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Chronic Chagas disease: Quantification of *Trypanosoma cruzi* in peripheral blood and dejections of *Triatoma infestans* fed by xenodiagnosis in patients with and without cardiopathy

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ABSTRACT

It is not currently known which individuals with chronic Chagas disease (ChD) will develop cardiopathy in a determined period and which will be maintained asymptomatic with normal routine laboratory tests all their lives. The parasite burden is a factor that could explain this different evolution. The objective of this study was to quantify *Trypanosoma cruzi* burden by real-time PCR in blood (qPCR-B) and dejections of triatomines fed by xenodiagnosis (qPCR-XD) in 90 individuals with chronic ChD untreated, classified according to XD results and the presence or absence of cardiopathy. All individuals came from hyperendemic areas of Chile and participated in the study under Informed Consent. The standard qPCR curves for qPCR-B and qPCR-XD were elaborated with a mixture of known concentrations of *T. cruzi* strains, performing DNA serial dilutions (1/10) with a dynamic range between 10^5 and 10^{-1} parasite equivalents/mL. The TaqMan® detection system was applied in a Stratagene Mx3000P thermocycler (Agilent Technologies, USA) with *cruzi* 1 and *cruzi* 2 satellite primers. 22.2% and 15.6% of cases with cardiopathy or without cardiopathy were XD positive. There was no significant difference between the groups. The positivity of qPCR-B and qPCR-XD in the positive XD group was 82.35% and 100%, respectively, while in the negative XD group was 55.26% and 42.10%, respectively. A superior qPCR value in chronic ChD patients with and without cardiopathy was determined for qPCR in cases with positive XD and positive qPCR-XD. The receiver operating characteristic (ROC) curve analyses show better accuracy for detecting parasite burden (area under the curve, AUC) for qPCR-XD in comparison to qPCR-B. That is to say, major performance in DNA samples obtained of positive XD (gold standard for viable *T. cruzi*) detected and quantified by qPCR-XD. A high percentage of cases with XD and qPCR-XD positive (80–100%) have result concordant with qPCR-B. In absence of XD, future challenges are especially related to the low parasitic load of chronic ChD patients treated with trypanocidal drugs and post-therapy parasitological evaluations by qPCR-B. Finally, no statistically significant differences were found between presence or absence of cardiopathy and XD, qPCR-B or qPCR-XD.

1. Introduction

Chagas disease (ChD) caused by the kinetoplastid protozoan *Trypanosoma cruzi*, is among the most neglected tropical disease in Latin American (Fernández et al., 2019). The endemic area for vector-borne transmission extends from the southern United States of America (USA) to southern Argentina and Chile (Sosa-Estani and Segura, 2015). Presently, the number of international infected people estimated by the World Health Organization amounts to 7–8 million and more than 10,000 deaths are assumed to happen yearly (Duschak, 2019). In Chile, the endemic area is between the Arica-Parinacota (18°30′S) and O´Higgins Regions (34°36′S), with a risk population of 873,415 persons (MINSAL, 2014). While the interruption of the vector transmission and improvement in the research systems is emphasized, the concern for this disease seems to be decreasing with less diagnostic efforts and lower education and by change in higher level. On the other hand the

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numbers show that the problem if it is not increasing, at least maintains its careless historical magnitude (Canals et al., 2017). In Chile, the biological vectors involved in the transmission of *T. cruzi* are insects of the subfamily Triatominae: *Triatoma infestans* of domestic habits and *Mepraia spinolai, Mepraia gajardoi* and *Mepraia parapatrica* of wild habits (Frías et al., 2017).

