Infection, Genetics and Evolution 83 (2020) 104328

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Real-time polymerase chain reaction based algorithm for differential diagnosis of Kinetoplastidean species of zoonotic relevance

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ARTICLE INFO

Keywords:
Trypanosomatids
Leishmania spp.
Diagnosis
Zoonosis
Triatomine
High-resolution melting analysis

ABSTRACT

Kinetoplastids are a group of flagellated protozoa that infect a vast repertoire of mammals and insect vectors. From a zoonotic point of view, domestic animals are critical reservoirs for transmission of Kinetoplastidean parasites. Due to their proximity to humans, they assume substantial epidemiological importance in the context of these zoonoses and consequently in public health. Their reliable identification is relevant to understand their eco-epidemiological involvement in transmission cycles. This work aimed to develop an algorithm based on sequential Real-Time PCR (qPCR) assays targeted to different loci (24S alpha rDNA, ITS1 and Hsp70) allowing distinction among *Trypanosoma cruzi*, *Trypanosoma rangeli*, *Trypanosoma evansi* and Leishmania species in biological samples collected from mammalian reservoirs and triatomine vectors. The algorithm includes a first qPCR test targeted to endogenous genes conserved within mammals and within triatomine vectors as internal controls of DNA sample integrity and/or qPCR inhibition. This algorithm was evaluated in biological samples from domestic cattle ($N = 14$), dogs ($N = 19$) and triatomines (N = 19). Analytical sensitivity of 24S alpha rDNA for detection of *T. rangeli* was 10 fg of DNA, with a linear range between 10 fg and 10 ng. For *T. cruzi* it varied depending on the Discrete typing unit. The ITS1 qPCR showed an analytical sensitivity of 100 pg/reaction and 100 fg/reaction of Leishmania spp. and *T. evansi* DNAs. In mammal field samples, four *T. cruzi* 24S alpha rDNA sequences and fourteen ITS1 amplicons specific for *T. evansi* were detected. qPCR-HRM analysis directed to the Hsp70 gene diagnosed two dogs with *Leishmania infantum* infection. Among 19 triatomine field samples, *T. cruzi* was detected in five; *T. rangeli* in eight and one specimen showed a mixed infection. This diagnostic algorithm can provide more accurate records of kinetoplastidean infection burden in vectors and reservoirs, relevant to update current eco-epidemiological maps in co-endemic regions.

1. Introduction

Kinetoplastids are a group of flagellated protozoa that can infect a large number of mammals, including humans. In many cases, these parasitic infections can become pathogenic with devastating health and economic effects. The most common zoonotic infections caused by Kinetoplastids include: i) human African trypanosomiasis (HAT; also known as African sleeping sickness) which is caused by infection with two of the three subspecies of *Trypanosoma brucei*; ii) infection by *Trypanosoma cruzi* causative of Chagas disease; iii) infection by *Trypanosoma rangeli,* a Kinetoplastid parasite considered non-pathogenic for humans but with epidemiological relevance because of overlapping geographical distribution with *T. cruzi*; iv) *Trypanosoma evansi* causative of "Surra" disease; v) *Trypanosoma vivax and Trypanosoma equiperdum* that cause equine and bovine diseases, and vi) infectious by different species of Leishmania, which cause three main forms of Leishmaniases: visceral (also known as kala-azar - the most serious form of the disease), cutaneous (the most common), and mucocutaneous (Mello et al., 1988; Vallejo et al., 1988; Burri and Brun, 2003; Grijalva et al., 2012; Guim et al., 2013; Santos et al., 2015; Villacís et al., 2015; Sasani et al., 2016; Arce-Fonseca et al., 2017; Greif et al., 2018; Parodi et al., 2018; Ribeiro et al., 2018; Travi, 2019).

All mammals are prone to get the infection from any of these causative agents; however, from a zoonotic point of view, domestic animals are critical reservoirs in the transmission of Kinetoplastids species. The predation and eventual encounters between these animals and wildlife

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https://doi.org/10.1016/j.meegid.2020.104328
Received 27 January 2020; Received in revised form 12 April 2020; Accepted 18 April 2020
Available online 21 April 2020

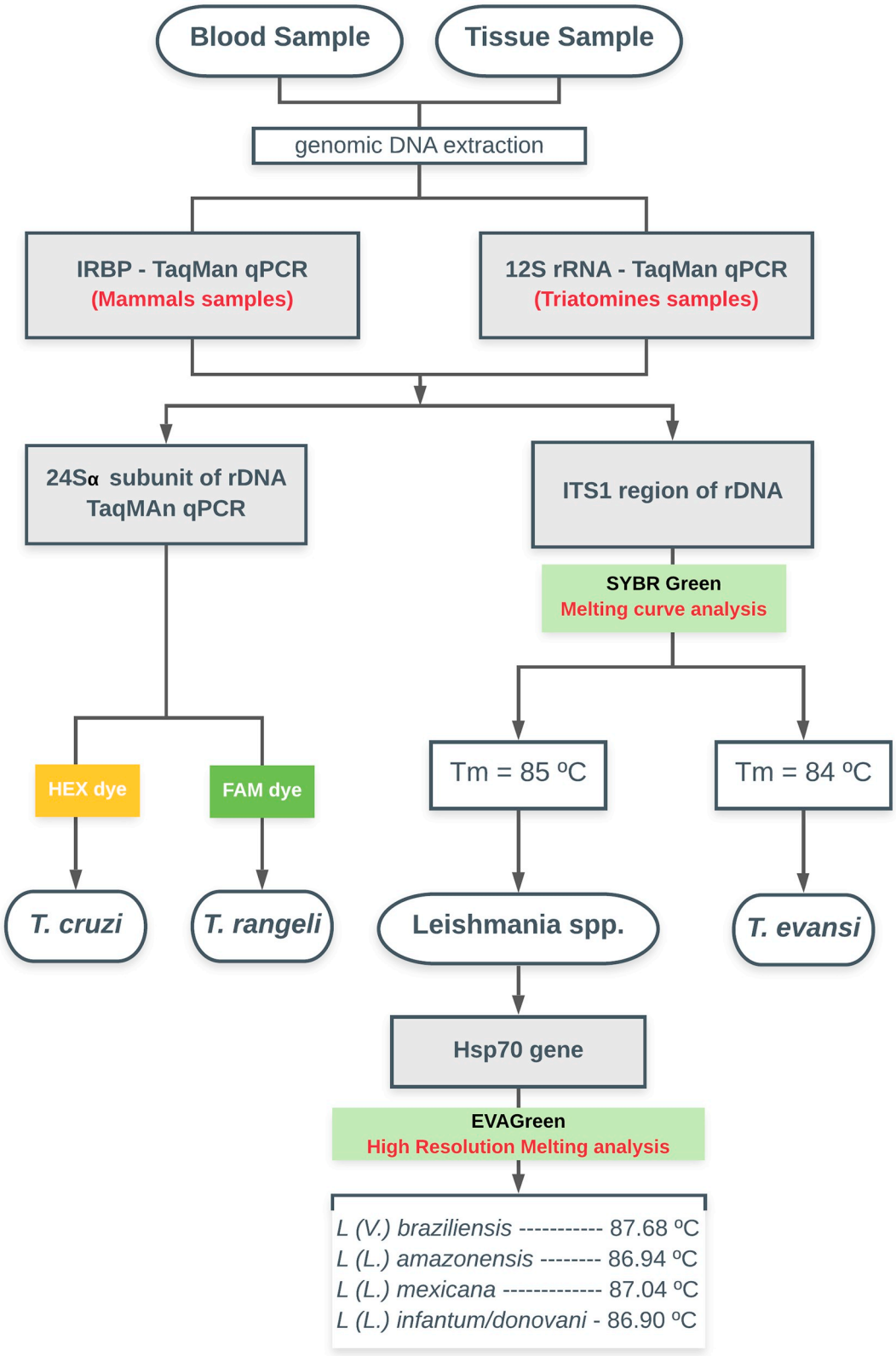


Fig. 1. Methodology Flow chart showing the different qPCR assays followed by Melting curves analysis designed to distinguish among *Trypanosoma cruzi*, *Trypanosoma rangeli*, *Trypanosoma evansi* and Leishmania spp., in biological specimens.

have been reported as a route of parasitic transmission in rural areas (Villacís et al., 2015; Daszak et al., 2000). In these environments, domestic animals function as sentinels to detect active transmission cycles before they involve humans, becoming reservoirs of peridomicile transmission cycles s. Due to their proximity to humans, domestic animals assume substantial epidemiological importance in the context of theses zoonoses, and consequently for public health (Thompson et al., 2009; Eloy and Lucheis, 2009). Consequently, the correct identification

Table 1
Genomic targets, probes and primer pair sequences used for qPCR assays.

