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# Can *Triatoma virus* inhibit infection of *Trypanosoma cruzi* (Chagas, 1909) in *Triatoma infestans* (Klug)? A cross infection and co-infection study

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ABSTRACT

Triatoma virus occurs infecting Triatominae in the wild (Argentina) and in insectaries (Brazil). Pathogenicity of Triatoma virus has been demonstrated in laboratory; accidental infections in insectaries produce high insect mortality. When more than one microorganism enters the same host, the biological interaction among them differs greatly depending on the nature and the infection order of the co-existing species of microorganisms. We studied the possible interactions between Triatoma virus (TrV) and *Trypanosoma cruzi* (the etiological agent of Chagas disease) in three different situations: (i) when Triatoma virus is inoculated into an insect host (*Triatoma infestans*) previously infected with *T. cruzi*, (ii) when *T. cruzi* is inoculated into *T. infestans* previously infected with TrV, and (iii) when TrV and *T. cruzi* are inoculated simultaneously into the same *T. infestans* individual. *Trypanosoma cruzi* infection was found in 57% of insects in the control group for *T. cruzi*, whereas 85% of insects with previous TrV infection were infected with *T. cruzi*. TrV infection was found in 78.7% of insects in the control group for TrV, whereas insects previously infected with *T. cruzi* showed 90% infection with TrV. A total of 67.9% of insects presented simultaneous infection with both types of microorganism. Our results suggest that TrV infection could increase adhesion of *T. cruzi* to the intestinal cells of triatomines, but presence of *T. cruzi* in intestinal cells would not increase the possibility of entry of TrV into cells. Although this study cannot explain the mechanism through which TrV facilitates the infection of triatomines with *T. cruzi*, we conclude that after TrV replication, changes at cellular level should occur that increase the adhesion of *T. cruzi*.

## 1. Introduction

Triatoma virus (TrV) was first isolated in 1987 from field colonies of *Triatoma infestans* (Hemiptera, Reduviidae) at the locality Dean Funes, Cordoba Province, Argentina (Muscio et al., 1987), and again in 2002 from *T. infestans* populations from the same locality; TrV was later found in seven Argentine provinces (Marti et al., 2009). It was also found naturally infecting six other Triatominae species in Argentina, and four other triatomine species in an insectary in Brazil (Ceccarelli et al., 2015; Marti et al., 2013; Susevich et al., 2012).

TrV is the only entomopathogenic virus classified and identified within the Triatominae, and it was recently classified as a new genus "Triatovirus" within the family Dicitroviridae (Echeverría et al., 2016). The pathogenicity of TrV in the laboratory has been shown in *T. infestans*, *T. guasayana* and *T. patagonica* (Muscio et al., 2000; Rozas-dennis et al., 2002). While insects collected in domiciles and peridomiciles in Argentina showed a TrV prevalence between 10 and 20% (Marti et al., 2009), accidental infections in insectaries produce high insect mortality, approaching 90% (Marti et al., 2015).

Although both horizontal and vertical transmission have been demonstrated (directly or indirectly) in the laboratory, the former is the main propagation route for TrV under laboratory conditions; individuals become infected when they extend the proboscis to feed and come into contact with faecal material released by other infected individuals (Muscio et al., 2000). Marti et al. (2015) demonstrated that incorporation of a TrV-positive insect pair into a colony with healthy individuals alone results in 100% infection after 45–60 days. To date, it is not known which of these two routes of transmission is more relevant

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in the dispersal and persistence of TrV in the wild.

The role of symbionts in the biology, ecology and evolution of insect species has been well documented recently, and this has led to suggestions regarding their use to control pests and disease vectors (Bourtzis et al., 2014). However, when more than one microorganism (pathogen or symbiont) enters the same host, the biological interaction between them differs greatly depending on the nature of the co-existing species of microorganisms: nematode-virus (Muttis et al., 2015), bacteria-protozoan (Moreira et al., 2009), fungus-protozoan (Moraes et al., 2001), or virus-protozoan (Bian et al., 2013; Hussain et al., 2013; Moreira et al., 2009; Van Den Hurk et al., 2012; Walker et al., 2011). These biological interactions are relevant because the presence of Triatoma virus in a *T. infestans* individual infected with *T. cruzi* could lead to the biological control of the insect vectors and/or the parasite.

