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Differential detection of *Blastocrithidia triatomae* and *Trypanosoma cruzi* by amplification of 24sα ribosomal RNA genes in faeces of sylvatic triatomine species from rural northwestern Argentina

A.G. Schijman [a,*], M.A. Lauricella [c], P.L. Marcet [b], T. Duffy [a], M.V. Cardinal [b], M. Bisio [a], M.J. Levin [a], U. Kitron [d], R.E. Gürtler [b]

[a] *Laboratorio de Biología Molecular de la Enfermedad de Chagas (LabMeCh), Instituto de Ingeniería Genética y Biología Molecular (INGEBI-CONICET), Buenos Aires, Argentina*
[b] *Laboratorio de Eco-Epidemiología, Departamento de Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina*
[c] *Instituto Nacional de Parasitología Dr. Mario Fatala Chabén, Buenos Aires, Argentina*
[d] *College of Veterinary Medicine, University of Illinois, Urbana-Champaign, IL, USA*

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Abstract

Flagellates indistinguishable from *Trypanosoma cruzi* were detected by microscopy in faecal samples of 2/110 *Triatoma guasayana* and 2/283 *Triatoma garciabesi* captured in a rural area of northwestern Argentina. Inoculation of faecal homogenates to mice followed by xenodiagnosis, haemoculture, histopathology and culture from cardiac homogenates, and PCR based on *T. cruzi* minicircle and nuclear sequences failed to detect *T. cruzi* infection, pointing to another trypanosomatidean. A PCR strategy targeted to the D7 domain of 24sα ribosomal DNA genes amplified a 250 bp sequence from one *T. guasayana* and one *T. garciabesi* faecal lysate. Sequence analysis revealed 100% identity with 24sα rDNA amplicons from *Blastocrithidia triatomae* obtained from faeces of reared *Triatoma infestans* bugs. Phylogenetic analysis clustered this sequence with *C. fasciculata* and *L. major*, separated from the *Trypanosoma* branch (bootstrap: 968/1000), in concordance with a Neighbour-joining dendrogram based on 18s rDNA sequences. This PCR procedure provides a rapid sensitive tool for differential diagnosis of morphologically similar trypanosomatids in field surveys of Chagas disease vectors and laboratory-reared triatomines used for xenodiagnosis.
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Keywords: *Triatoma guasayana*; *Triatoma garciabesi*; *Trypanosoma cruzi*; *Blastocrithidia triatomae*; Chagas disease; 24sα ribosomal RNA genes

Several sylvatic or peridomestic triatomine species, such as *Triatoma sordida* and *Triatoma guasayana* have been found infected with *T. cruzi* and accordingly implicated as secondary vectors of Chagas disease in endemic regions of northern Argentina and Bolivia (Noireau et al., 1995; Castanera et al., 1998; Cecere et al., 1999; Lauricella et al., 2005). *Trypanosoma cruzi* infection in triatomine bugs is currently detected through microscopic observation (MO) of flagellated forms in unstained fresh faecal preparations. However,

* Corresponding author at: Vuelta de Obligado 2490, Second Floor, Buenos Aires 1428, Argentina. Tel.: +54 11 47832871; fax: +54 11 47868576.
E-mail address: schijman@dna.uba.ar (A.G. Schijman).

