

Original Article

## A *Trypanosoma cruzi* strain from southern Mexico is more virulent for male mice in part by blocking the immune response

Celeste Amaranta Palma-González<sup>1</sup>, Héctor Israel Recinos-Vázquez<sup>1</sup>, Julio César Burguete-Gutiérrez<sup>1</sup>, José Antonio de Fuentes-Vicente<sup>1</sup>, María Adelina Schlie-Guzmán<sup>1</sup>, Dolores Guadalupe Vidal-López<sup>1</sup>, Jesús Díaz-Gómez<sup>1</sup>, Jorge Eugenio Vidal<sup>2</sup>, Javier Gutiérrez-Jiménez<sup>1</sup>

<sup>1</sup> *Biological Sciences Institute, University of Sciences and Arts of the state of Chiapas, Tuxtla Gutiérrez, Chiapas, México*

<sup>2</sup> *Department of Microbiology and Immunology, University of Mississippi Medical Center, Jackson, MS, United States*

### Abstract

**Introduction:** Chagas disease is a neglected disease in the American continent. The southern Mexican state of Chiapas has the highest incidence rate of Chagas disease in the country. The disease, mainly caused by *Trypanosoma cruzi* in Mexico, is more prevalent in males than in females but the scientific basis for the sex-related tropism is not completely understood. The objective of this study was to evaluate the pathogenicity of a *T. cruzi* strain in mice of both sexes and to assess certain elements of the immune response in the infected animals.

**Methodology:** Triatomines bugs were searched at Los Mezcales, Chiapas, Mexico and *T. cruzi* was identified by PCR and sequencing. A *T. cruzi* strain was isolated from the feces of triatomines bugs. Mice were infected with the strain and the virulence of the *T. cruzi* strain as well as the immune response against the infection was compared in male versus female mice.

**Results:** *T. dimidiata* was identified in all dwellings. 42.9% of the bugs were infected with *T. cruzi* lineage TcI. Male mice exhibited higher parasitemia than females, and developed leukopenia and lower levels of anti-*T. cruzi* antibodies compared to female mice.

**Conclusions:** The identification of the *T. cruzi* strain in this endemic region of Mexico revealed that male mice are prone to this infectious protozoan, in addition to manifesting a deficient immune response against infection. These findings may explain the greater number of cases of Chagas disease among men in this endemic region of Latin America.

**Key words:** Chagas disease; pathogenicity; southern Mexico; immune response.

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### Introduction

The causal agent of American Trypanosomiasis (Chagas disease), the protozoan parasite *Trypanosoma cruzi*, was identified by Carlos Justiniano Ribero das Chagas, a Brazilian physician [1]. Today, Chagas disease is endemic to 21 American countries, from the southern United States of America to Argentina's southern region. According to the World Health Organization (WHO), this disease is one of the most neglected globally and represents the fifth cause of death among these neglected infections [2]. Chagas disease affects 6–8 million people worldwide and induces 50,000 deaths yearly [3]. In Mexico, the number of cases reported of American trypanosomiasis is higher in central and southern Mexican states than that in northern states. The state-by-state seroprevalences (%) are as follows: Jalisco (12.01), San Luis Potosí (6.65), Chiapas (6.15), Estado de México (5.74), Querétaro (5.2), and Oaxaca (4.41) [4,5].

Accordingly, a retrospective analysis (2007–2016) demonstrated that the incidence rate of the disease (per 100,000 inhabitants) ranged between 1.1 and 4.4 in the states of Chiapas and Oaxaca, representing the highest burden of Chagas disease in the country [5]. In Mexico, for reasons that are not yet completely understood, Chagas disease is more prevalent in males >20 years of age than in females [5]. As reported in recent data on morbidity from 2019, the overall incidence rate of chronic American trypanosomiasis was higher than the acute form (0.78 vs. 0.07), and men exhibited higher rates of the disease (1.05 and 0.11 for chronic and acute forms, respectively) compared to women (0.52 and 0.03 for chronic and acute forms, respectively) [6]. *T. cruzi* infection in humans induces a complex immunologic response including effector and regulatory mechanisms [7]. The initial host response against *T. cruzi* includes an inflammatory reaction, activation of natural killer cells, and proliferation of T lymphocytes [8]; followed

by a concomitant secretion of polyclonal B cells [9]. However, the parasite has evolved mechanisms to evade the immune response, such as the co-expression of trypomastigote surface epitopes that delay the activation of neutralizing antibodies [9,10]. Also, the genetic heterogeneity of *T. cruzi* strains determines the variation of the immunological reactions [11].