To date, triatomine control is mainly carried out using pyrethroid insecticides (World Health Organization, 2013). Some sources have recently estimated that the share of microbial pesticides in the pesticide market reached 3% in 2014; one of the major forces driving this increase is the impact of European legislation restricting the residual levels of most synthetic chemical pesticides (Lacey et al., 2015). Vector control with insecticides is not 100% effective because insecticide resistance is increasing (Germano et al., 2010). In recent years, a number of wild foci of *T. infestans* have been described, mainly in the Inter-Andean Valleys of Bolivia, in the Gran Chaco of Argentina, Bolivia and Paraguay, and in a Metropolitan region in Chile (Bustamante Gomez et al., 2016). Extensive recent research on the biology, ecology and evolution of microorganisms associated with insect vectors of disease, suggests that such microorganisms could be used as tools to control pests and diseases (Usta, 2013). Thus, biological control may be a valid alternative for the eradication of kissing bugs in endemic areas. Introducing or increasing the density of natural enemies in kissing bug populations may result in reduction of the number of insect vectors to levels low enough to ensure a severe reduction, and even interruption, of the vectorial transmission of *T. cruzi* to the human population.

The dynamics of pathogen-host systems are complex and usually difficult to predict, unless the factors affecting the species interaction can be determined (Richards et al., 1998). In an analysis of more than 250 kissing bugs that were naturally infected with TrV, all of them were negative to *T. cruzi* (Ceccarelli et al., 2015; Marti et al., 2009; Susevich et al., 2012).

In this work we studied the possible interactions between TrV and *T. cruzi* in three different situations: i) when TrV is inoculated into *T. infestans* previously infected with *T. cruzi*, ii) when *T. cruzi* is inoculated into the triatomine insect previously infected with TrV, and iii) when TrV and *T. cruzi* are co-inoculated into the same insect simultaneously.

## 2. Materials and methods

### 2.1. Experiment 1: Effect of T. cruzi in insects previously infected with TrV

To obtain an initial pool of TrV-positive insects, 60 days before the beginning of the experiment 50 third-stage nymphs of *T. infestans* were infected with TrV by feeding them with TrV particles [0.2 μg/ml] in a human blood solution, using an artificial feeder (Marti et al., 2015). During those 60 days, all nymphs moulted to 4th stage.

All the moulted nymphs were TrV-positive (confirmed by RT-PCR), and were then infected with *T. cruzi* using the following procedure: two male mice were infected with *T. cruzi* with a dose of 10,000 metacyclic forms of discrete typing units (DTU) TcVI/mouse, 45 days before the beginning of the experiment. The DTU used is epidemiologically important in one of the areas with the highest incidence of Chagas in Argentina, since it is the most prevalent lineage in dogs (Monje-Rumi et al., 2015) and also present in the vector insect, frequently with DTUs TcI and TcV. Subsequently, 30 fourth-stage TrV-positive nymphs (moulted 48 h before starting the experience) were allowed to feed on those *T. cruzi* infected mice. To estimate the total blood volume ingested by the *T. infestans* nymphs, each insect was weighed before and after feeding. The control consisted of 30 fourth-stage nymphs (negative to TrV) fed on two mice infected with *T. cruzi* (with the same characteristics as the experimental group).

### 2.2. Experiment 2: Effect of TrV in insects previously infected with T. cruzi

In this test we estimated the infective capacity of TrV in insects previously infected with *T. cruzi*. Thirty-five days before beginning the experiment, 50 *T. infestans* third-stage nymphs were fed using *T. cruzi*-positive mice (previously infected as in Experiment 1). During the following 35 days all nymphs moulted to 4th stage. Twenty-five *T. cruzi*-positive 4th-stage nymphs (moulted 48 h before starting the experience, confirmed by PCR) were fed with a 3 ml solution of 2.8 ml human blood and 0.2 ml TrV [0.2 μg/ml] particles using the same artificial feeder as in Experiment 1. The control of this experiment consisted of 25 4th-stage nymphs fed with a 3 ml solution with 2.8 ml human blood and 0.2 ml TrV [0.2 μg/ml] particles using the same artificial feeder.