Gene target	Primer/Probe	Sequence 5′ - 3′	Reaction concentration	Amplicon size	Source
IRBP gene from mammalian genome	IRBP2 Fw	CCAAYACVACCACTGAGATCTG	0.6 µM	140 bp	Wehrendt et al. (2019)
	IRBP3 Rv	GCGCATCTGYTTGAGGATGTARG	0.6 µM		
	IRBPTq (Probe)	HEX-TGGTGGTCCTCACCAG-NFQ-MGB	0.05 µM		
Triatomine 12S ribosomal DNA	P2B Fw	AAAGAATTTGGCGGTAATTTAGTCT	0.3 µM	163 bp	Moreira et al. (2017)
	P6R Rv	GCTGCACCTTGACCTGACATT	0.5 µM		
	Triat (Probe)	VIC-TCAGAGGAATCTGCCCTGTA-NFQ-MGB	0.05 µM		
24Sα subunit of ribosomal DNA	D75b	ACACGAGTTGTTCGGATACTG	0.4 µM	140/175 bp	This work
	D76b	GGTTCTCTGTTGCCCCTTTT	0.4 µM		
	TrTq (Probe)	FAM-AAATGGTATGGGCCTCCTCTCTTCGG-BHQ1	0.05 µM		
	TcTq (Probe)	HEX-AAAGTGAAGGTGCGTCGACAGTGTG-BHQ1	0.1 µM		
ITS1 region of ribosomal DNA	ITS1 CF	CCGRAAGTTCACCGATATTG	0.2 µM	400/480 bp	Njiru et al. (2005)
	ITS1 BR	TGCTGCGTTCTTCAACGAA	0.2 µM		
Hsp70 gene from Leishmania spp.	Hsp70 Fw	GGAGAACTACGCGTACTCGATGAAG	0.2 µM	144 bp	This work
	Hsp70C Rv	TCCTTCGWCGCCTCCTGGTTG	0.2 µM		

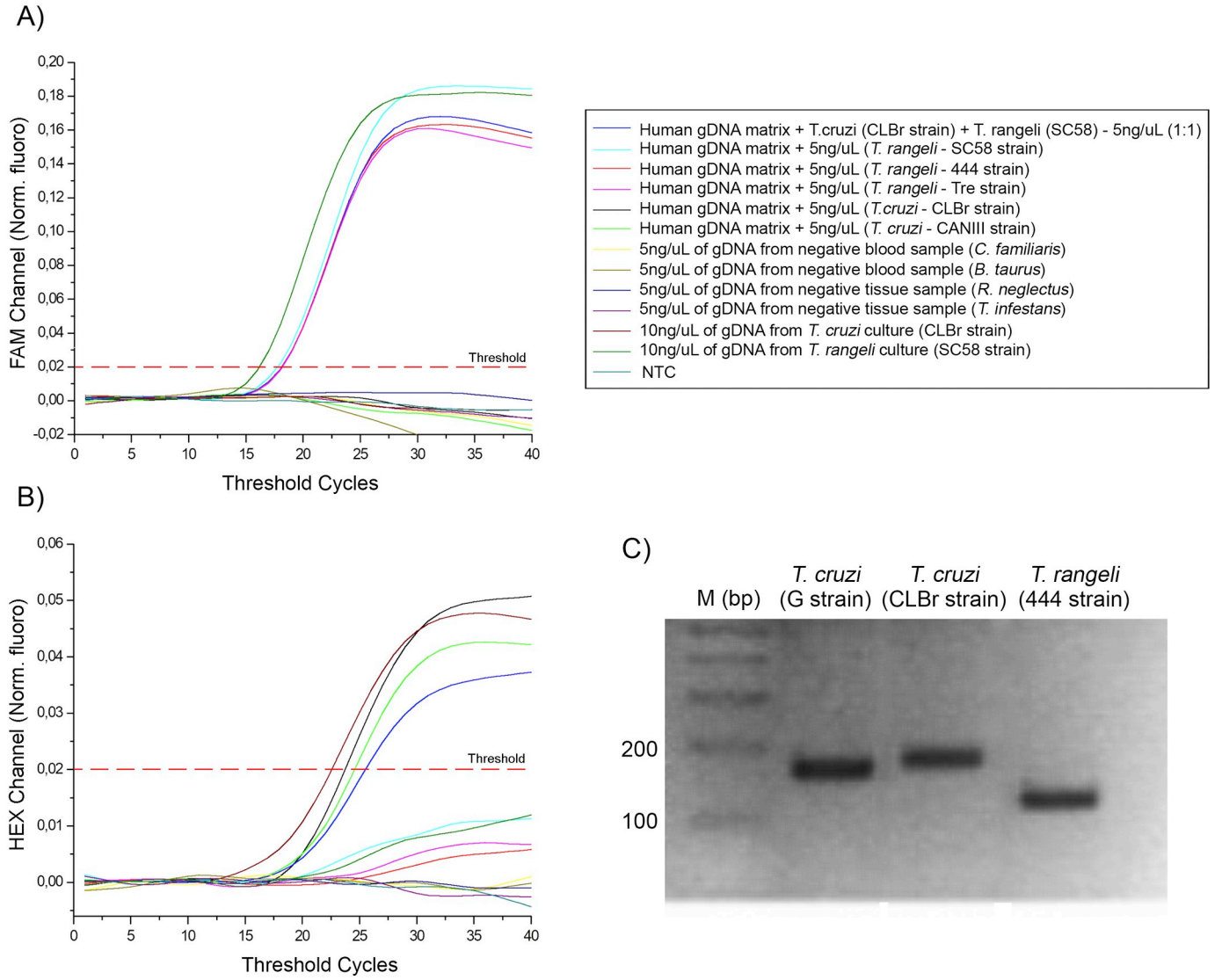


Fig. 2. 24Sα rDNA based TaqMan qPCR curves showing specific detection of (A) *Trypanosoma rangeli* and (B) *Trypanosoma cruzi* in the presence of human genomic DNA. Genomic DNA from uninfected *Bos Taurus, Canis familiaris, Rhodnius neglectus* and *Triatoma infestans* were included as specificity controls (C) 3% Agarose Gel electrophoresis showing qPCR amplicons. M: 100 bp molecular weight marker; NTC: non-template control.

of their parasitic infections, and those of insect vectors involved in the transmission cycles is important. Currently, to detect kinetoplastid infections in reservoirs, xenodiagnostics, serological and PCR tests have been used independently. However, the results vary due to the characteristics of the tests used as well as due to particular features of the infection in the different hosts (Vexenat et al., 1996; Desquesnes et al., 2002; Ndao, 2009; Medkour et al., 2020). Besides, distinction in triatomine vector samples, usually done by microscopic observation

Table 2
Inclusivity and Exclusivity of the 24Sα rDNA TaqMan qPCR.

Species	Strain	Cq	
		TcTq-HEX	TrTq-FAM
T. cruzi	G (TcI) [a]	20.87	ND
	K98 (TcI) [a]	19.28	ND
	Silvio X10 (TcI) [a]	21.01	ND
	Colombiana (TcI) [a]	23.17	ND
	Dm28c (TcI) [a]	19.75	ND
	Y (TcII) [a]	19.68	ND
	TU 18 (TcII) [a]	17.02	ND
	MAS cl1 (TcII) [a]	18.75	ND
	M5631 (TcIII) [a]	18.27	ND
	3663 (TcIII) [a]	23.88	ND
	X109/2 (TcIII) [a]	20.86	ND
	Can III (TcIV) [a]	16.79	ND
	4167 (TcIV) [a]	19.60	ND
	Dog Theis (TcIV) [a]	22.08	ND
	92122192R (TcIV) [a]	22.86	ND
	PAH 265 (TcV) [a]	19.18	ND
	MN cl2 (TcV) [a]	16.48	ND
	PAH179 (TcV) [b]	16.07	ND
	LL014 (TcV) [c]	30.78	ND
	CL Brener (TcVI) [a]	17.64	ND
	RA (TcVI) [a]	28.34	ND
	Tep7 (TcVI) [b]	16.64	ND
	LL052 (TcVI) [b]	28.45	ND
	Tcbat	18.27	ND
T. rangeli	444	ND	18.69
	SC-58	ND	19.54
	Tre	ND	16.28
L. (L.) mexicana	L1508	ND	ND
L. (L.) amazonensis	M2269	ND	ND
L. (L.) major	L1566	ND	ND
L. (V.) braziliensis	L1569	ND	ND
H. sapiens gDNA		ND	ND

ND: not detectable.
[a] Zingales et al. (2009).
[b] Lauthier et al. (2012).
[c] Cosentino and Agüero (2012).

may be cumbersome in case of morphologically similar trypanosomatids. Therefore, it becomes relevant to design precise, differential and sensitive diagnostic tools for parasitic diseases in overlapping transmission areas to detect and control their transmission cycles.

