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Antibodies against *Lutzomyia longipalpis* saliva in the fox *Cerdocyon thous* and the sylvatic cycle of *Leishmania chagasi*

Regis B. Gomes[a,b], Ivete L. Mendonça[c], Vladimir C. Silva[d], Jerônimo Ruas[e], Mauro B. Silva[d], Maria S.P. Cruz[d], Aldina Barral[a,b], Carlos H.N. Costa[d,*]

[a] *Faculdade de Medicina, Universidade Federal da Bahia, Salvador, Bahia, Brazil*
[b] *Centro de Pesquisas Gonçalo Moniz, Laboratório de Imunoparasitologia, Salvador, BA, Brazil*
[c] *Laboratório de Sanidade Animal — LASAN, Universidade Federal do Piauí, Teresina, PI, Brazil*
[d] *Laboratório de Leishmanioses, Instituto de Doenças Tropicais Natan Portella e Universidade Federal do Piauí, Teresina, PI, Brazil*
[e] *Universidade Federal de Pelotas, Faculdade de Veterinária, Pelotas, RS, Brazil*

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Summary Sera of 11 wild *Cerdocyon thous* foxes from an endemic area for American visceral leishmaniasis were tested for the presence of antibodies against salivary gland homogenates (SGH) of *Lutzomyia longipalpis*. All foxes had higher levels of anti-*Lu. longipalpis* SGH antibodies than foxes from non-endemic areas, suggesting contact between foxes and the vector of visceral leishmaniasis. Sera of humans and dogs living in the same area were also tested for reactivity against *Lu. longipalpis* SGHs and had a lower proportion of reactivity than foxes. Antibodies against *Leishmania chagasi* were not detected in any of the foxes, but three foxes showed the presence of parasites in the bone marrow by direct examination, PCR or by infecting the vector. Both humans and dogs had higher levels of anti-*Le. chagasi* IgG antibodies than *C. thous*. The finding of an antibody response against saliva of *Lu. longipalpis* among *C. thous* together with the broad distribution of the vector in resting areas of infected foxes suggests that the natural foci of transmission of *Le. chagasi* exists independently of the transmission among dogs and humans.

1. Introduction

American visceral leishmaniasis (AVL) is an important vector-borne human disease caused by the protozoa *Leishmania chagasi*. More common in northeast Brazil but expanding to the Amazon rain forest and to larger cities of the industrialised southeast, the disease kills 5—10% of over 4000

* Corresponding author. Tel.: +55 86 3221 3413; fax: +55 86 3221 2424.
E-mail address: chncosta@gmail.com (C.H.N. Costa).

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persons that become sick every year in this country. Patients almost uniformly present with fever, anaemia and hepatosplenomegaly. The main complications are opportunistic infections or severe bleeding (Jerônimo et al., 1994; Werneck et al., 2003).

In the New World, AVL is transmitted by the bite of *Lutzomyia longipalpis* after this sandfly has bitten an infected vertebrate host. Female sandflies inject saliva when probing for a blood meal (Ribeiro et al., 1986). The saliva contains a number of substances that are able to interfere with vertebrate haemostatic and inflammatory responses (Charlab et al., 1999; Titus and Ribeiro, 1990). It also triggers an immune response, which, as also happens upon exposure to saliva of other blood-feeding arthropods, leads to the production of specific antibodies. This response against salivary proteins has already been used as an epidemiological marker of exposure to vectors (Barral et al., 2000; Brummer-Korvenkontio et al., 1994; Feingold, 1968; Wikel, 1996).

