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Tamay-Segovia, Paulino; Blum-Domínguez, Selene C.; Núñez-Oreza, Luis A.; Martínez-Hernández, Fernando; Gómez-Solano, Mónica I.; Maldonado-Velázquez, María G.; Retana-Guiascón, Oscar G.

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SHORT COMMUNICATION

Presence of Virginia opossum (*Didelphis virginiana*) and Pic (*Triatoma dimidiata*) infected with *Trypanosoma cruzi* in urban areas: preliminary evaluation in the city of Campeche, Mexico

Paulino Tamay-Segovia[a*], Selene C. Blum-Domínguez[a], Luis A. Núñez-Oreza[a], Fernando Martínez-Hernández[b], Mónica I. Gómez-Solano[c], María G. Maldonado-Velázquez[c], Oscar G. Retana-Guiascón[d]

ABSTRACT. The aim of this study was to identify the infection prevalence of *Trypanosoma cruzi* in opossums and triatomines captured in urban areas of Campeche City, Mexico. We collected 25 specimens of marsupials of Virginia opossums (*Didelphis virginiana)* species showing a *T. cruzi* infection prevalence of 52% (13/25). Also, 36 specimens of Pic (*Triatoma dimiata*) vectors were collected, showing a *T. cruzi* infection prevalence of 41.6% (15/36). All *T. cruzi* isolates were TcI, which correspond to the predominant lineage in mammals of Mexico, irrespective of the mammalian host species or vector.

Key words: *Didelphis virginiana, Triatoma dimidiata, Trypanosoma cruzi,* Chagas Disease.

RESUMEN. El objetivo del estudio fue identificar la prevalencia de infección con *Trypanosoma cruzi* en zarigüeyas (*Didelphis virginiana)* y Pic (*Triatoma dimidiata*) capturados en áreas urbanas de la ciudad de Campeche, México. Se colectaron 25 zarigüeyas con una prevalencia de infección con *T. cruzi* del 52% (13/25). También se colectaron 36 vectores de Pic con una prevalencia de infección del 41,6% (15/36) con *T. cruzi.* Todos los aislados de *T. cruzi* fueron TcI de acuerdo con la predominancia de este linaje en México independientemente del huésped mamífero o vector.

Palabras clave: *Didelphis virginiana, Triatoma dimidiata, Trypanosoma cruzi,* Enfermedad de Chagas.

INTRODUCTION

Chagas disease is a public health issue in Latin America, affecting more than 8 million people who could experience cardiological manifestations of the disease (WHO 2010, Ribeiro *et al* 2012). The causal agent *T. cruzi* is transmitted through the faeces of hematophagous insects of the Triatominae subfamily transmit the causal agent *T. cruzi*. The insects acquire the infection by feeding from wild infected mammals which in turn act as reservoirs, sustaining the cycle of infection. It has been demonstrated that *T. cruzi* can infect almost any mammal that is in contact with its vector. One of the main wild hosts –considered as primary reservoir of *T. cruzi*– is an ancestral marsupial belonging to the Didelphidae family (WHO 2002). Common opossum *(Didelphis marsupialis)* and Virginia opossum (*Didelphis virginiana*) are widely distributed in Mexico and currently considered sympatric (Gardner 1973). Opossum species are the main reservoirs that maintain the transmission in the wild; however, the destruction of their ecosystem has triggered urban adaptation processes increasing the risk of transmission and dispersion in areas where it cohabitates with the insect vector.

Studies carried out in rural communities of Jalisco and Yucatan (Mexico) have shown the relevance of Virginia opossum as reservoir of *T. cruzi*, since the infection prevalence ranged from 24% up to 55% (Ruiz-Piña and Cruz-Reyes 2002, Villagrán *et al* 2011, Parada *et al* 2013). However, there are not studies that show the role of these mammals in the transmission of *T. cruzi* in more urbanized areas. In Mexico, the infection by *T. cruzi* in humans has been recognised in the states where the vector and reservoirs are present. In Campeche State, there are records of Pic (*Triatoma dimidiata*) infected by *T. cruzi*, patients with Chagas myocardiopathy, and the presence of both opossum species as potential reservoirs, but there are no studies connecting the cases or determining the role of these mammals in maintaining the risk of the disease. Therefore, the main objective of this study was to identify the prevalence of *T. cruzi* in subpopulations of opossums and triatomines in Campeche City, Mexico.