This paper reports the development of a sequential diagnostics algorithm based on Real-Time PCR assays (qPCR) coupled to High Resolution Melting (HRM) analysis aiming to provide a molecular detection tool for direct identification of *T. cruzi, T. rangeli, T. evansi* and Leishmania species of zoonotic relevance in biological samples from mammalian hosts and insect vectors.

2. Materials and methods

2.1. Biological specimens

2.1.1. Kinetoplastids strains

DNA samples from *Trypanosoma cruzi* (G, TU18, M5631, CANIII, PAH265 and CL-Brener strains), *Trypanosoma rangeli* (444, SC-58 and Tre strains), *Trypanosoma evansi*, *Trypanosoma brucei brucei*, *Leishmania (V.) braziliensis* (L1569 strain), *Leishmania (L.) amazonensis* (M2269 strain), *Leishmania (L.) mexicana* (L1508 strain), *Leishmania major* (L1566 strain) and the complex *Leishmania (L.) infantum/Leishmania (L.) donovani* were selected from stocks available at the DNA collection of LABMECH (INGEBI-CONICET) or kindly provided by Dr. Otacilio Moreira (Fiocruz, Brazil) and Dr. Felipe Guhl (CIMPAT, Universidad de los Andes, Bogotá, Colombia) and stored stably at −20 °C.

2.1.2. Mammalian reservoirs

Blood samples from domestic animal reservoirs were provided from Santiago del Estero, Chaco and Cordoba provinces (North-eastern Argentina) by Andrea Gomez-Bravo (Fundación Mundo Sano, Buenos Aires, Argentina), Dr. Horacio Lucero (Universidad Nacional del Nordeste, Resistencia, Chaco) and Dr. Matilde Giammaría (Universidad Nacional de Rio Cuarto, Córdoba).

2.1.3. Triatomine vectors

Triatoma infestans ($N = 10$) specimens were collected in Añatuya, Santiago del Estero province, Argentina in 2018 (Andrea Gomez-Bravo, Fundación Mundo Sano, Argentina). Faecal samples were obtained and stored at −20 °C. DNA was extracted within one year after collection and stored at −20 °C.

Rhodnius neglectus ($N = 19$) specimens were collected at Central Brazil (César Cuba Cuba, Núcleo de Medicina Tropical (NMT), University of Brasília, Brazil) in 2012 and tissues from the back of the abdomen were cut and preserved in 100 µL of PBS at 4 °C until DNA extraction.

2.2. DNA extraction methods

2.2.1. Mammalian blood samples

The algorithm was assayed using DNA extracted from thirty-three peripheral blood samples collected from domestic mammalian reservoirs captured in Argentina (Fundación Mundo Sano). Blood samples were treated with 6 M guanidine hydrochloride and 0.2 M EDTA buffer (GEB) in a proportion 1:3. The DNA was extracted from 300 µL GEB blood using the High Pure PCR Template Preparation Kit (Roche Diagnostic Corp, Indiana, USA) following manufacturer instructions.

2.2.2. Triatomines tissue samples

Genomic DNA from vector samples (gDNA) was extracted using the High Pure PCR Template Preparation Kit (Roche Diagnostic Corp, Indiana, USA), following the recommended protocol for mammalian tissue but extending the tissue lysis reaction for 24 h. The purified gDNAs were stored at −20 °C until use (Gurgel-Gonçalves et al., 2012).

DNA samples from *T. infestans* were negative for *T. cruzi* infection by means of a qPCR assay targeted to the highly repetitive satellite DNA sequence of *T. cruzi* (Duffy et al., 2013). Then, 10 µL of these gDNA lysates were spiked with 10 µL (5 ng/µL) of *T. rangeli* and *T. cruzi* DNAs to evaluate the ability of 24S qPCR to detect these trypanosomatids in artificial vector samples.

2.3. Real-time PCR assays (qPCR) procedures

A three-step qPCR protocol was proposed to detect Trypanosomatids and Leishmania spp., in biological samples (Fig. 1). Their oligonucleotide sequences, final concentrations in the qPCR reactions and cycling programs used are given in Table 1. All qPCR reactions were carried out using a Rotor-Gene Q HRM Platform (Qiagen, USA). Essential parameters for each assay were determined following the recommendations of MIQE guidelines (https://www.ncbi.nlm.nih.gov/pubmed/19246619).

2.3.1. Endogenous internal amplification standard qPCR assays

2.3.1.1. Mammalian internal amplification control. This qPCR assay was included in the diagnostics algorithm to provide an internal control of DNA integrity and/or PCR inhibition in mammalian DNA. It was directed to the highly conserved mammalian gene encoding for the interphotoreceptor retinoid-binding protein (IRBP) (Wehrendt et al., 2019). The reaction was performed in a final volume of 20 µL with FastStart Universal Probe Master Mix (Roche Diagnostics, Mannheim, Germany) and 5 µL of DNA, in a Rotor-Gene 3000 (Corbett Life Science, Cambridge, UK) or an ABI 7500 (Applied Biosystems, Foster City, CA) device. Primers IRBP Fw and Rv and IRBP probe were used. Their

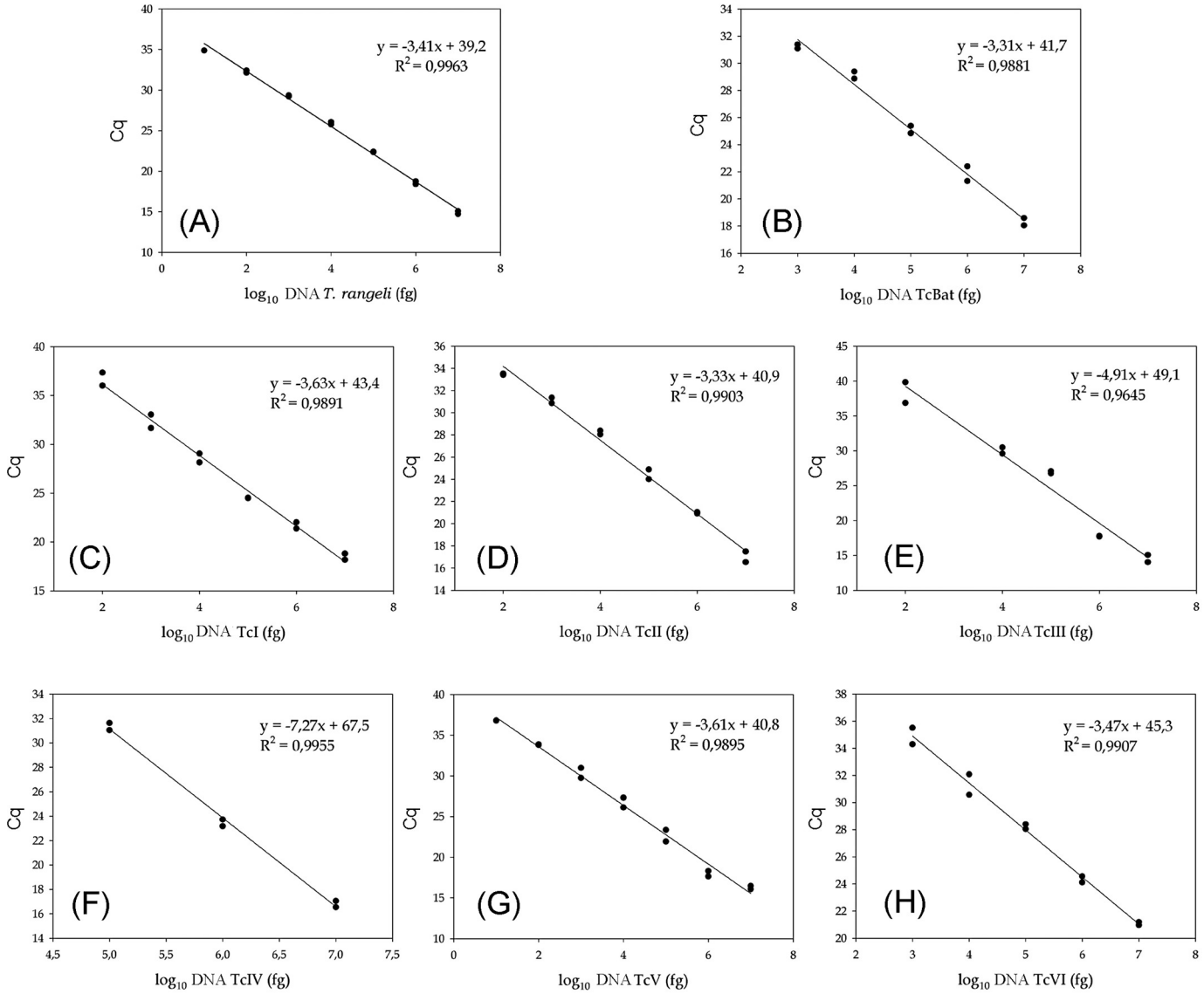


Fig. 3. Determination of the analytical sensitivity and linear range of the 24Sα TaqMan qPCR in different *Trypanosoma cruzi* DTUs, Tcbat and *Trypanosoma rangeli*. Standard reaction curves specify the relationship between the threshold cycle (Cq) and the amount of genomic DNA (log10) from *T. rangeli* (A), *Tcbat* (B) and *T. cruzi* (C to H).

sequences and final concentrations in the qPCR reaction are given in Table 1. Cycling conditions were an initial step of 10 min at 95 °C and 45 cycles at 95 °C for 15 s and 56 °C for 1 min.