Humans, dogs, wild canines and opossums are naturally infected with *Le. chagasi* (Deane and Deane, 1954a; Pearson et al., 1999; Sherlock et al., 1984). However, dogs are thought to be the most important source of infection for humans because they easily transmit *Le. chagasi* to the sandfly vector, they present a higher proportion of natural infection than humans (Evans et al., 1992) and they are more abundant and live closer to humans than wild animals (Costa, 1997). On the other hand, the early finding of infected *Pseudalopex vetulus* (= *Lycalopex vetulus*) foxes (Deane and Deane, 1954b) in northeastern Brazil and recently of the more abundant species of fox *Cerdocyon thous* also infected with *Le. chagasi* in the Amazon area (Lainson et al., 1969) suggests the existence of a sylvatic cycle that may have evolved before Europeans arrived to the New World (Lainson et al., 1987; Momem et al., 1987). However, sylvatic transmission of *Le. chagasi* has never been definitely demonstrated.

This issue has practical consequences. Since dogs are thought to be the main vertebrate reservoir, Brazil developed a huge programme of culling infected dogs (Lacerda, 1994). If transmission originates solely from dogs, reservoir control might be more easily achieved and eradication could be envisioned. However, if a wild cycle does exist, transmission from sylvatic animals to humans and dogs could dampen the effect of reservoir control measures.

Finding animals with antibodies specific for saliva of *Lu. longipalpis* would therefore indicate natural contact of the vertebrate host with the sandfly vector of AVL and suggest that both foxes and sandflies occupy the same environment. If so, together they could maintain a putative sylvatic cycle of transmission of *Le. chagasi*. Having captured many foxes in the surroundings of a city where AVL is highly endemic, we decided to verify the possibility of contact of foxes with the abundant local population of *Lu. longipalpis* and to compare the evidence of vector–fox contact with that obtained for contact of local dogs and humans with *Lu. longipalpis*.

2. Materials and methods

2.1. Study area and subjects

Data were collected from the years 2001–2003 in the environs of Teresina, Piauí, situated in middle–northern Brazil. Part of the study was undertaken at the Zoo Botanic Park, located approximately 500 m from the closest neighbourhood but still within the city limits. Most other data were collected approximately 6 km outside the city (Árvore Verde).

A total of 11 *C. thous* foxes were captured in both areas. Two were cubs and five were males. All but one adult female were healthy. The animals were weighed, a veterinarian evaluated the general health condition, and blood and bone marrow samples were collected from all of them. Ten to 20 female *Lu. longipalpis* were allowed to feed in the ear for 20–30 min. A radio-emitting collar was used to track the animals, allowing two more adults and one cub that had recently died to be found. The ethical committee of the Instituto de Doenças Tropicais Natan Portella approved the protocol for human evaluation and informed consent was obtained from each participant. The national institution for protection of the environment (IBAMA) authorised tagging, blood collection and bone marrow aspiration of the animals.

2.2. Bone marrow examination and PCR

Direct examination and culturing in NNN media of bone marrow from each fox was performed. Then, Giemsa-stained slides were scratched and DNA was extracted with phenol–chloroform. Using PCR, a 120 bp template of the constant (generic) region of kDNA was amplified using the primers 13A and 13B, as previously described (Rodgers et al., 1990).

2.3. Sera samples from foxes and controls

Sera were obtained from all 11 *C. thous* captured in Teresina. Eleven samples of *C. thous* were also studied from the non-endemic area of Pelotas, Rio Grande do Sul, at the extreme south of Brazil where *Lu. longipalpis* has never been found. Sera from 59 dogs from the study area and 8 dogs from a non-endemic area (Salvador, Brazil) previously tested as negative to sandfly saliva by ELISA were used as negative controls. The sera of 119 individuals from the study area were also analysed as well as 15 sera of persons from a non-endemic area for AVL in southern Bahia where *Lu. longipalpis* is not present.

Lutzomyia longipalpis comprises over 90% of sandflies captured in the woodlands where the foxes of Teresina area were found (Soares et al., 2004a, 2004b). The other species identified in the area were *Lu. lenti*, *Lu. goiana*, *Lu. evandroi* and *Lu. whitmani*. We evaluated the antibody response to salivary gland homogenates (SGH) of *Lu. longipalpis* and *Lu. whitmani* only, since we do not have established colonies of *Lu. lenti*, *Lu. goiana* or *Lu. evandroi*. Additionally, the serological response to *Lu. intermedia* SGH was also tested. Sera from BALB/c mice bitten by *Lu. whitmani* were used as positive controls to measure the antibody response against the SGH of this species of sandfly.

