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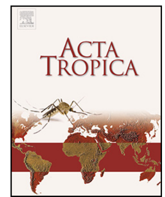

Genotyping of *Trypanosoma cruzi* DTUs and *Trypanosoma rangeli* genetic groups in experimentally infected *Rhodnius prolixus* by PCR-RFLP

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ABSTRACT

The specific detection and genetic typing of trypanosomes that infect humans, mammalian reservoirs, and vectors is crucial for diagnosis and epidemiology. We utilized a PCR-RFLP assay that targeted subunit II of cytochrome oxidase and 24Sα-rDNA to simultaneously detect and discriminate six *Trypanosoma cruzi* discrete typing units (DTUs) and two genetic groups of *Trypanosoma rangeli* (KP1+/KP1−) in intestinal contents of experimentally infected *Rhodnius prolixus*. The PCR assays showed that in 23 of 29 (79.4%) mixed infections with the six *T. cruzi* DTUs and mixed infections with individual DTUs and/or groups KP1+ and KP1−, both parasites were successfully detected. In six mixed infections that involved TcIII, the TcI, TcII, TcV, and TcVI DTUs predominated to the detriment of TcIII, indicating the selection of genetic groups. Interactions between different genetic groups and vectors may lead to genetic selection over TcIII. The elimination of this DTU by the immune system of the vector appears unlikely because TcIII was present in other mixed infections (TcIII/TcIV and TcIII/KP1+). Both molecular markers used in this study were sensitive and specific, demonstrating their usefulness in a wide geographical area where distinct genotypes of these two species are sympatric. Although the cellular and molecular mechanisms that are involved in parasite-vector interactions are still poorly understood, our results indicate a dynamic selection toward specific *T. cruzi* DTUs in *R. prolixus* during mixed genotype infections.

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1. Introduction

Trypanosoma cruzi is the causative agent of Chagas disease, which affects approximately 5–6 million people in Latin America (World Health Organization, 2015). This parasite is transmitted by 130 species of triatomine vectors, mainly the genera *Pantrongylus*, *Rhodnius*, and *Triatoma*, among a variety of more than 150 wild and domestic mammalian species, including humans (Coura et al., 1999; World Health Organization, 2002; Jansen and Roque, 2010; Zingales et al., 2012). Isolates from *T. cruzi* exhibit high genetic diversity and are classified into six discrete typing units (DTUs): TcI–TcVI. These genotypes are widely distributed throughout South and Central America and southern North America (Zingales et al., 2009, 2012). Recently, a new *T. cruzi* genetic group, called TcBaT, was described in bats in Brazil and Panama (Pinto et al., 2012; Zingales et al., 2009).

Trypanosoma rangeli is the second most common American trypanosome that infects triatomine vectors, sharing the same hosts with *T. cruzi*. Although genetically related to *T. cruzi*, *T. rangeli* is non-pathogenic to mammals, but human infection by this parasite induces serological cross-reactions with Chagas disease through shared soluble antigens (Moraes et al., 2008; Parada et al., 2010; Stoco et al., 2014). *T. rangeli* isolates are classified into two major genotypes, KP1+ and KP1−, the geographic distribution of which often overlaps with *T. cruzi* (Recinos et al., 1994; Vallejo et al., 2002;

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Guhl and Vallejo, 2003). KP1+ strains have been isolated from *R. prolixus*, *R. neglectus*, and *R. robustus*, and KP1- strains have been isolated from *R. pallescens*, *R. colombiensis*, and *R. ecuadoriensis* (Pullido et al., 2008; Vallejo et al., 2009).

Because of the sympatric occurrence of both parasites in large geographical areas, single or mixed infections by distinct *T. cruzi* DTUs or major genetic groups of *T. rangeli* have been reported in mammalian hosts and vectors (Coura et al., 1996; Saldaña et al., 2005; Sousa et al., 2008). In Panamá, where *T. cruzi* and *T. rangeli* are endemic, 50.7% of the mixed infections in *Triatoma dimidiata* have involved *T. cruzi* (TcI) and *T. rangeli* (Saldaña et al., 2012). Of the population of the *R. ecuadoriensis* vector that has been identified in Ecuador, 64.7% are infected with *T. cruzi*, 15.7% are infected with *T. rangeli*, and 8% are infected with both *T. cruzi* and *T. rangeli* (Grijalva et al., 2012). In Ceará, Brazil, natural mixed infection in *R. nasutus* has been reported (Dias et al., 2008), and infection in *Panstrongylus megistus* has been reported in the state of Santa Catarina (Steindel et al., 1994).

R. prolixus is a triatomine species that is well adapted to both domestic and wild habitats. It is related to human cases of *T. cruzi* infection that is acquired by oral transmission in the Brazilian Amazon and the main vector of Chagas disease in Venezuela and Colombia (Coura, 2015). It is also involved in mixed infections with *T. cruzi* and *T. rangeli* and has the ability to simultaneously transmit these parasites to various hosts (Vallejo et al., 2009). This vector was chosen because it has been widely used as an experimental model in several parasitological and physiological studies (Mello et al., 1995; Azambuja et al., 2005; Pulido et al., 2008; Araújo et al., 2014).

