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SEARCH FOR TRYPANOSOMA CRUZI IN URBAN BATS AND DOGS FROM BUENOS AIRES

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Chagas disease, the American trypanosomiasis, is caused by *Trypanosoma cruzi* infection. There are about 6 million of infected people in America, where de infection is endemic, but the dynamic of its epidemiology is changing due to migrations flows, especially to United States of America, Europe and Oceania (1). Among different infection routs, vector-borne is still the main one for *T. cruzi* transmission. In this eco-epidemiological scenario, domestic animals, rodents, and sylvatic mammals participate as reservoirs in parasite cycle maintenance. Moreover, cats and dogs infection is associated with human risk of infection in their shared dwellings (2,3). Currently, *T. cruzi* is classified in six discrete typing units widely distributed (DTUs, named TcI to TcVI) and Tcbat as a seventh group (4,5). Dogs' infection can be caused by the six DTUs and it prevalence can reach 50% in endemic regions (3,6). As domestic animals, their movements together with humans plays a role in dispersal infections from endemic to no-endemic regions. Due to their variable parasitemia that lead them to a heterogeneous infectiousness contribution, they can establish new infection foci because of vertical transmission (3,7). Bats (Chiroptera) are other *T. cruzi* reservoir that display special attention. They can be infected by different parasites DTUs, as TcI-IV, but also Tcbat, an almost bat specific group, unusually observed in human infections (5,8). Their gregarious behavior and their high densities communities nearby humans points this group suitable for pathogen emergence or re-emergence, as described for the endemicity of *T. cruzi* in Oklahoma, USA (9). Moreover, bats capability of move through long distances and, more important, the presence of vertical transmission of the infection in this group, make this flying mammals being able to transport and establish new foci of infection far from endemic regions (10,11). Among them, the free-tailed bat *Tadarida brasiliensis* (Molossidae, Chiroptera) was described as a *T. cruzi* reservoir and it is distributed across Americas from USA to Chile and Argentina (8,9). It show migratory behaviors of thousands of kilometers and is one of the main species living in Buenos Aires city, where is close to citizens, establishing colonies into human dwelling. Taking together, we decided to study the presence of *T. cruzi* infection in this bat specie.

Although Buenos Aires city belongs to a non-endemic region, the presence of the vector *Triatoma infestans* points the surveillance importance of feasible infected mammals (12). Herein, by means of a new set up multiplex PCR design and the trans-sialidase inhibition assay (TIA), we analyzed a group of dogs and bats to evaluate them as putative parasite reservoirs captured in Buenos Aires city.

MATERIALS AND METHODS

SAMPLES

Seventy-five bats and 45 dogs were admitted at Luis Pasteur Institute of Zoonosis (IZLP) of Buenos Aires for diagnosis of different zoonosis. All animals proceed from Buenos Aires city, carried to the IZLP by citizens that found them in public spaces. At IZLP, brain and blood were obtained for standardized screening of rabies and hemoparasites and bacteria (*Anaplasmataceae* family) from bats and dogs, respectively.

For molecular diagnosis of *T. cruzi* infection in bats, 50mg of liver, spleen, and lung mix of tissues of each specimen was processed for DNA isolation with High Pure PCR Template Preparation Kit (Roche®, Germany) following the manufacturer's instructions. For dogs' molecular diagnosis, DNA was extracted from blood by the same commercial kit. Purified

DNAs were store at -20ºC until use for PCR-based amplifications. Moreover, for TIA (*trans*-sialidase inhibition assay) diagnosis of *T. cruzi*, sera from dogs were also collected and sotred al -70C until use.

Identification and classification of bat species were carried out on their morphologic characteristics. All bat samples used in the present study showed previous negative findings for rabies diagnosis, carried out by PCR from brain extracted DNA (not shown).