2.4. Sandflies and preparation of salivary glands

Lutzomyia longipalpis Cavunge strain, *Lu. whitmani* and *Lu. intermedia* were regularly reared. The strain of *Lu. longipalpis* comes from an isolate from an area ~150 km from Salvador and approximately 1000 km apart from Teresina. Adult sandflies were offered cotton containing a sucrose

solution and females were fed on slightly anaesthetised hamsters. Salivary glands were prepared from previously unfed, 5—7-day-old laboratory female flies. After dissection, they were placed in PBS on ice and stored at −70 °C. Immediately before use the glands were sonicated, microfuged at 12 000 × *g* for 2 min and the supernatant was collected. *Lutzomyia longipalpis* fed on foxes were dissected after 5—6 days and examined for the presence of promastigotes.

2.5. Serology for sandfly saliva-specific antibodies

Anti-saliva antibodies were evaluated by ELISA. Briefly, plates were coated overnight at 4 °C with SGH (5 μg/ml) as the antigen. After washing with PBS—Tween and blocking with PBS—Tween 0.05% plus bovine serum albumin 5% for 2 h at room temperature, sera from control (1/50 dilution), foxes (1/50), dogs (1/50) and humans (control, 1/100; sample, 1/100) were incubated overnight at 4 °C. After washing, plates were incubated with alkaline phosphatase-conjugated anti-dog IgG antibody (Sigma, St Louis, MO, USA) and with alkaline phosphatase-conjugated anti-human IgG antibody (Sigma) followed by alkaline phosphate substrate (*p*-nitrophenyl phosphate; Sigma). The plate was read at an absorbance at 405 nm. Cut-off was set as the mean of negative controls plus two SDs.

2.6. Anti-*Leishmania chagasi* serologic testing

IgG responses to *Le. chagasi* antigen were measured by ELISA. Briefly, ELISA plates were coated for 1 h at 37 °C with antigen of *Le. chagasi* (10 μg/ml). After washing with PBS—Tween and blocking with PBS—Tween 0.1% overnight, sera from control and foxes (1/50 dilution) were incubated for 1 h at 37 °C. After washing, plates were incubated with alkaline phosphatase-conjugated anti-dog IgG antibody (Sigma) followed by alkaline phosphate substrate (*p*-nitrophenyl phosphate). The plate was recorded at an absorbance at 405 nm and the cut-off was set as above.

2.7. Western blot

Western blots of salivary gland antigens were performed as described elsewhere (Belkaid et al., 1998). Western blot was performed by electrophoresis of SGH (80 pairs of salivary glands) on 16% Tris-glycine gel containing a single long well (Invitrogen, Carlsbad, CA, USA). After transfer to nitrocellulose membranes, these were blocked with 5% non-fat milk in PBS 0.05% Tween overnight at 4 °C. Membranes were placed on a Mini-PROTEAN® II multiscreen apparatus (Bio-Rad Laboratories, Hercules, CA, USA), incubated with serum (1:10 dilution) in blocking buffer for 1 h at room temperature, followed by anti-dog IgG alkaline phosphatase-conjugated antibody diluted 1:1000 (Sigma) for 1 h. Bands were visualised using alkaline phosphatase substrate (Promega, Madison, WI, USA). The reaction was stopped by washing the membrane with deionised water.

2.8. Statistical analyses

Graphs were built and calculation of the cut-off and statistical tests were done with GraphPad Prism software (San Diego, CA, USA). The χ^2 and Fisher's exact tests were used to compare the proportions of serological results among foxes, dogs and humans.