In Mexico, the virulence of *T. cruzi* strains isolated from insect vectors or cases of human infection has been studied [12–16]. Despite the endemic status of the disease, research focused on strains isolated from the state of Chiapas remains scarce [15]. The aim of this study was to evaluate the pathogenicity of a strain of *T. cruzi* circulating in a remote region of Chiapas. We conducted the study in this region because (i) the population is small, and because (ii) accessibility is poor (i.e., virtually no travel typically exists from or to the study area for months if not years), giving us a unique opportunity to study Chagas disease in an isolated setting. Following recommendations by the Centers for Disease Control and Prevention (CDC) and the WHO, we conducted thorough screening to identify the source of triatomines in this region and then utilized phenotypic and molecular assays to identify and type the parasites. *T. cruzi* strains were then cultured *in vivo* using a mouse model and *in vitro* to investigate the molecular basis for the observed sex-related tropism. Identifying the pathogenic traits of this strain will allow us to better understand the parasite's pathogenic potential in the Latin American communities where this trypanosomatid species coexist.

## Methodology

### Isolation of *T. cruzi* strain

Triatomine bugs were collected in Los Mezcales in the Amatenango de la Frontera municipality as explained below. The climate of Los Mezcales is semi-warm and humid with heavy summer rains. The village is located 863 meters above mean sea level (mamsl) in

the Sierra Madre de Chiapas Mountain range; it borders Frontera Comalapa to the north, Guatemala to the east, Mazapa de Madero to the south, and Bejucal de Ocampo to the west [17]. Only 49 people live in this region, and they all present high levels of marginalization (Gini index = 1.37894); more than half of the inhabitants are illiterate (58.6%), 62.5% of their dwellings have earthen floors, and they do not have access to drinking water [18]. Prior informed consent was obtained, and structured interviews were conducted with the heads of families to collect personal and housing information.

Collecting triatomine bugs is one of the control measures for Chagas disease [19]. Hence, *Triatoma dimidiata* adult specimens or their traces (feces, eggs, or exuviae) were obtained by staff members of the Multidisciplinary Laboratory and Vivarium-UNICACH (in Spanish, *Laboratorio Multidisciplinario Experimental y Bioterio de la UNICACH*) and the personnel of Health Jurisdiction #X of Motozintla, Chiapas. The search method was intradomicile (e.g., wall surfaces, beds, areas behind furniture, and objects hanging on walls) and peridomicile (e.g., henhouse and woodpiles). The entomologic findings are shown in Figure 1A and included the identification of nymphs as well as adult triatomine bugs.

The insects were collected in plastic jars for their transportation to the laboratory, where they were kept in polycarbonate cages containing 15–18 adobe bricks and covered with mesh (Figure 1B). To feed the insects, healthy Balb/c mice (males and females 3–4 weeks of age) were anesthetized with xylazine (10 mg/kg)/ketamine (80 mg/kg) (following recommendations of the Mexican National Commission of Bioethics) and insects were allowed to feed on their blood for up to 20 minutes (Figure 1C). After feeding, the insects' feces were deposited on a sodium chloride solution (0.9% NaCl), and the presence of trypomastigotes in feces samples was analyzed using

**Figure 1.** *T. dimidiata* from Los Mezcales, Chiapas, México. A, nymph and female and male adults of *T. dimidiata* (bar for each bug structure is as follows: nymph = 1 mm; adult = 5 mm); B, blocks of adobe to maintain bugs in the laboratory; C, Feeding of *T. dimidiata* bugs with blood of an anesthetized mouse.



optical microscopy. Infestation, colonization, overcrowding, and natural infection entomological indices were calculated from the data on the entire sample of insects [19].

The propagation of strains in mice was carried out via intraperitoneal inoculation with  $10^5$  parasites/mL contained in the excreta of insects and using 0.9% sterile NaCl as a vehicle. For the *in vitro* propagation, a sample of total blood with a concentration of  $10^6$  parasites/mL- was obtained by cardiac puncture at the parasitemia peak and transferred to 10 mL of liver infusion tryptose (LIT) medium. After 15 days of culture, the parasites were concentrated by centrifugation at  $4,000\times g$  for 20 minutes followed by a 30 minutes period standing at room temperature. The preparation was then centrifuged again at  $4,000\times g$  for 10 minutes. The pellet of cells was resuspended in 10 mL of LIT medium (replacing the LIT medium every 10 days) and incubated at 25 °C for 40 days. The parasites were finally cryopreserved at -80 °C in LIT medium with 10% glycerol. Animal studies were approved by the Ethics Committee of the Chiapas University of Sciences and Arts (approval #048/2016).

