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Short Communication

The first canine visceral leishmaniasis outbreak in Campinas, State of São Paulo Southeastern Brazil

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ABSTRACT

Introduction: Early detection of American visceral leishmaniasis (AVL) outbreak in animals is crucial for controlling this disease in non-endemic areas. **Methods:** Epidemiological surveillance (2009-2012) was performed in Campinas, State of São Paulo, Brazil. **Results:** In 2009, *Leishmania chagasi* was positively identified in four dogs. Entomological research and three serological studies (2010-2012) were undertaken as monitoring measures; these approaches revealed a moderate prevalence of *Leishmania* present in 4% of the canine population. *Nyssomyia whitmani* and *Lutzomyia longipalpis* were the predominant species identified. **Conclusions:** Detection of an AVL outbreak in dogs in an area with an evolving natural landscape containing sand flies is crucial for control programs.

Keywords: Canine visceral leishmaniasis. Epidemiological surveillance. Control Program.

American visceral leishmaniasis (AVL) is a zoonosis caused by *Leishmania (Leishmania) chagasi* protozoa and transmitted by the Phlebotomus species, with *Lutzomyia longipalpis* as the main vector[1].

In urban environments, dogs act as a reservoir and source of infection for vectors. Dogs are frequently found in domestic and peridomestic environments and usually have a high prevalence of infection, which is often long-lasting. Disease symptoms in dogs are characterized by fever, weight loss, lymphadenopathy, and spleen and liver enlargement. Additionally, seropositive dogs without clinical disease signs may act as a source of infection for sand flies[1].

Canine visceral leishmaniasis cases usually precede human cases. Thus, the detection of new geographical areas of leishmaniasis transmission in dogs is a critical step to starting or improving the epidemiological surveillance of leishmaniasis[1].

In Latin America, over 90% of AVL cases have occurred in Brazil (in 21 Brazilian states). Historically known as a rural endemic, this disease has become endemic and epidemic in large Brazilian cities since the 1980s[2]. The State of São Paulo was considered free of AVL until late in the 1990s when an outbreak occurred in the western part of the state[3]. From that point, the disease spread to other regions of the state, and 100 municipalities had registered AVL transmission by 2011[4]. Campinas was added to the list of municipalities with established canine transmission maintained by the state's AVL Surveillance and Control Program in 2009 after the confirmation of the first autochthonous case of canine AVL[4,5].

Previous investigations of AVL transmission in areas around allochthonous canine cases were conducted before 2009 in Campinas; no clinical, epidemiological, or laboratory evidence was found in 10 canine serological surveys in different parts of the city.

According to the 2010 Census, the city population of Campinas was 1,080,999 people, making it the 14th most populous Brazilian city with more than 98% of the population living in urban areas. An estimate of the population of owned dogs, based on the human population in Campinas, was 156,548 animals.

The first report of the disease in Campinas occurred in a residential lot with good infrastructure in a community with high socioeconomic status. The lot is situated in an environmentally protected area in the east part of the city that displays patches of forest with wild fauna and characteristics of topography and vegetation that favor the presence of sand flies[5,6].

The present study aimed to describe the process of investigating the AVL canine outbreak in a previously non endemic area during the period of 2009 to 2012.

The first autochthonous canine case identified in Campinas was confirmed with reactive serological results and polymerase chain reaction (PCR) sequencing from biological specimens collected by necropsy (liver and spleen samples) and aspirates (bone marrow and lymph nodes).These samples tested positive for *Leishmania* when using the nested small subunit ribosomal

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deoxyribonucleic acid (SSU-rRNA)-based PCR strategy performed at the São Paulo Zoonosis Center Control. The fragments produced by S17 and S18 oligonucleotides were sequenced and identified as the *Leishmania chagasi* parasite[5].

Confirmation that the case was autochthonous led to a focused investigation in 2009 of the area where this dog resided. Blood samples were collected from 198 canines, and these samples were clinically examined. In this way, three additional canine cases were identified that were both symptomatic and serologically reactive to AVL, resulting in four cases and a prevalence of 2% in the tested population.

The clinical criteria to consider an animal as suspected of having AVL include the presence of one or more of the following signs: loss of weight or muscle mass, increased lymphatic ganglia, enlarged liver or spleen, onychogryphosis, and skin lesions[1,7].

Blood samples were tested for AVL using enzyme-linked immunosorbent assay (ELISA) and indirect fluorescence assay (IFA), both produced in Bio-Manguinhos; Rio de Janeiro; Brazil. The tests were performed at the Institute Adolfo Lutz (IAL), the reference public health laboratory of the State of São Paulo.

ELISA was used for screening to identify seronegative dogs. ELISA-reactive samples required confirmation by the IFA test and were considered reactive when the obtained values were greater than or equal to 1:40[7,8]. The ELISA results were considered reactive when the value of the optical density was at least 3-fold higher than the standard deviation of the cutoff of the negative control result.