2.3.1.2. Triatomine internal amplification control. A qPCR procedure to provide an internal control of DNA sample integrity and/or PCR inhibition in triatomine samples was included. It was targeted to the 12S ribosomal RNA gene of triatomines (Moreira et al., 2017). The qPCR was performed in a final volume of 20 µL using 2 µL of DNA, extracted from the triatomine samples, by the following cycling profile: 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 58 °C. Sequences and final concentrations of primers in the TaqMan qPCR reaction are given in Table 1. All qPCR reactions were carried out using a Rotor-Gene Q HRM Platform (Qiagen, USA).

2.3.2. 24Sα rDNA TaqMan qPCR for distinction between Trypanosoma cruzi and Trypanosoma rangeli

Once genomic DNA (gDNA) integrity is verified, the algorithm proposes the simultaneous search for *T. cruzi* and *T. rangeli* DNA in biological samples. The assay is based on TaqMan probes that recognize specific sequences within the 24Sα subunit of the rDNA gene of both Trypanosoma species (Supplementary Fig. 1). Primer and probe design were carried out after aligning *T. cruzi* 24Sα rDNA sequences from strains representative of the six Discrete Typing Units (DTUs) available at the databases (Genbank accession numbers: TcI L22334, TcII AY367118, TcIII AY367117, TcIV AY367115, TcV AY367122, TcVI U73959 and one from *T. rangeli* San Agustin strain (U73612). Moreover, we sequenced the homologous gene fragments from five *T. rangeli* stocks Rne290.3A (JN673224), Rne324A (JN673225), Rne966A (JN673235), TG383-1 (JN16745), bl711 (JN016743) and one *T. cruzi* isolate from bats (Tcbat, accession number MT258567).

The reaction was carried out in a final volume of 20uL, with 2uL of gDNA and the FastStart Universal Probe Master Mix (Roche Diagnostics, Mannheim, Germany). The sequences and final concentrations of primers in the qPCR reaction are given in Table 1. Cycling conditions were an initial step of 10 min at 95 °C and 40 cycles at 95 °C for 30s and 57 °C for 1 min.

Analytical sensitivity and linear range were determined testing ten-fold serial dilutions of DNA samples from *T. cruzi* stocks G (TcI), Tu18 (TcII), M5631 (TcIII), CanIII (TcIV), PAH265 (TcV), CL Brener (TcVI),

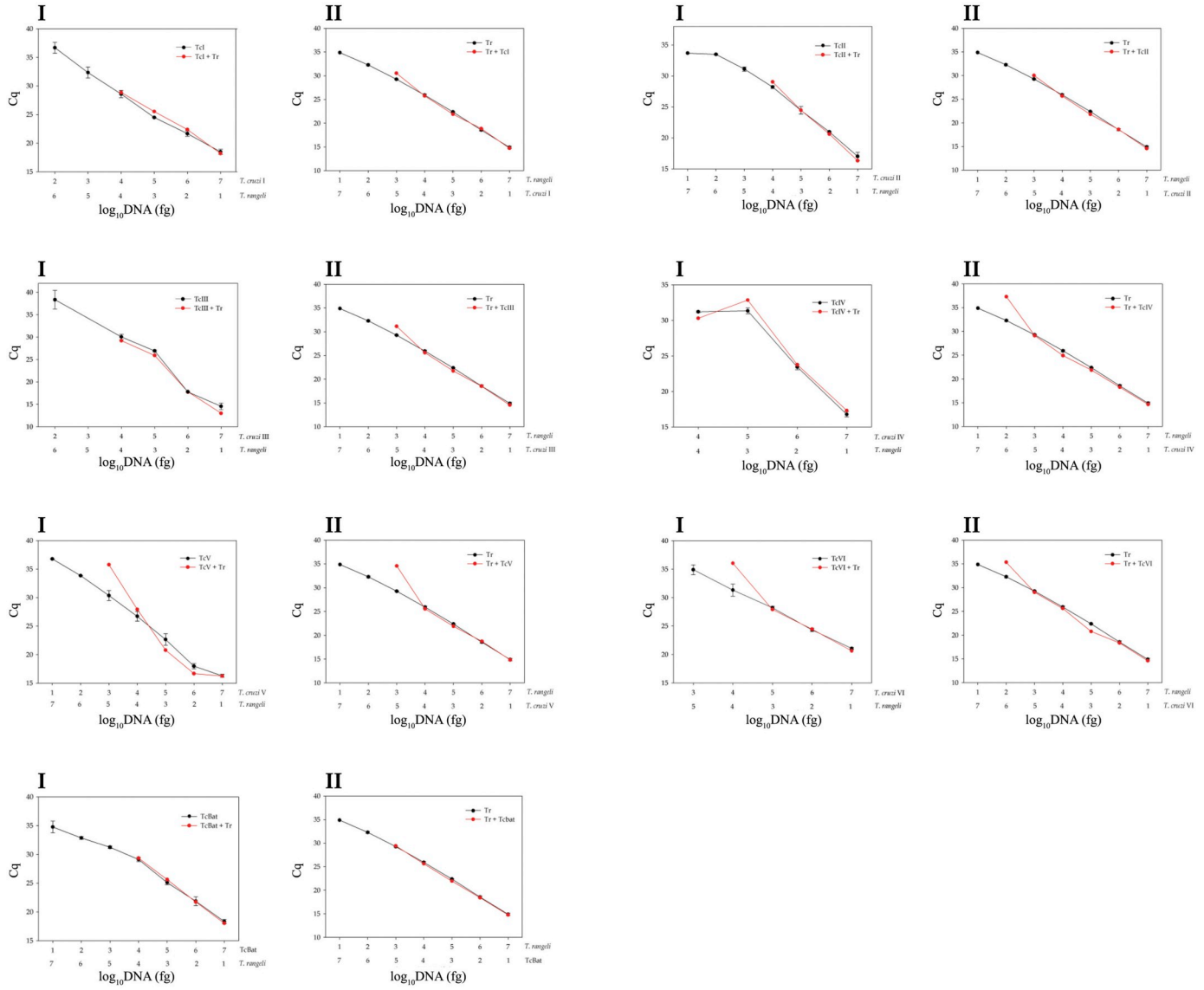


Fig. 4. Sensitivity of 24Sα TaqMan qPCR to detect mixtures of *T. cruzi* and *T. rangeli*. A. TcI (G) + *T. rangeli* (444). B. TcII (Tu18) + *T. rangeli* (444). C. TcIII (M5631) + *T. rangeli* (444). D. TcIV (CanIII) + *T. rangeli* (444). E. TcV (PAH265) + *T. rangeli* (444). F. TcVI (CL Brener) + *T. rangeli* (444). G. Tcbat + *T. rangeli* (444). Cq values of HEX (I) and FAM (II) detection for different mass ratios of *T. cruzi*/*T. rangeli* DNAs expressed in $\log_{10}$.(I) Red lines represent fluorescence signals obtained by amplification of *T. cruzi*/*T. rangeli* mixtures and black line represents HEX fluorescence signals obtained by amplification of samples containing only *T. cruzi*. (II) Red lines represent fluorescence signals obtained by amplification of *T. cruzi*/*T. rangeli* mixtures and black line represents FAM fluorescence signals obtained by amplification of samples containing only *T. rangeli*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Tcbat and *T. rangeli* strain 444, within a range of 1 fg to 10 ng per reaction tube,

2.3.3. ITS1 qPCR for distinction between Trypanosoma evansi and Leishmania species

A conserved region of the internal transcribed spacer (ITS1) gene (Njiru et al., 2005) was amplified by a SYBR Green qPCR assay for distinction between *T. evansi* and Leishmania spp. on the basis of differential melting temperature profiles (Tm) of their corresponding amplicons. The reaction was carried out in a final volume of 20uL, with 3uL of gDNA and 1× of SYBR GreenER qPCR SuperMix universal (Invitrogen, USA). Sequences and final concentrations of primers used in the qPCR reaction are given in Table 1. Cycling conditions were an initial step of 5 min at 95 °C, 45 cycles at 95 °C for 30s,57 °C for 40s and 72 °C for 90s. After amplification, an analysis of the melting curve between 70 and 90 °C with 0.5 °C increments was performed.