3. Results

When bitten by *Lu. whitmani*, mice developed an antibody response to *Lu. whitmani* SGH but not to *Lu. longipalpis* saliva (data not shown). Sera from all 11 *C. thous* foxes from Teresina reacted to SGH from *Lu. longipalpis*, but sera from foxes from the non-endemic area of Pelotas did not ($P < 0.001$) (Figure 1A). In contrast, both groups of foxes had low levels of antibodies to *Lu. whitmani* SGH (Figure 1B). However, among foxes from Teresina, a slightly higher reactivity was seen against SGH from *Lu. intermedia* compared with that of the foxes from Pelotas (Figure 1C).

We found significant differences in the numbers of foxes, dogs and humans that reacted with salivary antigens of *Lu.*

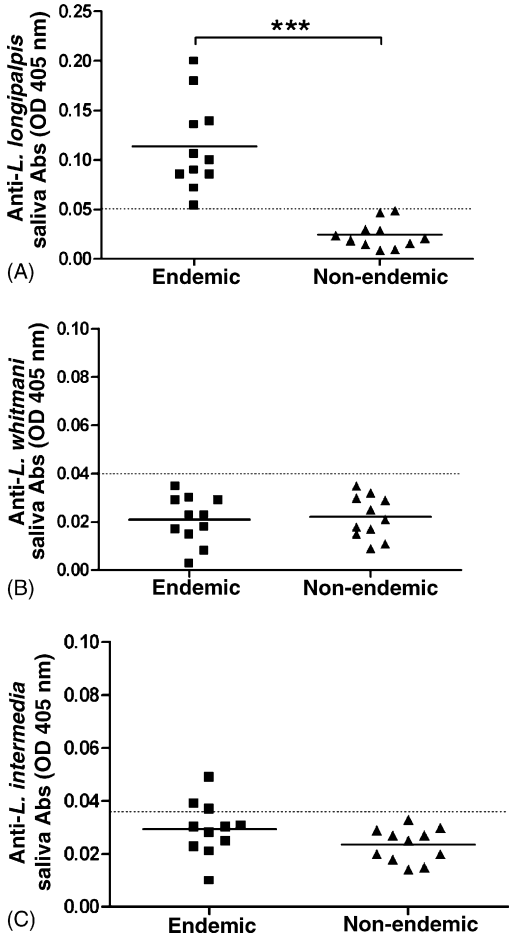

Figure 1 *Cerdocyon thous* antibodies reactive against sandfly saliva. Levels of serum IgG antibodies were determined by ELISA with homogenates of salivary glands from (A) *Lutzomyia longipalpis* (cut-off = 0.051), (B) *Lu. whitmani* (cut-off = 0.040) and (C) *Lu. intermedia* (cut-off = 0.036). The cut-off (mean ± 2 SD) of negative controls (*C. thous* from Pelotas) is indicated by the dotted line. ***Significant difference ($P < 0.001$) between values for endemic and non-endemic groups. Abs: antibodies; OD: optical density.

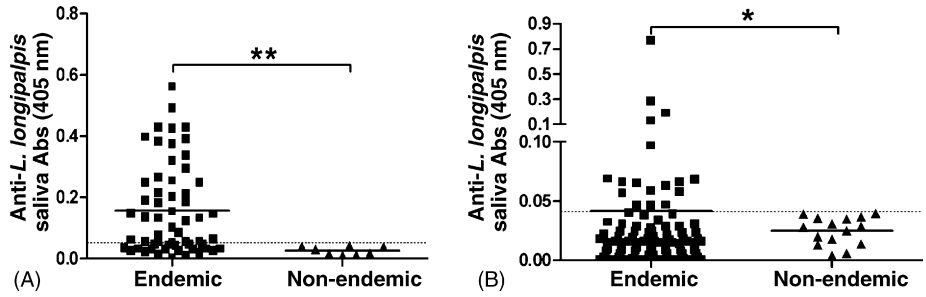

Figure 2 Antibodies reactive against saliva of *Lutzomyia longipalpis* from (A) dogs and (B) humans. Levels of serum IgG antibodies against *Lu. longipalpis* were determined by ELISA in the serum of (A) dogs (cut-off = 0.052) and (B) humans (cut-off = 0.041) living in the surroundings of Teresina. The cut-off (mean ± 2 SD) of negative controls (dogs from Salvador and humans from southern Bahia) is indicated by the dotted line. Significant difference ($^{*}P = 0.0366$; $^{**}P = 0.0029$) between values for endemic and non-endemic groups. Abs: antibodies; OD: optical density.

