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DETECTION OF *Trypanosoma cruzi* AND *Trypanosoma rangeli* INFECTION IN TRIATOMINE VECTORS BY AMPLIFICATION OF THE HISTONE H2A/SIRE AND THE SNO-RNA-CL1 GENES

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SUMMARY

Trypanosoma rangeli is non pathogenic for humans but of important medical and epidemiological interest because it shares vertebrate hosts, insect vectors, reservoirs and geographic areas with *T. cruzi*, the etiological agent of Chagas disease. Therefore, in this work, we set up two PCR reactions, TcH2AF/R and TrFR2, to distinguish *T. cruzi* from *T. rangeli* in mixed infections of vectors based on amplification of the histone H2A/SIRE and the small nucleolar RNA Cl1 genes, respectively. Both PCRs were able to appropriately detect all *T. cruzi* or *T. rangeli* experimentally infected-triatomines, as well as the S35/S36 PCR which amplifies the variable region of minicircle kDNA of *T. cruzi*. In mixed infections, whereas *T. cruzi* DNA was amplified in 100% of samples with TcH2AF/R and S35/S36 PCRs, *T. rangeli* was detected in 71% with TrF/R2 and in 6% with S35/S36. In a group of *Rhodnius colombiensis* collected from Coyaima (Colombia), *T. cruzi* was identified in 100% with both PCRs and *T. rangeli* in 14% with TrF/R2 and 10% with S35/S36 PCR. These results show that TcH2AF/R and TrF/R2 PCRs which are capable of recognizing all *T. cruzi* and *T. rangeli* strains and lineages could be useful for diagnosis as well as for epidemiological field studies of *T. cruzi* and *T. rangeli* vector infections.

KEYWORDS: *Trypanosoma cruzi*; *Trypanosoma rangeli*; *Rhodnius prolixus*; *Rhodnius colombiensis*; PCR, Histone H2A; SIRE; sno-RNA -Cl1.

INTRODUCTION

Chagas disease, caused by the flagellate parasite *Trypanosoma cruzi*, affects fifteen countries throughout Latin America. The number of new cases has been estimated at 200,000 per year and about 21,000 chagasic patients die each year from the disease[17,42]. In Colombia, Chagas disease is a major public health problem. It is estimated that 5% of the population is at a high risk of being infected and that approximately 700,000 people are currently infected[17]. Chagas disease is transmitted to humans by bloodsucking triatomine bugs, from which 23 species have been reported in Colombia. The most important vector species that circulate in the domestic cycle in this country are *Rhodnius prolixus, Triatoma dimidiata, Triatoma maculata,* and *Triatoma venosa* whereas in the sylvatic regions *Rhodnius pallescens* and *Rhodnius colombiensis* are the prevalent ones[12,15].

Based on phenotypic and genotypic characters, *T. cruzi* has been divided into two principal lineages: *T. cruzi* I and *T. cruzi* II[1]. In addition, *T. cruzi* II is divided into five subgroups, named IIa-e[2]. While *T. cruzi* I, associated with opossums and an arboreal ecology, predominates from the Amazon basin northwards, *T. cruzi* II is associated with armadillos and a terrestrial ecology and predominates in southern cone countries of South America[43].

On the other hand, although *Trypanosoma rangeli* infection in humans is harmless, this parasite is a serious concern for the epidemiology and diagnosis of Chagas disease due to its morphological similarity and immunological cross-reactivity with *T. cruzi*[14]. Moreover, these trypanosomes are sympatric and share triatomine insects as well as vertebrate hosts, allowing the occurrence of mixed infections[7]. Colombia is one of the countries in which *T. cruzi* shares vectors and reservoirs with *T. rangeli*[7,13,14]. Recently, two important epidemiological groups of *T. rangeli* have been described: KP1(-) strains, associated with the adaptive line of *Rhodnius*, represented by *R. colombiensis, R. pallescens*, and *R. ecuadoriensis,* and KP1(+) strains, associated with *R. prolixus*[30-32]. These two groups have been defined on the basis of independent mitochondrial (minicircle profile dimorphism obtained by kDNA PCR amplification) and nuclear (mini-exon PCR amplification) markers.