SAT-BA PCR

A multiplex PCR, Sat-bA PCR, was designed to amplify the 188bp *T. cruzi* satellite DNA specific region (oligonucleotides TCZ1 CGAGCTCTTGCCCACACGGGTGCT and TCZ2 CCTCCAAGCAGCGGATAGTT CAGG and the mammal 289bp fragment of exon III of beta-actin gene (oligonucleotides β-actin-F ACCCACACTGTGCCCATCTA and β-actin-R CGGAACCGCTCATTGCC). PCR multiplex mix was carried out on 25 *µ*l final volume containing reaction buffer 1X (Promega), 3 mM of $MgCl_2$, 250 µM each dNTP, 0.5 µM TCZ1 and TCZ2, 0.25 µM β-actin-F and β-actin-R, and 0.75 units of GoTaqpol (Promega). Cycling program consisted on 5 min 94°C, 40 cicles of 45 sec 94°C - 30 sec 58°C - 45 sec 72°C, ending with 10 min 72°C.PCR products were observed by 2% agarose gel electrophoresis and UV visualization. Sensitivity of Sat-bA PCR was determined by spiking DNA isolated from a *T. cruzi* negative dog with different amounts of *T. cruzi* DNA from CL Brener strain.

kDNA PCR

Amplification of the 330bp variable region of the *T. cruzi* kinetoplastid minicircle DNA was performed in a touch down PCR. Briefly, reaction was carried out in 25 µL with 5 µL of reaction buffer 5x (GoTaq Promega); 3 mM final concentration $MgCl_2$; 250 µM each dNTP; 2.5 µM oligonucleatides 121 (5'AAATAATGTACGGG(T/G)GAGATG CATGA-3') and 122 (5'-GGTTCGATTGG GGTTGGTGTAATATA-3'); 0,6U of GoTaq pol 5U/µl in a cycling program of 94°C 3m; 3 cycles 94°C 40s, 64°C 30s; 72°C 30s; 35 cycles 94°C 40s, 61°C 40s, 72°C 40s; and one final extension step at 72°C for 10 min. PCR products were analyzed by agarose gel electrophoresis under UV visualization.

TIA

TIA (*trans*-sialidase inhibition assay) for diagnosis of *T. cruzi* infection was carried out as described Leguizamón (13). Briefly, for detection of serum TS (*trans*-sialidase) neutralizing antibodies, recombinant TS was incubated with serum samples and the remnant TS activity was assayed by measuring the transfer of sialic acid from sialyl-lactose (1 mM) to [D-glucose-1-14C]lactose (12 M, 54.3 mCi/mmol; GE Healthcare), using 20 mM Tris buffer (pH 7.6). Counts per minute (cpm) were quantified in a β-scintillation counter. The inhibition value was calculated as follows: % inhibition = (1 - [cpm sample/ cpm negative control]) X100. As positive controls, sera from infected dogs, positive by ELISA and IHA, were used.

RESULTS

SAT-BA MULTIPLEX PCR

To achieve a simplified analysis, a multiplex PCR was designed for simultaneous amplification of the 188bp *T. cruzi* satellite DNA specific sequence and the 289bp fragment of exon III of mammal beta-actin gene. This reaction allowed detection of specific *T. cruzi* DNA while DNA integrity and quality during isolation is checked by amplification of the beta-actin gene. In a spiked assay, multiplex Sat-bA PCR allowed detection of 1fgr of *T. cruzi* DNA in the context of mammal DNA (Figure).

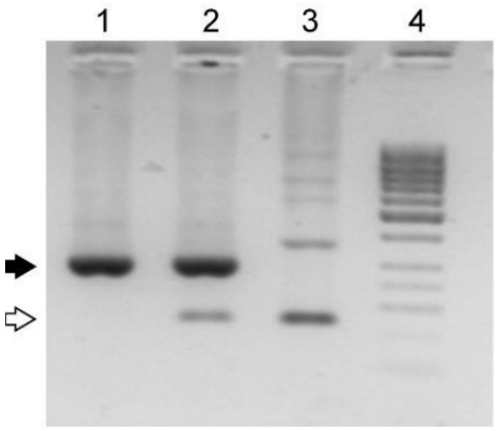

Figure.

Agarose gel electrophoresis (2%, Bret stained, UV visualized) of Sat-bA multiplex PCR. Black arrow: 289bp amplicon from exon III of mammal beta-actin gene; White arrow: 188bp amplicon from *T. cruzi* satellite DNA; Different Sat-bA multiplex reactions are shown: Lane 1: DNA of uninfected bat; Lane 2: DNA of uninfected bat spiked with 1 fgr of *T. cruzi* DNA; Lane 3: 1 fgr of *T. cruzi* DNA; Lane 4: 50pb Marker.

DOGS AND BAT DIAGNOSIS

Forty-five dogs taken from streets of Buenos Aires and carried to the IZLP by citizens were tested for *T. cruzi* infection by means of Sat-bA PCR and TIA analysis carried out from blood purify DNA and serum samples, respectively. All of them showed negative findings by means of both analysis strategies.