After the acute phase of infection, ChD enters a chronic phase, initially with an indeterminate or latent form. Subsequently 20–30% of patients develop cardiac abnormalities, 10% digestive disturbances, 3–4% neurologic alterations and 2–3% mixed forms (cardiac and digestive) (Rassi et al., 2012). This phase is characterized by low, subpatent and fluctuating parasitemia evidenced by some parasitological tests such xenodiagnosis (XD), specific procedure but of low performance. Nevertheless, the application of conventional Polymerase Chain Reaction (PCR) in dejections of triatomines used in XD applied in patients with ChD, has been shown to have high sensitivity in the detection of *T. cruzi* (Silber et al., 1997; Campos et al., 2007a; Zulantay et al., 2011). In recent years, the quantitative modality of PCR or real-time PCR (qPCR) has been successfully applied to detect, quantify and/or genotype *T. cruzi* in the same reaction (Piron et al., 2007; Duffy et al., 2009; Schijman et al., 2011; Ramírez et al., 2015; Muñoz et al., 2017).

In Saavedra et al. (2016), we described the *T. cruzi* parasitic load determined by qPCR in peripheral blood (B) and dejections of triatomine fed by XD in individuals with chronic ChD. The objective of this work was to determine in 90 cases with chronic ChD classified according presence or absence of cardiopathy in pre-therapy condition, the *T. cruzi* parasite burden in these types of biological samples received under Consent Inform.

2. Material and methods

2.1. Type design study and population

This study is a quasi-experimental descriptive type. The studied population corresponded to 90 non-treated chronic ChD, 45 with cardiopathy (13 women and 32 men, between 30–79 years, average 57.8), and 45 without cardiopathy (11 women and 34 men, between 20 and 76 years, average 47.1). All came from rural and urban zones of Illapel, Salamanca and Combarbalá, Coquimbo Region, Chile (located between 29°02´ and 32°16´ South latitude in the area of transverse valleys of Chile). The *T. cruzi* infection was established by the application of conventional serological tests ELISA and IFI IgG for *T. cruzi* (Muñoz et al., 2019).

2.2. Determination of cardiopathy

To all the patients of this study a twelve-lead electrocardiogram (ECG) was performed. Each electrocardiographic tracing included the following parameters: P axes, P duration, P-R space, R spaces, QT value, QTc calculation, QRS axis, T axis, ventricular gradients, RV1 intrensicoide deflexion, SV1, RVS, Sokolow index and electrocardiographic diagnosis. The final interpretation of this test data was performed by a specialist cardiologist following the double-blind protocols recommended by the World Health Organization (Maguire et al., 1982). The non-cardiopaths corresponded to individuals with clinical and physical evaluation, normal ECG without radiological or echotomographic study, according to current norms (MINSAL, 2017).

2.3. Xenodiagnosis (XD)

Was applied using the technique described by Schenone (1999). Two cylindrical boxes, each containing seven infection-free *Triatoma infestans* nymph of instars III and IV, were placed on of each of the patients studied. The XD boxes were maintained at 27 °C with a relative humidity of 70% in fasting conditions and after 30, 60 and 90 days of incubation, the fecal samples (FS) of each insect were microscopically examined to detect trypomastigote forms of *T. cruzi*.

2.4. Blood (B) and XD samples

Five mL of venous B of the 90 study subjects were received in the same volume of 6 M guanidine chlorhydrate 0.2 M EDTA, pH 8.0 (GEB). The GEB solution was incubated at 98 °C for 15 min to break the minicircles DNA of *T. cruzi* and stored at 4 °C until B-DNA extraction. On the other hand, the dejections of XD were prepared as described (Zulantay et al., 2011). Briefly, the dejection samples obtained at 30, 60 and 90 days, were received in 500 µL PBS buffer pH 7.2, incubated for 15 at 98 °C and centrifuged for 3 min at 3500 rpm. A total of 200 µL from each XD sample was recovered and stored at −20 °C until XD-DNA extraction.

2.5. B-DNA and XD-DNA extraction

The B-DNA extraction was performed in an initial volume of 200 µL, using the QIAamp® Blood Mini (Qiagen, Valencia, CA), according to the manufacturer's instructions, while the XD-DNA purification was performed using an initial volume of 100 µL with the Favor Prep Blood Mini Kit Genomic DNA (Favorgen, Biotech Corp.), modified by the omission of lysis step of cells with proteinase K. The eluted samples were maintained at −20 °C until qPCR-*T. cruzi* and qPCR-X12 were performed in both samples.