#### *T. cruzi* inoculum preparation

To quantify trypomastigotes in the blood of the mice, a 10- $\mu$ L sample of blood was collected by tail puncture and diluted 1:10 with 3.8% sterile sodium citrate in PBS buffer (pH 7.2). The number of trypomastigotes/mL was determined by multiplying the number of parasites counted, using a Neubauer chamber, by  $10^5$ . The morphology and presence of the trypomastigotes in the blood samples were confirmed using 10% Giemsa-stained thin blood smears (Figure 2A).

#### *Dissection of the spleens and hearts of infected mice*

Mice were euthanized at 45 days post-infection (p.i.) with CO<sub>2</sub> for 3 minutes [24], and the spleen and heart were aseptically removed, weighed using an analytical balance (Ohaus, New Jersey, USA), and introduced into a 10% sterile PBS buffer. The extraction and purification of total DNA were accomplished as described below.

#### *Molecular identification of T. cruzi.*

Genomic DNA was obtained from the feces of the infected insects as well as the organs obtained from infected mice (i.e., the spleen and heart) using commercial kits (Quick DNA Fecal/Soil Microbe and Quick DNA Miniprep kits, respectively, Zymo, California, USA) following the manufacturer's

specifications. Amplification of DNA sequences (293–340 bp) from the kinetoplast DNA was conducted to detect *T. cruzi* [20,21]. PCR reactions were performed using a final volume of 25  $\mu$ L using PCR Master Mix 2X (Thermo Scientific, Massachusetts, USA), 0.4  $\mu$ M of the oligonucleotides KNS1 (5'-GGG GTT CGA TTG GGG TTG GTG TA-3') and KNS2 (5'-AAA GTT GAA CGC CCC TCC CAA A-3'), 25 ng of template DNA (previously digested with 0.5 u NsiI enzyme [Promega, Wisconsin, USA] at 37 °C for 3 hours), and nuclease-free water. Reactions were cycled 34 times at 94 °C for 1 minute, 56 °C for 1 minute, and 72 °C for 1 minute, followed by a final elongation at 72 °C for 7 minutes. Amplicons were analyzed by electrophoresis on a 1.5% agarose gel using TAE buffer and run for 40 minutes at 100 V; afterward, the gel was stained with 0.5  $\mu$ g/mL ethidium bromide. The genetic lineage was determined through the amplification of the intergenic region segment of the mini-exon gene of *T. cruzi*. If the length of the intergenic region was 350 bp, it was assigned to the lineage TcI; if the intergenic region was 300 bp, it was assigned to lineage TcII [22]. PCR reactions were then adjusted and conducted using a final volume of 25  $\mu$ L, using PCR Master Mix 2X (Thermo Scientific, Massachusetts, USA), 2  $\mu$ L of genomic DNA, 20 pmol/ $\mu$ L of each primer (Tc: 5'-CCC CCC TCC CAG GCC ACA CTG- 3', Tc1: 5'-GTG TCC GCC ACC TCC TTC GGG CC-3', and Tc2: 5'-CCT GCA GGC ACA CGT GTG TGT G-3'), and nuclease-free water. The first cycle consisted of a denaturation step at 94 °C for 1 minute. Subsequently, 27 cycles were performed at 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds, followed by a final elongation cycle at 72 °C for 10 minutes. The analysis of the amplicons was conducted as described above. DNA from the Y strain of *T. cruzi* (kindly provided by Dr. José Luis Rosales Encina from CINVESTAV) was used as a control for the TcII lineage.

#### *Parasitological load in the murine model*

Female (n = 16) and male (n = 16) Balb/C mice were inoculated intraperitoneally with  $10^5$  trypomastigotes. The animals were kept in polycarbonate boxes at  $24 \pm 2^\circ$  C and a photoperiod of 12/12 light/dark and provided with water and food *ad libitum*. The control group consisted of 16 mice (8 males and 8 females) inoculated with 0.9% sterile saline solution. According to established criteria, we evaluated the following parameters every 48 or 72 hours [23]: i) prepatent period (time between inoculation and the detection of the parasites in the

distal bloodstream of the rodent); ii) parasitemia, which quantifies the presence of parasites in the blood using a Neubauer chamber, as described above; iii) cell tropism (by detecting specific gene sequences of *T. cruzi* in some organs); and iv) mortality (the number of deaths per experimental group). The care of the animals was performed as established previously [24].