Considering the environmental characteristics of the region, other potential reservoirs were investigated. Forty wild animals were captured alive in wooded areas through baited traps. After administration of an anesthetic, biological samples were collected, and the animals were released back into the wild. The 40 wild animals captured belonged to the following species: *Nectomys squamipes* (South American water rat), *Didelphis albiventris* (opossum), *Callithrix penicillata* (marmoset), and *Gracilinanus agilis* (brown four-eyed opossum). None of the captured animals showed evidence of infection by *Leishmania infantum chagasi* using (PCR) tests, aside from a positive slide of *Didelphis albiventris* for trypanosomatids.

The PCR test results described in the present study were attained by the reference public health laboratory for Leishmaniasis in State of São Paulo, the Institute Adolfo Lutz,which currently adopts the PCR protocol described by Gomes et al[8].

The entomological investigation was performed by the Laboratory of Entomology of the Endemic Control Superintendence (SUCEN: *Superintendência de Controle de Endemias*) using the technique of manual capturing through aspiration of phlebotomine sand flies after dusk inside and around the residence in which the infected dog had lived and in other at-risk residences (residences without flooring or with abundant vegetation, organic matter, or other domestic animals).

A total of 85 houses were searched, of which 16 (18.8%) had phlebotomine sand flies. *Lutzomyia longipalpis* was found in three (3.5%) houses. Nine *L. longipalpis* females were dissected in saline, identified, stored in a culture medium, and sent for detection of *Leishmania* with PCR at IAL-SP[8]. Two out of the 9 (22.2%) females tested positive for *L. chagasi.* Two *N. whitmani* females of 27 (7.4%) tested positive for a trypanosomatid of another genus.

Canine survey

In the subsequent years of 2010 to 2012, the area was monitored by a yearly census serological canine survey in more than 90% of the estimated total population of 800 canines in the area.

During the first 2 years, the serological tests performed were ELISA and IFA. In 2012, the Brazilian Ministry of Health changed the serological techniques routinely used in reference laboratories by introducing the Dual-Path Platform (DPP®; Bio Manguinhos; RJ; Brazil) CVL rapid test for detecting K26/K39-reactive antibodies[99].

In some cases of asymptomatic reactive animals, more specific tests were conducted, such as PCR and parasitological exams from material obtained by necropsy (liver and spleen samples) and aspirates (bone marrow and lymph nodes)[9].

The direct parasitological examination to detect the presence of intracellular amastigote forms of *Leishmania* vertebrate hosts, conducted at the Center for Control of Zoonosis of Campinas and validated by IAL, was performed using samples prepared on slides treated with Giemsa staining and examined under an optical microscope (100x)[9].

The first canine serological census occurred in 2010. From 210 serological samples collected, 11 (5.2%) were reactive (**Table 1**). The complementary laboratory investigation of samples from six reactive asymptomatic dogs with parasitological techniques and PCR confirmed infection in five (83.3%) of the animals.

The second canine serological census in 2011 was performed in an area broadened to include adjacent residences, based on proximity to wooded areas. Of the 528 samples collected, 21 (4%) were reactive (**Table 1**). PCR and parasitological techniques confirmed infection in five of 13 (38.5%) asymptomatic reactive dogs.

The third canine serological census took place in 2012 with 606 samples collected, 19 (3.1%) of which were serologically reactive (**Table 1**). There was no complementary laboratory investigation in 2012.

Entomological research

In May, October, and November of 2010 and 2011, phlebotomine fauna was monitored by entomological research in fixed units in households at risk. Traps with luminescent bait that was exposed for 12h were set at dusk and removed at dawn the next day. Captured specimens were sent to SUCEN for species identification. The predominant species identified were *Lutzomyia (Nyssomyia) whitmani* (69.5%) and *Lutzomyia longipalpis* (22.5%) (**Table 2**).

The canine outbreak in Campinas was identified in residences located near areas of residual native forest where anthropic activities have been changing the natural landscapes.

TABLE 1 - Results of the serologic tests for canine American visceral leishmaniasis in blood samples of dogs in the study area, Campinas, State of São Paulo, from 2009-2012.

Results	2009*	2010*	2011*	2012**	Total
Positive serological samples/total samples	4/198	11/210	21/528	19/606	55/1542
Reactive samples (%)	2.0	5.2	4.0	3.1	3.6

***ELISA** and **IFA:** enzyme-linked immunosorbent assay and indirect fluorescence assay. ****DPP®CVL** and **ELISA:** Dual-Path Platform canine visceral leishmaniasis and enzyme-linked immunosorbent assay. **Source:** Center for Zoonosis Control of Campinas, SP.