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such infections were referred to as "*T. cruzi*-like trypanosomes" (Wisnivesky-Colli et al., 1993) or "flagellates" (Noireau et al., 1995) because light microscopy has limited specificity to discriminate between *T. cruzi* and other overlapping and morphologically similar trypanosomatids, such as *Blastocrithidia triatomae* or *Trypanosoma rangeli* (Cerisola et al., 1971; Chiurillo et al., 2003). Nucleic acid amplification of polymorphic regions within conserved genes of the order Kinetoplastida may provide a rapid laboratory tool to univocally identify flagellates directly from faecal samples of naturally or experimentally infected triatomines (Breniere et al., 1995; Souto et al., 1999; Chiurillo et al., 2003). As part of a wider eco-epidemiological project conducted in a well-defined rural area of northwestern Argentina (Marcet et al., 2006), we sought to assess the distribution of *T. cruzi* infection in sylvatic triatomine species. Accordingly, 124 *T. guasayana* and 317 *T. garciabesi* specimens were captured in domiciles and peridomestic and sylvatic sites in October 2002. Diluted faecal drops obtained by abdominal compression were thoroughly examined for active trypanosomes with a microscope at 22–40×. Flagellates indistinguishable from *T. cruzi* were microscopically observed in only two *T. guasayana* and two *T. garciabesi* specimens. To confirm *T. cruzi* infection, the rectal ampoule from each MO-positive bug was obtained to prepare homogenates for: (1) bi-phasic culture into four to six tubes (50–100 μl homogenate/tube) containing 3 ml nutrient agar Difco (31 g/l)–0.5 ml defibrinated rabbit blood (penicillin 200 U/ml–streptomicin 200 U/ml) and 2 ml brain heart infusion-10% fetal calf serum (BHI-FCS) (Bioser, Buenos Aires) and (2) intraperitoneal inoculation in groups of Balb-C suckling mice (20–30 days of age, 8 g) with 0.2 ml of faecal homogenates in BHI-FCS 10% (Lauricella et al., 2005). One mouse from each group was studied by xenodiagnosis and examined 30 and 60 days post-feeding, as described (Cerisola et al., 1974); after xenodiagnosis and under anesthesia, heparinized blood was extracted by cardiac puncture and inoculated into two to three tubes per mouse and into other mice. The remaining mice of each group were tested by haemoculture and histopathological analysis, 30–45 days after inoculation, as described elsewhere (Lauricella et al., 2005). All these attempts at culturing and isolating *T. cruzi* or detecting histologic evidence of chagasic infection were negative (Table 1). PCR strategies targeted to *T. cruzi* minicircle DNA (kDNA), satellite DNA (Sat-DNA) and the intergenic regions of miniexon genes (SL-DNA) were carried out from DNA extracts obtained

Table 1
Studies in mice and PCR-based identification of flagellates from microscopically positive faecal samples of sylvatic triatomines

	Triatomine species			
	T. garciabesi		*T. guasayana*	
	LA-9-1[a]	CD-13-3[a]	6-11[a]	PE-6-2[a]
Developmental stage	Adult male	Fifth instar nym.	Adult male	Adult female
Capture site	Tree	Pig corral	Cemetry (LT)	Goat Corral
Microscopic examination	Positive	Positive	Positive	Positive
Culture	Negative	Positive	Not done	Negative
Studies in mice				
Xenodiagnosis[b]	Negative	Not done[c]	Negative	Not done
Haemoculture	Negative	Negative	Negative	Not done
Histology[d]	Negative	Negative	Negative	Negative[e]
Heart culture	Negative	Negative	Negative	Not done
PCR studies				
k-DNA	Negative	Negative	Negative	Negative
Sat-DNA	Negative	Negative	Negative	Negative
SL-DNA	Negative	Negative	Negative	Negative
24S alpha rDNA	Negative	250 bp (F + C)	250 bp (F + C)	Negative
Inhibition[f]	Yes	No	No	Yes

F: faeces; C: culture; LT: light trap; Nym.: nymph. k-DNA: kinetoplastid DNA; Sat-DNA: satellite DNA; SL-DNA: spliced leader DNA.
[a] Specimen identification.
[b] Four boxes containing four *T. infestans* third instar stage/box.
[c] Two mice died 14 dpi; heart homogenates were inoculated into other two mice, with negative findings.
[d] Intestine, skeletal muscle, lymph nodes, lung, liver and kidney.
[e] Histologic examination performed from tissues obtained after a second passage to mice.
[f] PCR inhibition was checked in all negative PCR DNA samples, as described in Schijman et al. (2003).

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(A) B.triatomae      GCAGATCTTGGTTGGCGTAGCAAAGATCTAACGGAGATACAATC-AACATGCAACGTTGG
    T.rangeli        GCAGATCTTGGTTGGCGTAGCAAAGATCTAACGGAAAATTACTTTAACATGCAACGTTGG
    T.cruzi-II       GCAGATCTTGGTTGGCGTAGCAAAGATCTAACGGAAAACTATTT-AACATGCAACGTTGG
    T.cruzi-I        GCAGATCTTGGTTGGCGTAGCAAAGATCTAACGGAAAACTATTT-AACATGCAACGTTGG
                     ************************************ *   * *  ***************
                             D 75
    B.triatoma       ATACTGGAGCGGGGAAGGATTTCGTGCCAACGGCACTCGTACACGAGTTGTTCGGATACT
    T.rangeli        ATACTGGAGCGGGGAAGGATTTCGTGCCAACGGCACTCGTACACGAGTTGTTCGGATACT
    T.cruzi-II       ATACTGGAGCGGGGAAGGATTTCGTGCCAACGGCACTCGTACACGAGTTGTTCGGATACT
    T.cruzi-I        ATACTGGAGCGGGGAAGGATTTCGTGCCAACGGCACTCGTACACGAGTTGTTCGGATACT
                     ********************************************************* ***