#### Determination of total leucocyte count and IgG anti-*T. cruzi* antibodies

Quantification of leukocytes was performed with 10 µL of blood from infected or uninfected mice (diluted to 1:20 with 2.5% acetic acid) using a Neubauer chamber [25]. The level of anti-*T. cruzi* antibodies in the serum of mice was assessed with the indirect ELISA technique [26]. Briefly, microplate wells (Nunc MaxiSorp™, Massachusetts, USA) were covered with 100 µL of cell lysate from the wild-type *T. cruzi* strain isolated in this study. Non-specific sites were blocked with 1% skim

milk and 100 µL of serum from infected mice, diluted 1:100 with PBS/T, was added, followed by 100 µL of anti-mouse IgG (diluted 1:1,500) labeled with peroxidase (Sigma, Taufkirchen, Germany). The reaction was developed using o-phenylenediamine (40 mg in 100 µL of 0.1 M citric acid; 0.2 M basic sodium phosphate, pH 5; and 100 µL of 30% H<sub>2</sub>O<sub>2</sub>). After incubation for 20 minutes at 37 °C, the reaction was stopped by adding 2.5 M HCl. Subsequently, absorbance at 490 nm was determined with a microplate reader (Biorad, California, USA). Serum from uninfected mice was used as a negative control. Assays were performed in triplicate.

#### Statistical analysis

Descriptive statistics were used to analyze the continuous variables. The Shapiro–Wilk test was used to analyze the normality of the data. Parasitological load and white blood cells (WBC) were compared using the Mann–Whitney U test. The weights of the mice and their organs were compared with a Student’s *t*-test or a Mann–Whitney U test. Levels of anti-*T. cruzi* antibodies were compared with a Student’s *t*-test. Statistical significance was defined as  $p \leq 0.05$ . SigmaPlot® (California, USA) software, version 12, was used for all statistical analyses and the preparation of graphics.

#### Results

A total of 11 dwellings was present in the marginalized locality of Los Mezcales, Chiapas. Five houses were abandoned; therefore, only the remaining six households (54.5%) were screened. The total number of people living in the examined ( $n = 6$ ) dwellings was 49. The average age of the inhabitants of these houses was 29.5 years, and 55.1% were women. Inhabitants were categorized into the following age groups: children (44.9%), adults (46.9%), and the elderly (8.2%). Many of the residents were natives of Los Mezcales. Most of the population was illiterate, followed by those with incomplete elementary education. Most men were engaged in agriculture, and the majority of women were dedicated to household tasks (Table 1).

#### Presence of insect vectors in households and propagation of the *T. cruzi* strain

*T. dimidiata* insects were collected from all houses; in half of them, insects were only identified in the peridomicile, while in the remaining households, insects were found in both intradomicile and peridomicile. A total of 15 triatomines were captured: 2

**Table 1.** Characteristics of inhabitants and households of Los Mezcales, Chiapas, Mexico

Inhabitants (N = 49)	n (%)
<b>Age (years) -- Mean (± SD)</b>	29.5 (23.2)
<b>Age groups (years)</b>	
under 5	7 (14.3)
6-11	8 (16.3)
12-19	7 (14.3)
20-24	3 (6.1)
25-60	20 (40.8)
>60	4 (8.2)
<b>Sex</b>	
Male	22 (44.9)
Female	27 (55.1)
<b>Origin</b>	
Los Mezcales, Chiapas, Mexico	33 (82.5)
Other municipalities of Chiapas, Mexico	6 (15.0)
Guatemala	1 (2.5)
<b>Education level</b>	
illiteracy	13 (31.0)
incomplete primary	6 (14.3)
Primary	5 (11.9)
Secondary	5 (11.9)
<b>Housing characteristics</b>	
Number of rooms per household -- Mean (± SD)	1.67 (0.81)
inhabitants per household -- Mean (± SD)	5.4 (3.04)
<b>Ceiling</b>	
wood	1 (16.7)
metal sheet	5 (83.3)
<b>Wall</b>	
reed	3 (50.0)
adobe	2 (33.3)
reed/cement	1 (16.7)
<b>Floor</b>	
ground/cement	4 (66.7)
ground	1 (16.7)
cement	1 (16.7)

adult males, 6 adult females, and 7 nymphs. We identified the presence of *T. cruzi* in the feces of 6 specimens out of the 15 triatomines collected and calculated a natural colonization index of 40%. The index of infestation and colonization was 100%, and the overcrowding index was 2.5.

*Identification of the T. cruzi strain*

The native *T. cruzi* strain isolated in this study was designated as IDIM/MX/16/Mezcales according to standardized nomenclature [19]. An amplicon of approximately 300 bp was obtained from the strain containing the kinetoplast sequence of *T. cruzi*, while a PCR product of 350 bp, which is specific to the TcI lineage, was also amplified (Figure 2B). The sequence of the 350 bp PCR product (www.eurofins.com) corresponded to the mini-exon gene of *T. cruzi* and was deposited in GenBank® with the accession number KX784052.

