Experimental infection of *Rhodnius robustus* Larrousse, 1927 (Hemiptera, Reduviidae, Triatominae) with *Trypanosoma cruzi* (Chagas, 1909) (Kinetoplastida, Trypanosomatidae) IV

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Abstract

Vector competence of triatomines (kissing bugs) for *Trypanosoma cruzi* transmission depends on the parasite-vector interaction and the genetic constitution of both. This study evaluates the susceptibility and vector competence of *Rhodnius robustus* experimentally infected with *T. cruzi* IV (TcIV). Nymphs were fed on infected mice or an artificial feeder with blood containing culture-derived metacyclic trypomastigotes (CMT) or blood trypomastigotes (BT). The intestinal contents (IC) and excreta of the insects were examined by fresh examination and kDNA-PCR. The rate of metacyclogenesis was also determined by differential counts. Fifth instar nymphs fed with CMT ingested a greater blood volume (mean of 74.5 μL) and a greater amount of parasites (mean of 149,000 CMT/μL), and had higher positivity in the fresh examination of the IC. Third instar nymphs fed with CMT had higher positivity (33.3%) in the fresh examination of the excreta. On the 20th day after infection (dai), infective metacyclic trypomastigote (MT) forms were predominant in the excreta of 3/4 experimental groups, and on the 30th dai, the different parasitic forms were observed in the IC of all the groups. Higher percentages of MT were observed in the excreta of the 5th instar nymphs group (84.1%) and in the IC of the 3rd instar nymphs group (80.0%). *Rhodnius robustus* presented high susceptibility to infection since all nymphs were infected, regardless of the method used for blood meal, in addition these insects demonstrated vector competence for TcIV with high rates of metacyclogenesis being evident.

Keywords: Chagas disease, vectors, parasite, interaction, susceptibility to infection, vector competence.

Introduction

Trypanosoma cruzi (Chagas, 1909), the etiological agent of Chagas disease (CD) or American trypanosomiasis, affects approximately 6-7 million people, mainly in Latin America, where the disease is endemic (WHO, 2021). This hemoflagellate protozoan is widely distributed in nature, circulating between vector insects (Hemiptera, Reduviidae, Triatominae) and various classes of mammals, including humans (Catalá et al., 2017; de Fuentes-Vicente et al., 2018). Infection of humans and other mammals mainly occurs by contact of injured skin or intact mucosa of the vertebrate host with the feces/urine of the triatomine naturally infected with *T. cruzi* (Catalá et al., 2017; Justi and Galvão, 2017).

T. cruzi strains are classified into six distinct genetic lineages or discrete typing units (DTUs), from TcI to TcVI (Zingales et al., 2012, 2009). TcIV, just like TcI, is associated with the wild transmission cycle and specimens have been isolated from CD outbreaks in the Brazilian Amazon region associated with oral infection in humans (Marcili et al., 2009; Monteiro et al., 2012).

Previous studies have shown that infection of mice using TcIV strains from Amazonas state of Brazil produces a varied susceptibility profile to benznidazole (BZ) (Teston et al., 2013), giving rise to blood forms that are predominantly slender compared to infections using TcI and TcII strains, in addition to a lower rate of *in vitro* metacyclogenesis (Abegg et al., 2017). In comparison with TcII strains from Paraná state, TcIV strains have lower pathogenicity (ability to cause inflammatory processes and parasitism in different tissues) in mice inoculated by the intraperitoneal route (IP) (Meza et al., 2014; Gruendling et al., 2015). It was also reported that the oral infection of mice with metacyclic trypomastigote (MT) forms of the TcIV strains, obtained from culture, is more severe than IP infection, as characterized by later and higher parasitemia (Teston et al., 2017).

Different strains of *T. cruzi* are co-evolving with the triatomine insect vector and this could explain why there is such variation in susceptibility to infection, even among strains belonging to the same DTU, but from different geographic origin. The evaluation of the vectorial capacity of triatomines of the species *Triatoma pallidipennis* (Stål,1872) infected with two strains of TcI from different geographical origins showed that insects infected with one strain grew more than those infected with the other strain (Cordero-Montoya et al., 2019). However, survival was reduced, with subsequent increased in oviposition and egg hatching, suggesting the insect mounts an adaptive response to *T. cruzi* infection

to produce offspring before the end of their lifespan. That is, even using the same DTU, the geographic origin can influence the triatomine-*T. cruzi* interaction, impacting the vector capacity (Cordero-Montoya et al., 2019).

Species of the genus *Rhodnius* Stål, 1859 are among the main vectors of *T. cruzi* in some countries of South America and the northern region of Brazil specifically (Justi and Galvão, 2017; Mosquera and Lorenzo, 2020). *Rhodnius robustus* is a paraphyletic taxon divided into four distinct genetic groups (I-IV) (Monteiro et al., 2003; Pavan et al., 2013; Justi and Galvão, 2017; Barnabé et al., 2018). It is mostly related to the wild environment, as it inhabits different palm tree species, with a wide distribution in the Amazon region and high rates of trypanosomatid infection. Human residences close to palm trees are often invaded by winged adults (Abad-Franch et al., 2015, 2009; Barreto-Santana et al., 2011; Rocha et al., 2001). In the Brazilian Amazon, *R. robustus* and *R. brethesi* have been frequently found harboring DTUs TcI and TcIV, while *R. pictipes* and *R. pallescens* (Colombia), harbor only TcI (Marcili et al., 2009; Monteiro et al., 2012, 2013; Julião et al., 2021).