2.6. Quantification B-DNA and XD-DNA

Was carried out using the kit AccueBlue™ dsDNA Quantification (Biotum Inc.). Briefly, the protocol started by mixing the dsDNA Quantitation Solution and the 100× Enhancer Solution in a 100:1 proportion. Then 40 µL of this mixture was loaded in each tube. After this step, 2 µL from each of the eight DNA standards provided by the kit (0, 0.5, 1, 2, 4, 6, 8 and 10 ng/µL dsDNA) were added to each tube. Finally, after mixing and incubating for 5 min in darkness at 25 °C, the samples were scanned using the Quantitative Plate Read and the FAM filter of the Mx3000P thermocycler (Agilent Technologies) (Bravo et al., 2012).

2.7. Endogenous (qPCR-B) and exogenous internal control (qPCR-XD)

As described Pirón et al. (2007) and Saavedra et al. (2016) a human qualitative internal control amplification in qPCR-B-*T. cruzi* was used. The primers for cromosome 12 (X12) were designed by N. Jullien using the AmplifX v.1.5.4 software and compared with Nucleotide BLAST (National Library of Medicine) to discount any other unspecific amplification (N. Nazal, personal communication) (Table 1). Also, X12 was used as qualitative exogenous internal control for qPCR-XD adding 50 µg of negative human blood DNA for *T. cruzi* before FS-DNA isolation. As described (Bravo et al., 2012), the human DNA is degraded completely by the triatomines within 30 days, for this reason, a system novel primers using a segment of a DNA sequence which codifies for X12 was designed, allowing ruling out inhibition and false negative results due to DNA loss during the process of extraction.

2.8. qPCR-B and qPCR-XD standard curve

The standard curve for absolute quantification of *T. cruzi* was performed using a stock of epimastigote forms of Dm28c (TcId) and Y (TcII) strains, of highest and lowest number of copies, respectively. This because to the variability described in the number of copies of the nuclear satellite DNA (Kooy et al., 1989; Moreira et al., 2013). The quantification of total *T. cruzi*-DNA was performed with the same methodology as for B-DNA and XD-DNA samples. A *T. cruzi*-DNA concentration equivalent to 1×10^6 epimastigotes/mL was adjusted, considering that 1 parasite cell contains 200 fg of DNA (Duffy et al., 2009).

Table 1
Primers and probe used for *Trypanosoma cruzi* qPCR-TaqMan® in blood (B) and XD (dejection samples of triatomines) of individuals with non-treated chronic Chagas disease.

Target	Primers or probe	Sequence (5´−3´)	Cycle amplification
T. cruzi	cruzi 1*	Forward (5′-ASTCGGCTGATCGTTTTCGA-3′)	10 min preincubation 95 °C
	cruzi 2*	Reverse (5′-AATTCCTCCAAGCAGCGGATA-3′)	40 amplification cycles (95 °C for 15 s, 60 °C for 1 min).
	cruzi 3*	Probe (5–6-FAM-CACACACTGGACACCAA-NFQ-MGB-3′)	
X12	N1 × 12**	Forward (5´-AGCTGGCTAGACTGTCAT-3´)	
	N2 × 12**	Reverse (5´-CTTTGCCGTTGAAGCTTG-3´)	
	N3 × 12**	Probe (5´/56-FAM/TGGGACTTC/ZEN/AGAGTAGGCAGATCG/3IABkFQ/ − 3´)	

* (Piron et al., 2007).
** (Bravo et al., 2012).

So, *T. cruzi* free human GEB sample or dejection samples of triatomines free of infection for *T. cruzi* were spiked with 1×10^6 parasite equivalents/mL (par-eq/mL). Later, the standard curve was performed with a 10-fold serial dilution of the DNA extracted from both initial points with range between 10^5 and 0.1 par-eq/mL. Both curves were maintained at −20 °C until use. Previously, 100 DNA samples of non-chagasic patients and 20 DNA samples obtained of XD without infection by *T. cruzi* were extracted with the same conditions of study group and evaluated by qPCR-B-TaqMan® system detection. All the samples were qPCR negative.