*Dynamics of parasitemia in the murine model*

The prepatent period comprised 9 days for both female and male mice. Hence, the patent period lasted from day 10 to day 33 p.i. and was characterized by the presence of trypomastigotes in the bloodstream (Figure 2A). During the patent period, the number of trypomastigotes in the blood of male mice was significantly higher on almost every day p.i. for which the parasite load was measured (i.e., 14, 17, 19, 21, 24, 26, and 28 days p.i.) compared with that in female mice. Thus, the mean number of trypomastigotes in the blood of male mice also was significantly higher than that in the blood of female mice ( $3.8 \times 10^6$  parasites/mL  $\pm 3.3 \times 10^6$  and  $1.79 \times 10^6$  parasites/mL  $\pm 1.46 \times 10^6$  for males and females, respectively). The parasitemia peak

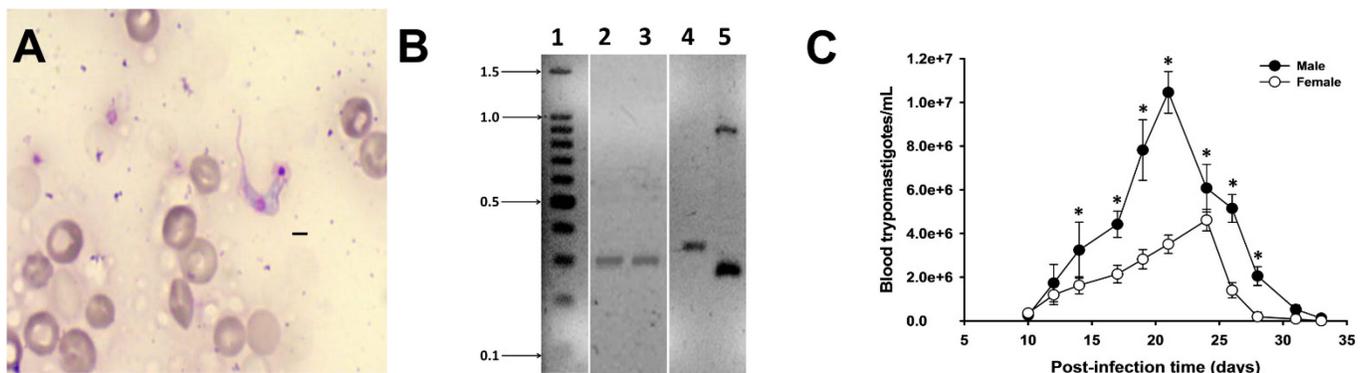
was reached on day 21 p.i. ( $1.04 \times 10^7$  parasites/mL  $\pm 9.5 \times 10^5$ ) and day 24 p.i. ( $4.61 \times 10^6$  parasites/mL  $\pm 4.92 \times 10^5$ ) in males and females, respectively (Figure 2C).

During the patent period, the average body weight of infected mice was significantly lower compared with uninfected mice in both females and males (Table 2). Moreover, infected female and male mice developed splenomegaly, which was characterized by an increased weight of the spleens of infected animals. Consistent with this, the spleen of infected animals was colonized by *T. cruzi* parasites, as the PCR amplified the gene encoding the mini-exon using DNA purified from spleen specimens (data not shown). There was no significant difference in the weight of hearts collected from infected mice compared with those of uninfected animals (Table 2). All infected male mice exhibited bristly hair, while 18.7% of them showed hind limb paralysis. None of the female mice exhibited bristly hair or limb paralysis. None of the infected mice died or was considered for euthanasia during the 42 days p.i.

*Evaluation of certain elements of the immune system in infected mice*

The median leukocyte count of uninfected females was  $15,759$  WBC/mm<sup>3</sup>  $\pm 3,549$ , whereas that of uninfected males was  $16,814$  WBC/mm<sup>3</sup>  $\pm 1,738$ . From day 10 through 33 p.i., infected female mice displayed a significantly higher mean leukocyte level ( $20,533$  WBC/mm<sup>3</sup>  $\pm 5,324$ ) in comparison with infected males ( $16,701$  WBC/mm<sup>3</sup>  $\pm 7,941$ ;  $p < 0.001$ ). From day 10 to 17 p.i., the infected males showed leukopenia, whereas healthy mice did not. However, from days 19 to 28 p.i., infected males exhibited leukocytosis, and uninfected males did not. (Figure 3A). From day 12 to 21 p.i.,

**Figure 2.** Identification and dynamics of infection of *T. cruzi* IDIM/MX/16/Mezcales from Los Mezcales, Chiapas, México. A, A blood trypomastigote from an infected mouse (bar = 2 μ); B, Specific PCR products of *T. cruzi*. Lanes: 1, Molecular weight marker, fragments at left are shown in kb; 2-3, ~300 pb of kinetoplast PCR amplicons using as a template DNA from IDIM/MX/16/Mezcales strain and Y strain of *T. cruzi*, respectively; 4, a 350 pb PCR amplicon of the mini-exon gene from IDIM/MX/16/Mezcales strain of *T. cruzi*; 5, a 300 bp PCR amplicon of the mini-exon gene from Y strain of *T. cruzi*; C, Comparison of blood trypomastigotes load in infected mice by sex. \*  $p < 0.05$ , according to the Mann-Whitney U Test.