In studies on the interaction of different *T. cruzi* strains with the main vector species, variation both in the protozoa population density and the infection rates of the insects has been observed (Alvarenga and Bronfen, 1997; Coura and Borges-Pereira, 2012; Julião et al., 2021); infection rates can vary from 9.8% (*Triatoma dimidiata*, Latreille, 1811) to 91.4% (*Triatoma pseudomaculata*, Corrêa and Espínola, 1964), with a mean rate of 67.3%. This wide variation in the positivity rate across vector species can be explained by proximate and ultimate mechanisms. The first refers to the biochemical and physiological explanations, as temperature oscillations and nutritional state of the invertebrate host that can affect the parasite development, multiplication, and differentiation into MT (Melo et al., 2020). Triatomines are resistant to temperature variation (18 ºC to 40 ºC), however, experimental studies show that when there is colonization by *T. cruzi*, high temperatures can influence the parasite-vector relationship, through greater development and increased rates of reproduction by insects. In addition, we have activation of an enzymatic cascade (propenoloxidase and phenoloxidase) aimed at immunological protection against invading pathogens, such as *T. cruzi* (González-Rete et al., 2019). In addition, the microbiome composition, which is different for each host species, tends to be more diverse in triatomine species positive for *T. cruzi* (Rodríguez-Ruano et al., 2018). Ultimate mechanisms refer to the

112 evolutionary explanations. In parasite-host relationships the former manipulates the latter so that the
113 parasite can enhance its own success, as in the case of *T. pallidipennis*, which become more attracted to
114 human odors when infected. Therefore, the use of evolutionary ideas to understand the parasite-host
115 relationship is a potential area for controlling both the parasite and the insect vector (Córdoba-Aguilar,
116 2020).

117 The transmission of *T. cruzi* to hosts depends on the vectorial capacity of the triatomine, i.e., its
118 susceptibility to the parasite strain, the time interval between the blood meal and defecation, and the
119 frequency of repasts (Galvão et al., 1995; Cordero-Montoya et al., 2019). It also depends on its vector
120 competence, that is, the ability of the insect to feed on an infected host and to generate infective forms
121 capable of reaching the vertebrate host (Dye, 1992). Susceptibility to *T. cruzi* infection and
122 metacyclogenesis, i.e., the transformation of non-infective forms of the parasite into infective forms,
123 which occurs in the digestive tract of the vector, are unknown for *R. robustus*, both in natural and
124 experimental infection (Barreto-Santana et al., 2011; Coura and Borges-Pereira, 2012). The vector
125 competence of this species is also not completely understood, and there are few studies on experimental
126 infection of *R. robustus* with TcIV (Dworak et al., 2017). In the present study, in addition to the
127 variables of susceptibility to infection (determined by the protozoa population density in the intestinal
128 contents of insects and the rate of infection) and vectorial capacity (ability to generate and eliminate
129 infective forms of *T. cruzi* in the excreta), the survival of the insects was also evaluated, since this can
130 also be influenced by the parasite-vector interaction.

131 Our working hypothesis is that the triatomine species *R. robustus* experimentally infected with
132 the TcIV strain reproduces what is observed in nature in terms of susceptibility to infection and vectorial
133 capacity. Using insects belonging to a known lineage (genetic group II), the current study can provide
134 subsidies on its relevance as a vector of *T. cruzi*, both by the classical vector transmission pathway and
135 by the oral route. The results obtained here on *T. cruzi* vector competence could also provide support to
136 the epidemiological reasoning of orally acquired CD in the Amazon region where outbreaks of infection
137 by this *T. cruzi* DTU have occurred.

138

139 **Materials and methods**

Ethical aspects

The use of the *T. cruzi* strain obtained from human was approved by the Fundação de Medicina Tropical Dr. Heitor Vieira Dourado ethical committee (registration number 360/07). The use, maintenance, and care of the mice followed the guidelines of the Conselho Nacional de Controle de Experimentação Animal (CONCEA) and were approved by the Ethics Committee on the Use of Animals at the Universidade Estadual de Maringá (UEM) (registration number 023/2014).

Trypanosoma cruzi strain

In this study, the *T. cruzi* strain AM14 (TcIV) was used, which was first obtained from a patient in the acute phase of infection during an outbreak of oral CD that occurred in Coari, Amazonas, Western Brazilian Amazon (Monteiro et al., 2012b). The strain is maintained in LIT (Liver Infusion and Tryptose) medium (Camargo, 1964) and cryopreserved as part of the collection of trypanosomatids at the Chagas Disease Laboratory (LDCh) at UEM.

Triatomines and their maintenance

During their life cycle, from egg to adult insect, members of the Triatominae subfamily go through five nymphal instars. The 3rd and 5th instar nymphs of *R. robustus* were used. The colonies of these insects are derived from Loreto, Peru and were kindly donated by the Laboratório Nacional e Internacional de Referência em Taxonomia de Triatomíneos do Instituto Oswaldo Cruz/Fiocruz. Insects were maintained under controlled conditions of temperature (~26 ^{o}C), relative air humidity (~60%), and light/dark cycle (12/12 h), in the Parasitology Sector of the Department of Basic Health Sciences at UEM.

Sequencing of the R. robustus lineage

The DTU (genetic lineage) of *T. cruzi*, as well as the genetic group to which the triatomine species belongs, can influence the parasite-vector interaction. Therefore, the *R. robustus* genetic lineage was determined by sequencing the mitochondrial cytochrome b (Cytb) gene, as described by Monteiro et al., (2003) and Pavan and Monteiro, (2007). The sequencing of the Cytb gene of *R. robustus* showed that the specimens used in this study belongs to genetic group II.

Experimental groups and infective meal

In total, forty nymphs of *R. robustus* were used, with four experimental groups that varied in terms of the age at which the insects were infected, and the way infection took place. Furthermore, culture-derived metacyclic trypomastigote (CMT) forms were used to infect insects from groups G1 and G2, and blood trypomastigote (BT) forms for insects from groups G3 and G4. Thus, the groups, containing 10 nymphs each, were as follows: Group 1 (G1), 3rd instar nymphs infected through an artificial feeder with CMT; Group 2 (G2), 5th instar nymphs infected through an artificial feeder with CMT; Group 3 (G3), 5th instar nymphs infected through an artificial feeder with BT; and Group 4 (G4), 5th instar nymphs infected with BT by direct feeding on an infected mouse. The 5th instar nymphs of G2 were used since the 3rd instar nymphs (G1) were small, which makes handling them difficult during the experiment compared to the 5th instar nymphs from group 2. Hence, insects of this age were used in the subsequent groups, G3 and G4, varying only the method of infection between these groups.