### 2.3. Experiment 3: Evaluation of simultaneous co-infection with T. cruzi and TrV

For this experiment, 30 4th-stage nymphs free of TrV and *T. cruzi* were fed (using the same artificial feeder) on a solution of 3 ml blood composed by: 1.8 ml human blood, 0.2 ml TrV [0.2 μg/ml], and 1 ml mouse blood with *T. cruzi*. For this experiment we set two control groups: (1) *Control for T. cruzi:* Thirty 4th-stage nymphs of *T. infestans* fed on a solution of 2 ml human blood and 1 ml mouse blood with *T. cruzi*, using the same artificial feeder; and (2) *Control for TrV:* Thirty 4th-stage nymphs of *T. infestans* fed on a 3 ml solution composed of 2.8 ml human blood and 0.2 ml TrV [0.2 μg/ml], using the same artificial feeder.

The human blood for the artificial feeder was provided by the "Instituto de Hemoterapia" of La Plata City, Argentina. From the second feeding on, all insects in all experiments were followed, and fed on 30 days-old male healthy mice C57BL/6J (TrV and *T. cruzi* free). In all experiments, the variable measured was TrV and/or *T. cruzi* positive/negative (see next section).

### 2.4. Marking procedure and positivity analysis

All insects in the three experiments were individually identified with marks made with synthetic enamel on the connexivum and placed in 60 $cm^3$ individual containers. Paper strips were placed at the bottom of these containers to collect the insects' faecal material, potentially infected with TrV. These paper strips were monitored daily and the faecal material was used to check for TrV infection by RT-PCR (Marti et al., 2008).

Every 30 days from the start of each experiment until the death of all individuals, faecal material was obtained from the insects by abdominal compression. The faecal material of insects exposed to *T. cruzi* was analysed by light microscopy to confirm the presence of parasites, which was later confirmed by PCR (Gomes et al., 1998).

### 2.5. Statistical analysis

As our results are composed by categorical data from classifying objects in positive vs negative, we can examine the significance of the association (contingency) between 2 × 2 contingency tables by their p-value using Fisher's exact test; we applied this test to determine possible significant differences between the experiments and their respective controls, as well as between experiments.