During method optimization, specific amplification of each SYBR Green qPCR product was checked in 2% agarose gel electrophoresis. The gels were stained with ethidium bromide and visualized on a UV transilluminator.

2.3.4. Heat shock protein 70 (Hsp70) qPCR for distinction among Leishmania species

The specific primers that amplify the Hsp70 gene were modified from the sequences proposed by Zampieri et al., 2016. The alignment of the amplified fragment among different Leishmania species is shown in Supplementary Fig. 2.

The reaction was carried out in a final volume of 20uL, with 3uL of gDNA and 1× of Type-it HRM qPCR Kit (Qiagen). Sequences and final concentrations of primers in the qPCR-HRM analysis are given in Table 1. Cycling conditions were an initial step of 5 min at 94 °C and 40 cycles at 94 °C for 30s and 60 °C for 30s and 72 °C for 90s. EvaGreen-

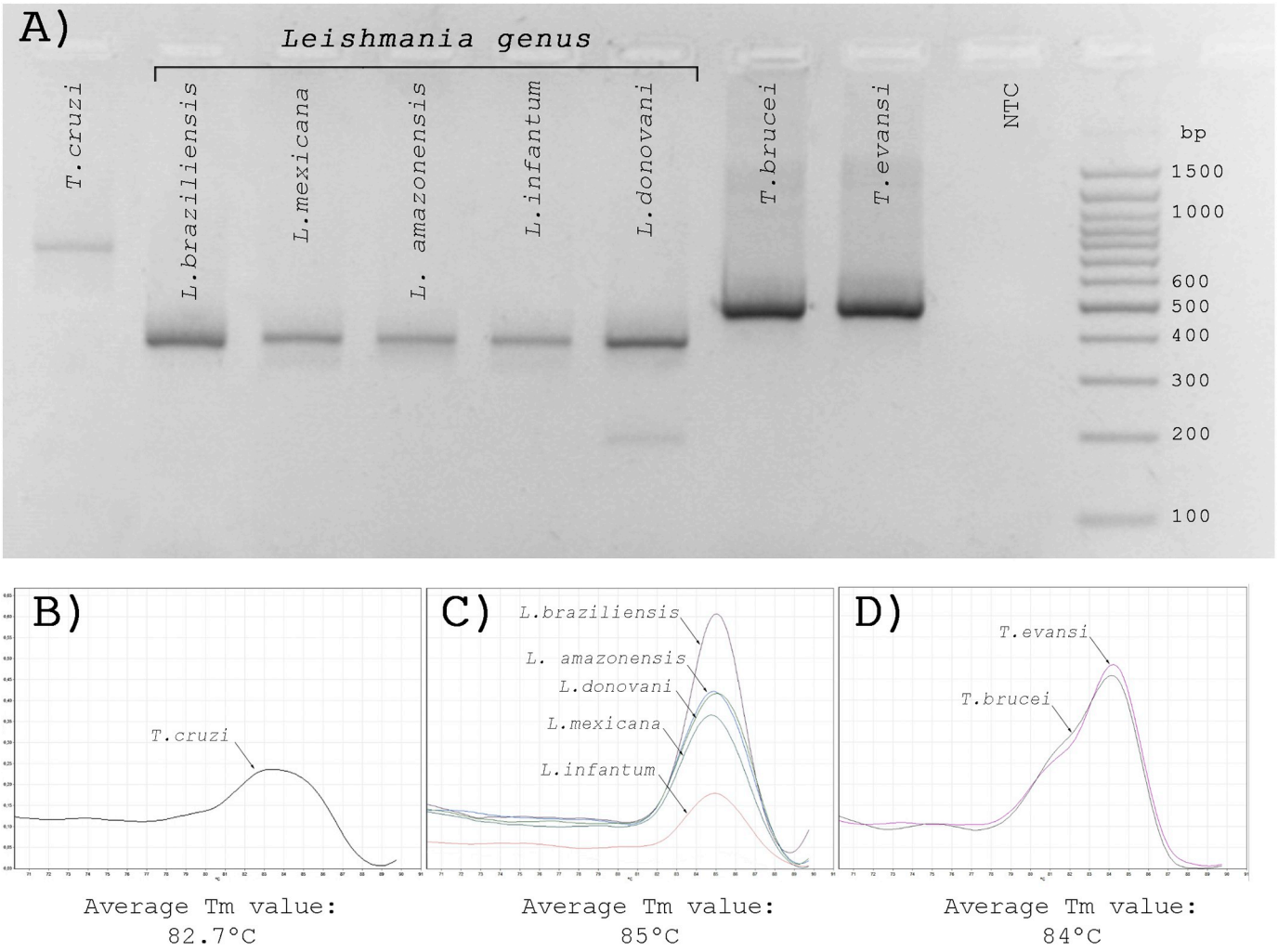


Fig. 5. Differential diagnosis of *Trypanosoma evansi* and Leishmania species by SYBR Green ITS 1 Real-Time PCR assay. (A) Amplified products analysed in 2% agarose gel electrophoresis, (B) Melting curve obtained for *T. cruzi* (C) Melting curves profiles and Tms for Leishmania species, (D) Melting curve profiles and Tms for *Trypanosoma brucei/Trypanosoma evansi*. Each qPCR test was repeated at least three times to test for repeatability. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

HRM analysis was performed at the end of each qPCR run in order to distinguish among *Leishmania (V.) braziliensis*, *Leishmania (L.) amazonensis*, *Leishmania (L.) mexicana*, and the complex *Leishmania (L.) infantum/Leishmania (L.) donovani*.

The amplicons dissociation analysis was performed by capturing fluorescence signals at 0.2 °C intervals and holding for 10 s in each range of the Tm (between 80 °C to 95 °C). The acquisition of fluorescence data and the construction of dissociation profiles were performed using the Rotor-Gene Q Software, HRM software normalizes melting curves relatively to values from pre and post-melting points assigned as 100% and 0%, respectively. The graphs containing the means and standard deviations of the Tm values obtained by HRM analysis were made using the OriginLab software.

2.4. Analytical parameters tested for the qPCR assays

The analytical sensitivity and linear range were estimated performing three replicates from serial dilutions covering from 1×10^8 to 10 fg of specific gDNAs from each parasitic species.

Inclusivity and exclusivity experiments were carried out in the novel qPCR procedures included in the algorithm. Also, analytical evaluation of the novel qPCR procedures was carried out in blind biological samples spiked with known quantities of parasitic DNAs. The samples were spiked by a laboratory operator, labelled with a different code and analysed by a second operator.

Positive qPCR controls were quantified DNAs obtained from cultured parasites and negative controls included non-spiked biological samples and Non-Template PCR reaction tubes.

2.5. Ethical issues

The study was approved by the IRB of the participating institutions.

The study does not include human subjects. Only DNA from a non-infected person's blood sample was used as a control and it was taken from a previous study, in which the written informed consent included the use of archival samples for future studies without revealing the identity of the person/patient (Besuschio et al., 2017).