The feeding of the insects with the infective blood meal was carried out in the following ways: 1) by using an artificial feeder containing blood of mouse collected with heparin and inoculated with 2×10^6 CMT/mL of culture in LIT medium (G1 and G2); 2) by using an artificial feeder containing blood inoculated with 2,800 BT/0.1 mL of blood (Alvarenga and Bronfen, 1997) (G3); 3) by placing the insects directly on a mouse infected with *T. cruzi* (with parasitemia of 1,400 BT/0.1 mL) (Brener 1962) (G4). Insects of G1 and G2 were fed individually while G3 and G4 were fed in groups. The parasite concentrations of the inocula were chosen based on our experience in the inoculation of mice and triatomines with *T. cruzi*, as a much higher infectivity is observed for BT forms compared to CMT forms. In addition, the concentrations of the BT forms in G3 and G4 was smaller than for CMT forms in G1 and G2 to more closely mimic the transmission cycle occurring in nature.

Evaluation of the volume of blood ingested

The nymphs were weighed in a semi-analytical balance (BK 300 GEHAKA) before and after the infective meal. The person that recorded weight of the insects, as well as the person that examined the excreta and the IC, did not know which group the insects belonged to. Insects with weight gain ≥1 mg after meal were considered fed according to Alvarenga and Bronfen (1997). To determine the hematophagic capacity of the species, the volume and number of parasites ingested were estimated,

194 considering that 1 mg of weight gain was equivalent to 1 µL of blood ingested. For G1 and G2, an
195 inoculum of 2×10^6 CMT/mL was used, corresponding to 2×10^3 CMT/µL.

196 As the insects of G3 and G4 were fed in groups, these parameters could not be calculated.

197 *Analysis of the excreta*

198 The insects were maintained for a total period of 120 days following the infective meal and were
199 fed every 20 days on an uninfected mouse that had been anesthetized with ketamine (100 mg/kg) +
200 xylazine (5-10 mg/kg) (1:2). During or after the blood meal, the excreta (feces/urine) were collected and
201 diluted in 100 µL of 0.15M PBS (pH 7.2). For the fresh examination (FE) in glass slides, aliquots of 5
202 µL of excreta for each insect we collected for groups 1 and 2, and a pool of excreta (approximately 50
203 µL) was collected for groups 3 and 4. The material was examined using an optical microscope with 400x
204 magnification (Brener 1962). In addition, global counts (GC) in a Neubauer chamber were performed
205 using 10 µL of the excreta (Alvarenga and Bronfen, 1997). From these analyses, the percentage of
206 positive insects in the fresh examination (%+FE) and the percentage of positive insects in the global
207 count (%+GC) could be determined.

208 A differential count (DC) was performed using 10 µL of excreta stained with Giemsa to
209 determine the number and proportion (%) of metacyclic trypomastigotes (MT), epimastigotes (EP), and
210 spheromastigotes (ST) forms for each insect. In the DC, the whole smear was examined on a microscope
211 at 100x magnification, due to the low amount of parasitic forms (PF) visualized.

212 *Analysis of intestinal contents*To evaluate the susceptibility of *R. robustus* to experimental
213 infection with the AM14 strain of TcIV, the insects were evaluated every 30 days following the infective
214 meal, two insects of each group were dissected, and their intestines were removed and macerated with
215 100 µL of 0.15 M PBS. From this macerate of the IC, 5 µL was used to perform the Brener (1962)
216 technique of FE, 10 µL for the GC, and 10 µL for Giemsa-stained smears for the DC. The same
217 parameters previously described (%+FE, %+GC and %+DC) were analyzed. Alcohol 70 °GL (500 µL)
218 was added to the remaining excreta and IC samples, which were stored in a freezer at -20 °C until DNA
219 extraction.

220 *Conventional polymerase chain reaction (cPCR)*

T. cruzi DNA was extracted from the excreta and IC of the insects using the conventional phenol/chloroform method described by Macedo et al. (1992). Primers 121 (5-AAATAATGTACGGG(T/G)GAGATGCATGA-3) and 122 (5-GTTCGATTGGGGTTGGTGTAATATA-3) were used to amplify a 330 base pair (bp) fragment of the kinetoplast minicircle DNA (kDNA), and subjected to 35 cycles of amplification in a Techne TC-512 thermal cycler. PCR products were visualized on 4.5% polyacrylamide gel and stained with silver, as described by Miyamoto et al. (2008). A 100 bp DNA molecular marker from Amresco (Ohio, USA) was used to confirm the correct fragment size.

Determination of infectivity and mortality rates

The infectivity rate was obtained by the ratio between the number of infected insects and the number of insects submitted to the infective blood meal × 100. The insect that presented a positive result in at least one of the techniques used (FE, GC, DC, and cPCR) was considered infected.

The analysis of survival of the insects in the different experimental groups was performed on the feeding and dissection days (20, 30, 40, 60, 80, 90, 100 and 120 days after infection) throughout the experiment in order to plot the Kaplan-Meier curve and obtain the cumulative mortality rate (%MORT). The dissected insects were not used for the mortality rate, since this was not a death related to the infection or experimental conditions.

Statistical analysis

Data were tabulated and statistically analyzed using Statistica Single 13.2 and R 3.0.2 software. In the two-by-two comparison between groups, the non-parametric Mann-Whitney test was used for quantitative variables and Fisher's exact test for qualitative variables. The level of significance adopted was 5% ($p < 0.05$).

First, the hematophagic capacity of the insects, in terms of the volume of blood ingested, will be described, followed by the number of PF (metacyclic trypomastigotes, MT, or blood trypomastigotes, BT) ingested.