TABLE 2 - Species of phlebotomine sand flies collected in traps and manually captured in a sample of households for American visceral leishmaniasis testing, Campinas, State of São Paulo, from 2009-2010.

Species	Males	Females	Number	Percentage*
Lutzomyia longipalpis	66	36	102	22.5
Nyssomyia whitmani	220	95	315	69.5
Evandromyia lenti	3	3	6	1.3
Pintomyia fischeri	1	0	1	0.2
Migonemyia migonei	8	6	14	3.1
Psathyromyia aragaoi	0	1	1	0.2
Nyssomyia neivai	2	1	3	0.7
Psathyromyia pascalei	1	0	1	0.2
Evandromyia cortelezzii	2	3	5	1.1
Martinsmyia minasensis	0	1	1	0.2
Martinsmyi aalphabetica	0	1	1	0.2
Pintomyia pessoai	1	1	2	0.4
Expapillata firmatoi	0	1	1	0.2
Total	304	149	453	100.0

*Percentage of each species respective to the total number of phlebotomine sandflies. **Source:** Laboratory of Entomology of the Endemic Control Superintendence (**SUCEN:** *Superintendência de Controle de Endemias*).

That landscape alteration and the adaptation of the vector to new ecological scenery may be seen as important determinants in the establishment of autochthonous focuses of AVL, as demonstrated in this study.

In Campinas, where autochthonous canine cases occurred, residences were situated less than 100m from a wooded area where sand flies and wild animals were identified. Previous studies show a correlation between cases of AVL and the distance between forest and residences in which the risk of infection by *L. chagasi* was higher in dogs living in areas of dense vegetation compared to dogs living at a distance of 100m or farther from forest areas[10,11]. Although autochthonous canine cases of AVL have been identified in this region only since 2009, an outbreak of human American tegumentary leishmaniasis (ATL) was documented previously in this same area between 1993 and 1994[12]. Cases of ATL were associated with the presence of riparian forest or small fragments of wooded areas. The great majority of cases suggested that the residence itself was the probable site of infection, and these residences were always situated close to or inside the forest[12, 13].

Entomological investigation conducted in 1993-1994 and 2009-2010 showed that *L. whitmani*, an implied vector in cases of ATL in the American continent[14] was the predominant species of the phlebotomine fauna in that region[12].

Given that several cases of ATL have already been recorded in this same region in which AVL transmission is now observed, it could be hypothesized that some seropositive dogs show false positive results for AVL because of a previous exposure to ATL-associated *Leishmania* species. This effect could occur due to cross reactions among different species of trypanosomatids, especially in asymptomatic animals and in those whose infection by *L. chagasi* could not be determined[9,15].

The specificity of serological testing is impaired by any cross reactions with other diseases, especially those caused by trypanosomatids[9,15]. Therefore, the results of canine serological

surveys must not be used as indicators of specific infection by *Leishmania*, given that in one of the wild animals (opossum), a slide tested positive for trypanosomatids. Moreover, one of the phlebotomine sandflies dissected during the investigation conducted in Campinas also tested positive.

Another factor that may suggest limitations in the interpretation of serological tests was the 52.6% disparity between reactive serological results and negative PCR and/or parasitological exam results. In other words, among the 19 animals that were tested, 10 were positive in the serological examination but negative in more specific tests, which could suggest that they were false-positives. Although false-positive results are expected in serological tests in areas with low disease prevalence, they constitute a potential problem for the control program, especially if dog culling is recommended by the Brazilian program for control and prevention of AVL of every dog that tests seropositive in areas of established transmission[7].

The results of this study do not support a definite affirmation that the autochthonous focus of American visceral leishmaniasis identified in Campinas has its origins in a previously existing wildlife focus. Because there is no control in Brazil over the migration and transportation of domestic animals, most of the epidemiological evidence suggests that the geographical expansion of the AVL occurs due to transport of infected dogs to areas where the vector *L. longipalpis* already occurs or in areas where the environmental destruction facilitates direct contact between humans, natural vector breeding habitats, and wild reservoirs[2,11].

In other parts of the country, endemic human AVL arose from the preceding canine enzooties[2,7]. However, 4 years after the detection of the first autochthonous cases in Campinas, there has been no identification of human cases of this disease. This finding may be related to the efforts to maintain awareness of the potential for the occurrence of human cases through intensified education of the medical and local community. The reasons for this finding may include factors like canine prevalence, virulence of implied *Leishmania* strain, vector competence, human population characteristics, and environmental conditions[11]. For this reason, additional studies should be conducted to better understand the determinants associated with the emergence and maintenance of AVL transmission in this new endemic area.

This study was approved by the Ethics in Research Committee of the FCM//UNICAMP, process 63307 (CAAE: 01196312.6.0000.5404).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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