Am. J. Trop. Med. Hyg., 96(1), 2017, pp. 178–181
doi:10.4269/ajtmh.16-0191

Vector Competence of *Lutzomyia cruzi* Naturally Demonstrated for *Leishmania infantum* and Suspected for *Leishmania amazonensis*

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Abstract. Corumbá city is one of the oldest visceral leishmaniasis–endemic foci in the state of Mato Grosso do Sul, Brazil, where the transmission of *Leishmania infantum* has been attributed to *Lutzomyia cruzi*. Aiming at investigating the parameters of the vectorial capacity of *Lu. cruzi* for *L. infantum*, a project was undertaken in this city. Among these parameters, vector competence was investigated and the results obtained are reported herein. Of the 12 hamsters exposed to feed wild-caught female sandflies, two developed infection with *L. infantum* and surprisingly, one with *Leishmania amazonensis*. In addition, hamsters with *L. infantum* infection were bitten only by females of *Lu. cruzi*, whereas the hamster infected with *L. amazonensis* was bitten by 124 *Lu. cruzi* females and one of *Evandromyia corumbaensis*. Although there is a strong suspicion regarding the competence of *Lu. cruzi* in transmitting *L. amazonensis* naturally, it was not demonstrated.

Corumbá city, in the state of Mato Grosso do Sul, Brazil, is the oldest known visceral leishmaniasis–endemic focus, where the transmission of the agent *Leishmania infantum* Nicolle, 1908 (syn. of *Leishmania chagasi* Cunha and Chagas, 1937) has been attributed to *Lutzomyia cruzi* (Mangabeira, 1938).[1,2] The high population density of this sandfly associated with human and canine cases of visceral leishmaniasis, and the presence of its females naturally infected with *L. infantum* in other midwestern Brazilian areas, suggest its role as a possible vector for *L. infantum*.[3,4] However, its vector competence, a parameter that should be evaluated before its incrimination as a vector for this parasite, has not yet been demonstrated.

Vector competence of a sandfly can be described as the ability of its population to become infected with a *Leishmania* species, and transmit it to a naive host. Its demonstration is a proof that a particular sandfly species is a vector of a specific *Leishmania* species. This parameter is one of the criteria to be evaluated in studies of vectorial capacity, in which the interaction between vectors, host reservoirs, and parasites involved in the ecoepidemiology of leishmaniasis is investigated.[5,6] Therefore, we sought to investigate the vector competence of *Lu. cruzi* for *Leishmania* by analyzing the natural transmission of the parasites through bites of wild-caught sandflies.

The wild-caught sandflies used in the present investigation were collected from Corumbá city (19°00′33″S; 57°39′12″W; 118 m a.s.l.) between March 2013 and December 2014, from the peridomiciles of two residences (private lands) located in Maria Leite (19°00′45″S; 57°37′31″W) and Nova Corumbá (19°02′47″S; 57°39′21″W) neighborhoods.

The specimens, aspirated using a Castro aspirator and/or an electric aspirator from a chicken coop (residence located in Maria Leite) and a black modified Shannon trap[7] (residence located in Nova Corumbá), were transferred to nylon cages, each with a metal frame (30 × 30 × 30 cm) covered with a dark cloth. In each cage, a naive hamster, anesthetized with xylazine and ketamine in adequate doses based on the weight of the animal, was offered as blood source for 1 hour. Four of the 12 hamsters (male and female), 30 to 50 days of age, were exposed twice to feed the wild-caught female sandflies, whereas the others were exposed once.

The black modified Shannon traps, described by Galati and others,[7] were made up of cotton fabric. They consisted of a rectangular roof (1.40 × 1.60 m) with flaps (0.40 m tall) on each side. In the middle of the roof, a fabric sheet (1.50 m high × 1.60 m wide) was attached. The free end of the sheet was almost 0.30 m above the ground, and the roof of the trap was suspended by ropes around 1.80 m above the ground. Apart from the light of the lantern held by a person, the presence of the person during the captures also helped to attract the sandflies.

The hamsters exposed to insect bites were kept safe on a ventilated rack, equipped with mini-isolators, in cages floored with sterilized sawdust, and plentiful food and water. The cages were cleaned weekly and the general physical state (weight loss, skin integrity, and changes in fur) of the hamsters was examined. Six months was the maximum follow-up period for the evaluation of possible *Leishmania* infection. When clinical signs suggestive of infection were found, the affected hamster was euthanized using carbon dioxide, and necropsy was performed to remove the spleen. Spleen tissue samples were used for preparing imprint slides using the Giemsa staining method (direct diagnosis), seeding in a culture medium, and were stored in microtubes at –20°C for further identification of *Leishmania* DNA using polymerase chain reaction (PCR).

For the isolation of the parasite, the tissue samples obtained during necropsy were seeded in artificial Neal–Novy–Nicolle medium with the liquid phase of Schneider's insect medium (Sigma-Aldrich, St. Louis, MO), supplemented with 20% fetal bovine serum (Cultilab, Sao Paulo, Brazil) and 140 μg/mL of gentamicin (Sigma-Aldrich), and incubated at 25°C. After the 7th day, the cultures were examined weekly for four consecutive weeks, investigating the presence of promastigotes.