Second, the variables related to susceptibility to infection are reported. That is, the presence and number of PF in the intestinal contents (IC) of the insects, in relation to their age (3rd or 5th instar nymphs) and the method of infection (by artificial feeder or on an infected mouse). Based on the results

of the fresh examination (FE), the global count (GC), PCR, and the cumulative mortality rates, the infectivity rates of each experimental group were determined.

Third, the variables related to vectorial capacity will be discussed. That is, the number of infective forms (MT) present in the IC. This is important in the oral transmission of *T. cruzi*, as the insect can be crushed during food processing thereby exposing the PF present in its IC. Whereas the variable "number of infective forms in excreta" is important in cases of transmission via the classic vector route, where contamination of the vertebrate host occurs through contact of injured skin or mucosa with the insect's excreta after the blood meal.

The differential counts and metacyclogenesis rates (%MC) of the experimental groups will be determined. Finally, the survival analysis were performed using GraphPad Prism 8.0.1

Results

Volume and average number of trypomastigote forms ingested per insect

The hematophagic capacity of *R. robustus* was evaluated by determining the volume of blood ingested by the 5th instar nymphs that were fed the infective blood meal using the artificial feeder (G2, n=10). Differences in the pre- and post-infective meal weight (3,10; p=0.0010) were observed, with the nymphs ingesting an average of 74.5 mg of blood. As the concentration of the inoculum was known, the number of PF ingested by the insects could be determined, which corresponded to 149,000 CMT/µL.

The hematophagy capacity and efficiency of infection was compared between insects fed on the artificial feeder and the pre-infected mouse. For the artificial feeding, a parasite concentration of 2,800 BT/0.1 mL was obtained by mixing blood collected from TcIV-infected mice, around the day of the parasitaemia peak, with blood from uninfected mice. For the feeding on mice pre-inoculated with the AM14 (TcIV) strain, all insects were fed directly on the same animal, which had a parasitemia of 1.400 BT/0.1 mL. The insects fed on the artificial feeder (G3) ingested on average 198.0 mg of blood, which corresponds to a mean of 555.5 BT/µL, while the insects fed directly on the mouse (G4) ingested on average 130.0 mg of blood, which corresponds to a mean of 92.2 BT/µL.

The hematophagic capacity was also compared between the insects of the G2 (CMT) and G3 (BT) groups, which were both fed on an artificial feeder. There was a statistical difference in the weight

277 gain between the groups (3.06; p=0.0073), with insects of G3 exhibiting a greater weight gain (**Table 1**).
278 Although the method of infection was the same for G2 and G3, the origin and concentration of the
279 trypomastigotes in the inoculum differed, preventing the comparison of the average number of ingested
280 forms. Furthermore, the volume and number of PF ingested by the 3rd instar nymphs (G1, n=10) was not
281 calculated due to their small size, which prevented individual specimens from being weighed.
282

Table 1 Average weight gain (mg) for 5th instar nymphs of *Rhodnius robustus* fed on an artificial feeder containing mouse blood inoculated with either *Trypanosoma cruzi* (AM14-TcIV) metacyclic trypomastigotes obtained from culture (G2 group) or blood trypomastigotes (G3 group).

G2 (n=10)			G3 (n=10)			*p* value
Mean	±	Std. Dev.	Mean	±	Std. Dev.	
0.07	±	0.07	0.20	±	0.08	0.0073*

*significant p-value by the Mann-Whitney test considering the level of significance of 5%.

283

284 *Susceptibility to infection*

285 In the IC, there was a significant difference in FE positivity between the four experimental groups
286 (p=0.0001), being greater for G2 (33.3%) and negative (0.0%) for G1 (**Table 2**). The IC of the insects in
287 G1 and G2 were submitted to a lower number of FE over the 120 days, as this method was applied
288 starting from the 60th day after infection (dai) for these groups, as opposed to 30 dai for G3 and G4. In
289 addition, there was variation in the total number of exams performed per group due to insect deaths that
290 occurred throughout the experiment (**Table 2**). GC positivity did not significantly differ between groups
291 G1 and G2.
292

Table 2 Infection positivity rates based on fresh examination (+FE) and global counts (+GC) of the intestinal contents of 3rd instar (G1) and 5th instar (G2) nymphs of *Rhodnius robustus* infected with metacyclic trypomastigotes of *Trypanosoma cruzi* (AM14-TcIV), obtained from culture or blood, through an artificial feeder (G3) and by feeding directly on a pre-infected mouse (G4).

Number of positive tests/Total of tests performed (%)					
Groups	G1	G2	G3	G4	*p* value*
+FE	0/4 (0.0)	2/6 (33.3)	1/8 (13.0)	2/8 (25.0)	0.0001
+GC	3/5 (60.0)	3/8 (38.0)	NP	NP	NS

*Fisher's exact test, considering significance level of 5%; NP = not performed; NS = not significant.

293

294 After the dissections of the digestive tract of the insects, it was possible to observe both MT and
295 EP forms concomitantly, regardless of the day after infection on which the insect was examined (**Fig. 1**).
296 Over the period of 120 days following the infective blood meal, a variation in the average number
297 of PF found in the IC of the four experimental groups was observed. On the first IC test performed (30th
298 dai), MT were visualized for all groups, with greater amount in the G2 group (**Table 3**). However, from
299 the 30th to the 60th dai, there was a decrease in the number of MT for groups G2, G3, and G4, and a
300 slight increase for G1. On the 90th dai, all groups had the lowest number of PF, which remained low
301 until the 120th dai, except for G2, which displayed a modest increase in the number of MT and EP
302 forms. A significant increase in the number of EP forms, reaching more than 350, occurred on the 60th
303 dai in the G2 group. On the 120th dai, another lower MT peak was observed for groups G1, G2, and G3,
304 but not for G4.