Different DNA sequences for detecting and differentiating *T. cruzi* from *T. rangeli* have been proposed (Souto et al., 1999; Vallejo et al., 1999; Fernandes et al., 2001; Chiurillo et al., 2003; Pavia et al., 2007), but none of these are able to simultaneously discriminate different parasite genotypes. Several molecular markers have been proposed to assess specific DTUs of infective *T. cruzi* isolates (Spitzner et al., 2007; Lewis et al., 2009; Abolis et al., 2011; Hamilton et al., 2011). However, such methods depend on parasite isolation, which can lead to the selection of populations (Pena et al., 2011) or cannot define the hybrid nature of some *T. cruzi* DTUs (Lewis et al., 2009).

In the present study, two recognized molecular markers (restriction fragment length polymorphism of subunit II of cytochrome oxidase [PCR/RFLP-COII] and 24Sα rDNA) were used to simultaneously detect and discriminate the six known *T. cruzi* DTUs (TcI–TcVI) and two *T. rangeli* genetic groups (KP1+ and KP1−) in the intestinal contents of *R. prolixus* that was experimentally infected with these parasites in single and mixed parasite combinations.

2. Material and methods

2.1. Parasites

The following *T. cruzi* strains and clones, representing the parasite DTUs, were used: PR-150 (TcI), PR-1256 (TcII), 222 (TcIII), CanIII (TcIV), SO3cl5 (TcV), and CL Brener (TcVI). The *T. rangeli* strains that represented the two major genotypes were Choachí (KP1+) and SC-58 (KP1−). The geographical origins of the hosts and vectors and genetic characterization of each strain are presented in Table 1.

2.2. Experimental triatomine infection

Groups of 10 fifth-instar nymphs of *R. prolixus* were fed using an artificially feeding apparatus containing 1×10^6 culture epimastigotes/mL of each parasite species in LIT medium supplemented with 10% human blood at 37° C for 1 h (Friend and Cartwright, 1963). After feeding, insects were maintained at 26° C and 75% relative humidity and fed on anesthetized mice at the 20th day post-infection. Infection of the insects by the six *T. cruzi* DTUs and two *T. rangeli* genotypes resulted in 37 distinct experimental groups: control groups (single infection with 10 nymphs of each *T. cruzi* DTU [TcI–TcVI], single infection with the two *T. rangeli* genotypes [KP1+ and KP1−], and insects that were fed non-infected blood [negative control]) and experimental groups (mixed infection with 29 different combinations of *T. cruzi* DTUs and *T. rangeli* genotypes [Table 2]). On day 35 post-infection (p.i.), the midgut and hindgut of all insects were removed and checked by microscopy for the presence of parasites. The intestinal contents of all insects were stored in 70% ethanol prior to DNA extraction.

All animal procedures were approved and performed in accordance with the guidelines established by the State University of Maringá (Universidade Estadual de Maringá [UEM]) Committee of Ethics for the Use of Animals (Protocol no. 076/2013).

2.3. DNA extraction

After removing the ethanol by centrifugation, the pellet underwent DNA extraction using a standard phenol-chloroform method (Macedo et al., 1992). Briefly, the pellet was resuspended in 500 μL of lysis solution (1% sodium dodecyl sulfate, 5 M NaCl, and 0.5 M ethylenediaminetetraacetic acid [EDTA]) supplemented with 10 mg/mL of proteinase K (Invitrogen) at 37° C overnight. Following phenol extraction and ethanol precipitation the DNA was resuspended in 10 mM Tris–HCl/1 mM EDTA pH 8.0 (TE) and digested with 10 mg/ mL Ribonuclease A (Invitrogen) at 37° C for 2 h. Following a further round of phenol extraction and ethanol precipitation DNA was again resuspended in TE buffer and DNA concentration was determined using a Qubit Fluorometric Quantification Kit (Invitrogen).

2.4. PCR/RFLP-COII

The assay was performed as described by Sá et al. (2013) using Platinum *Taq* Polymerase (Invitrogen) and the restriction enzyme *Alu*I (New England BioLabs) in NEB 4 buffer as indicated by the manufacturer.

2.5. Polymerase chain reaction determination of 24Sα rDNA

The amplification of 24Sα rDNA was performed as described by Souto et al. (1996) using a final volume of 12.5 μL containing 0.125 U of Platinum *Taq* DNA Polymerase (Invitrogen), 3.1 pmol of each primer (D71 and D72), 2 ng of genomic DNA, and 3.5 mM $MgCl_2$ in buffer provided by the manufacturer. The amplified fragments were resolved on 6% polyacrylamide gels, stained with silver nitrate, and digitally recorded. The 24Sα rDNA marker has been applied previously to differentiate *T. cruzi* TcV and TcVI DTUs (Lewis et al., 2009).

3. Results

All *T. cruzi* and *T. rangeli* strains were able to infect *R. prolixus* as revealed by positive microscopic examination of the triatomine intestinal contents 35 post infection. Two independent PCR reactions that targeted the COII and 24Sα rDNA genes were performed for each sample that was collected from the control and experimental groups. The assay presented the expected band patterns for each *T. cruzi* DTU and *T. rangeli* genotype (Figs. 1–3, Table 1).

The PCR/RFLP-COII and 24Sα rDNA analyses of the intestinal content of infected *R. prolixus* showed that 23 of 29 groups (79.4%) presented mixed infections by the six *T. cruzi* DTUs and KP1+ and KP1− *T. rangeli* genotypes. TcI was the only DTU that was detected in

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