MATERIAL AND METHODS

STUDY AREA

The survey was carried out in Campeche City, capital of homonymous state, which is located at 19°50' latitude north and 90°32' longitude west of the Yucatan Peninsula. It displays an annual average temperature and precipitation of 28 °C and 1,100 mm, respectively (INEGI 2009).

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[a]Centro de Investigaciones Biomédicas, Universidad Autónoma de Campeche, Campeche, México.

[b]Departamento de Ecología y Agentes Patógenos del Hospital General "Dr. Manuel Gea González", Tlalplan, México.

[c]Facultad de Ciencias Químico Biológicas, Universidad Autónoma de Campeche, Campeche, México. [d]Centro de Estudios de Desarrollo Sustentable y Aprovechamiento de la Vida Silvestre, Universidad Autónoma de Campeche, Campeche, México.

[*]Corresponding author: P Tamay-Segovia; Campus de la Salud, Av. Patricio Trueba de Regil S/N, Col. Lindavista, San Francisco de Campeche, Cam., México; pautamay@hotmail.com

Opossums captures were performed between March and June in twelve housing areas, which were selected beforehand based on surrounding vegetation criteria. Tomahawk traps set with mango and almonds as bait were placed at sunset and checked the next day early in the morning.

IDENTIFICATION OF OPOSSUMS

Basic information was registered for all mammals, such as the color on the tail base and age (adult or juvenile) and the sequence of premolar and molar eruption as well as dental wearing (Petrides 1949).

BLOOD EXTRACTION

It was carried out by cardiac punction (5 mL); in order to perform the procedure the animals were previously anesthetised with a mixture of 0.25 mg/kg xylazine and 25 mg/kg ketamine (Pietrzak and Pung 1998). Following blood extraction, opossums were marked on the right ear and liberated after observing total recovery. The blood samples (2 mL) were centrifuged and the obtained serum was placed on vials and maintained at –70 °C until processing.

FRESH SAMPLE PREPARATION

A drop of blood with anticoagulant was added onto a glass slide with isotonic saline solution, mixed and observed by light microscopy.

Isolation of *T. cruzi:* All samples from heart blood (3 mL) were cultured in Liver Infusion Tryptose medium (LIT), enriched with hemin, 10% fetal bovine serum, and de-complemented at 56 °C. All cultures were incubated at 28 °C.

MOLECULAR AND SEROLOGICAL DIAGNOSIS

The serological diagnosis was performed using ELISA (Voller *et al* 1979), with modifications to determine *T. cruzi* antibodies in opossums. The antigen for ELISA testing was prepared by means of a mixture of epimastigotes (Luquetti *et al* 2009) of three strains of *T. cruzi* (total extract) isolated from triatomines captured in the city of Campeche from a previous study. The mixture was cultivated in LIT media until logarithmic phase was reached, lysed and centrifuged posteriorly. The assay was carried out on polystyrene 96 well plates (Nunc) sensibilised with 200 µL of solution containing 0.5 µg/µL of the carbonated buffered total extract at pH 9.6; working serums were diluted 1:20 and the IgG Goat Anti-Opossum HRP conjugate at 1:1000 (Alpha Diagnostic International); O-Phenylenediamine Assay Chromogenic substrate was added. The reaction was stopped by adding 50 µL of H_2SO_4 2.5 N and read in ELISA (Biotek) reader at 490 nm absorbance.

DNA EXTRACTION

Five milliliters from the culture were centrifuged at 13,000 x g, the pellet was resuspended in 1000 µL TE buffer (10 mM Tris-HCL, pH 8.0, 10 mM EDTA) with 50 µL Proteinase K at a concentration of 20 mg/mL and incubated at 56 °C overnight. The extraction from the lysed product was performed with Phenol:Chloroform:IsoAmyl alcohol (25:24:1); for DNA precipitation 3 M sodium acetate and absolute ethanol were added (Sambrook *et al* 1989).