Due to direct microscopic detection of trypanosomes, the traditional method for assessment of infection in vectors is not able to distinguish *T. cruzi* from *T. rangeli* infection, several polymerase chain reaction techniques have been developed[3,4,5,6,9,13,19,28,33,36,40,41]. However, current PCR assays used for mixed infection detection show some disadvantages such as the amplification of bands of similar size both in *T. cruzi* and *T. rangeli*[27,28], the amplification of polymorphic

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fragments[11,19], and bias to *T. cruzi* in the case of mixed *T. cruzi* and *T. rangeli* infection[9,33,36]. Therefore, it is necessary to develop new techniques as better options to specifically identify each of these parasite species in mixed infections. In this study we aimed to determine the capacity of two previously standardized PCR tests to detect specifically *T. rangeli* and *T. cruzi* based on the small nucleolar RNA-C11 (sno-RNA-C11) gene and the SIRE (short interspersed repetitive element) sequence inserted into the histone H2A gene, respectively[18,20]. Our results indicate that the use of both PCR tests allow a specific identification of *T. cruzi* and *T. rangeli* in experimental and natural triatomine infection.

MATERIALS AND METHODS

Parasites: Artificial and experimental infections were performed with the Colombian strains MHOM/CO/01/DA and IRHO/CO/85/MTA, belonging to *T. cruzi* I, and the Colombian KP1(+) strain IRHO/CO/86/CH, of *T. rangeli*. These strains were provided by Laboratorio de Parasitología, Instituto Nacional de Salud, (INS) (Bogotá, Colombia). Both parasites species were characterized by isoenzymes[24] and PCR using miniexon and minicircle sequences as targets[5,31,33]. The bulk parasite mass was cultivated in REI modified liquid medium, supplemented with 2% FCS and 100 µg/mL of gentamicin, and incubated at 24 °C.

Triatomines: Experimental infections were carried out using non-infected fifth stage nymphs of *R. prolixus*. Fourteen specimens were fed on mice previously infected with 1 x 10^6 flagellates/mL of *T. cruzi*. Twenty-two insects were infected with *T. rangeli* by intrafemoral inoculation of 1 x 10^5 parasites/mL. Seventeen triatomines were fed on mice inoculated intraperitoneally with 1 x 10^6 of both trypanosomes per mL. Forty non-infected vectors were used as controls and distributed equally among each group. On the other hand, twenty-nine specimens of *R. colombiensis* were collected from *Attalea butyracea* palm trees in Coyaima, Department of Tolima (Colombia) and 21 *T. maculata* specimens were collected from the Department of Bolivar (Colombia). Each experimentally infected triatomine was dissected 15 and 45 days post-infection to obtain the intestinal tract, feces, hemolymph and salivary glands respectively after exposure to UV light for one hour. Samples were homogenized in 240 µL of phosphate buffered saline (PBS). A 40 µL aliquot was examined by direct microscope observation (DMO) and Giemsa stained. The DNA from the rest of the sample was extracted three times with phenol-chloroform-isoamylic alcohol (25:24:1), followed by ethanol precipitation[33]. Finally, DNA obtained was amplified with the different PCR methods. Field-collected triatomines were treated and analyzed as described above. To simulate vector infection, the digestive tracts of non-infected adult triatomines (*R. prolixus*) were removed (100 µL) and mixed with 100 µL of 1 to 10^4 epimastigote forms of *T. cruzi* or 2 x 10^6 to 12 x 10^6 epimastigote forms of *T. rangeli.* Then, DNA was extracted and amplified with different PCR tests.