## 3. Results

Fig. 1 summarizes the results of all experiments. In experiment 1, *T.*

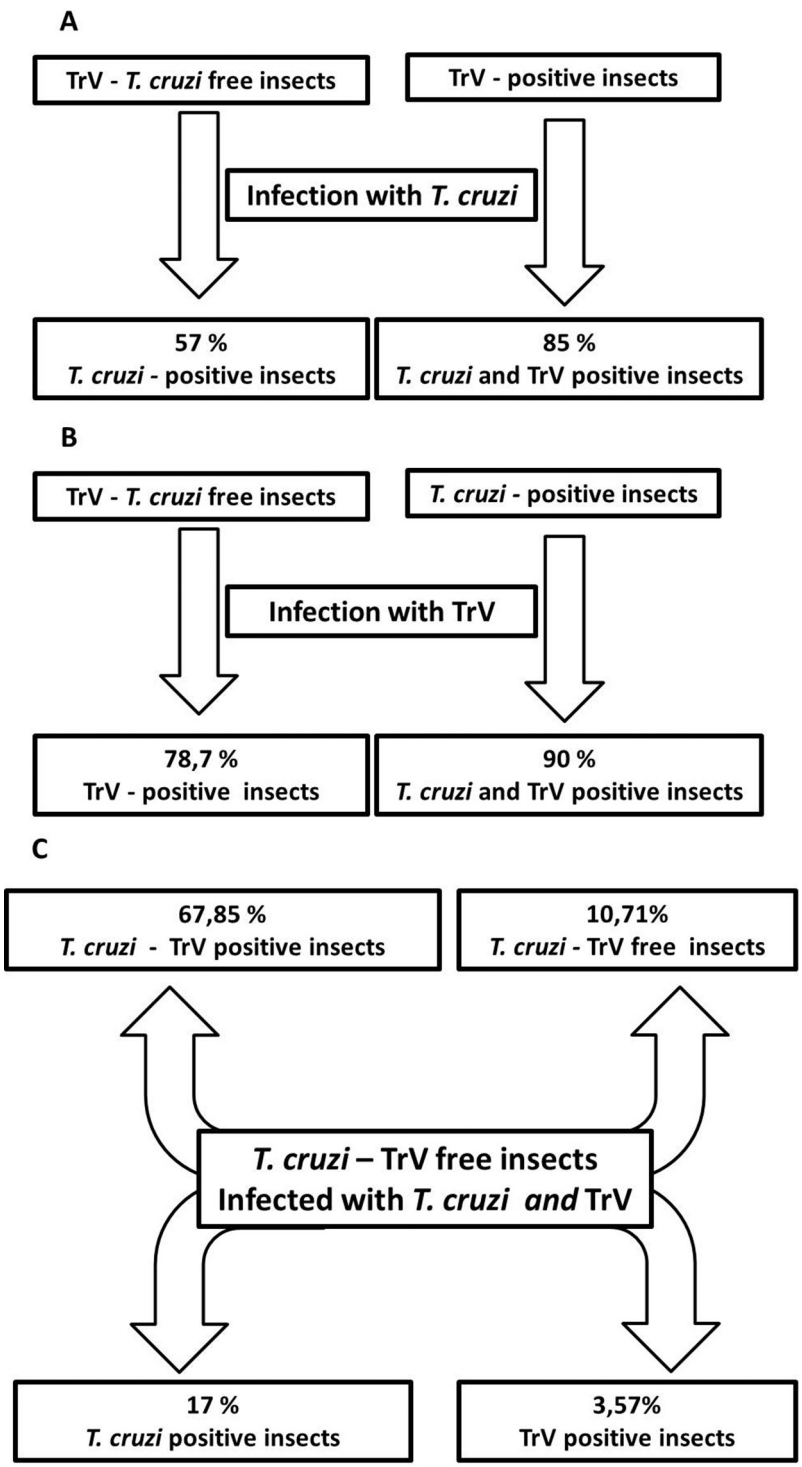


**Fig. 1.** Diagram summarizing results of TrV and *T. cruzi* infections. A (experience 1) and B (experience 2): % of individual microorganism infection (TrV or *T. cruzi*). C (experience 3): % of simultaneous infection with both microorganisms.

*cruzi* infection was found in 57% of the insects (control group for *T. cruzi*), whereas 85% of the insects with previous TrV infection were infected with *T. cruzi* (Fig. 1A); there was a significant difference between this experiment and its control ($p = 0.046$, Fisher's Exact Test). There was an increase of 30% of *T. cruzi* infection in TrV-positive insects.

In experiment 2, TrV infection was found in 78.7% of the insects (control group for TrV), whereas insects previously infected with *T. cruzi* had 90% of infection with TrV (Fig. 1B); although virus infection increased by 12% no significant difference between this experiment and its control was found ($p = 0.65$, Fisher's Exact Test). In this experiment TrV infection was detected at 55.5 ± 25.4 days (mean ± standard deviation) by RT-PCR, but TrV detection by this method occurred much earlier in those insects previously infected with *T. cruzi* (34.4 ± 14.8 days, mean ± standard deviation).

In experiment 3, 67.9% of insects presented simultaneous infection with both microorganism species, while 17.9% were infected with *T. cruzi* only, 3.6% with TrV only, and 10.7% of insects showed no infection by either microorganism (Fig. 1C). The number of kissing bugs infected only with *T. cruzi* showed no significant difference with that of insects infected with both microorganisms (57% to *T. cruzi*, 67% to both microorganisms, $p = 0.46$, Fisher's Exact Test); similarly, no significant differences were found between the number of insects infected with TrV only and with both microorganisms (78% to TrV, 67% to both

microorganisms, $p = 0.39$, Fisher's Exact Test).