2.9. qPCR-T. cruzi and qPCR- X12

The TaqMan® detection system was applied in a Stratagene Mx3000P™ thermocycler (Agilent Technologies) under conditions suggested by the manufacturer. In the Table 1, are described the sequences of primers for qPCR-*T. cruzi* and qPCR-X12 with the common amplification cycles.

The reaction mixture consisted in 2 μL of B-DNA or XD-DNA, 10 μL Brilliant Multiplex qPCR (Stratagene), 0.5 μL of a 1:500 dilution of a reference dye (ROX), 0.5 μL of each nuclear primers cruzi 1 and cruzi 2, 0.2 μL cruzi 3 probe, 0.2 μL BSA (100×) and 6.1 μL Molecular Biology Grade Water free of nucleases (Mo Bio Laboratories Inc) in a final volume of 20 μL. The controls used for qPCR-*T. cruzi* were as follows: negative control, DNA of a non-chagasic patient confirmed by serology (IIF and ELISA), evaluated previously with qPCR equipment and positive controls, DNA of an individual with ChD with confirmed parasitemia by PCR and evaluated previously in qPCR equipment. The controls used for qPCR-X12 were: negative control, DNA of dejection samples of *T. infestans* free of infection by *T. cruzi* and dejection samples of *T. infestans* with infection by *T. cruzi*, both without and with presence of mobile tripomastigotes under microscopic examination, respectively. Also, in qPCR-B and qPCR-XD were included: two controls of *T. cruzi*, to evaluate contamination in the preparation of the mixture reaction or unspecific qPCR reactions, a control mixture of 20 μL of mixture reaction for *T. cruzi* (without the sample under study), water control with 2 μL of water free of nucleases (Mo Bio Laboratories Inc.) and control *T. rangeli* DNA. The measurement of emitted fluorescence was performed at 60 °C at the end of each cycle. All the samples and controls were tested in duplicate. The MxPro v 4.1 (Agilent Technologies) software delivered automatically the par-eq/mL data.

2.10. Statistical analysis

The description of the data was performed using graphs (GraphPad Prism 7.0 for Windows) and tables. The frequencies of positive XD between cardiopaths and non cardiophats were compared by χ_1^2 ($\alpha = 0.05$). The qPCR value between the different groups was compared by three-way ANOVA analysis (Tukey test $\alpha = 0.05$), considering the qPCR value as response variable and as independent categorical variables: presence of cardiopathy (cardiopaths v/s non cardiopaths), xenodiagnóstico (positive v/s negative) and qPCR type (qPCRB v/s qPCR-XD). As the variable response did not meet with the normality criteria, logarithmic transformation was applied (log(qPCR + 1)). A receiver operating characteristic (ROC) analysis was performed for qPCR-XD and qPCR-B, determining the area under the curve (AUC), a measure of the goodness of the methods. AUC near 100% are good, being 50% that expected by simple chance. The break point of the curve determines the threshold value to decide a positive result and their respective sensitivity (*S*) and specificity (Sp).

2.11. Ethics statement

The patients participated under Informed Consent, approved by the Ethical Committee of the Faculty of Medicine of the University of Chile (Protocols 046/2011 and 048/2011).

3. Results

In this study, the difference between the ages of the cardiopaths and non cardiopaths were significant ($F_{1,86} = 12.34$, $p < 0.001$). No difference between the ages of men and women was detected. XD was applied in 90 individuals with non-treated chronic ChD. Globally, in 17 of them (18.9%), mobile trypomastigotes of *T. cruzi* were observed under microscopy (positive XD). In detail, 10/45 (22.2%) and 35/45 (77.8%) of non cardiopaths group were positive and negative XD, respectively. In the cardiopaths group, 7/45 (15.6%) and 38/45 cases (84.4%) were positive and negative XD, respectively. No statistically significant differences were found in XD results of both groups ($\chi_1^2 = 0.43$, $p = 0.51$, 95% CI) (Table 2).