PCR conditions: For *T. cruzi* detection the following primers were used: TcH2AF (5'-GAGAGTGATCGTGGGAGAGC-3'), and TcH2AR (5'-AGTGGCAGACTTTGG GGTC-3'). These primers amplify a 234 bp fragment present in the 3' non-encoding region of the 1.2 kb unit encoding for histone H2A from *T. cruzi* (GenBank accession number X67287), corresponding to the 16-248 nucleotides of SIRE sequence[20,21]. PCR reactions were performed in a 25 µL final volume, containing: 5 µL of DNA from triatomines (diluted 1:5 or 1:10), 1X reaction buffer (10 mM Tris-HCl, pH 8.5, 500 mM KCl), 1.25 U/µL of *Taq* DNA polymerase, 1.5 mM of $MgCl_2$, 200 µM of deoxynucleoside triphosphate (dNTP) mixture, and 20 pmol of each primer. The reaction was carried out on a MJ Research PTC-100 DNA thermal cycler, using the following profile: 95 °C/5 min, 15 cycles of 95 °C/30 s, 72 °C/1 min, and 30 cycles of 95 °C/30 s, 65 °C/30 s and 72 °C/30 s, and a final incubation of 72 °C for five min. 15 µL of the reaction products were electrophoresed in 1.5% agarose gels and stained with ethidium bromide. For *T. rangeli* detection, we used a modified version of a TrF/R2 PCR assay targeted to the sno-RNA-Cl1 genes (GenBank accession number AY028385) which amplifies a 620 bp fragment[18]. PCR reactions were made in a 25 µL final volume, containing: 5 µL of DNA from triatomines (diluted 1:5 or 1:10), 1X reaction buffer (100 mM Tris-HCl, pH 8.5, 500 mM KCl), 1.25 U/µL of *Taq* DNA polymerase, 1.5 mM of $MgCl_2$, 200 µM of dNTP mixture, and five pmol of each primer. The reaction was carried out on a MJ Research PTC-100 DNA thermal cycler, using the following profile: 95 °C/5 min, 15 cycles of 95 °C/30 s, 63 °C/1 min and 72 °C/30 s and 20 cycles of 95 °C/30 s, 60 °C/1 min, 72 °C/30 s, and a final incubation of 72 °C for 5 min. 15 µL of the reaction products were electrophoresed in 1% agarose gels and stained with ethidium bromide. In addition, all DNA samples were amplified with the S35 (5'-AAATAATGTACGGGTGGAGATGCATGA-3'), and S36 (5'-GGGTTCGATTGGGGTTGGTGT-3') primers based on conserved regions of the minicircles from kDNA from *T. cruzi*[29] (GenBank accession number X04680). These primers amplify two fragments of 300 and 450 bp in *T. rangeli*, besides a 330 bp band amplified in *T. cruzi*. PCR reactions were performed according to previously described[33].

RESULTS

Artificial infections: To asses the sensitivity of TcH2AF/R and TrF/R2 PCRs, an artificial infection was simulated by mixing *T. cruzi* or *T. rangeli* with the intestinal tract and feces from non-infected triatomines. For *T. cruzi*, the appropriately sized amplification product was obtained from a single parasite (Fig. 1A, lane 5). For *T. rangeli*, the assay detected DNA from 2 x 10^6 parasites (Fig. 1B, lane 6).

Experimental infection of *R. prolixus* with *T. cruzi:* Parasites were detected 15 days post-infection in 100% (14/14) and 78% (11/14) of insects by DMO and Giemsa staining, respectively. TcH2AF/R and S35/S36 PCR reactions showed the presence of *T. cruzi* in 100% of insects (14/14) with the expected PCR products (Table 1). The kappa index between both PCR reactions was 1.0 (95% Confidence Interval), (95% CI: 0.95-1.0), interpreted as a perfect concordance[25]. The group of nine non-infected vectors all tested negative.

Experimental infection of *R. prolixus* with *T. rangeli:* The presence of parasites in the haemolymph 15 days post-infection, was 100% (22/22) determined by DMO and Giemsa staining. *T. rangeli* was detected in 100% (22/22) in the haemolymph with both PCR tests showing the expected amplification products (Table 2). The kappa index between both tests was 1.0 (95% CI: 0.95-1.0), interpreted as a perfect concordance[25]. On the other hand, non-infected triatomines were tested negative. After 45 days post-infection, 21 out of 22 *T. rangeli* infected

