solteira (Figure 1), this area can be considered of high risk for dispersion of infected vectors and consequent onset of the disease, with subsequent dispersion into the city, thereby increasing the numbers of dogs or even humans infected with visceral leishmaniasis. Corroborating this hypothesis, Costa et al. (2007) reported that abandoned dogs on the periphery of cities can be also infected by wild reservoirs, thus increasing the chances of amplifying disease transmission to other domestic dogs and humans.

Out of the 32 dogs living on the 12 properties studied here, 31.25% (10/32) were positive for CVL by means of ELISA or IFAT. Table 1 shows the numbers of dogs that were positive or negative for CVL, correlated with the number of male or female *L. longipalpis* sand flies caught using the CDC light traps on each rural property.

No direct statistical association ($p \leq 0.05$) between the presence of *L. longipalpis* and CVL in dogs was observed in the present study (Table 2), even though 66.7% (8/12) of the farms had serum-positive dogs, while only 4/12 properties (33.3%) had negative dogs. However, França-Silva et al. (2005) reported that *L. longipalpis* was important in the dynamics of CVL transmission

Table 1. Numbers of *Lutzomyia longipalpis* (males and females) and dogs that were positive or negative for CVL on 12 rural properties during the period from September 2012 to August 2013, in a rural area named "Cinturão Verde" in the municipality of Ilha Solteira, São Paulo, Brazil.

Identification of each property	Numbers of *L. longipalpis*		Numbers of dogs*	
	Male	Female	Negatives for CVL	Positives for CVL
1	3	1	2	0
2	5	1	3	0
3	5	1	3	1
4	2	2	2	0
5	36	11	3	1
6	2	0	2	0
7	1	0	2	1
8	7	8	1	1
9	2	0	2	1
10	0	1	0	2
11	1	0	1	2
12	1	0	1	1
Total	65	25	22	10

*Dog serum positivity by ELISA or IFAT diagnostic tests.

in an endemic area in the state of Minas Gerais in Brazil, which demonstrated that there was a positive correlation between the population density of *L. longipalpis* and visceral leishmaniasis in dogs. Although the statistical analysis in our study did not reveal any significant results, the biological factor and the importance of the presence of the disease and the vector on these properties must be emphasized. One reason for this lack of statistical association was probably the low density of sand flies collected in each place. Moreover, even though all the properties were positive at least on one occasion over the year, they were not consistently positive during the year, and many of them were negative most of the time.

Lutzomyia longipalpis was caught on 100% (12/12) of the rural properties evaluated on at least one occasion during the year (September 2012 to August 2013), thus indicating that the area studied was favorable for vector breeding. Property number 5 (Table 1), which was located very close to the banks of the Paraná River, was where the greatest numbers of sand flies (36 males and 10 females) were collected. The peridomestic area of this household had not been cemented over the ground and it was damp and dirty, with a lot of organic matter on the ground. There were also some bushes, native trees and fruit trees (mango, orange and *jabuticaba*), and dogs, cats, pigs and poultry had free access to this area, which favored the conditions for breeding sites and food sources for *L. longipalpis* survival. Michalsky et al. (2009) emphasized that lack of hygiene or basic sanitary conditions, no rainwater drainage or irregular garbage collection in association with presence of domestic animals, are factors that contribute towards the presence of *L. longipalpis*. Property number 8 was considered to be the second site in terms of the number of sand flies caught, totaling 15 insects. In this place, there was a kennel with a great concentration of captured dogs and cats that had been abandoned by their owners. Silva et al. (2014) found that 89% of these dogs were infected with CVL, thus showing that this place had a potential risk of maintaining and disseminating the disease.

The importance of these vectors in the epidemiology of leishmaniasis is a factor that has motivated studies on their infectivity and transmission to susceptible reservoirs (SARAIVA et al., 2006), and on the vector population density and environmental factors (CAMARGO-NEVES et al., 2001). In the present study, the occurrences of *L. longipalpis* sand flies were not correlated with climatic data (temperature, rainfall and humidity) in the municipality of Ilha Solteira. This study showed that there were increased numbers of *L. longipalpis* during the months of December 2012, February 2013, May 2013 and July 2013 and slightly decreased numbers during the months of January 2013, March 2013, April 2013, June 2013 and August 2013. As shown in Figure 2, the months in which the lowest numbers of sand flies were recorded were those with the highest rainfall. However, as soon as the precipitation rate became lower, the population of sand flies increased. In contrast, the average monthly temperatures did not change much over the year, ranging from 22 °C to 28.1 °C. Similarly, Macedo et al. (2008) and Almeida et al. (2010) noted higher numbers of *L. longipalpis* during the rainy season or after the peak rainfall. In addition, many studies in diverse regions of Brazil have indicated that rain was one of the most important climatic factors (high humidity) that could influence sand fly vector breeding and survival (BARATA et al., 2004). However, our results showed that the climatic data (temperature, humidity and rainfall) did not show any statistical association ($p \leq 0.05$) with the sand fly population density in the area.

The presence of *L. longipalpis* in this rural area is a reason for needing to remain alert to the possibility of visceral leishmaniasis outbreaks in the municipality of Ilha Solteira, especially because this is the most abundant and important vector for CVL in the state of São Paulo, Brazil.

Table 2. Univariate statistical analysis on correlations regarding the presence of *Lutzomyia longipalpis* sand flies caught over a 12-month period using CDC light traps placed on rual properties in a rural area named "Cinturão Verde", in the municipality of Ilha Solteira, state of São Paulo, Brazil.

	Numbers/Percentages of properties		
	Serum positive dogs for CVL	**Presence of *Lutzomyia longipalpis***	**P* value**
Yes	8/66.67	12/100	0.2482ns
No	4/33.33	0/0	
Total	12/100	12/100	

*Test Chi-square; ns = not statistical significant (p > 0.05).

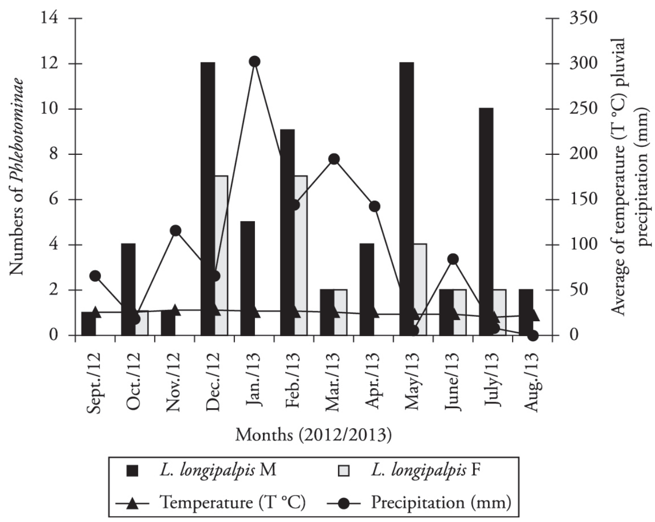

Figure 2. Numbers of *Lutzomyia longipalpis* sand flies collected in the rural area named "Cinturão Verde", in Ilha Solteira, São Paulo, Brazil, from September 2012 to April 2013, the average monthly temperature (°C) and rainfall (mm). Climatic data obtained from UNESP, Ilha Solteira, SP, Brazil.

Conclusion

The results from the present study showed that the main vector for CVL transmission (*L. longipalpis*) was found on 100% of the rural properties (small farms) located in the rural area called "Cinturão Verde". On these properties, 31.3% of the dogs were serum reactive for visceral leishmaniasis, but no direct associations between vector density, occurrence of the disease in dogs and climatic factors were identified.

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