**Table 2.** Effects on weight and organs of infected mice with *T. cruzi* IDIM/MX/16/Mezcales.

Body weight (g)	Male			Female		
	Infected	Uninfected	p	Infected	Uninfected	p
Mean (±SD)	25.9 (3.78)	32.1 (4.34)	< 0.001 <sup>b</sup>	23.9 (3.02)	28.5 (2.11)	< 0.001 <sup>b</sup>
<b>Organ weight (g)</b>						
Heart						
Mean (±SD)	0.175 (0.057)	0.220 (0.103)	0.165 <sup>a</sup>	0.256 (0.115)	0.190 (0.099)	0.120 <sup>b</sup>
Spleen						
Mean (±SD)	0.275 (0.085)	0.110 (0.031)	< 0.001 <sup>b</sup>	0.181 (0.040)	0.100 (0.0)	< 0.001 <sup>b</sup>

<sup>a</sup> p value calculated with the two-tailed Student T test; <sup>b</sup> p value calculated with the U-Mann Whitney test.

infected females showed leukocytosis whereas uninfected females did not (Figure 3B).

We next investigated the levels of specific IgG anti-*T. cruzi* antibodies. Apart from the overall leukopenia observed in infected male mice, levels of IgG anti-*T. cruzi* antibodies were similar in infected female and male mice on day 10 p.i. However, after day 10 p.i., there was a tendency for an increased level of anti-*T. cruzi* antibodies in both female and male mice, and the level of IgG antibodies against the *T. cruzi* IDIM/MX/16/Mezcales strain was significantly higher on day 21 p.i. in female mice compared with that in male mice (Figure 3C). Levels of antibodies remained significantly higher in females compared with male mice 31, 35, 38, and 42 days p.i.

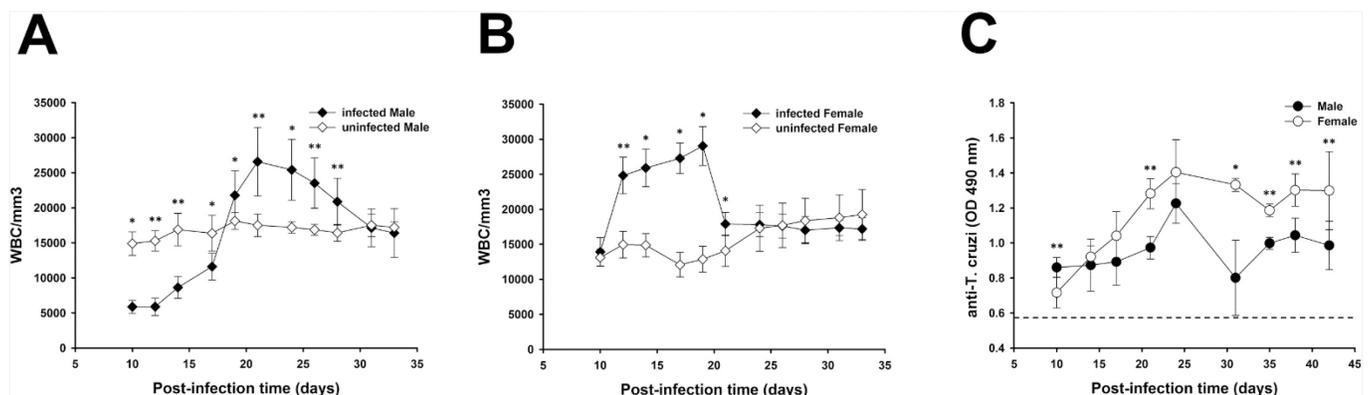
**Discussion**

Chagas disease is a neglected infection that impacts the health of 5,742,167 people in Latin America [27]. In 2015, it was estimated that nearly 4 million Mexicans were infected with *T. cruzi* and developed Chagas disease [4]. Determining the factors that allow the spread of the parasitic disease might help to reduce its burden. These factors include close contact with humans in the ecological niches where the insect vector resides as well as poverty and marginalization affecting

human populations [28]. In the current study, we identified socioeconomic conditions of the residents of Los Mezcales, including illiteracy, overcrowding, and housing situations (i.e., dwellings built with adobe bricks) that may have increased the presence of *T. dimidiata* in the residents’ households. Reports suggest that this insect is the most frequent species in the Chiapas state and one of the best insect vectors of *T. cruzi*, which is chiefly due to the favorable environmental conditions in the region [29]. For example, an investigation showed that the virulence of a *T. cruzi* strain isolated from *T. dimidiata* was greater when the insects were collected at 700 mamsl in contrast to specimens retrieved at 300 or 1,400 mamsl [15]. Another study conducted in rural areas of Colombia showed that the risk of an infestation of *T. dimidiata* was seven times higher in homes with walls constructed with adobe bricks versus those with walls built with concrete [30]. Similarly, a study carried out in the Gran Chaco region in Argentina exhibited a *T. infestans* infestation rate of 30.4% in mud-walled buildings, along with a direct association between the infestation rate and overcrowding [31].