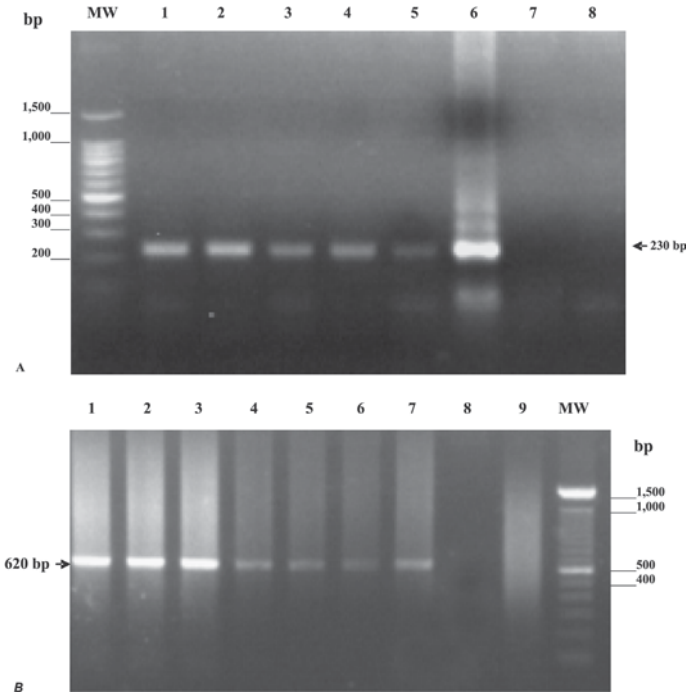

Fig. 1 - A: PCR TcH2AF/R of epimastigotes from *T. cruzi* IRHO/CO/85/MTA mixed with the intestinal tract and feces of *R. prolixus* visualized in ethidium bromide-stained 1.5% agarose gel. Fifteen µL of PCR products obtained from 10^4 (1), 10^3 (2), 10^2 (3), 10^1 (4), and 10^0 (5) parasites; DNA of *T. cruzi* IRHO/CO/85/MTA as positive control (6), uninfected feces of *R. prolixus* (7), and distilled water (8) as negative controls. 100 bp (Promega) was used as molecular weight marker and its sizes are indicated on the left. **B**: TrF/R2 PCR of epimastigotes from *T. rangeli* IRHO/CO/86/CH mixed with the intestinal tract and feces of *R. prolixus*. Ethidium bromide-stained 1% agarose gel containing 15 µL of the PCR product of 12 x 10^6 (1), 10 x 10^6 (2), 8 x 10^6 (3), 6 x 10^6 (4), 4 x 10^6 (5), 2 x 10^6 (6) parasites; DNA of *T. rangeli* KP1(+) IRHO/CO/86/CH as positive control (7), uninfected feces from *R. prolixus* (8), and distillated water (9) as negative controls. 100 bp (Promega) was used as molecular weight marker and its sizes are indicated on the right.

vectors and three controls were decontaminated and dissected obtaining the salivary glands. Parasites were observed in 90% (19/21) and 67% (14/21) by DMO and Giemsa staining, respectively. TrF/R2 positive PCR reaction for *T. rangeli* was found in 33% (7/21) of vectors while 48% of insects were positive by the S35/S36 PCR (10/21) (Table 3). The kappa index between both PCR reactions was 0.76, interpreted as a good concordance (95% CI: 0.52-0.90)[25]. It is important to bear in mind that positive triatomine bugs by PCR were also positive with the conventional tests. The group of non-infected vectors tested negative.

Experimental infection of *R. prolixus* with *T. cruzi* and *T. rangeli:* The presence of parasites in the intestinal tract and feces 15 days post-infection was detected by ODM and Giemsa staining in 100% (17/17) and 76% (13/17) of cases, respectively. *T. cruzi* was observed in 100% (17/17) with both PCR tests (Table 4). *T. rangeli* was detected with TrF/R2 PCR in 71% of vectors (12/17) (Fig. 4C) and 6% (1/17) of insects with S35/S36 PCR. The group of five non-infected vectors tested negative.

Detection of *T. cruzi* and *T. rangeli* in triatomine field samples: Having studied the detection capacity of TcH2AF/R and TrF/R2 PCRs in vectors, the next step was to evaluate the *T. cruzi* and *T. rangeli* detection in naturally infected triatomines. Two groups of field triatomines, *R. colombiensis* and *T. maculata* were collected and their intestinal tract and feces were analyzed. In *R. colombiensis*, trypanosomes were detected in the intestinal tract and feces of 83% (23/29) by DMO and in 86% (25/29) by Giemsa staining, whereas in salivary glands no parasites were observed.

Table 1
Experimental infection of *Rhodnius prolixus* with *T. cruzi*

Vector	ODM	Giemsa	TcH2AF/R	S35/S36
1	+	+	Pos	+
2	++	+	Pos	+
3	+	+	Pos	+
4	++	+	Pos	+
5	++	-	Pos	+
6	++	+	Pos	+
7	++	+	Pos	+
8	+++	+	Pos	+
9	++	+	Pos	+
10	++	+	Pos	+
11	++	+	Pos	+
12	++	+	Pos	+
13	+	-	Pos	+
14	++	-	Pos	+
15	-	-	Neg	-
16	-	-	Neg	-
17	-	-	Neg	-
18	-	-	Neg	-
19	-	-	Neg	-
20	-	-	Neg	-
21	-	-	Neg	-
22	-	-	Neg	-
23	-	-	Neg	-

T. cruzi inoculated vectors: 1-14, non-inoculated vectors: 15-23. The level of parasitosis in DMO (Direct microscope observation) and Giemsa corresponds to: (-): non parasites, (+): 0-1 parasites, (++): 1-3 parasites, (+++): 3-6 parasites per microscopic field. Positive amplification signal: (Pos); Negative amplification signal: (Neg).