305

Table 3 Number of parasitic forms in the intestinal contents of 3rd instar (G1 group) and 5th instar (G2 group) nymphs of *Rhodnius robustus*, infected with *Trypanosoma cruzi* (AM14-TcIV) metacyclic trypomastigotes obtained from culture, or with blood trypomastigotes (BT) (G3 group), through artificial feeder; and infected with BT by feeding directly on a pre-infected mouse (G4 group).

Days after infection	G1			G2			G3			G4		
	MT	EP	ST	MT	EP	ST	MT	EP	ST	MT	EP	ST
30	6	1	0	141	22	0	3	3	7	32	37	12
60	15	2	0	107	354	0	0	2	2	0	0	0

90	8	5	0	9	3	0	0	1	0	0	0	0
120	11	2	0	21	9	0	6	1	0	1	2	0
Total	40	10	0	278	388	0	9	7	9	33	39	12

MT = metacyclic trypomastigote, EP = epimastigote and ST = spheromastigote.

306

307 *Molecular analysis*

308 Amplification of the *T. cruzi* minicircle kDNA from the IC and excreta was performed by cPCR
309 for the insects in G4 only, to verify the infection as this group displayed a low infectivity rate by the
310 visual examination methods. The presence of *T. cruzi* DNA was detected through the visualization of the
311 330 bp fragments in both the IC and excreta samples of all insects (100% positivity).

312

313 *Infectivity and mortality*

314 The insects of G1 and G2 were fed and examined individually, making it possible to determine
315 the infectivity rate, which was 100.0% for both. This parameter was not obtained for groups G3 and G4,
316 since it was examined a pool of the biological materials of the insects within both groups. Regarding the
317 cumulative mortality, the groups G1 (20th, 40th, and 60th dai; 3/10) and G2 (40th dai; 2/10) had rates of
318 30.0 and 20.0% respectively, whereas, for the G3 (20th and 40th dai; 2/10) and G4 (40th, 60th, and 80th
319 dai; 3/10) these rates were 20.0 and 30.0%, respectively, with no statistical differences. The Kaplan-Meier
320 with the survival curve are shown in **Fig. 2**.

321 *Vectorial capacity*

322 The vectorial capacity of *R. robustus* was determined by evaluating the number of infective MT
323 forms present both in the IC and in the excreta of the insects, and by the rate of metacyclogenesis.

324 The average proportion of MT in the IC was 80.0% for the 3rd instar nymphs fed on an artificial
325 feeder with CMT (G1); 42.0% for the 5th instar nymphs fed on an artificial feeder with CMT (G2);
326 22.0% for the 5th instar nymphs fed on an artificial feeder with BT (G3); and 39.0% for the 5th instar
327 nymphs fed directly on an infected mouse (G4), with significant difference between the groups
328 ($p=0.0002$).

As with IC of the insects, FE positivity in the excreta varied between the four experimental groups (p=0.0023), with the highest rate for G1 (33.3%), and negativity (0.0%) for G3 (**Table 4**). On the other hand, the GC positivity of the excreta did not vary between G1 and G2.

Table 4 Infection positivity rates based on fresh examination (+FE) and global count (+GC) of the excreta of 3rd instar (G1) and 5th instar (G2) nymphs of *Rhodnius robustus* infected with trypomastigotes of *Trypanosoma cruzi* (AM14-TcIV), obtained from culture or blood, through artificial feeding (G3) and by feeding directly on a pre-infected mouse (G4).

	Number of positive tests/Total of tests performed (%)				
Groups	G1	G2	G3	G4	*p* value*
+FE	2/6 (33.3)	2/10 (20.0)	0/25 (0.0)	2/30 (7.0)	0.0023
+GC	3/18 (16.7)	5/25 (20.0)	NP	NP	NS

*Fisher's exact test, considering significance level of 5%; NP = not performed; NS = not significant.

The average number of PF found in the excreta varied between the four experimental groups throughout the 100 days following the infective blood meal. On the 20th dai, a predominance of MT forms was observed for G1, G2, and G3; however, no parasitic form was detected in G4 at this time (**Table 5**). There was a decrease in the amount of MT in G1 and G2 from the 20th dai until the 60th dai, at which point the number increased again, reaching a peak on the 80th dai. This was not the case for G3 in which the MT numbers continued to decrease to the end of the evaluation period. The highest number of MT for the G4 group occurred on the 40th dai. However, only G2 showed PF in its excreta by the 100th dai (**Table 5**).

Table 5 Number of parasitic forms in the excreta of 3rd instar (G1 group) and 5th instar (G2 group) nymphs of *Rhodnius robustus*, infected with *Trypanosoma cruzi* (AM14-TcIV) metacyclic trypomastigotes obtained from culture, or with blood trypomastigotes (BT) (G3

group), through an artificial feeder; and infected with BT by feeding directly on a pre-infected mouse (G4 group).

Days after infection	G1			G2			G3			G4		
	MT	EP	ST	MT	EP	ST	MT	EP	ST	MT	EPI	ST
20	29	16	0	140	31	0	10	5	0	0	0	0
40	24	2	0	26	2	0	8	0	0	20	1	1
60	1	1	0	11	2	0	7	3	0	15	5	0
80	15	5	0	109	19	0	0	0	0	8	11	1
100	0	0	0	6	1	0	0	0	0	0	0	0
Total	69	24	0	292	55	0	25	8	0	43	17	2

MT = metacyclic trypomastigote, EP = epimastigote and ST = spheromastigote.

The average proportion of MT forms in the excreta was 74.2% for the 3rd instar nymphs fed on an artificial feeder with CMT (G1); 84.1% for the 5th instar nymphs fed on an artificial feeder with CMT (G2); 75.8% for the 5th instar nymphs fed on an artificial feeder with BT (G3); and 69.4% for the 5th instar nymphs fed directly on an infected mouse (G4), with significant variation (0.86; p=0.0022).