3. Results

3.1. 24Sα rDNA duplex TaqMan qPCR

Inclusivity and Exclusivity: Following the recommendations of the MIQE guidelines, the ability of the duplex TaqMan qPCR to

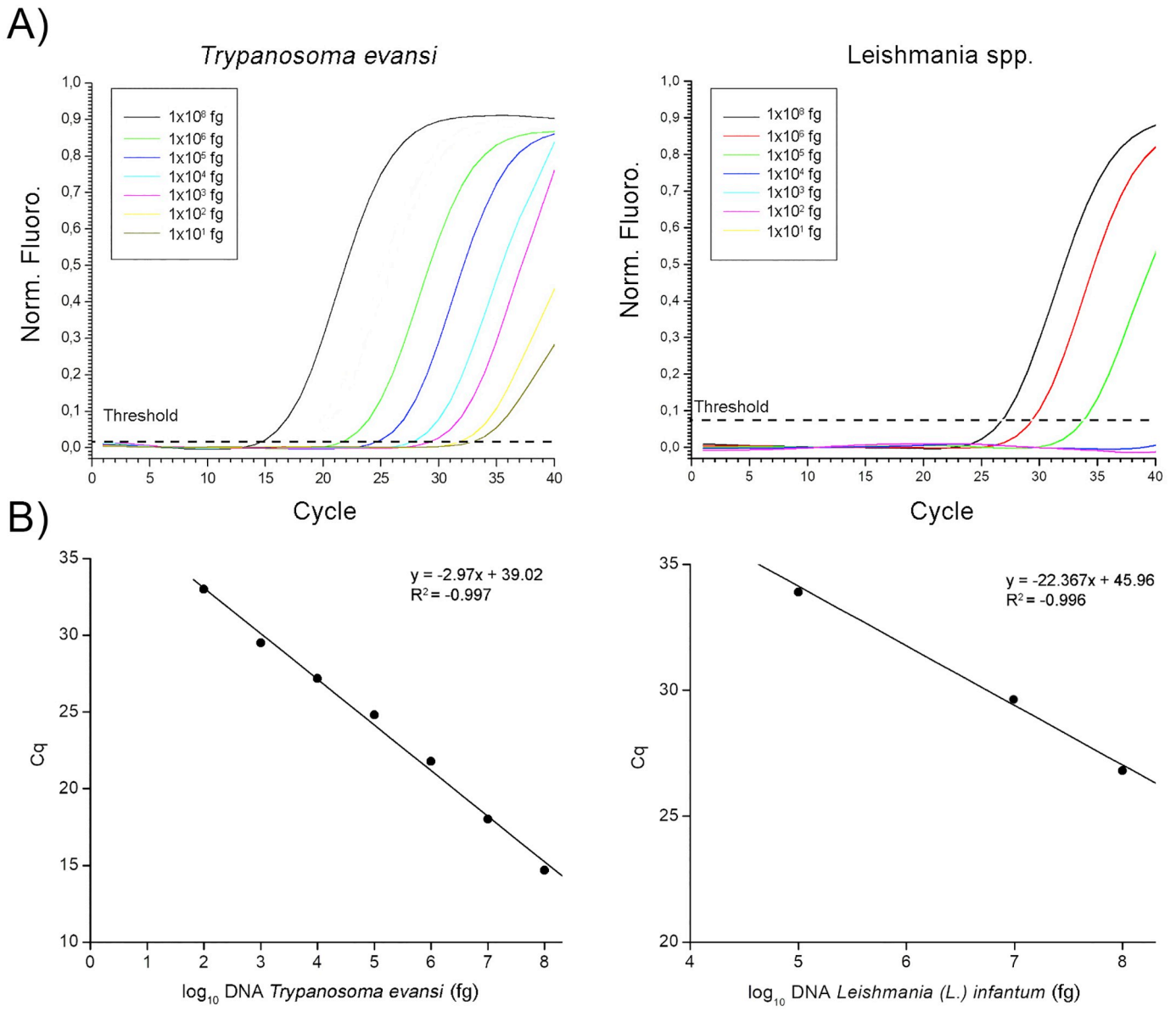


Fig. 6. Linearity of the ITS 1 qPCR assay to distinguish between *Trypanosoma evansi* and Leishmania spp.

differentially detect *T. cruzi* (including the six DTUs: I to VI and Tcbat) and *T. rangeli* was firstly evaluated in reference strains from different geographical regions (Fig. 2 and Table 2). The products were confirmed in 3% agarose gel electrophoresis, obtaining a single 140 bp amplicon for *T. rangeli* and a single 150 bp or 175 bp products for TcI and TcII/V/VI strains respectively (Fig. 2C). Additionally, we verified the exclusivity of the reaction using DNA from other related trypanosomatids, such as Leishmania sp. and human gDNA from a non-infected person (Table 2).

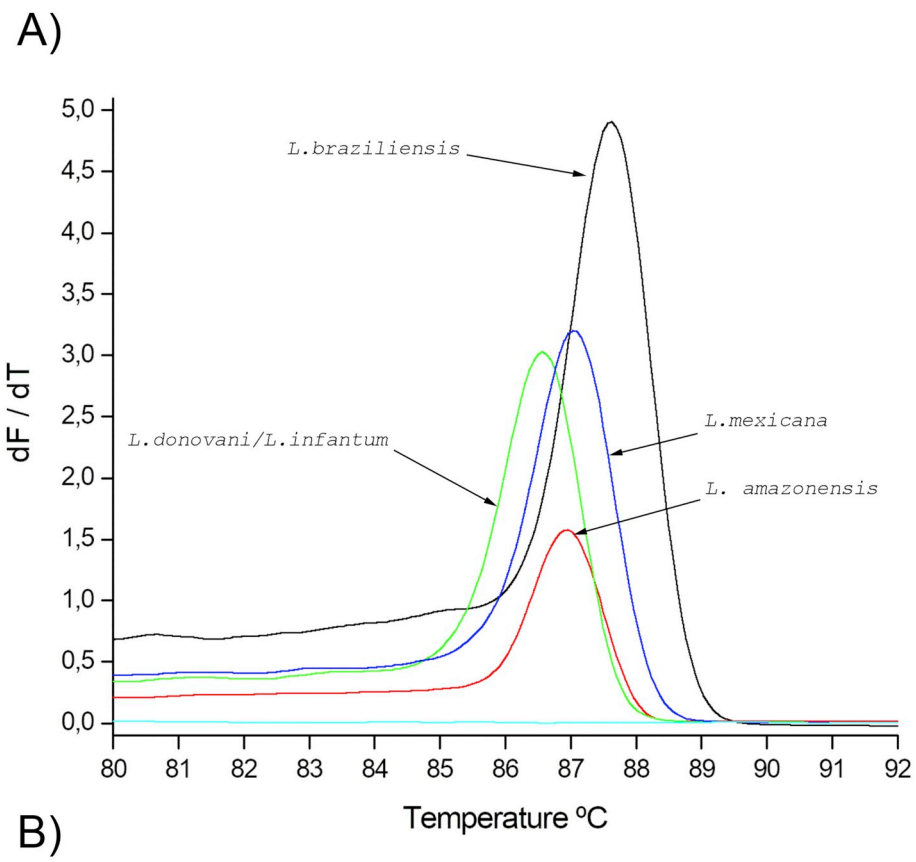
Only the HEX fluorescence signal was detected when the reaction contained *T. cruzi*, while in the samples spiked with *T. rangeli* DNA only the FAM signal was detected (Fig. 2A–B). This TaqMan qPCR test allowed detection of *T. cruzi* stocks representative of the six DTUs and Tcbat, as well as *T. rangeli* 444, SC-58 and Tre strains (Table 2). The human DNA control and the different Leishmania species evaluated did not show detectable signals, indicating reaction specificity and exclusivity.

Lineal range and analytical sensitivity: The qPCR analytical sensitivity for the detection of *T. rangeli* was 10 fg of DNA per reaction tube, with a linear range between 10 fg and 10 ng (Fig. 3A). In the case of *T. cruzi*, the qPCR analytical sensitivity was 10 fg for TcV (Fig. 3G), 100 fg/reaction tube for TcI, TcII and TcIII DNA (Fig. 3C-E), 1 pg/reaction tube for Tcbat (Fig. 3B) and TcVI (Fig. 3H); followed by 100 pg for TcIV (Fig. 3F).

Furthermore, we evaluated the performance of the qPCR for concomitant detection of *T. cruzi* and *T. rangeli*, in artificial mixtures of gDNA of different strains covering the six DTUs and Tcbat with *T. rangeli* in the following proportions per reaction tube: 10 fg/10 ng, 100 fg/1 ng, 1 pg/100 pg, 10 pg/10 pg, 100 pg/1 pg, 1 ng/100 fg and 10 ng/10 fg for *T. cruzi* and *T. rangeli* respectively (Fig. 4).

Simultaneous detection of both *T. cruzi* and *T. rangeli* in mixtures was possible within the mass ratio 1:1 to 100:1 for the combinations TcI + Tr, TcII + Tr, TcIII + Tr and Tcbat + Tr), within the ratio 1:100 to 100:1 for TcV + Tr) and 1:1 to 1000:1 (TcIV + Tr and TcVI + Tr) (Fig. 4).