The presence of *T. cruzi* in the intestinal tract and feces was detected in 100% (29/29) of insects with both PCR tests (Table 5), while *T. rangeli* was observed in intestinal tract and feces with TrF/R2 PCR in 14% (4/29) (Table 5) and 10% (3/29) with S35/S36 PCR. Remarkably, in eight salivary glands studied, this parasite was not observed with the tests performed, even when three out of eight specimens were positive in feces with both conventional and PCR techniques. All tests yielded negative results for both parasites in 21 *T. maculata* specimens.

DISCUSSION

The detection of *T. rangeli* and *T. cruzi* in naturally infected vectors has been subject to study because in intestinal infections both species of flagellates can be found and the distinction between them is difficult[35].

Table 2
Experimental infection of *R. prolixus* with *T. rangeli* (Haemolymph)

Vector	ODM	Giemsa	TrF/R2	S35/S36
1	++++	++++	Pos	Pos
2	++++	++++	Pos	Pos
3	++++	++++	Pos	Pos
4	++++	++++	Pos	Pos
5	++++	++++	Pos	Pos
6	++++	++++	Pos	Pos
7	++++	++++	Pos	Pos
8	++++	++++	Pos	Pos
9	++++	++++	Pos	Pos
10	++++	++++	Pos	Pos
11	++++	++++	Pos	Pos
12	++++	++++	Pos	Pos
13	++++	++++	Pos	Pos
14	++++	++++	Pos	Pos
15	++++	++++	Pos	Pos
16	++++	++++	Pos	Pos
17	++++	++++	Pos	Pos
18	++++	++++	Pos	Pos
19	++++	++++	Pos	Pos
20	++++	++++	Pos	Pos
21	++++	++++	Pos	Pos
22	++++	++++	Pos	Pos
23	-	-	Neg	Neg
24	-	-	Neg	Neg
25	-	-	Neg	Neg
26	-	-	Neg	Neg
27	-	-	Neg	Neg
28	-	-	Neg	Neg

T. rangeli inoculated vectors: 1-22, non-inoculated vectors: 23-28. The level of parasitosis in DMO (Direct microscope observation) and Giemsa corresponds to: (-): non parasites, (+): 0-1 parasites, (++): 1-3 parasites, (+++): 3-6 parasites, (++++): 6-8 parasites per microscopic field. Positive amplification signal: (Pos); Negative amplification signal: (Neg).

Table 3
Experimental infection of *R. prolixus* with *T. rangeli* (Salivary glands)

Vector	ODM	Giemsa	TrF/R2	S35/S36
1	+	-	Neg	Neg
2	+	+	Neg	Neg
3	++	+	Neg	Neg
4	-	-	Neg	Neg
5	++	-	Neg	Neg
6	++	-	Neg	Neg
7	+	-	Neg	Neg
8	-	-	Neg	Neg
9	++	+	Neg	Neg
10	+	-	Neg	Neg
11	++++	++++	Pos	Pos
12	++++	++	Pos	Pos
13	++++	+++	Pos	Pos
14	++++	+	Pos	Pos
15	++	+	Neg	Pos
16	++++	++	Pos	Pos
17	++++	+	Neg	Neg
18	+	+	Neg	Neg
19	++++	++	Pos	Pos
20	++	+	Neg	Neg
21	++++	++	Pos	Pos
22	-	-	Neg	Neg
23	-	-	Neg	Neg
24	-	-	Neg	Neg

T. rangeli inoculated vectors: 1-21, non-inoculated vectors: 22-24. The level of parasitosis in DMO (Direct microscope observation) and Giemsa corresponds to: non parasites, (+): 0-1 parasites, (++): 1-3 parasites, (+++): 3-6 parasites, (++++): 6-8 parasites per microscopic field. Positive amplification signal: (Pos); Negative amplification signal: (Neg).

Bearing this in mind, in this work we evaluated the use of TcH2AF/R[20] and TrF/R2 PCRs[18] to identify these trypanosomes by comparing them with conventional techniques and with the S35/S36 PCR, the most sensitive PCR described for *T. cruzi*[3,4,40,41] in the two last decades. Despite the S35/S36 PCR being described as a specific tool for the amplification of kDNA from *T. cruzi* at the beginning[8,29], sequencing of kDNA minicircles of *T. rangeli* found a high degree of homology among conserved regions between the two species. Therefore, these primers anneal and amplify kDNA from both trypanosomes[33,34].