The standard curve of qPCR-X12 presented average values of coefficient of determinations (R^2) = 0.993, slope = −3.440 and efficiency (Eff) = 95.6%. All the qPCR-B-X12 and qPCR-FS-X12 had an acceptable qualitative signal for X12 amplification, discarding false negatives results generated by PCR inhibitors in the samples that did not amplify for *T. cruzi*. The Ct value obtained for the amplifications of qPCR-XD-X12 from 50 ng of DNA added to a sample of non-infected triatomine-FS was 29.30 and the range obtained in the 90 samples of study group was average 27.70. In relation to qPCR-B-X12, the average Ct value obtained for the study group was 25.80.

The qPCR-*T. cruzi* standard curve had average values of R^2: 0.999, slope: −3.359 and Eff: 98.5% and dynamic range 10^5 to 10^{-1} par-eq/

Table 2
Xenodiagnosis results in 90 non-treated chronic Chagas disease individual with and without cardiopathy.

ECG	Negative	Xenodiagnosis		%	*N*	Total
		%	Positive			%
Non cardiopaths	35	77.8	10	22.2	45	100
Cardiopaths	38	84.4	7	15.6	45	100
TOTAL	73	81.1	17	18.9	90	100

$\chi_1^2 = 0.43$, $p = 0.51$ (95% CI).

Table 3
Parasitic load of *Trypanosoma cruzi* determined by real-time PCR in samples of *Triatoma infestans* used in xenodiagnosis (qPCR-XD) and in peripheral blood (qPCR-B) in 45 chronic chagasic individuals with cardiopathy.

Parasitic load range par-eq/mL	Xenodiagnosis							
	Positive n = 7				Negative n = 38			
	qPCR-B		qPCR-XD		qPCR-B		qPCR-XD	
	n	%	n	%	n	%	n	%
No Ct	1	14.3	0	0	22	57.9	30	78.9
0.1–0.99	4	57.1	0	0	15	39.5	6	15.79
1–9.9	2	28.6	4	57.1	1	2.63	1	2.63
10–99	0	0	2	28.6	0	0	1	2.63
100–1000	0	0	1	14.3	0	0	0	0

Table 4
Parasitic load of *Trypanosoma cruzi* determined by qPCR in samples of *Triatoma infestans* used in xenodiagnosis (qPCR-XD) and peripheral blood (qPCR-B) in 45 chronic chagasic patients without cardiopathy.

Parasitic load range par-eq/mL	Xenodiagnosis							
	Positive n = 10				Negative n = 35			
	qPCR-B		qPCR-XD		qPCR-B		qPCR-XD	
	n	%	n	%	n	%	n	%
No Ct	2	20	1	10	30	85.7	27	77.1
0.1–0.99	7	70	2	20	4	11.4	3	8.6
1–9.9	1	10	1	10	1	2.9	3	8.6
10–99	0	0	3	30	0	0	1	2.9
100–1000	0	0	3	30	0	0	1	2.9

mL. Parasitic load of *T. cruzi* determined in the study group by qPCR-B and qPCR-XD are described in Tables 3 and 4.

In Table 3 is observed the quantification of *T. cruzi* by qPCR-B and qPCR-XD in the 45 cases with cardiopathy, 7 (15.6%) with positive XD and 38 (84.4%) with negative XD.

In the positive XD group, the positivity of qPCR-B and qPCR-XD was 85.7% and 100%, respectively, with different ranges of parasitemia. Four (57.1%) and two (28.6%) cases with positive XD had parasitic load between 0.1–0.99 and 1–9.9 par-eq/mL, respectively. One case with positive XD was No Ct in qPCR-B. In this case, only 1/3 box of XD was positive at 90 days of incubation (see S1). In relation to quantification of *T. cruzi* by qPCR-XD, was possible to establish that in 4 of 7 cases with positive XD (57.1%), the parasitic load fluctuated between 1 and 9.9 par-eq/mL. In this group the highest parasitic load fluctuated between 100 and 1.000 par-eq/mL (14.3%).