In six out of seven *T. cruzi* strains (TcI, TcII, TcIII, TcIV, TcVI and Tcbat) mixed with *T. rangeli*, *T. cruzi* was detectable (HEX signal) in mixtures containing equivalent amounts (10 pg *T. cruzi* /10 pg *T. rangeli*) as well as higher *T. cruzi* DNA amounts respect to *T. rangeli*. The TcV strain was detectable in mixtures with *T. rangeli* in reactions



Leishmania species	Melting Temperature (ºC)
L. (L.) braziliensis	87.68
L. (L.) mexicana	87.04
L. (L.) amazonensis	86.94
Complex L. donovani / L. infantum	86.60

Fig. 7. EvaGreen qPCR coupled to High Resolution Melting analysis, (A) Melting curve profiles corresponding to the average of three replicates for each DNA sample (50 ng DNA per reaction tube), (B) Melting Temperature values for each Leishmania species.

Table 3
Molecular detection of Kinetoplastids species in *T. infestans* samples spiked with *T. cruzi* and *T. rangeli* DNAs.

Tested specimens (Code sample)	Trypanosoma species DNA (5 ng/ µL each) spiked into Triatoma DNA samples	12S rDNA	Duplex TaqMan Real-Time PCR (Cq value)	
		Cq value	TcTq	TrTq
T. infestans (TI42)	None	22.12	ND	ND
T. infestans (TI47)	*T. cruzi*	21.87	22.66	ND
T. infestans (TI62)	*T. rangeli*	18.52	ND	17.57
T. infestans (TI63)	*T. cruzi*	18.25	23.35	ND
T. infestans (TI70)	*T. cruzi/T. rangeli*	19.31	24.04	17.60
T. infestans (TI71)	*T. rangeli*	21.11	ND	17.19
T. infestans (TI84)	*T. rangeli*	22.25	ND	17.64
T. infestans (TI85)	None	16.48	ND	ND
T. infestans (TI90)	None	19.37	ND	ND
T. infestans (TI113)	None	24.28	ND	ND

containing at least 1 pg TcV /100 pg *T. rangeli*) (Fig. 4I).

Detection of *T. rangeli* (FAM signal) was positive in mixtures with TcI, TcII, TcIII, TcV and Tcbat in all combinations containing equivalent or higher proportions of *T. rangeli* In mixtures of *T. rangeli* plus TcIV or TcVI strains, *T. rangeli* amplification signals were detectable at a 1000:1 *T. cruzi/T. rangeli* amount ratio, in agreement with the higher sensitivity of the method observed for single *T. rangeli* DNA samples (Fig. 4(II)).

3.2. ITS-1 based qPCR assay

The second step of the diagnostics algorithm was to evaluate the ITS1 region of rDNA, which has been extensively used in phylogenetic studies and as a molecular epidemiological marker for these parasites. This region presents multiple copies within the organisms studied, guaranteeing adequate sensitivity for analysis of biological samples.

The SYBR Green Real-Time PCR assay targeted to ITS1 region coupled to a Melting Curve allowed amplification of a unique profile for *T. cruzi*, *T. brucei/T. evansi* and Leishmania spp. (Fig. 5B–D). These amplification profiles were corroborated by means of conventional PCR followed by agarose gel electrophoresis. Indeed, a band size of 480 bp was obtained for *T. brucei/T. evansi,* whereas a same pattern of bands of approximately 400 bp was observed for Leishmania spp. (Fig. 5A).

The ITS 1 qPCR linearity was assessed using a panel of seven DNA dilutions from each parasite stock mixed with gDNA isolated from

Table 4
Molecular Detection of Trypanosoma species in archival *R. neglectus* field samples.

Tested specimens (Code sample)	12S rDNA[a]	Duplex TaqMan Real-Time PCR (Cq value)		Kinetoplastidean species detected
	Mean Cq value	TcTq	TrTq	
R. neglectus (197.1)	Not done	33.41	ND	*T. cruzi*
R. neglectus (197.8)	Not done	ND	ND	None
R. neglectus (198.2)	Not done	23.79	ND	*T. cruzi*
R. neglectus (198.3)	Not done	ND	ND	None
R. neglectus (198.8)	Not done	18.09	ND	*T. cruzi*
R. neglectus (1154)	Not done	30.72	34.70	*T. cruzi/T. rangeli*
R. neglectus (324)	Not done	ND	19.41	*T. rangeli*
R. neglectus (331)	Not done	ND	24.98	*T. rangeli*
R. neglectus (328)	Not done	ND	15.06	*T. rangeli*
R. neglectus (443)	Not done	ND	25.02	*T. rangeli*
R. neglectus (RN1612)	Not done	37.24	ND	*T. cruzi*
R. neglectus (290.3)	Not done	ND	18.75	*T. rangeli*
R. neglectus (723.1)	Not done	ND	19.07	*T. rangeli*
R. neglectus (794)	Not done	ND	33.01	*T. rangeli*
R. neglectus (RN01)	Not done	ND	ND	None
R. neglectus (RN1614)	Not done	38.19	ND	*T. cruzi*
R. neglectus (RN6-802.12)	Not done	ND	35.14	*T. rangeli*
R. neglectus (RN1165)	Not done	ND	ND	None
R. neglectus (PM Fercal)	Not done	ND	ND	None

[a] Not enough archival sample volume was available for 12S rDNA qPCR, then in samples that neither *T. rangeli* nor *T. cruzi* DNAs was detected, DNA inhibitors or degradation could not be discarded.

pooled blood collected from five non-infected dogs, covering from 1×10^8 to 10 fg of gDNA and tested in triplicate (Fig. 6A,B).

The analytical sensitivity of the ITS1 qPCR was 100 pg/reaction for reference strains of Leishmania spp., and 100 fg/reaction for *T. evansi*, with a linear range up to 100 ng for both organisms. The efficiency values of the qPCR within the reportable range were 98% (*T. evansi*) and 95% (Leishmania spp.).

3.3. Hsp70 qPCR-HRM analysis

As ITS 1 qPCR did not show differences among Leishmania species, obtaining a common Tm of 85 °C for all them (Fig. 5C), we designed an EvaGreen qPCR coupled to HRM analysis targeted to the Leishmania Hsp70 gene. This procedure allowed differentiation among *L. (V.) braziliensis*, *L. (L.) mexicana*, *L. (L.) amazonensis*, and the complex *L. (L.) donovani/L, (L.) infantum* (Fig. 7A). Melting temperatures (Tm) for each Leishmania species are shown in Fig. 7B.

3.4. Evaluation of the multiplex qPCR algorithm in samples from insect vectors

The qPCR based algorithm was evaluated firstly in a blind panel made of DNA extracted from abdominal tissue samples from *T. infestans* ($N = 10$) spiked with *T. cruzi* and *T. rangeli* gDNAs (Table 3). In these spiked *T. infestans* samples the qPCR detected correctly *T. cruzi* or *T. rangeli* DNA as well as both parasite DNAs in one sample spiked with equivalent proportions of both Trypanosoma species.

Moreover, archival DNA obtained from faecal samples of naturally infected *R. neglectus* specimens (N = 19) were analysed (Table 4). The 24Sα rDNA TaqMan qPCR allowed identification of *T. cruzi* in five out of 19), *T. rangeli* in eight out of 19 and an mixed infection in one sample. The remaining five samples did not amplify parasite DNA. Unfortunately, the quality of these DNA samples could not be addressed due to lack of material to perform the 12 s rDNA PCR.

3.5. Evaluation of the multiplex qPCR algorithm in samples from naturally infected mammals

Peripheral blood from domestic cattle (*Bos Taurus*, N = 14) and dogs (*Canis lupus familiaris*, N = 19) from North-eastern Argentina was tested in duplicates using the qPCR algorithm (Table 5). The IRBP TaqMan qPCR Cq values were between 22.62 and 27.51 ± 1.12. The ITS1 based qPCR allowed detection of 13 out of 33 samples infected with *T. evansi* (seven samples from cattle and six from dogs). Moreover, in two samples from dogs, we could detect Leishmania spp. The next step in the algorithm was to identify the Leishmania species by means of Hsp70 qPCR-HRM analysis assay, which allowed detection of *L. (L.) infantum* in C15 and C22 (Table 5). It is noteworthy that sample C22 was co-infected with *T. evansi*.

4. Discussion

T. cruzi, T. evansi and Leishmania spp. are multi-host parasites, capable of parasitizing a large number of domestic and wild mammalian species. Animals infected with Kinetoplastids show a series of clinical manifestations compatible with various diseases that not necessarily are related to parasitic infections and so a differential diagnosis is difficult to achieve. Typically, diagnosis is mainly based on finding the parasites in smears, wet blood films or by the microhematocrite centrifugation technique. However, these techniques are operator dependent and low sensitive, particularly in detecting infections with low levels of parasitemia (Brun et al., 1998).

Therefore, an accurate laboratory tool is essential for prompt differential diagnosis and eco-epidemiological surveillance. Additionally, in wild environments, it is common to find multi-parasitized animals, making accurate diagnosis even more challenging.