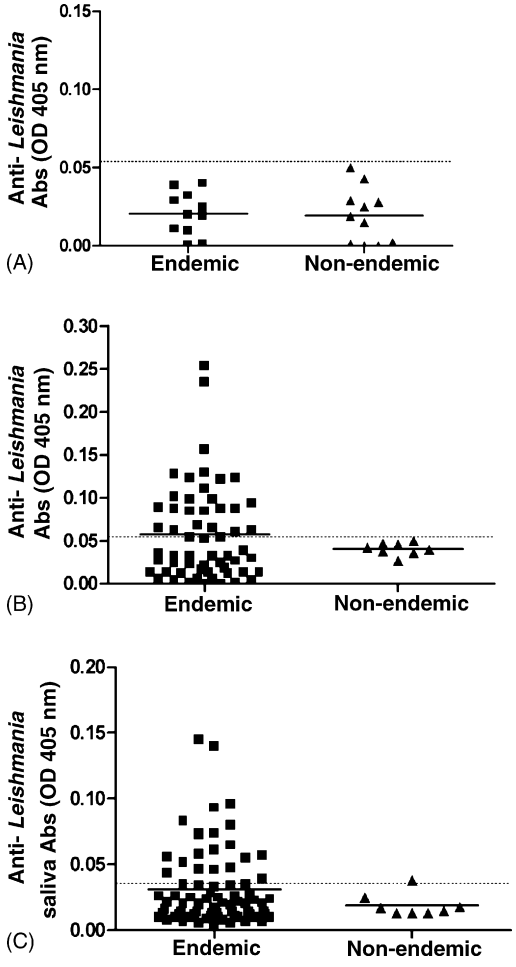

Figure 3 Antibodies reactive against *Leishmania chagasi* antigens. Levels of serum IgG antibodies against *Le. chagasi* were determined by ELISA in the serum of (A) *Cerdocyon thous* (cut-off = 0.054), (B) dogs (cut-off = 0.055) and (C) humans (cut-off = 0.036). The cut-off (mean ± 2 SD) of negative controls is indicated by the dotted line. Abs: antibodies; OD: optical density.

longipalpis: all 11 of the Teresina foxes reacted to saliva, in contrast to 38 of 59 dogs (64%; Figure 2A; χ^2 test, $P < 0.05$) and 18 of 119 individuals (15%; Figure 2B; χ^2 test, $P < 0.001$) living in the area.

To determine whether these groups had also been exposed to the parasite, we evaluated the anti-*Le. chagasi* antibody levels in serum samples from all foxes, dogs and humans. Anti-*Le. chagasi* antibodies were not detected in any fox either from endemic or from the non-endemic areas (Figure 3A). In contrast to the uniform negativity of sera from foxes, many sera from dogs and humans from the same area where foxes were radio-tracked reacted to *Le. chagasi*; 25 of 59 dog sera (42%) tested against *Le. chagasi* antigen (Figure 3B) and 22 of 119 human sera (18%) (Figure 3C) were positive.