On the other hand, in 15/38 (39.5%) cases with negative XD, qPCR-B showed a parasitic load between 0.1 and 0.99 par-eq/mL. In this group, a single case presented a parasitic load of 1–9.9 par-eq/mL. For the contrary, in 6 of 38 cases (15.79%) with negative XD, qPCR-XD had a parasitic load between 0.1 and 0.99 par-eq/mL. The highest load fluctuated between 10 and 99 par-eq/mL (2.63%). In 22 (57.9%) and 30 cases (78.9%) with negative XD, *T. cruzi* was not detected by qPCR-B and qPCR-XD, respectively. The global positivity of qPCR-B and qPCR-XD in the negative XD group was of 42.13% and 21.05%, respectively.

In Table 4 is observed the quantification of *T. cruzi* by qPCR-B and qPCR-XD in the 45 cases without cardiopathy, 10 (22.2%) with positive XD and 35 (77.8%) with negative XD.

In this positive XD group, the positivity of qPCR-B and qPCR-XD was 80.0% and 90%, respectively. In 8/10 cases (8.0%) qPCR-B fluctuated between 0.1 and 9.9 par-eq/mL. When qPCR-XD was applied, was possible to establish that in 3 cases (30%), the parasitic load fluctuated between 0.1 and 9.9 par-eq/mL. The highest parasite burden was

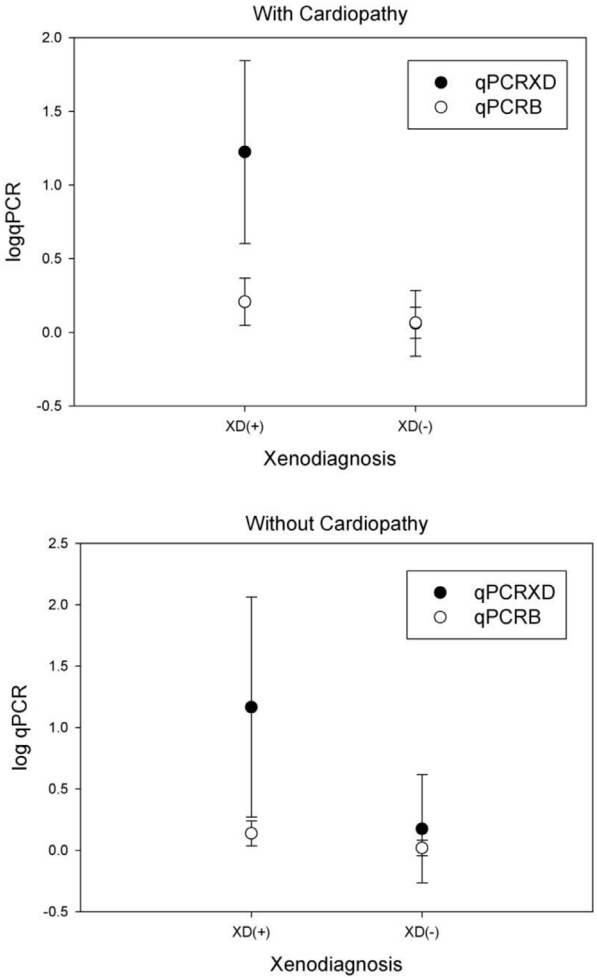

Fig. 1. qPCR value in chronic ChD patients with and without cardiopathy positive v/s negative XD with qPCR-B and qPCR-XD. Different letters represents differences in Tukey comparisons at $\alpha = 0.05$.

detected in 6 cases (30%), with parasitic load between 10 and 1.000 par-eq/mL. In one case with positive XD *T. cruzi* was not detected by qPCR-XD.

In the cases with negative XD, 4 cases (11.4%) had between 0.1 and 0.99 par. eq. /mL and only 1 case 1–9.9 par-eq/mL (2.9%). In 30 cases (85.7%), *T. cruzi* was not detected by qPCR-B. Finally, in cases with negative XD, qPCR-XD showed that in 6% of them (17.14%) had a parasitic load between 0.1 and 9.9 par-eq/mL. The highest *T. cruzi* load fluctuated between 10 and 1.000 par-eq/mL (5.8%). The global positivity of qPCR-B and qPCR-XD in the negative XD group was of 14.3% and 23%, respectively.