Our molecular analysis of the *T. cruzi* IDIM/MX/16/Mezcales isolated from *T. dimidiata* bugs revealed that it belongs to the lineage TcI (Figure 2B).

**Figure 3.** Immune response of infected mice with *T. cruzi* IDIM/MX/16/Mezcales . A, WBC counts among infected male mice; B, WBC counts among infected female mice; C, Comparison of levels of antibodies anti-*T. cruzi* in infected mice by sex (dashed lines: cut-off value at OD<sub>490 nm</sub> = 0.57 ± 0.06 calculated from serum of uninfected mice). \* p < 0.05, according to the Mann-Whitney U Test; \*\* p < 0.05, according to the Student t test.



This lineage is considered the most frequent genetic variant in Mexico, as documented previously [32]. Additionally, recent research indicated that of 334 specimens, 38% (n = 126) of *T. dimidiata* adults collected in Central American countries were infected with *T. cruzi*, 94% of them belonging to the lineage TcI [33]. However, the genetic diversity of *T. cruzi* in Mexico may be larger. A study conducted in the central region of Veracruz revealed that 27% of the *T. dimidiata* insects captured in the area were infected with the *T. cruzi* strain TcI, while 73% of the insects carried strains of different lineages, such as TcII, TcIII, TcIV, and TcV [34].

Bloodstream trypomastigotes of the *T. cruzi* IDIM/MX/16/Mezcales were observed on day 10 p.i. Male mice showed higher levels of parasites at peak parasitemia than females (Figure 2C). In the present work, the prepatent period was similar to that of other reports of *T. cruzi* strains isolated from central and southern Mexico, although the parasitic loads of the animal hosts in a previous study were lower than the quantity presented here [12]. These differences may be linked to the genetic lineage of *T. cruzi*, which is related to the pathogenicity exhibited by the different strains [35, 36]. In a murine model, researchers demonstrated that the Y (TcI lineage) and VFRA (TcVI lineage) strains were more virulent than the Sc43 (TcV lineage) strain, and Y and VFRA strains caused greater damage to the myocardium in comparison with the Sc43 strain [37]. However, another study pointed out that *T. cruzi* strains of different genetic lineages (TcI to TcVI) could share common pathogenic traits in a syngeneic murine model [38]. We did not observe colonization of the heart in our study, as evaluated by PCR. However, histological damage to the myocardium was not evaluated in the current study. Undetectable sequences of *T. cruzi* in the heart may indicate that the parasite was not yet present in the organ at the time of the euthanasia or that the limit of detection of our assay was not ideal to identify the parasite in the heart.

On the other hand, mice infected with the *T. cruzi* IDIM/MX/16/Mezcales developed splenomegaly and lost weight. Considering all the characteristics that we used to evaluate the pathogenicity of this strain, we observed a very intriguing, enhanced virulence against male mice in comparison with infected female mice. Whereas the enhanced virulence in males was in part related to an early leukopenia along with a decreased level of anti-*T. cruzi*-specific antibodies, the specific mechanism by which male mice are more susceptible to infection with *T. cruzi* parasites requires further research. Similar clinical signs have been reported in

male Swiss Webster mice infected with the *T. cruzi* Y strain; males of this breed presented additional signs such as stooped posture, closed eyes, low ears, peeling of the skin, and prostration [39]. The hind limb paralysis we observed in infected male mice, absent in females, indicates that *T. cruzi* parasites migrated to the skeletal muscle. We hypothesize that the significantly higher density of the parasite in the bloodstream of males compared with that in infected females could have stimulated the migration of the parasite to the skeletal muscle. Alternatively, tissue-specific components in the skeletal tissue of males might have caused the tropism leading to the observed hind limb paralysis. Although not investigated in the current study, a recent publication demonstrated amastigote nests in the striated muscle of *T. cruzi*-infected mice that developed hind limb paralysis [14]. To examine this, it would be necessary to demonstrate the presence of the protozoan pathogen in the hind limbs of mice.