    B.triatomae      GAGCACAACGTTACATCGTTTTGTTAGGAAAGTGAAGGTGTGTCGGCGGAATTGCAG---
    T.rangeli        GAGCACAACGTTACACCGTTTTGTTAGGAAAGTGAAGGTGCGTCAAATGGTATGGG----
    T.cruzi-II       GAGCACAACGTTACACCGTTTTGTTAGGAAAG--AAGGTGCGTCGACAGTGTGGGAGTCC
    T.cruzi-I        GAGCACAACGTTACACCGTTTTGTTAGGAAAGTGAAGGTGCGTCGACAGTGTGGGGGAGT
                     ************* * ***************  ****** ***    *

    B.triatomae      -------CTCTTCCCTCTCTTCAC---------------------------------GGG
    T.rangeli        ----------CCTCCTCTCTTCGG-------------------------------------
    T.cruzi-II       TTTTTTTTTTCTACCTCTCTTTCTCTCTCTTCACGGGGGGGTTATATAGAGGGAGTAGGA
    T.cruzi-I        CTCTTCTTCTTTCCCCCTCTCTCCTTTT---------GGTGT---------GGGGTGTGG
                             *  ***
                                                                  D 76
    B.triatomae      GAGGACCTAGACTGCTTTCGACTGGCCATAATTGAAAAGGGGCAACAGAGAACC 250 bp
    T.rangeli        -----AAAGGAGCCTTACCCTTTGGCCATTACTGAAAAGGGGCAACAGAGAACC 239 bp
    T.cruzi-II       AAAAAAAAAGCCCCTTACTGT-CGGCCATT-CTGAAAAGGGGCAACAGAGAACC 289 bp
    T.cruzi-I        GAGGAAGAAGCCCCTTACTGTTCGGCCATT-CTGAAAAGGGGCAACAGAGAACC 274 bp
                           *     *       ******   **********************
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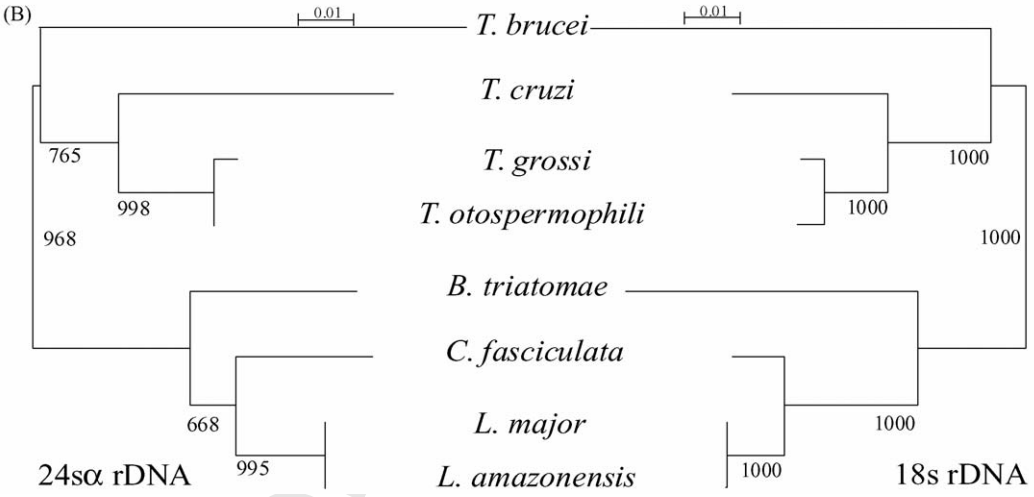