Otherwise, given the ability of triatomines to become infected with several flagellate species, including *T. cruzi*, the evaluation of the level of infection in wild, peridomiciliary and domiciliary environments is relevant for control programs aiming to reduce human infections (Shikanai-Yasuda et al., 1996; Moser et al., 1989).

Here, we have described a multiplex qPCR-based diagnostics algorithm that in a few reactions allows distinction among different species of American *Trypanosomes* as well as Leishmania species in biological samples from mammalian reservoirs as well as triatomine vectors. The algorithm includes as a first step the amplification of an endogenous gene; useful as DNA integrity/inhibition control that allows discarding inadequate samples that otherwise would lead to false negative results. In the case of mammals we have chosen the IRBP gene, previously demonstrated to detect a vast number of mammalian species that can become infected by these parasites (Wehrendt et al., 2019; Ferreira et al., 2010). In the case of insect vectors, we have chosen the 12rRNA gene that is highly conserved in triatomines (Moreira et al., 2017). As this algorithm allows the detection of Leishmania spp., a slight modification of the primers and the probe based on the 12S rDNA gene would allow extending the use of this locus as an endogenous control for the analysis of DNA integrity in samples of Phlebotomus spp. and Lutzomia spp. sandflies. Additionally, previous research has shown that by amplifying and sequencing this molecular target, Phlebotomus and Lutzomyia species of sandflies can be systematically identified and related (Haouas et al., 2007; Baeti et al., 2004).

The 24S rDNA qPCR allowed distinction between *T. cruzi* and *T. rangeli*, being higher sensitive for *T. rangeli* (10 fg/reaction tube) and TcV (10 fg), decreasing for the remaining DTUs. Then, simultaneous detection of mixtures of *T. rangeli* and *T. cruzi* depended on the amount ratios between *T. rangeli* and the *T. cruzi* strain present in the mixture. This may be related to a different copy number or the 24S rDNA genes in the different DTUs. For the *T. cruzi* strains with lower copies, there had to be at least equivalent amounts of *T. cruzi* and *T. rangeli* to allow

Table 5
Detection of Kinetoplastid species in mammalian samples.

Sample code	Species	Mean Cq values			ITS1 region	Hsp70 gene	Diagnosis
		IRBP	Duplex TaqMan Real-Time				
		Mean Cq value	TcTq	TrTq	Mean Tm	Mean Tm	
C1	*Bos taurus*	24.67 ± 0.02	ND	ND	84 °C ± 0.43	–	*T. evansi*
C2	*Bos taurus*	24.21 ± 0.30	ND	ND	84 °C ± 0.11	–	*T. evansi*
C3	*Bos taurus*	23.95 ± 0.10	ND	ND	84 °C ± 0.24	–	*T. evansi*
C4	*Bos taurus*	24.01 ± 0.04	ND	ND	ND	–	
C5	*Bos taurus*	24.12 ± 0.02	ND	ND	84 °C ± 0.28	–	*T. evansi*
C6	*Bos taurus*	23.90 ± 0.04	ND	ND	84 °C ± 0.13	–	*T. evansi*
C7	*Bos taurus*	23.81 ± 0.01	ND	ND	ND	–	
C8	*Bos taurus*	24.00 ± 0.12	ND	ND	ND	–	
C9	*Bos taurus*	24.30 ± 0.08	ND	ND	84 °C ± 0.01	–	*T. evansi*
C10	*Bos taurus*	24.01 ± 0.04	ND	ND	84 °C ± 0.21	–	*T. evansi*
C11	*Bos taurus*	24.15 ± 0.04	ND	ND	ND	–	
C12	*Bos taurus*	24.10 ± 0.05	ND	ND	ND	–	
C13	*Bos taurus*	24.40 ± 0.02	ND	ND	ND	–	
C14	*Bos taurus*	25.04 ± 0.05	ND	ND	ND	–	
C15	*C.familiaris*	23.01 ± 0.01	ND	ND	85 °C ± 0.42	86.60 ± 0.2	*L. infantum*
C16	*C.familiaris*	24.54 ± 0.10	ND	ND	ND	–	
C17	*C.familiaris*	25.00 ± 0.21	ND	ND	ND	–	
C18	*C.familiaris*	25.54 ± 0.04	ND	ND	ND	–	
C19	*C.familiaris*	25.9 ± 0.06	ND	ND	ND	–	
C20	*C.familiaris*	25.02 ± 0.04	ND	ND	ND	–	
C21	*C.familiaris*	26.00 ± 0.07	ND	ND	ND	–	
C22	*C.familiaris*	22.97 ± 0.07	ND	ND	84/85 °C ± 0.18	86.62 ± 0.44	*T. evansi/L. infantum*
C23	*C.familiaris*	22.62 ± 0.09	ND	ND	84 °C ± 0.05	–	*T. evansi*
C24	*C.familiaris*	25.43 ± 0.21	ND	ND	84 °C ± 0.7	–	*T. evansi*
C25	*C.familiaris*	26.62 ± 0.13	ND	ND	84 °C ± 0.3	–	*T. evansi*
C26	*C.familiaris*	25.32 ± 0.01	ND	ND	84 °C ± 0.42	–	*T. evansi*
C27	*C.familiaris*	25.9 ± 0.04	ND	ND	84 °C ± 0.09	–	*T. evansi*
C28	*C.familiaris*	26.26 ± 0.06	ND	ND	ND	–	
C29	*C.familiaris*	25.02 ± 0.06	ND	ND	ND	–	
C30	*C.familiaris*	27.51 ± 0.04	ND	ND	ND	–	
C31	*C.familiaris*	25.62 ± 0.08	ND	ND	ND	–	
C32	*C.familiaris*	25.42 ± 0.02	ND	ND	ND	–	
C33	*C.familiaris*	26.63 ± 0.14	ND	ND	84 °C ± 0.05	–	*T. evansi*

ND: not detectable.
Tm: Melting temperature.

detection of both parasitic species and in general *T. rangeli* in lower amounts than *T. cruzi* was always detectable in mixtures (Fig. 5).

The new sequencing technologies are providing important information about various genetic variations in most organisms. In particular, sequencing of ITS1 region present in rDNA has been used to investigate genetic diversity in several protozoa, including trypanosome species (Cacciò et al., 2010; Lollis et al., 2011). This marker has been very useful for differential diagnosis of African trypanosomes (Njiru et al., 2005), allowing assessment of inter and intra phylogenetic relationships among species such as *T. brucei brucei*, *T. brucei gambiense*, *T. cruzi*, *T. rangeli* and *T. evansi* (Beltrame-Botelho et al., 2005; Areekit et al., 2008; Tian et al., 2011). Additionally, it has been experimentally found that the ITS1 molecular marker is also capable of specifically differentiating *T. cruzi*/*T. rangeli*, though with lower sensitivity with respect to other *T. cruzi* specific target sequences (Gurgel-Gonçalves et al., 2012).

Among several targets described for Leishmania identification, the Hsp70 coding gene has been useful to identify different species. A few qPCR-HRM analysis based assays have been reported for identification of American Leishmania species (Hernandez et al., 2014; Zampieri et al., 2016 and this work). It can differentiate several Leishmania species than can circulate in overlapping regions of America. The exception is in samples infected with *L.(L) mexicana* or *L.(L) amazonensis*, because the Tms of the Hsp70 amplicon are similar due to the high homology between their sequences (only three polymorphic SNPs within the amplified fragment). If the method is to be used in places where infections by both species overlap, distinction between them should be done by sequencing of the obtained amplicons.

Furthermore, the qPCR platform permits quantification of parasitic loads in infected samples, which can be useful to estimate the potential infectivity of the tested reservoirs, as well as to follow-up response to treatment or vaccine monitoring.

Finally, it is expected that this sensitive and specific molecular diagnostics algorithm will provide more accurate records of trypanosomatidean infections in animal reservoirs. Indeed, four different species of trypanosomatidean pathogens that may circulate in overlapping geographical regions and infect a same host species can be identified in only two qPCR reactions. This strategy may offer information to update the current epidemiological maps of these zoonotic infections, some of them causative of severe neglected diseases for animals and humans.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2020.104328.

Declaration of Competing Interest

No conflict of interest.

Acknowledgments

To Dr. Cesar Cuba Cuba and Dr. Rodrigo Gurgel-Gonçalves (Faculty of Medicine, University of Brasilia), for field work and facilitating samples from *Rhodnius neglectus* specimens from Central Brazil. PICT 2014-1188 (ANPCyT, MINCyT, Argentina) and Fundación Mundo Sano, Argentina

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