When comparing the methods of *T. cruzi* infection, i.e. feeding on parasitized mice or artificial feeder (mixture of human and parasitized-mice blood), no significant differences were found between the two methods ($p = 0.64$, Fisher' s Exact Test); 57% of insects were infected by feeding on parasitized mice vs. 58% using artificial feeder.

## 4. Discussion

At present, there are no vaccines or effective drugs for the treatment of chronic patients of Chagas' disease. Thus, some of the main efforts toward reducing Chagas' disease by *T. cruzi* transmission are currently focused on controlling vector populations, and thgis is done by using insecticides (World Health Organization, 2013).

While triatomine behaviour has been extensively studied, how *T. cruzi* infection may affect this behaviour has only recently been investigated (Lazzari, 1991; Lazzari et al., 2013). Studies with different triatomine species have shown that insects infected with *T. cruzi* exhibit behavioural and/or physiological changes, such as an increase in feeding, in biting frequency, or in shorter defecation time; these changes seem to result from the parasites consuming some of the blood ingested by the hosts (Botto-mahan et al., 2006; Guarneri and Lorenzo, 2017; Marliére et al., 2015). Individuals infected with TrV show physiological changes, such as leg paralysis or moulting failure, in the long term, but with no short-term manifestations (Muscio et al., 1987). However, as the behavioural aspects were not part of our objectives, physiological and behavioural changes were not measured nor recorded.

This is the first report for a triatomine species showing that when two microorganisms enter the same individual host, one of them could be favoured by the subsequent entry of the other. In general, it is well known that the entry of one microorganism favours the entry of another microorganism but not *vice versa* (depending which was the microorganism first to infect). For example, Muttis et al. (2015) showed that the entry of nematodes into mosquito cells favours the subsequent entry of iridoviruses; Moreira et al. (2009) and Bourtzis et al. (2014) showed that preexistent *Wolbachia* infection reduces the ability of some arboviruses (Dengue, Chikungunya, West Nile and Yellow fever) and malaria parasites (protozoa) to successfully infect mosquitoes.

The 78.7% TrV infection is a similar percentage to that of insects infected with TrV when exposed to both pathogens. However, when insects had been previously infected with *T. cruzi*, the percentage of infection with TrV reached 90%, i.e. an increase greater than 10%. Therefore, insects infected with *T. cruzi* could increase the proportion of TrV infection, albeit only marginally.

*T. cruzi* epimastigotes are anchored by adhesion of the flagellum to the surface of the perimicrovillar membrane of the triatomine intestinal cells to carry out cell division and continue their life cycle without causing damage to the intestinal cells (Kollien et al., 1998). On the contrary, TrV replicates in the cytoplasm of intestinal cells produce cell lysis (Marti et al., 2015; Muscio et al., 1988). As 57% of healthy insects exposed to *T. cruzi* were infected, a percentage higher than when insects were exposed to both pathogens (67.8%), and as with insects previously infected with TrV, infection with *T. cruzi* was found in 85% of cases, we conclude that both previous infection with TrV and simultaneous exposure to both microorganisms, result in an increased infection of triatomines with *T. cruzi*.

Based upon these results we hypothesize that TrV infection could increase adhesion of *T. cruzi* to the intestinal cells of triatomines, but the presence of *T. cruzi* in the intestinal cells would not facilitate the entry of TrV into cells. We postulate this hypothesis because, after TrV replication, some changes at the cellular level occur that may increase the adhesion of *T. cruzi* (Muscio et al., 1988).

Although there are some published analyses on the interactions of *T. cruzi* with fungi and bacteria (Moraes et al., 2001; Soares et al., 2015), this is the first study focused on the interaction between *T. cruzi* and a virus in an insect vector.

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