Fig. 1. (A) Multiple alignment of the 24sα rDNA sequence from D75 to D76 amplified DNA of *B. triatomae* with equivalent regions of *C. fasciculata*, *T. rangeli*, *T. cruzi* I and *T. cruzi* II, obtained from the Genbank. Sequences were aligned using CLUSTAL W algorithm (Thompson et al., 1994), with a gap opening penalty value of 15.00 and a gap extension penalty value of 3.00. Numbers at the end of the sequences denote amplicon lengths. Asteriks indicate consensus sequences and dashes denote gaps. Sequences from *B. triatomae* were deposited in the Genbank under the accession numbers AY820895, AY820896 and AY820897. (B) Neighbour-joining dendrograms based on trypanosomatidean 24sα rDNA (left tree) and 18s rRNA genes (right tree). Numbers at the branches show bootstrap values after 1000 replications. Bar at the top denotes distance. Genbank accession numbers for 24sα rDNA sequences: *L. major* AC005806, ~~*T. grossi*~~ AB175623, *T. otospermophili* AB190228, *T. brucei* X14553, *C. fasciculata* Y00055, *T. cruzi* M28885 and *L. amazonensis* U73615. Genbank accession numbers for 18S rDNA genes: *L. major* AC005806, ~~*T. grossi*~~ AB175623; *T. otospermophili* AB190228; *T. brucei* M12676; *C. fasciculata* Y00055; *T. cruzi* M31432; *B. triatomae* AF153037 and *L. amazonensis* X53912. ~~Sequences from *B. triatomae* were deposited in the Genbank under the accession numbers AY820895, AY820896 and AY820897.~~

from the mentioned faeces, as described (Schijman et al., 2000; Marcet et al., 2006) but no amplification products were obtained (Table 1), suggesting that other trypanosomatideans could be responsible for the MO-positive findings in the sylvatic triatomine faecal samples. Consequently, a PCR strategy based on the amplification of the polymorphic D7 domain of the 24Sα rRNA genes was applied, using oligonucleotides D75 GCAGATCTTGGTTGGCGTAG (position 01–20) and D76 GGTTCTCTGTTGCCCCTTTT (position 279–298) which match conserved sequences within the trypanosomatidean genomes (Briones et al., 1999; Souto et al., 1999) in a 50 μl volume reaction containing 4 μM of each primer, 250 μM dNTPs, 3 mM $MgCl_2$ and 1.25 U Taq Platinum (Invitrogen, USA). Amplification consisted of an initial denaturation for 3 min at 94 °C, a five-step-touch-down PCR, ranged from 60 to 52 °C, with four rounds of three cycles each one, consisting of 1 min at 94 °C, 1 min annealing at 60, 58, 56 and 54 °C from the first to the fourth round respectively and 1 min elongation at 72 °C. The fifth round consisted of 35 cycles of 1 min at 94 °C, 1 min at 52 °C and 1 min at 72 °C. A final elongation step at 72 °C for 10 min ended the program. *T. cruzi* lineage I, *T. cruzi* lineage IIb and *T. rangeli* DNA samples were used as controls. Amplification products of 250 bp (Fig. 1A) were obtained from the faecal lysates of one *T. guasayana* (6–11, Table 1) and one *T. garciabesi* (CD-13-3, Table 1), as well as from its corresponding culture isolate (Table 1). The other two MO-positive samples, one from *T. guasayana* (PE-6-2) and the other one from *T. garciabesi* (LA-9-1) were PCR negative, but as their DNA preparations carried PCR inhibitors (Table 1) these negative findings are invalid.

Noteworthy, the length of the amplicons did not correspond to any other known 24Sα rDNA fragment from other trypanosomatids, namely *T. cruzi* I, 270 bp; *T. cruzi* II, 290 bp, *T. rangeli*, 240 bp (Fig. 1A), *C. fasciculata* or *Leishmania* spp., 225 bp (Souto et al., 1999). Comparison between the DNA sequences of the 250 bp amplicons from *T. guasayana* and *T. garciabesi* samples revealed 100% of nucleotide identity, but did not match to any other known 24Sα rDNA sequence. On the basis of previous studies describing *B. triatomae* as a monogenetic kinetoplastid of triatomine species (Cerisola et al., 1971; Schaub and Breger, 1990; Fernandes et al., 1993; Maslov et al., 1996; Cecere et al., 1999), we attempted to amplify the equivalent 24Sα rDNA fragment from *B. triatomae* obtained from faeces of laboratory-reared *T. infestans* specimens. This control also amplified the 250 bp 24Sα rDNA sequence, which was identical to those originally characterized from the field-captured sylvatic triatomines (Fig. 1A).

A neighbour-joining (NJ) tree was constructed from the alignment of the novel *B. triatomae* 24sα rRNA sequence with homologous ones from *L. major*, *L. amazonensis*, *T. grosi*, *T. otospermophili*, *T. brucei*, *C. fasciculata* and *T. cruzi* available at the Genbank (Fig. 1B, left tree) and compared to another NJ tree based on published full-length 18s rDNA sequences of these eight kinetoplastidean protozoans (Fig. 1B, right tree). Both dendrograms were concordant, clustering the blastocrithidial sequence together with those from *C. fasciculata* and *Leishmania* spp. and separated from the *Trypanosoma* clades (bootstrap: 968/1000 for 24sα rRNA and 1000/1000 for 18s rDNA, Fig. 1B).