TcH2AF/R amplification is a highly specific test for detecting DNA from *T. cruzi* I and *T. cruzi* II strains which yields an amplification product of the same size in both *T. cruzi* groups. In addition, this PCR does not amplify *T. rangeli* DNA as well as DNA from other trypanosomatids, humans, mice or vectors[20]. In this study, we could detect the presence of *T. cruzi* DNA by PCR in artificial infections from a single parasite. Also, we detected *T. cruzi* in intestinal tract and feces in 100% of the insects infected experimental and naturally, results that were similar to those obtained with the S35/S36 PCR. These results together with the finding that TcH2AF/R PCR can detect an equivalent to 1/200th of *T. cruzi* cells[20], suggest this PCR can be considered as one of the main techniques which display a great power of detection in insect vectors among those that use nuclear DNA as target. The high sensitivity of this PCR might be explained because this PCR amplifies a fragment corresponding to the 16-248 nucleotides of SIRE, a short interspersed repetitive element, present in *T. cruzi*[21,38,39]. SIRE, a sequence of 428 bp is repeated about 1500 to 3000 times per genome depending on the parasite strain but to date it has not been reported in *T. rangeli*[23,38,39]. However, recent studies show the presence of a retrotransposon inserted in nucleotide 182 of SIRE in *T. cruzi*[16,37] and *Trypanosoma brucei*[16], rising to a VIPER (Vestigial interposed retroelement) of 4480 bp in length[16]. This element, a tyrosine recombinase retroelement, is composed by three open reading frames (ORF) flanked by the first 182 bp of SIRE in the 5' region and by the last 226 bp of SIRE in the 3' region. Therefore, in the case that VIPER would be present in the genome of *T. rangeli* this retroelement should not amplify with the TcH2AF/R primers because SIRE is blocked at the nucleotide 182 by the specific 4072 bp from VIPER. Interestingly, ELIAS *et al*. (2003)[10] and SCHIJMAN *et al*. (2004)[26] reported a nested PCR based on SIRE which is able of detecting parasite DNA in the heart tissue of chronic chagasic patients.

Table 4
Experimental infection of *R. prolixus* with *T. cruzi* and *T. rangeli*

Vector	DMO	Giemsa	TcH2A	TrF/R2	S35/S36 T.c	S35/S36 T.r
1	+++	+	Pos	Pos	Pos	Pos
2	+++	++	Pos	Pos	Pos	Pos
3	++++	++	Pos	Pos	Pos	Pos
4	++	-	Pos	Pos	Pos	Pos
5	++++	+	Pos	Pos	Pos	Pos
6	++	-	Pos	Neg	Pos	Pos
7	+	-	Pos	Neg	Pos	Pos
8	+	++	Pos	Neg	Pos	Pos
9	+	+	Pos	Neg	Pos	Pos
10	++	+	Pos	Neg	Pos	Pos
11	++	+	Pos	Pos	Pos	Pos
12	+++	+	Pos	Pos	Pos	Pos
13	++	+	Pos	Pos	Pos	Pos
14	++++	++	Pos	Pos	Pos	Pos
15	++	+	Pos	Pos	Pos	Neg
16	+	+	Pos	Pos	Pos	Pos
17	++	-	Pos	Pos	Pos	Pos
18	-	-	-	-	-	
19	-	-	-	-	-	
20	-	-	-	-	-	
21	-	-	-	-	-	
22	-	-	-	-	-	

T. cruzi and *T. rangeli* inoculated vectors: 1-17, non-inoculated vectors: 18-22. The level of parasitosis in DMO (Direct microscope observation) and Giemsa corresponds to: (-): non parasites, (+): 0-1 parasites, (++): 1-3 parasites, (+++): 3-6 parasites, (++++): 6-8 parasites per microscopic field. Positive amplification signal: (Pos); Negative amplification signal: (Neg).

On the other hand, the TrF/R2 PCR, based on the repetitive genes encoding for the sno-RNA-Cl1, is a specific test that amplify a product of the same size in both *T. rangeli* KP1(+) and KP1(-) strains and does not present any amplification signal either with the DNA from both *T. cruzi* and DNA from human, mouse or even other trypanosomatids[18]. In this work, with this PCR *T. rangeli* was detected in a specific way in artificial infections from 2 x 10^6 parasites. In addition, in experimental infections this parasite was detected in the hemolymph of 100% of the insects using both TrF/R2 and S35/S36 PCR, while in salivary glands the parasite was detected in only 33% with TrF/R2 and in 48% with S35/S36. These results are evidence of a lower sensitivity for both PCR tests to detect *T. rangeli* in salivary glands in comparison with conventional methods, fact that could be due to the presence of PCR inhibitors. Otherwise, it is important to mention that detection of single infections in vectors using TrF/R2 is lower compared to the S35/S36 PCR probably due to the higher copy number of the S35/S36 target than TrF/R2 target.