Discussion

This is the first time that it was possible to observe that the 3rd and 5th instar nymphs of *R. robustus* became infected with *T. cruzi* IV (AM14 strain) following experimental feeding using either an artificial feeder containing CMT or BT forms, or feeding directly on an experimentally infected mouse. Regardless of the nymphal instar, this triatomine species presented susceptibility to infection with this DTU under laboratory-controlled conditions, since all the insects analyzed were infected (100% infectivity).

Three experimental groups consisted of 5th instar nymphs (G2, G3 and G4) were infected with either CMT (G2) or BT (G3 and G4) forms, by means of an artificial feeder (G2 and G3) or infected mouse (G4). Even taking into account the differences in the type of infection, the insects of G2 ingested a greater amount of infective forms (mean 149,000 CMT/µL). The larger ingestion of infective forms subsequently influenced the protozoa population density in the insect, as G2 presented a higher total number of PF in the IC over the 120 days of infection (666 PF), as compared to G3 (25 PF) and G4 (84 PF) (**Table 3**). These results differed from those of other authors who used mice infected with *T. cruzi*

363 strains belonging to three different DTUs (Zingales et al., 2009) to infect triatomines of the species *T.*
364 *infestans* (Alvarenga and Bronfen, 1997). They observed that despite insects ingesting different amounts
365 of infective forms of the same strain, they had practically the same number of MT, indicating a trend
366 towards numerical stabilization of the population of flagellates throughout the intestinal tract of
367 triatomines. In addition to maintaining the infection for longer, the TcI (YuYu) and TcVI (CL) strains,
368 both isolated from naturally infected *T. infestans* from Rio Grande do Sul, southern Brazil, had a higher
369 number of MT than those infected with TcII (Y strain). These data suggest that both the invertebrate host
370 species and the geographic origin of the *T. cruzi* strain could influence the parasite-vector interaction.
371 Triatomines with these infective forms inside, when crushed together with foods such as palm fruits, can
372 contaminate them, favoring the oral transmission of *T. cruzi*.

373 The forms EP, MT, and ST of *T. cruzi* were observed in both the excreta and in the IC of insect
374 vector. The proportion of each of these PF in the different compartments of the insect's digestive tract
375 was not evaluated in this study. However, it is known that EP and ST forms are generally observed
376 colonizing the initial and middle portions of the digestive tract, while MT are predominant in the distal
377 portions and in the Malpighi tubes (Brener, 1979; Dias, 2006; Dworak et al., 2017).

378 In the comparison of the results obtained for the experimental groups of the present study, it was
379 possible to observe variations in the analyzed parameters (susceptibility to infection and vector
380 competence). However, due to the low weight of the 3rd instar nymphs of G1, it was not possible to
381 obtain some parameters for this group. For the groups with the 5th instar nymphs, G2 showed a greater
382 susceptibility to infection and higher vector competence than G3 and G4. The highest number of PF
383 were found for G2, both in the excreta and in the ICs, which may be related to the parasite concentration
384 of the inoculum used. When evaluating this last parameter over 120 days following the infective blood
385 meal, there was a tendency towards a decrease in the number of PF from the 60th dai. The insects of G2,
386 however, differed from the other groups, with a greater number of both EP and MT forms, which peaked
387 on the 60th dai and were still present on the 120th dai. The decline in the abundance of *T. cruzi* can be
388 explained by immunological mechanisms in the intestinal lumen of the triatomines that, when activated,
389 try to eliminate the parasite, as has been observed in experimental infections of *R. prolixus* with TcII (Y
390 strain) (Garcia et al., 2010). A worse parasite-vector interaction was observed between the Y strain with

391 *T. infestans*, since flagellates were not present in more than 25% of insects at 30 days after infection
392 (Alvarenga and Bronfen, 1997). These data suggest that the protozoa population density in the IC of the
393 insect may be influenced by the *T. cruzi* DTU.

394 Metacyclogenesis, which is the ability to differentiate from the EP form of the parasite into the
395 MT form, is a relevant biological parameter in the study of *T. cruzi* strains since it is a process involving
396 the transformation of a non-infective form into an infective form (Alvarenga and Bronfen, 1997; Brener,
397 Z, 1979; Contreras et al., 1985; Dias, 2006). This process is directly related to the epidemiology of the
398 infection. In the present study, the rate of metacyclogenesis (%MC) was 84.1% (on the 20th dai) in the
399 excreta, and 80.0% (on the 30th dai) in the IC. These data demonstrate a high susceptibility to infection
400 and high vector competence of *R. robustus* for the AM14 strain. The vector competence of triatomines
401 experimentally infected with different *T. cruzi* strains were previously reported by our team (Dworak et
402 al., 2017), with infection rates being higher between sympatric pairs, i.e., *R. robustus* and TcIV, and
403 *Triatoma sordida* and TcII. For the non-sympatric pair of *R. robustus* and TcII, no vector competence
404 was observed since infective forms were not present in their excreta (Dworak et al., 2017). In addition,
405 an infectivity rate of 100% was obtained when mice were orally inoculated with MT of TcIV strains,
406 including AM14, both derived from *R. robustus* and culture, confirming the vector competence of *R.*
407 *robustus* for these strains (Teston et al., 2017; Zanusso et al., 2018). In the Brazilian Amazon region,
408 specimens of this triatomine species have already been found naturally infected with this DTU and were
409 captured close to the locations where outbreaks of acute CD by oral transmission occurred in both the
410 states of Pará (Marcili et al., 2009) and Amazonas(Monteiro et al., 2012).