The existing taxonomy of kinetoplastids is based on morphology and life cycles, which do not reflect the true genetic affinities in these organisms (Maslov et al., 2001). Accordingly, caution should be taken when diagnosing *T. cruzi* infection based only on microscopic observation of unstained fresh preparations of triatomine faeces. "False positives" may lead to invalid incrimination of a vector species and invalid inferences on the relationships between sylvatic and domestic Chagas disease transmission cycles. The MO positive *T. guasayana* specimen infected with *B. triatomae* was a female adult captured by light trapping in a cemetery at the locality of Amamá and the *T. garciabesi* specimen was a fifth-instar nymph captured in a pig corral at the locality of Central Dolores, in the same localities where *T. cruzi* was detected in faecal samples of peridomestic and domestic *T. infestans* specimens (Marcet et al., 2006).

It is noteworthy the yield of Blastocrithidia in biphasic culture, known to be difficult to obtain in conventional media (Reduth et al., 1989).

To our knowledge, this is the first PCR-based report showing the differential identification of *B. triatomae* and *T. cruzi* infections directly in faeces of field-captured triatomines. This strategy may also provide a rapid and sensitive tool for monitoring laboratory-reared triatomine bugs used for xenodiagnosis, for differential identification of infections by *T. cruzi* and *T. rangeli* in Chagas disease vectors from overlapping endemic areas (Souto et al., 1999; Chiurillo et al., 2003), and for differential diagnosis of opportunistic infections with lower monoxenous trypanosomatids in patients with severe AIDS (Chicharro and Alvar, 2003).

Uncited references

Canale et al. (2000), Cecere et al. (1997), Chuit et al. (1992) and Souto et al. (1996).

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References

Breniere, S.F., Bosseno, M.F., Telleria, J., Carrasco, R., Vargas, F., Yaksic, N., Noireau, F, 1995. Field application of polymerase chain reaction diagnosis and strain typing of *Trypanosoma cruzi* in Bolivian triatomines. Am. J. Trop. Med. Hyg. 53, 179–184.

Briones, M.R., Souto, R.P., Stolf, B.S., Zingales, B., 1999. The evolution of two *Trypanosoma cruzi* subgroups inferred from rRNA genes can be correlated with the interchange of American mammalian faunas in the Cenozoic and has implications to pathogenicity and host specificity. Mol. Biochem. Parasitol. 104, 219–232.

Canale, D.M., Cecere, M.C., Chuit, R., Gürtler, R.E., 2000. Peridomestic distribution of Triatoma garciabesi and *Triatoma guasayana* in north-west Argentina. Med. Vet. Entomol. 14, 383–390.

Castanera, M.B., Lauricella, M.A., Chuit, R., Gürtler, R.E., 1998. Evaluation of dogs as sentinels of the transmission of *Trypanosoma cruzi* in a rural area of north-western Argentina. Ann. Trop. Med. Parasitol. 92, 671–683.

Cecere, M.C., Gürtler, R.E., Canale, D.M., Chuit, R., Cohen, J.E., 1997. The role of the peridomiciliary area in the elimination of *Triatoma infestans* from rural Argentine communities. Pan Am. J. Public Health 1, 273–279.

Cecere, M.C., Castanera, M.B., Canale, D.M., Chuit, R., Gürtler, R.E., 1999. *Trypanosoma cruzi* infection in *Triatoma infestans* and other triatomines: long-term effects of a control program in rural northwestern Argentina. Pan Am. J. Public Health 5, 392–399.

Cerisola, J.A., Rohwedder, R., Bozzini, J.P., Del Prado, C.E., 1971. *Blastocrithidia triatomae*. sp. found in *Triatoma infestans* from Argentina. J. Protozool. 18, 503–506.

Cerisola, J.A., Rohwedder, A., Segura, E., Del Prado, C.E., De Martini, G.W., 1974. El Xenodiagnóstico. Normalización. Utilidad. Sec Est Salud Pública Ed., Buenos Aires, pp.127.

Chicharro, C., Alvar, J., 2003. Lower trypanosomatids in HIV/AIDS patients. Ann. Trop. Med. Parasitol. 97, 75–78.