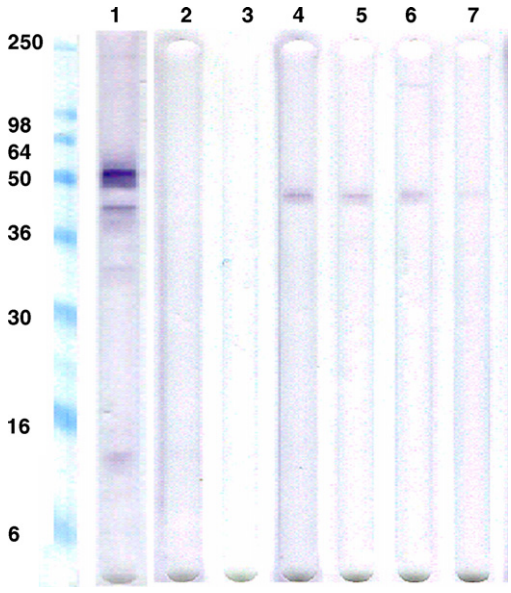

Figure 4 Western blot of *Lutzomyia longipalpis* salivary proteins reacting with IgG antibodies from foxes and dogs. As a negative control, a pool of serum samples from foxes and a pool of serum samples from dogs living in a non-endemic area were used. Lane 1: dogs from endemic area (pool); lane 2: dogs from non-endemic area (pool), lane 3: foxes from non-endemic area (pool); and lanes 4—7: foxes from endemic area. Molecular weight markers in kDa.

One fox was sick. It was stunted and had an ulcer on its snout. Despite being seronegative, amastigotes were identified in a smear made from its bone marrow and the kDNA of *Leishmania* sp. was also detected in this material. However, none of the 15 laboratory-reared *Lu. longipalpis* fed on it showed promastigotes after dissection. Two other healthy *C. thous* also were PCR-positive (data not shown) but were bone marrow smear-negative. However, 1/12 sandflies fed on one of the foxes had promastigotes identified in the midgut. Bone marrow cultures of all three foxes were negative.

Using Western blot we evaluated the number and pattern of *Lu. longipalpis* salivary proteins recognised by the serum samples of foxes and dogs. Among four selected serum samples from foxes that presented a positive anti-*Lu. longipalpis* response by ELISA, three foxes recognised one salivary protein of 44 kDa. A pool of dog sera recognised five proteins and strongly reacted to 44 kDa and 45 kDa salivary proteins, and others of 32 kDa and 15 kDa (Figure 4).

4. Discussion

In this report we have detected anti-*Lu. longipalpis* saliva antibodies among *C. thous*, dogs and humans from an endemic area of AVL. These antibodies recognise the 44 kDa salivary protein, which is one of the major antigens recognised by humans (Barral et al., 2000; Gomes et al., 2002) and dogs. They were specific for *Lu. longipalpis*, since no *C. thous* showed cross-reactivity to the salivary proteins of the local *Lu. whitmani*. Although some reactivity was noticed against antigens of *Lu. intermedia*, the specificity to *Lu. longipalpis* cannot be disregarded by this finding since *Lu. intermedia* is not present in Teresina. Cross-reactivity with other local species that were not evaluated, such as *Lu. lenti*, *Lu. goiana* and *Lu. evandroi*, seems unlikely because they comprise only 5% or less of the captures, which is vastly dominated by *Lu. longipalpis* (Soares et al., 2004a, 2004b). Therefore these findings offer support to the idea that *Lu. longipalpis* feeds on *C. thous* in the wilderness. This conclusion is supported by another study which showed that frequent exposure of humans to *Culex quinquefasciatus* led to a progressive increase of antibody concentration against the mosquito saliva (Peng and Simons, 2004). If the same kind of response occurs to sandfly saliva among *C. thous*, one may conclude that the high proportion of reactivity found in our study indicates regular and frequent exposure of *C. thous* to *Lu. longipalpis* in the woodland of the surroundings of Teresina.

Antibody reactivity to sandfly saliva was found among endemic foxes but not among those from Pelotas where *Lu. longipalpis* is unknown. Also, the proportion of reactivity was significantly higher among *C. thous* than among the local dogs and humans. This finding suggests that the contact of *Lu. longipalpis* with vertebrate hosts may be higher in the wilderness than in domestic settings. In fact, in the sylvatic areas where *C. thous* are found are plenty of *Lu. longipalpis*, as demonstrated by previous captures using foxes and dogs as bait (Lainson et al., 1990; Soares et al., 2004a, 2004b).