411 In the present study, the biochemical and physiological mechanisms that could affect the
412 morphogenesis of the parasite, such as the temperature and nutritional state of the invertebrate host
413 (Melo et al., 2020), were kept constant, with the insects of all groups being regularly fed every 20 days
414 on an uninfected mouse. The microbiome composition was also kept constant, since we use insects of the
415 same species and genetic group. A previous study concerning *in vitro* metacyclogenesis of TcI, TcII, and
416 TcIV strains, including AM14, using different culture media, reported variation in the %MC depending
417 on the strain and DTU of the parasite (Abegg et al., 2017). It was demonstrated that the AM14 strain,
418 despite what was found in this study with experimentally infected triatomines (whose %MC was ≥80%),

had a low %MC, which varied from 2.7% (in M16 medium) to 4.6% (in LIT medium). These data show that, in addition to the genetic lineage and geographical origin of the parasite and the triatomine species, metacyclogenesis and, consequently, vector competence, could be influenced by other factors, and evolutionary mechanisms may be acting on parasite-host relationships (Córdoba-Aguilar, 2020). *R. robustus* has been considered one of the main vectors in the Brazilian Amazon region and *T. cruzi* strains of the DTU TcIV have been frequently isolated from triatomines of this species in this region, where they have been co-evolving (Marcili et al., 2009; Monteiro et al., 2012). For the triatomine species and *T. cruzi* strain evaluated, no significant difference was observed in the survival rate of insects from the different experimental groups despite differences in nymph age, type of infection and origin of the infective forms used in the study. This also suggests a good adaptation of strain AM14 (TcIV) to *R. robustus*.

The genetic constitution of the invertebrate host may be directly related to its immunological and physiological mechanisms. Physiological alterations can occur in the digestive tract of the insect vector since the parasite feeds on hemoglobin and other substances present in the ingested blood. In addition, the amount of hemoglobin present in the blood and the action of digestive and hemolytic enzymes influence the process of parasite differentiation, stimulating or inhibiting its development (Alvarenga and Bronfen, 1984, 1997; Garcia and Azambuja, 1991; Garcia et al., 1995).

The results of the kDNA-cPCR molecular analysis of the excreta and IC of *R. robustus* 5th instar nymphs that directly fed on an infected mouse (G4 group) were positive on all the days in which these samples were collected. These data confirm the sensitivity of this method for detection of *T. cruzi* DNA in these biological materials (Miyamoto et al., 2008; Dworak et al., 2017).

The techniques used in this study indicated high rates of positivity; however, the specificity and sensitivity presented by the smear stained with Giemsa validate it as the best parasitological method for this type of study, since it allows a differential count of the developmental stage of *T. cruzi*. On the other hand, cPCR confirmed infection with the TcIV DTU in all insects. It is known that the genetic lineage of the vector, and not only the species, may influence its ability to transmit *T. cruzi* and its DTUs. Therefore, susceptibility to infection and vector competence must be determined for other cryptic species of *R. robustus*.

Conclusion

Rhodnius robustus had high susceptibility to the experimental infection with *T. cruzi* IV, as evidenced by the presence of proliferative forms (epimastigotes) in its intestinal contents. It also demonstrated vector competence, characterized by the presence of infective forms (metacyclics) in their feces and urine, in addition to high rates of metacyclogenesis (up to 84.1%). In this experimental model, on the 20th day after infection, it was already possible to observe infective metacyclic forms in the excreta of the 5th instar nymphs fed on an artificial feeder with culture-derived metacyclic trypomastigotes. Our data also confirm the potential of the cPCR technique to be used in epidemiological studies in sympatric areas of TcIV and *R. robustus* presence, enabling the detection of *T. cruzi* in nature.

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Conflict of interest statement

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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List of abbreviations

Base pairs - bp

Blood trypomastigote - BT

Chagas disease - CD

Culture-derived metacyclic trypomastigotes - CMT

Days after infection - dai

Deoxyribonucleic acid - DNA

Differential count - DC

Discrete typing units – DTUs

Epimastigote - EP

Fresh examination - FE

Global count - GC

Gram - g

Group 1 - G1

Group 2 - G2

Group 3 - G3

Group 4 - G4

Intestinal content - IC

Kinetoplast DNA - kDNA

Liver infusion tryptose - LIT

Metacyclic trypomastigote - MT

Metacyclogenesis rate - %MC

653 Microliter - μL

654 Milligram - mg

655 Milliliter - mL

656 Millimolar – mM

657 Parasitic forms - PF

658 Percentage of positive insects in differential count - %+DC

659 Percentage of positive insects in fresh examination - %+FE

660 Percentage of positive insects in global count - %+GC

661 Phosphate buffer solution - PBS

662 Polymerase chain reaction - PCR

663 Spheromastigote - ST

664 **FIGURES LEGENDS**

665

666 **Fig. 1** Developmental stages of *Trypanosoma cruzi* observed by optical microscopy in the intestinal
667 content of an experimentally infected triatomine of the species, *Rhodnius robustus* (A) Engorged fifth
668 instar nymph; (B) epimastigote, (C) metacyclic trypomastigote, and (D) spheromastigote forms (1000X
669 magnification). Arrows indicate parasitic forms.