A higher value was found in the qPCR for the cases who had positive XD in comparison with the cases who had negative XD ($F_{1,172} = 89.76$, $p < 0.0001$). The higher values were found with qPCRXD ($F_{1.172} = 74.22$, $p < 0.0001$). A relationship was also found between the positivity of XD and the biological sample used for qPCR ($F_{1,172} = 55.20$, $p < 0.0001$) explained by a greater change in the values of qPCR positive XD with the technique qPCRXD with respect to negative XD (Fig 1).

The ROC curve analyses show a betters AUC for qPCR-XD (AUC = 0.902; $IC_{0.95}$ = [0.762–0.98]) than qPCR-B (AUC = 0.747; $IC_{0.95}$ = [0.571–0.914]). S and specificity Sp according to test: qPCR-XD S = 94.1% and Sp = 86.3% and for qPCR-B S = 82.4% and Sp = 72.6% (Fig 2).

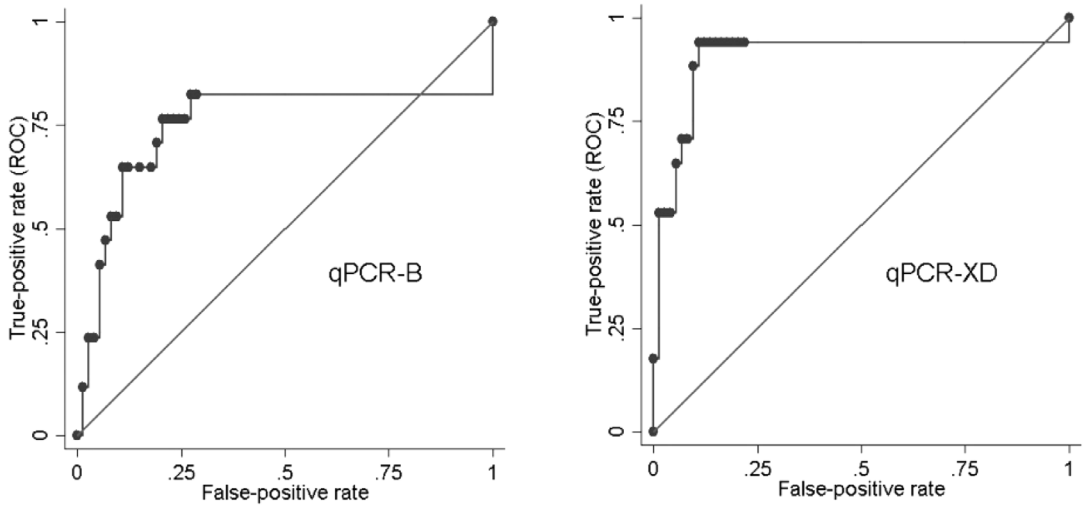

Fig. 2. ROC curves for qPCR-B and qPCR-XD in 90 non-treated chronic Chagas disease.

4. Discussion

In this study, the older ages of the cardiopaths is explained because the evolution of heart involvement is a slow process that takes years (Rassi et al., 2012). The XD positivity (17.5%), is the expected for individuals with chronic ChD, considering that with two boxes of XD the sensitivity of circulating *T. cruzi* is 21.7% (Schenone, 1999). Previous studies of our group described 21% and 14% of positive XD in groups of different patients with chronic ChD (Zulantay et al., 2011; Saavedra et al., 2016). No statistically significant differences was found between the positivity of XD and the presence or absence of cardiopathy ($\chi^2_1 = 0.43$, $p = 0.51$, 95% CI).

It is important to note that although XD is a valuable tool for the detection of viability of *T. cruzi*, this technique is archaic (Tarleton and Curran, 2012) and dependent operator, however, the main advantage of conventional XD is its potential to confirm the presence of viable *T. cruzi* (Amato Neto, 2012; Saavedra et al., 2013), a vital aspect in evaluations of chemotherapeutic efficacy of treated patients. In the present study, like so many others, is evidenced the capacity of the biological vector of *T. cruzi* to allow its multiplication (Kollien and Schaub, 2000). Nevertheless, due to rejection of some patients, in our lasted study protocols, conventional XD technique is no longer included.