Only three *C. thous* were found to be infected by *Leishmania* spp., either by kDNA amplification from bone marrow smear (all three foxes), direct identification of amastigotes in the bone marrow of one fox, or by the finding of promastigotes in one sandfly fed on another PCR-positive fox. Unfortunately, the tests are not specific for *Le. chagasi*. However, three facts argue in favour of infection with *Le. chagasi*: the poor general clinical state of the bone marrow smear-positive fox, the rarity of cutaneous leishmaniasis in Teresina, and the finding of promastigotes in the midgut rather than in the hindgut of the infected *Lu. longipalpis*. This finding of natural infection of *Le. chagasi* among *C. thous*, but apparently at a lower proportion than that seen among dogs, suggests that despite a higher exposure to the vector, the force of transmission of *Le. chagasi* in the wild depends on other factors. For instance, if a vector with catholic feeding habits such as *Lu. longipalpis* is searching for food in a habitat where there are large numbers of naturally resistant hosts, then the chances of successful transmission are greatly reduced.

Contradicting earlier findings, Courtenay et al. (2002) failed to transmit *Le. chagasi* from 27 infected *C. thous* to *Lu. longipalpis*. They concluded that foxes are a dead end on the transmission cycle of *Le. chagasi* and hence that they acquire infection not from sandflies that have bitten other foxes but from those fed on infected dogs. This was a surprising result, since Deane and Deane (1954b) described one *P. vetulus* fox naturally infected with *Le. chagasi* that was infective to 10 of 10 *Lu. longipalpis*. Also, Lainson et al. (1990) were able to infect 4 of 54 *Lu. longipalpis* fed on one *C. thous* that had been experimentally infected by sandfly bites; here we add another infectious fox. Moreover, humans and hamsters, phylogenetically more distant from dogs than foxes, are infective to sandflies (Costa et al., 2000; Deane and Deane, 1955, 1962; Montoya-Lerma et al., 2003). Finally, there is no description of a specific biological factor that prevents a heavily infected vertebrate species transmitting a parasite to its natural vector. Therefore, it must be concluded that this new finding of the lack of infectivity of *C. thous* should wait for confirmation or clarification and, in the meanwhile, the ability to transmit *Le. chagasi* should still be assumed to be a typical feature of infected foxes. Therefore, it may be concluded from the present findings that a sylvatic cycle of *Le. chagasi* exists among *C. thous* in the environs of the highly endemic city of Teresina. The explanation for its lower transmission rate may be due to the lower density and proportion of susceptible hosts in the wilderness.

The search for antibodies against the saliva of blood-feeding insects for the purpose of identification of parasite reservoirs is a novel approach. These antibodies were shown to be specific not only for New World sandflies but also for vectors involved in the transmission of leishmaniasis in Eurasia (Volf and Rohousova, 2001). This would be particularly useful in situations where the insect vector is well known and when more than one vertebrate is susceptible to infection. However, more studies are necessary to elucidate the dynamics of the anti-saliva antibody response, such as the length and intensity as a function of exposure to insect bites. With this knowledge, the prevalence of antibody responses in different animals would not only help to incriminate suspected hosts but also to identify resistant vertebrate host species, which would protect susceptible species (Rodgers, 1991). It could also detect areas where vector–host contact is more intense and transmission poses a higher risk. Such

information would help the understanding of parasite ecology and the design of better strategies for the control of vector-borne diseases.

5. Conclusions

Cerdocyon thous foxes developed a specific response to the saliva of *Lu. longipalpis* in the environs of the city of Teresina where AVL is endemic. This finding indicates the presence of a sylvatic cycle of AVL that is independent of transmission among dogs and humans in Brazil and that this cycle deserves attention when developing control policies for this disease. Antibody response to vector proteins is a promising method for identification of reservoirs.

Conflicts of interest statement
The authors have no conflicts of interest concerning the work reported in this paper.

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