PCR Technique was used for the molecular determination of *T. cruzi* isolates. The amplification of an intergenic region of the mini-exon gen was carried out using a mixture of 2 oligonucleotides and a common sequence present in both lineages of *T. cruzi.* The oligonucleotides used were: TC1: 5'-GTGTCCGCCACCTCCTTCGGGCC; TC2: 5'-CCTGCAGGCACACGTGTGTGTG; TCC: 5'-CCCCCCTCCCAGGCCACACTG in 50 µL reaction mixture containing 1ng/μL DNA, 5 µL 10× buffer, 1 mM $MgCl_2$, 2.5 mM each dNTPs (Sigma-Aldrich, St. Louis, MO, USA), 100 pmol of each primers, and 0.5 U/µL Taq DNA polymerase (Invitrogen, Carlsbad, California, USA). Thermal profile with modifications in Eppendorf thermocycler was 94 °C/l0 min; 35 cycles of 94 °C/30 s, 55 °C/30 s, 72 °C/30 s; and a final extension at 72 °C/10 min. Amplification products were analysed by electrophoresis in 1.5% agarose gels. These oligonucleotides amplify a 300 and 350 base pairs band for *T. cruzi* Lineage II and *T. Cruzi* Lineage I, respectively (Souto *et al* 1996).

DETECTION OF *T. CRUZI* IN TRIATOMINES

Triatomines were captured in households and surroundings with the participation of the inhabitants and researchers. The insects captured were placed in labeled flasks, and identified to species using the keys of Lent and Wigodzinsky (1979). The determination of *T. cruzi* infection in the collected Pic was performed directly by observing the faeces under light microscope (40X). The trypanosome identification was confirmed applying the same PCR protocol as for the opossums.

ETHICAL ASPECTS

The opossums capture and management was performed following the Mexican Official Norm NOM-062-ZOO-1999 and NOM-059-SEMARNAT-2010 under previous approval of the Ethical Committee of the General Hospital of Medical Specialties "Dr. Javier Buenfil Osorio" from Campeche City, number of document 05-EXT-13.

RESULTS AND DISCUSSION

A total of 25 opossums were captured in 9 out of 12 residential areas in Campeche City, where Virginia opossum was the solely species found. Ten juvenile (40%)

and 15 (60%) adults composed the capture; among them there were sixteen females, 7 males and 2 undetermined. Up to 31.25% (5/16) of females had an offspring of 3 to 9 individuals.

Prevalence of infection with *T. cruzi* was 52% (13/25). By serological diagnosis (table 1) and hemoculture 13 opossums tested were positives. On the other hand, parasites were detected only in six samples by light microscopy, suggesting low levels of parasitemia (Brown *et al* 2010). We consider that the high prevalence found in a mono-species in this case, increase the role of Virginia opossum as reservoir of *T. cruzi* and the consequent morbidity of the disease in humans. Similar prevalence of infection, 53.9% and 55%, were reported recently in Virginia opossum from Yucatan, Mexico (Ruiz-Piña and Cruz-Reyes 2002, Parada *et al* 2013). Other studies from Louisiana and Florida in USA reported a prevalence of 60% and 52%, respectively (Brown *et al* 2010, Houk *et al* 2010), which confirms that the opossums act as *T. cruzi* reservoirs in several geographic zones.

One of the offspring of a female opossum infected with *T. cruzi* was tested by direct search of *T. cruzi*, blood culture, PCR and ELISA; results were negative.

Regarding the infection per age class, we found that adults had a higher prevalence (84.61%) than juveniles (15.38%) with females displaying the highest rate 61.5%, although there was no significant difference (table 1). These results agree with other studies reported from tropical areas such as Yucatan (Parada *et al* 2013) and Venezuela (Telford and Tonn 1982). Similar prevalence were found in Georgia and Florida (Brown *et al* 2010) with significant differences and it is likely that the wider sample size influenced their results in comparison to our study.

Table 1. Results of the opossum tested for *T. cruzi* parasites by light microscopy, hemoculture and serological diagnosis.