However, in mixed infections with both parasites, *T. rangeli* was detected in intestinal tract and feces in 71% of insects with TrF/R2 primers and in 6% with S35/S36 PCR. In the same way, the analyses of intestinal tract and feces of 29 *R. colombiensis* showed a *T. cruzi* amplification profile that overlapped the presence of *T. rangeli*.

Table 5
Detection of *T. cruzi* and *T. rangeli* in triatomine field samples

Vector	DMO	Giemsa	TcH2AF/R	TrF/R2	S35/S36 T.c	S35/S36 T.r
1	++	+	Pos	Neg	Pos	Neg
2	++	+	Pos	Neg	Pos	Neg
3	+++	+	Pos	Neg	Pos	Neg
4	+++	++	Pos	Neg	Pos	Neg
5	++	++	Pos	Neg	Pos	Neg
6	+	+	Pos	Neg	Pos	Neg
7	+	+	Pos	Neg	Pos	Neg
8	+	+	Pos	Neg	Pos	Neg
9	+++	++	Pos	Neg	Pos	Neg
10	++++	+++	Pos	Neg	Pos	Neg
11	+++	++	Pos	Neg	Pos	Neg
12	+++	++	Pos	Neg-	Pos	Neg
13	+	+	Pos	Neg	Pos	Neg
14	++	+	Pos	Neg	Pos	Neg
15	++++	+++	Pos	Pos	Pos	Neg
16	+	+	Pos	Neg	Pos	Neg
17	-	-	Pos	Neg	Pos	Neg
18	-	-	Pos	Neg	Pos	Neg
19	-	+	Pos	Neg	Pos	Neg
20	++	+	Pos	Neg	Pos	Neg
21	-	-	Pos	Neg	Pos	Neg
22	-	-	Pos	Neg	Pos	Neg
23	-	+	Pos	Neg	Pos	Neg
24	+	++	Pos	Neg	Pos	Neg
25	++	+	Pos	Pos	Pos	Pos
26	++++	+++	Pos	Pos	Pos	Pos
27	+++	++	Pos	Pos	Pos	Neg
28	+++	++	Pos	Neg	Pos	Neg
29	++++	+++	Pos	Pos	Pos	Pos

The level of parasitosis in DMO (Direct microscope observation) and Giemsa corresponds to: (-): non parasites, (+): 0-1 parasites, (++): 1-3 parasites, (+++): 3-6 parasites, (++++): 6-8 parasites per microscopic field. Positive amplification signal: (Pos); Negative amplification signal: (Neg).

These results show that despite the minor copy number of snoRNA-Cl1 genes than minicircles sequences, the TrF/R2 PCR does not present primer interference with *T. cruzi* genome renders this PCR a very useful to detect the presence of *T. rangeli* in co-infected insects. Besides, this findings are in accordance with those of VALLEJO *et al*. (1999)[33] and VARGAS *et al*. (2000)[36] who observed that the amplification of *T. cruzi* with the S35/S36 primers are dominant in most mixed infections, probably because the minicircles annealing sites of *T. cruzi* are present in greater quantity and could compete for the annealing of primers generating a typical *T. cruzi* profile that overlaps the presence of *T. rangeli*. It is important to highlight that eight salivary glands analyzed from the 29 *R. colombiensis* tested negative by PCR as well as by conventional methods, suggesting that at the time of the analysis, the parasite had not yet invaded the salivary glands of insects. Besides, the negativity for *T. cruzi* and *T. rangeli* of the 21 specimens of *T. maculata* collected in Bolívar with both TcH2AF/R and TrF/R2 PCR's as well with the conventional techniques and the S35/S36 PCR, confirms the specificity of the TcH2AF/R and TrF/R2 PCR tests. Of special interest

is the fact that the presence of *T. cruzi* DNA does not interfere with the amplification of the *T. rangeli* DNA despite the smaller copy number of the snoRNA-Cl1 genes in comparison to the TcH2AF/R targets. In addition, these two PCRs amplify different nuclear targets whose amplification product have sizes easily differentiated in a single agarose gel of 1.5%.