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PCR was performed for the molecular analysis of the tissues and cultures (when positive). The parasite was identified using restriction fragment length polymorphism analysis (PCR-RFLP). DNA was extracted from the samples with the Wizard DNA Purification Kit (Promega, Madison, WI), following the manufacturer's instructions. PCR was performed by targeting a 300–base pair region of the internal transcribed spacer 1 of the *Leishmania* ribosomal gene, as described by El Tai and others[8] and Oliveira and others.[9]

Reactions containing water without DNA and DNA from nonfed F_1 females were used as negative controls. DNA samples of *L. infantum* (strain MHOM/BR/1972/BH46) and *Leishmania amazonensis* (strain IFLA/BR/1967/PH8) extracted from their cultures were used as positive controls.

The PCR products were analyzed using 1.5% agarose gel electrophoresis in 100 mL Tris-borate-ethylenediaminetetraacetic acid (TBE) buffer, and stained with GelRed™ (Biotium, Hayward, CA). The electrophoretic run was performed at 100 V for 100 minutes in concentrated TBE buffer. The bands were viewed under ultraviolet light with a 300-nm filter.

The PCR products from the positive samples were subjected to *Hae*III restriction enzyme digestion to identify the species of *Leishmania*, according to Shönian and others.[10]

Sequencing of PCR products amplified and identified as *L. amazonensis* by PCR-RFLP was carried out in both directions using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The sequencing reaction consisted of the BigDye premix, 0.25 pmol of either forward or reverse primer, and the purified PCR product in a final volume of 10 µL. The primers used for PCR were also used for sequencing. Labeling reactions were performed using an LGC XP Cycler thermocycler (Bioer Technology Co., Ltd., Hangzhou, Zhejiang, China) with an initial denaturing step at 96°C for 3 minutes, followed by 25 cycles of 96°C for 10 seconds, 55°C for 5 seconds, and 60°C for 4 minutes. Nucleic acid sequence analyses were performed on an automated ABI PRISM 3500 Genetic Analyzer (Applied Biosystems). The sequencing was performed only for samples positive for DNA of *L. amazonensis*, as this was the first description of transmission of this parasite by *Lu. cruzi*.

This study received the approval of the Animal Experimentation Ethics Committee of the Federal University of Mato Grosso do Sul (Brazil), under process number 491/2013. The research group has a permanent license for the collection of zoological material, issued by the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA: SISBio 25952-1). The field studies were carried out on two private properties, and both owners gave permission to conduct the study in their respective peridomiciliary areas. In addition, the field studies did not involve any endangered or protected species.

A total of 12 hamsters were used as blood meal sources for 1,954 wild-caught female sandflies, whose mean feeding rate was 40.10%. As these females were destined for the maintenance of the sandfly colony, they were clarified after oviposition and death for identification of their species. Thus, the specimens were not dissected in the search of flagellates nor submitted to molecular analysis for *Leishmania* DNA research.

Of the 12 hamsters exposed, two were infected with *L. infantum* and one with *L. amazonensis* (Table 1).

TABLE 1

Summary of information on the natural transmission of *Leishmania infantum* and *Leishmania amazonensis* to hamsters through sandfly bites

Hamster	Sandfly species no. of females exposed (fed)	Collection sites of sandflies*	Collection method of sandflies	Collection date of sandflies	Time between exposure to bites and necropsy	Diagnostic tests			*Leishmania* species	General state of the hamster
						Direct	Culture	Molecular		
1	*Lutzomyia cruzi* 5 (5)	Nova Corumbá (19°02′47″S; 57°39′21″W)	Aspiration from a black Shannon trap	September 8, 2014	4 months	Pos	Pos	Pos	*L. infantum*	Cachexia and enlarged spleen (4.5 cm)
2	*Lu. cruzi* 87 (53)	Maria Leite (19°00′45″S; 57°37′31″W)	Aspiration from a chicken coop	September 30, 2013	6 months	Neg	Neg	Pos	*L. infantum*	Accentuated weight loss, ascites, and enlarged spleen (8.5 cm)
3†	*Lu. cruzi* 299 (124) *Lutzomyia forattinii* 1 (0) *Evandromyia corumbaensis* 3 (1)	Maria Leite (19°00′45″S; 57°37′31″W)	Aspiration from a chicken coop	First exposure: November 11, 2013 Second exposure: March 29, 2014	6 months	Neg	Neg	Pos	*L. amazonensis*	No clinical signs

Neg = negative; pos = positive.
*The collections were undertaken in the peridomiciliary areas.
†This animal was exposed twice to wild-caught sandfly females: specimens of *Lu. forattinii* and *Ev. corumbaensis* were exposed to the hamster in the first exposure along with 63 other specimens of *Lu. cruzi*.

The natural transmission of *L. infantum* was found in two hamsters that were used only once to feed *Lu. cruzi* females. Both animals exhibited clinical signs suggestive of infection with *L. infantum*, such as accentuated weight loss, ascites, and an enlarged spleen.