Stage	Sex	Light Microscopy	Hemoculture	ELISA O.D.
Adult	Male	–	+	0.325
Adult	Male	+	+	0.406
Adult	Female	–	+	0.436
Adult	Female	+	+	1.092
Adult	Male	–	+	0.428
Adult	Female	+	+	0.944
Adult	Male	–	+	0.659
Adult	Female	–	+	0.999
Adult	Female	–	+	1.281
Juvenil	Female	+	+	0.298
Juvenil	Female	+	+	0.270
Adult	Male	+	+	2.418
Adult	Female	–	+	2.752

O.D. = Optical Density
Cutoff: 0.216.

Most of the opossums infected were captured during the dry season, probably because their activities increase during that time of the year, which was also observed by Ruiz-Piña *et al* (2002). The high infection prevalence and the detectable parasitosis are a potential infection risk for vectors as well as other mammals including man.

This is the first time that a study of *T. cruzi* infection in its natural reservoirs is being carried out in an urban area in this region; similar studies had been previously performed in rural areas only. Our findings suggest that from the public health perspective, there might be a moderate risk of infection in the city, considering the presence of the opossum in most of the localities studied with the Pic vector.

On other hand, the ADN amplification of all *T. cruzi* isolates obtained from opossums generated a 350 bp (figure 1), which corresponded to *T. cruzi* Lineage I similar to the predominance of TcI found by Bosseno *et al* (2002) in 7 Mexican isolates of opossums from 3 other Mexican states.

This TcI Lineage association with the *Didelphis* species has been demonstrated by several data analysis (Matthew *et al* 2005). The predominance of *T. cruzi* Lineage I in Mexico is not limited to just marsupials; it has also being found in humans and vectors (Ruíz-Sánchez *et al* 2005, Gómez-Hernández *et al* 2011). Our findings contrast with those from South American countries were *T. cruzi* lineage II is the main genotype associated with human infections (domestic cycle) (Bosseno *et al* 2002).

On the other hand a total of 36 Pic vectors were captured mainly in the household surrounding areas, from

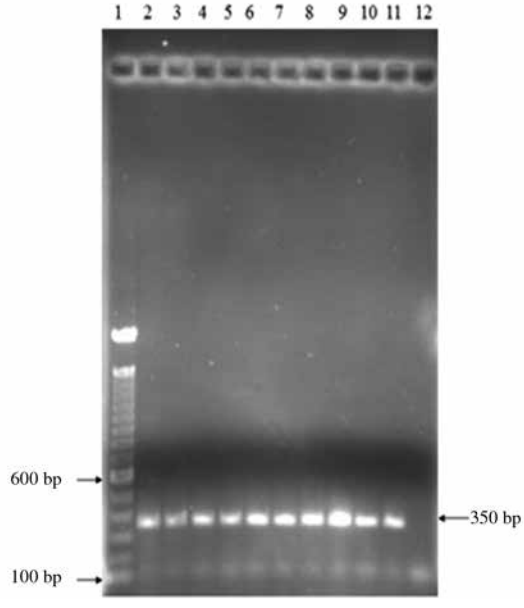

Figure 1. Electrophoresis in 1% agarose gel: Lane 1, MWM 100 bp; Lanes 2-10, samples of DNA from different isolates of de *T. cruzi*; Lane 11, positive control (Ninoa TcI Strain); Lane12, Negative control (water).

which 17 were males, 17 females and 2 were nymphs of the fifth stage. The search of the parasite in triatomines faeces resulted in a total infection prevalence of 41.66 (15/36), confirmed by the PCR method as *T. cruzi*. From this 29.41% (5/17) were males and 58.82% (10/17) were females. The months reporting higher capture, registering 66% (24/36), were those corresponding to the dry season (March-April). This is the only arthropod vector reported for the Campeche state, which seems to be more abundant during the dry season, as reported by Ruiz Piña and Cruz-Reyes (2002) and Hernández *et al* (2010). Activities of both reservoirs coincide on the dry season.

In conclusion, Virginia opossum and Pic coexist in urbanized areas of Campeche City, both with high prevalence of individuals infected with *T. cruzi*, were the dissemination of Chagas disease may reach its peak during the dry months. As a result, an emerging public health risk could be establish for the population of Campeche City. These findings should be taking into account whether a vector control program is enforced.

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