Seventy-five bats, identified as *Tadarida brasiliensis* based on their morphological characteristics, were captured from citizens´ dwellings and carried by them to IZLP of Buenos Aires city. After confirm their negative diagnosis for rabies infection, they were incorporated to the current *T. cruzi* molecular diagnosis study. DNA extractions were carried out from a liver, spleen, and lung tissue mix of each animal. Among them, 20 bat samples showed unspecific amplifications of different sizes, not expected for the *T. cruzi* satellite DNA amplicon. In a deepen study of those samples, a PCR targeted to the *T. cruzi* kinetoplastid minicircle (kDNA-PCR) was carried out. Out of them, five samples depicted amplifications, although there were not exactly of the expected molecular weight (330bp). Based on these findings, satellite amplicons obtained from these samples were sequenced and the *T. cruzi* origin of those amplicons was discarded.

DISCUSSION

Domestic reservoir of *T. cruzi* infections are usually used to estimate the transmission risk to human. In endemic regions, this study is appropriate to evaluate insecticide spraying campaigns effectiveness. Out of those areas, because animals can transport the infection by moving themselves or together with humans, the study of its infected status is needed for evaluating the establishment of new foci of infection that could involving human beings. Here we evaluated dogs and bats from Buenos Aires city as putative reservoirs for *T. cruzi* infection. Although it is a non-endemic region, the presence of the insect vector *Triatoma infestans* requires surveillance to detect the installation of possible local sources of infection (12).

In our study, all 45 analyzed dogs were negative for *T. cruzi* infection analyzed by means of PCR satellite DNA amplification and TIA serological analysis. Domestic dogs are able of keeping the pathogen, turning them into a *T. cruzi* reservoir in the domestic environment, and a risk factor for human infection (2,3). *Trypanosoma cruzi* PCR based diagnosis of domestic animals were carried out both in endemic and non-endemic regions with different performance, strongly associated to parasitemia levels, and the transmission incidence (14). On the other hand, dogs´ infection was also analyzed by TIA based on recombinant TS neutralization, a not-detected enzyme in coendemic parasites, which presents a higher sensitivity

and specificity level respect to conventional serology to detect chronic *T. cruzi* infection in different mammalian hosts (13).

Different bats families of America as *Molossus, Desmodus, Carollia*, *Artibeus*, *Myotis* and *Phyllostomus* were observed as *T. cruzi* reservoirs (11,15). *Tadarida brasiliensis*, also described as reservoir, was the unique analyzed in this study due to its prevalence in Buenos Aires. It is a insectivorous bat species found from USA to Argentina which shows long migratory routes, near 2,000 kilometers, to avoid winter temperatures (9,10). Although with low infection prevalence, this species acts as reservoir and transporter of the parasite (8). Different tissues samples were used for DNA extraction to *T. cruzi* PCR screening researches. Blood, heart, uropatagium and plagiopatagium, among other tissues were successfully used (9,11). Here we extracted DNA from a mix of liver, spleen, and lung samples, as parasite target tissues. Blood samples were impossible to withdrawn because bats were trapped, and usually killed, by citizens in their dwellings before carrying to IZLP. There, their organs were kept at -20°C for further analysis, until rabies negative diagnosis from brain tissue was confirmed. In our study, the analysis of DNA extracted from 75 bats showed negative *T. cruzi* findings. Other studies of *Tadarida brasilensis* bats infections carried out in non-endemic regions of USA, thousands kilometers from endemic areas, reached 0.27% of prevalence. Although it infection rate is low, bat was pointed as potential contributor to the endemicity of *T. cruzi* in Oklahoma and to possible future enzootic expansion of infection to USA (9). In this scenario, reliable and highly sensitive methods are required for a correct parasite detection. We carried out the diagnosis by means of a set up multiplex PCR designed to amplify *T. cruzi* specific satellite sequence and a fragment of the mammal beta-actin gene. This strategy allowed us to get control of quality and integrity of DNA extraction at the same time to amplify *T. cruzi* DNA with a burden sensitivity of 1 fgr, equivalent to the genetic load of 1/200 parasite cell. The dynamics of infection with participation of mammals' movements and presence of vectors requires surveillance of putative parasite reservoirs by means of sensitive techniques to avoid the increment or the installation of new infecting areas.

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