Among different research groups, different PCR detecting systems have been reported for detecting *T. cruzi* and *T. rangeli* in the intestinal content from experimental and naturally infected vectors. In this sense, DORN *et al*. (1999)[9] showed that the TC1/TC2 minicircle targed PCR assay was biased to *T. cruzi* since *T. rangeli* must constitute at least 75% of the sample in the presence of *T. cruzi* for detection by the PCR.

Later on, VARGAS *et al*. (2000)[36] used three PCR systems: S35/S36 (kDNA), D72/D75/RG3 (Variable domain of the ribosomal subunit (LSU) of trypanosomatids), and R1/R2 (specific repetitive element P542), found in 50% of *R. colombiensis* examined (6/12), *T. rangeli* was masked by the amplification of *T. cruzi* using the S35/S36 primers, concluding that this PCR does not allow the diagnosis of mixed infections in most of the insects. Afterwards, RAMÍREZ *et al*. (2002)[22] detected *T. cruzi* and *T. rangeli* in an endemic area of Brazil using five PCR systems: S35/S36, D72/D75/RG3, R1/R2, D71/D72 (D7 domain of LSU gene from *T. cruzi*) and multiplex PCR for intergenic regions in the mini-exon gene. The use of the S35/S36 primers in cultured parasites showed that in 11% of the samples (2/18), *T. cruzi* profiles masked *T. rangeli.* With this, we can conclude that those primers do not allow the detection of mixed infections. A duplex PCR assay based on telomeres sequences have been developed to determine the presence of both parasites, nevertheless, since the number of samples analyzed was reduced to a single triatomine bug[6], more studies are needy in order to asses the lack of interference or competition between *T. cruzi* and *T. rangeli* DNA telomeric sequences in field samples.

In order to avoid the interaction between the DNA of both parasites, it is recommended to use two independent PCR systems to detect mixed infections by *T. rangeli* and *T. cruzi.* The use of PCR reactions based on genes encoding for H2A/SIRE (TcH2AF/R) and sno-RNA-C11 (TrF/R2) to detect *T. cruzi* and *T. rangeli,* as described in the present work, provide the conditions of reliable tests to be implemented in detection of mixed infections in naturally infected triatomines since they yield non-polymorphic amplification products of different size in all *T. cruzi* and *T. rangeli* strains and lineages, and are capable of detecting both parasites in natural mixed infections. Therefore, these techniques might fulfil the conditions to become powerful tools for the study and understanding the epidemiology of Chagas disease. At present these PCR techniques are being tested as an alternative diagnostic method in humans.

RESUMO

Detecção da infecção por *Trypanosoma cruzi* e *Trypanosoma rangeli* em vetores triatomíneos através da amplificação dos gens de histona H2A/SIRE e sno-RNA-C11

Embora o *Trypanosoma rangeli* não seja patogênico para o homem, sua importância médica e epidemiológica reside no fato de compartilhar vetores, reservatórios e áreas geográficas com o *Trypanosoma cruzi*, agente causal da Doença de Chagas. Neste estudo, para distinguir *T. cruzi* de *T. rangeli* em vetores com infecções mistas, se utilizaram duas amplificações de PCR; TcH2AF/R para o gen da histona H2A/SIRE e TrFR2, para um gen repetitivo de ARN nucleolar Cl1 (sno-RNA-Cl1). Assim como a PCR S35/S36, ambas as reações foram capazes de detectar corretamente a presença de *T. cruzi* ou *T. rangeli* em triatomíneos infectados experimentalmente. Nas infecções mistas, o ADN de *T. cruzi* foi amplificado em 100% das amostras quando se utilizaram TcH2AF/R e S35/S36, enquanto *T. rangeli* foi detectado em 71% delas com os iniciadores TrF/R2 e em 6%, com S35/S36. Adicionalmente, em um grupo de *Rhodnius colombiensis* coletados na região de Coyaima (Tolima), *T. cruzi* foi identificado em 100% com ambas PCRs e *T. rangeli* em 14% delas com os iniciadores TrF/R2 e em 10%, com S35/S36. Estes resultados mostram que as reações de PCR TcH2AF/R e TrF/R2, capazes de reconhecer todas as cepas e linhagens de *T. cruzi* e *T. rangeli,* podem ser úteis no diagnóstico e também nos estudos epidemiológicos do campo com vetores infectados pelo *T. cruzi* e *T. rangeli*

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