One of these hamsters presented positive results in its diagnostic tests (direct, culture, and molecular diagnosis). This animal was euthanized 4 months after its exposure to the wild-caught sandflies. Only five of the wild *Lu. cruzi* females, caught on the black Shannon trap, bit this animal, and all of them were engorged.

The infection with *L. infantum* in the other hamster could be diagnosed only by molecular test. This animal was euthanized 6 months after exposure to 87 *Lu. cruzi* caught in the chicken coop, 53 of them becoming engorged.

The third hamster was naturally infected with *L. amazonensis* (confirmed by PCR-RFLP and by sequencing). The infection could be diagnosed only by molecular testing. No clinical signs suggestive of infection by *Leishmania* were observed, and the hamster was euthanized 6 months after its second exposure to wild-caught sandflies. This animal was exposed twice to 303 wild-caught sandfly females captured in the chicken coop. Although *Lu. cruzi* predominated (98.7%), two other species participated in one of the two blood meals: *Lutzomyia forattinii* (Galati, Rego, Nunes and Teruya, 1985) (one female) and *Evandromyia corumbaensis* (Galati, Nunes, Oshiro and Rego, 1989) (three females).

Of the 125 engorged females, 124 (99.2%) were *Lu. cruzi* and only one (0.8%) was *Ev. corumbaensis*.

The present study demonstrated the vector competence of *Lu. cruzi* for *L. infantum* by showing natural transmission from naturally infected wild-caught females. Although the natural transmission of *L. amazonensis* by wild-caught female sandflies was observed, the presence of four females of the other two sandfly species in one of the groups of insects that bit the hamster that developed the infection, does not allow us to attribute the transmission to *Lu. cruzi*, despite the strong evidence due to its high frequency (98.68%), as compared with that of the other species which participated in the blood meal, and the previous report of the finding of wild *Lu. cruzi* infected by *L. amazonensis*.[9]

Lutzomyia cruzi is considered to be a sibling species within the *Lutzomyia longipalpis* (Lutz and Neiva, 1912) complex, and shares morphological and epidemiological characteristics with the other members of the complex. Its females are indistinguishable from those of some of the other species, but the respective males are easily distinguishable from one another.[11,12] In Corumbá city, although Santos and others[13] reported the collection of three *Lu. longipalpis* males, other studies have only found *Lu. cruzi*.[1,2,14,15]

Since the first demonstration of the experimental transmission of *L. infantum* through bites of *Lu. longipalpis* reared in the laboratory,[16] studies have been conducted with Neotropical species of *Leishmania* in combination with different sandfly species.[17–19] In recent years, however, few studies have been published demonstrating the experimental or natural transmission of *Leishmania*[18,20] or the vectorial capacity of sandflies suspected of participating in the transmission of the parasite.[21] Due to lack of similar studies involving *Lu. cruzi*, little is known regarding the *Leishmania*/sandfly interaction of this dipteran.

Some authors have suggested that *Lu. longipalpis* may naturally transmit *L. amazonensis* in regions endemic to cutaneous leishmaniasis, the etiology of which includes this parasite.[19,22,23] Considering that *Lu. longipalpis* and *Lu. cruzi* are phylogenetically closely related, it is possible that the latter exhibits the same behavior, as suggested by the evidence reported herein. Epidemiologically, it should also be considered that the species of genus *Evandromyia* are not known to be attracted to humans, and the specimens exposed to the hamster were captured from a chicken coop.

The hamster infected with *L. amazonensis* exhibited no clinical signs of infection, such as nodular lesions. Moreover, the DNA of the parasite was identified on the basis of spleen tissues, suggesting possible visceralization. Cases of visceral leishmaniasis in Brazil attributed to *L. amazonensis* have been reported in dogs[24] and humans.[25] The viscerotropism of *L. amazonensis* found in the city of Jacobina, Bahia State, Brazil, led Sherlock[19] and Warburg and others[26] to suggest the hypothesis that elements of the saliva of *Lu. longipalpis* could modify the behavior of this parasite, altering its tropism so that it causes visceral rather than cutaneous leishmaniasis.

The sympatry of *L. infantum* and *L. amazonensis* in Corumbá city and the possibility of their transmission by *Lu. cruzi*, a highly predominant species, underscores the need for study of the etiology of visceral leishmaniasis and possible coinfection. It should be noted that the visceral leishmaniasis lethality coefficient recorded in Corumbá in 2014 was 75%.[27]

Received March 9, 2016. Accepted for publication July 4, 2016.

Published online November 14, 2016.

Acknowledgments: We are grateful to the Centro de Controle de Zoonoses do Município de Corumbá for their technical assistance and help during the capture of sandflies and to the Centro de Controle de Zoonoses do Município de Campo Grande, which granted authorization for the use of the dogs in the experimental infection assays. We are also grateful to Manoel Sebastião da Costa Lima and Rosianne Tsujisaki for their help in the molecular analysis.

Financial support: This study was supported by grants from the São Paulo Research Foundation (FAPESP 2011/23414-0) and the Foundation for Development Support of Education, Science and Technology of the State of Mato Grosso do Sul (FUNDECT/DECIT-MS/CNPq/SES N° 04/2013—PPSUS-MS—23/200.537/2013).

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