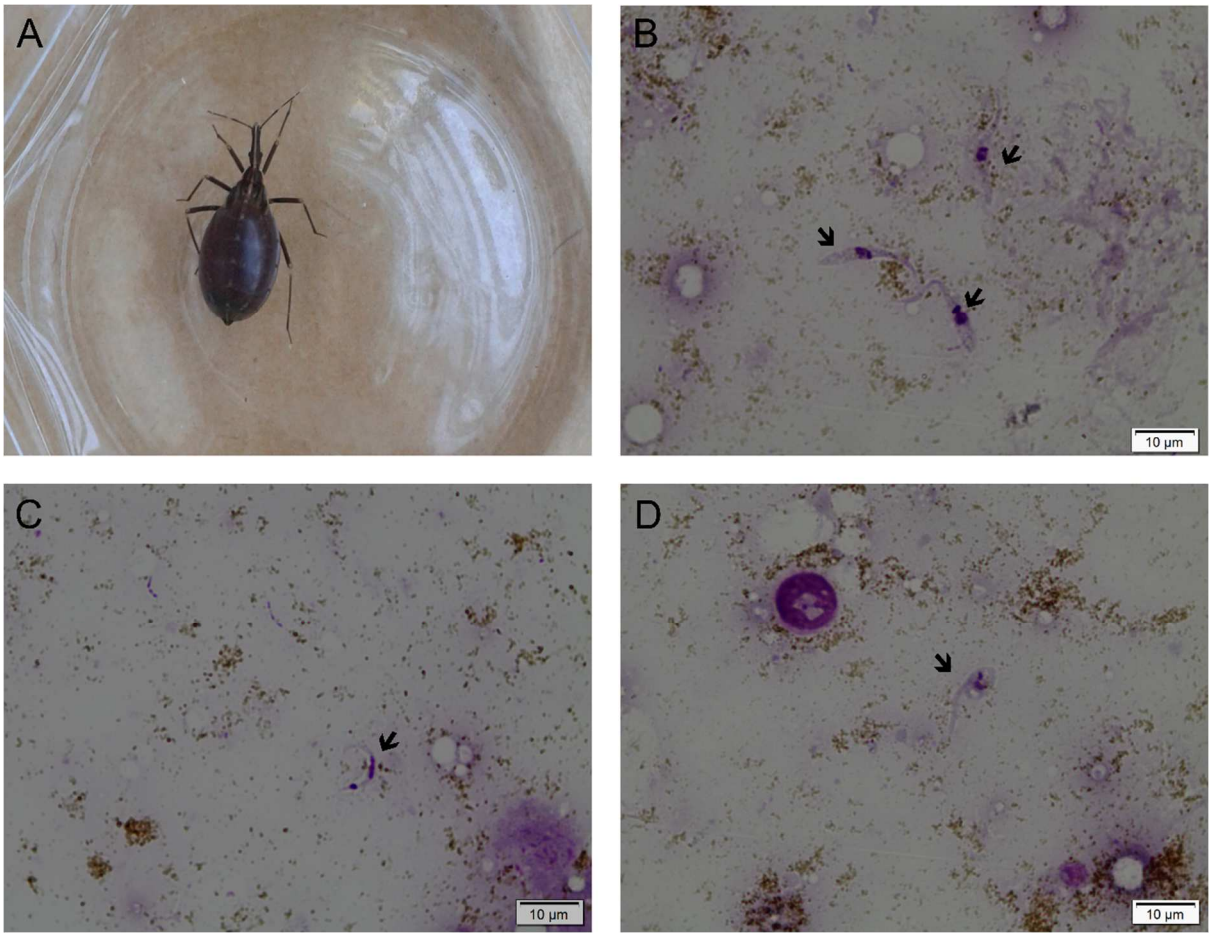
670

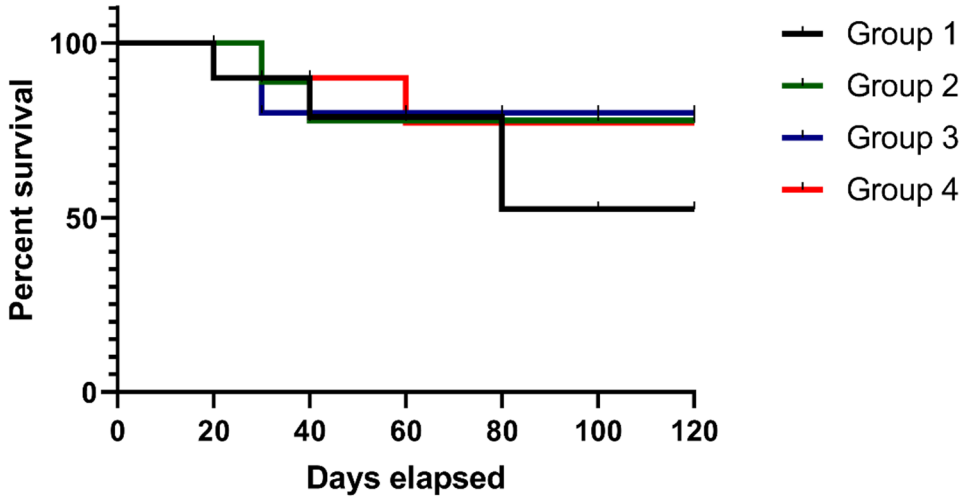
671 **Fig. 2** Kaplan-Meier curve with the survival rate 3rd instar (G1 group) and 5th instar (G2, G3 and G4
672 group) nymphs of *Rhodnius robustus* infected with *Trypanosoma cruzi* (AM14-TcIV) was performed on
673 the feeding and dissection days (20, 30, 40, 60, 80, 90, 100 and 120 days after infection) throughout the
674 experiment.

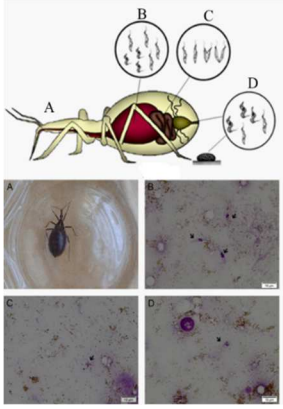
675

676 **Graphical abstract** Image of the digestive tract of the triatominae and the biological cycle of
677 *Trypanosoma cruzi* in an undetermined host. Source: http: //chagas.fiocruz.br/fisiologia/

678


A
B
10 µm
C
10 µm
D
10 µm


Percent survival
100
50
0
0
20
40
60
80
100
120
Days elapsed
Group 1
Group 2
Group 3
Group 4



Number of parasitic forms in the excreta

Days after infection	G1			G2			G3			G4		
	MT	EP	ST	MT	EP	ST	MT	EP	ST	MT	EPI	ST
20	29	16	0	140	31	0	10	5	0	0	0	0
40	24	2	0	26	2	0	8	0	0	20	1	1
60	1	1	0	11	2	0	7	3	0	15	5	0
80	15	5	0	109	19	0	0	0	0	8	11	1
100	0	0	0	6	1	0	0	0	0	0	0	0
Total	69	24	0	292	55	0	25	8	0	43	17	2

MT = metacyclic trypomastigote, EP = epimastigote and ST = spheromastigote.