Chiurillo, M.A., Crisante, G., Rojas, A., Peralta, A., Dias, M., Guevara, M., Añez, N., Ramírez, J.L., 2003. Detection of *Trypanosoma cruzi* and *Trypanosoma rangeli* Infection by Duplex PCR Assay Based on Telomeric Sequences. Clin. Diag. Lab. Immunol., 775–779.

Chuit, R., Paulone, I., Wisnivesky-Colli, C., Bo, R., Perez, A.C., Sosa-Stani, S., Segura, E.L., 1992. Results of a first step toward community-based surveillance of transmission of Chagas disease with appropriate technology in rural areas. Am. J. Trop. Med. Hyg. 46, 444–450.

Fernandes, A.P., Nelson, K., Beverley, S.M., 1993. Evolution of nuclear ribosomal RNAs in kinetoplastid protozoa: perspectives on the age and origins of parasitism. Proc. Natl. Acad. Sci. U.S.A. 90, 11608–11612.

Lauricella, M.A., Stariolo, R.L., Riarte, A.R., Segura, E.L., Gürtler, R.E., 2005. Distribution and pathogenicity of *Trypanosoma cruzi* isolated from peridomestic populations of *Triatoma infestans* and *Triatoma guasayana* from rural western Argentina. Mem. Inst. Oswaldo Cruz 100, 123–129.

Marcet, P.L., Duffy, T., Cardinal, M.V., Burgos, J.M., Lauricella, M.A., Levin, M.J., Kitron, U., Gürtler, R.E., Schijman, A.G., 2006. PCR-based screening and lineage identification of *Trypanosoma cruzi* directly from faecal samples of triatomine bugs from northwestern Argentina. Parasitology 132, 57–65.

Maslov, D.A., Podlipaev, S.A., Lukes, J., 2001. Phylogeny of the kinetoplastida: taxonomic problems and insights into the evolution of parasitism. Mem. Inst. Oswaldo Cruz 96, 397–402.

Noireau, F., Bosseno, M.F., Carrasco, R., Telleria, J., Vargas, F., Camacho, C., Yaksic, N., Breniere, S.F., 1995. Sylvatic triatomines (Hemiptera: Reduviidae) in Bolivia: trends toward domesticity and possible infection with *Trypanosoma cruzi* (Kinetoplastida: Trypanosomatidae). J. Med. Entomol. 32, 594–598.

Reduth, D., Schaub, G.A., Pudney, M., 1989. Cultivation of *Blastocrithidia triatomae* (Trypanosomatidae) on a cell line of its host *Triatoma infestans* (Reduviidae). Parasitology 98, 387–393.

Schaub, G.A., Breger, B., 1990. Pathological effects of *Blastocrithidia triatomae* (Trypanosomatidae) on the reduviid bugs *Triatoma sordida*, *T. pallidipennis* and *Dipetalogaster maxima* after coprophagic infection. Parasitol. Res. 76, 306–310.

Schijman, A.G., Vigliano, C., Burgos, J.M., Favaloro, R., Perrone, S., Laguens, R., Levin, M.J., 2000. Early diagnosis of recurrence of *Trypanosoma cruzi* infection by polymerase chain reaction after heart transplantation of a chronic Chagas' heart disease patient. J. Heart Lung Transpl. 19, 1114–1117.

Schijman, A.G., Altcheh, J., Burgos, J.M., Biancardi, M., Bisio, M., Levin, M.J., Freilij, H., 2003. Etiological treatment of congenital Chagas disease diagnosed and monitored by the polymerase chain reaction. J. Ant. Chemother. 52, 441–449.

Souto, R.P., Fernandes, O., Macedo, A.M., Campbell, D.A., Zingales, B., 1996. DNA markers define two major phylogenetic lineages of *Trypanosoma cruzi*. Mol. Biochem. Parasitol. 83, 141–152.

Souto, R.P., Vargas, N., Zingales, B., 1999. *Trypanosoma rangeli*: discrimination from *Trypanosoma cruzi* based on a variable domain from the large subunit ribosomal RNA gene. Exp. Parasitol. 91, 306–314.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence waiting, position—specific gap penalties and weight matrix choice. Nucl. Acids Res. 22, 4673–4680.

Wisnivesky-Colli, C., Gürtler, R.E., Solarz, N.D., Schweigmann, N.J., Pietrokovsky, S.M., Alberti, A., Flo, J., 1993. Dispersive flight and house invasion by *Triatoma guasayana* and *Triatoma sordida* in Argentina. Mem. Inst. Oswaldo Cruz 88, 27–32.

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