Comparable with the results of this investigation, male Swiss albino mice infected with the *T. cruzi* Tulahuen strain exhibited a higher parasitemia than females [40]. The current investigation revealed that in the prepatent period, leukocytosis was present in female mice on the first 10 days, whereas males displayed leukopenia (Figure 2A-B). It is not completely clear why this leukopenia was produced only in infected male mice early during the infection. However, the decrease of leukocytes in circulation in a very short time (< 10 days p.i.) may indicate that the parasite specifically targets this cell lineage in males. Likewise, sex-related hormones are related to different immune responses. Estradiol has been found to stimulate both the innate and adaptive immune systems, whereas testosterone does not; consequently, males are more prone to infectious diseases than females [41]. Accordingly, sex hormones could explain the development of leukopenia and the lower level of specific anti-*T. cruzi* antibodies in males in comparison with females (Figure 3). Like the present findings, male *Calomys callosus* rodents infected with two *T. cruzi* strains developed higher parasitemia levels and amastigote nests in the cardiac tissue and adrenal glands; by contrast, females demonstrated enhanced immunity [42]. Other animal models have demonstrated the role of sex hormones on the immunological control of *T. cruzi* infection. One of these studies highlighted that testosterone deficiency improves immune responsiveness, evidenced by an increase in total leukocyte count, T lymphocytes CD3+ and CD8+, and IL-2 in orchietomized male rats infected with the *T. cruzi* Y strain compared with non-orchietomized rats infected with the parasite [43]. A

clinical trial found that estrogen may regulate the protozoan parasitic disease, as ovariectomized *Mus musculus* specimens infected with the *T. cruzi* Y strain exhibited increased parasitemia and amastigote nests, in addition to anemia, thrombocytopenia, leukopenia, and decreased lytic antibodies, relative to non-ovariectomized animals [44]. These data were confirmed by research on orchietomized and ovariectomized *C. callosus* rodents infected with the *T. cruzi* Y strain [45]. Male rodents' higher susceptibility to infection has also been documented by the parasitic load and greater mortality caused by *Trypanosoma brucei rhodesiense* (a species that elicits sleeping sickness) in comparison with females [46]. Our study is fully aligned with the evidence presented from other settings. By investigating the specific virulence traits of *T. cruzi* IDIM/MX/16/Mezcales, this research paves the way for further studies aimed at elucidating the pathogenesis of strains isolated in endemic regions of Mexico. Furthermore, it serves as the basis to expand knowledge of the parasite's biology, the behavior of its insect vector, and their environments.

## Conclusions

The present study identified a *T. cruzi* strain (TcI group) isolated from a marginalized region of the Mexican southeast. The vector of this parasite, *T. dimidiata*, was found in all evaluated dwellings, with almost half of the insects carrying the parasite. Given the ecologic and socioeconomic conditions of the Los Mezcales locality, the residents are particularly prone to Chagas disease. As a control measure to eradicate the triatomines in this area, the houses were fumigated with bendiocarb (0.4 g active ingredient per square meter surface area) by personnel of Health Jurisdiction X of Motozintla, Chiapas. A surveillance program is in place to detect triatomines and *T. cruzi* strains in the study area. Research on Chagas disease among the inhabitants of Los Mezcales is in progress; therefore, the results obtained will reveal the infection's impact on the population and the inhabitants' roles as potential carriers of the disease, an issue that may be problematic considering migratory flows to neighboring countries (i.e., the USA or Guatemala). This examination of the pathogenicity of *T. cruzi* IDIM/MX/16/Mezcales using a murine model indicates that male mice are more susceptible to infection and that the enhanced virulence in males is partly attributable to a deficient immune response.

The susceptibility of men to Chagas disease in Mexico and specifically in the state of Chiapas is clearly seen in epidemiological data. The incidence rates in

men with the acute and chronic forms of the infection (0.65 and 0.22, respectively) are more than three times higher than those in women (0.21 and 0.07, respectively) [6]. Hence, local health authorities must establish action frameworks to eradicate the insect vector and disseminate knowledge related to this neglected disease among the population; this action will aid in the prevention of Chagas disease in marginalized regions of Mexico and the rest of the world.

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## Author contributions

CAPG, HIRV, JCBG, JDG: Performed the experiments. JADF, MASG, DGVL, JEV: Wrote the article. JGJ: Conceived the study, performed the statistical analysis and wrote the article.

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### Corresponding author

Professor Javier Gutiérrez-Jiménez, PhD

Laboratorio de biología molecular y genética, Instituto de Ciencias Biológicas, Universidad de Ciencias y Artes de Chiapas, Tuxtla Gutiérrez, Chiapas, 29039, México.

Phone: +529616170440 Ext. 4303

Fax: +529616170440

Email: javier.gutierrez@unicach.mx

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