It has been demonstrated that the limited sensitivity of direct observation of *T. cruzi* by conventional XD in man and different mammals it is improved when qualitative PCR (semi-automated technique) is applied in triatomine FS previously fed by XD (Breniere et al., 1995; Dorn et al., 2001; Campos et al., 2007b; Pizarro et al., 2007; Zulantay et al., 2011; Saavedra et al., 2013; Egaña et al., 2014; Oda et al., 2014; Santana et al., 2014). This is possible because *T. cruzi* contain satellite and kinetoplastid DNA (kDNA) with many repetitive and abundant sequences that are well suited for the application of PCR (Sturm et al., 1989; Avila et al., 1993; Requena et al., 1996; Elias et al., 2003; Duffy et al., 2009; Qvarnstrom et al., 2012). This biological property has been used for the successful standardization of qPCR what has allowed to determine the parasitic load by *T. cruzi* (Piron et al., 2007; Duffy et al., 2009; Moreira et al., 2013; Ramírez et al., 2015). Previous studies have allowed us applied qualitative PCR in DNA extracted from FS used in XD (Zulantay et al., 2011; Muñoz et al., 2013; Saavedra et al., 2013; Ortiz et al., 2015), allowed to increase the sensitivity of XD and to confirm the presence of viable *T. cruzi*. In this study, the parasitic load in *T. infestans* fed by XD was quantified by qPCR-XD and qPCR-B in patients that were classified according the presence or absence of cardiopathy. This clinical antecedent was considered because currently no biomarkers to assess which patients with chronic indeterminate ChD will develop heart disease and which will spend their entire life in this state (Apt et al., 2015).

In the group of patients with cardiopathy, the positivity of qPCR-B and qPCR-XD in cases with positive XD was 85.7% and 100%, respectively, while the positivity of qPCR-B and qPCR-XD in cases with negative XD was 42.13% and 21.05%, respectively. In patient without cardiopathy, something similar is observed. In cases with positive XD, qPCR-B and qPCR-XD detected *T. cruzi* in 80.0% and 90% of the cases, respectively, while the positivity of qPCR-B and qPCR-XD in the negative XD group was of 14.3% and 23%, respectively. Considering the global positivity, the major efficiency in to detect *T. cruzi* was qPCR-XD in patients with positive XD, is to say, XD was efficient to amplify high parasitemias. The statistical analysis evidenced the major qPCR value in the positive XD with the qPCR-XD in relation with the negative XD ($F_{1,172} = 55.20$, $p < 0.0001$). Nevertheless, if we considered at XD as gold standard technique, three cases were false negative in the group of patient without cardiopathy. The case 1 (see S1) was qPCR-B and qPCR-XD negative, with positive XD at 90 days of incubation of the triatomines, suggesting a low but detectable parasitemia or an error in the microscopic observation of the operator. It is also possible that the sample of peripheral blood received at the same time as the application of XD did not have circulating parasites. Finally, as demonstrated in this study, qPCR-B it is less sensitivity that qPCR-XD (ROC curve analyses, Fig. 2). The case 7 was positive XD at 30, 60 and 90 days of incubation of triatomines with high burden parasite quantified by qPCR-XD (149.5 par-eq/mL). Clearly, the negative result in qPCR-B is not compatible with the positive results of XD and qPCR-XD, nevertheless, the qPCR-B remained negative despite repeated trials. Was *T. infestans* able to efficiently amplify an eventual low parasitemia? The possible answer could be in some of the interesting aspects discussed in this topic: number of boxes used, insects ingestion capacity (Castro et al., 1983; Schenone, 1999); efficiency of the replication in the insect and the initial inoculum (Apt et al., 2013); diuresis of triatomines after the high initial blood supply (Kollien and Schaub, 1989), complexity of the metabolome in feces of triatomines (Antunes et al., 2013), bacterial microbiota in the insect gut (Gumiel et al., 2015), influence of starvation on the development of *T. cruzi* in *T. infestans* or *M. spinolai* (Kollien and Schaub, 1998; Cabe et al., 2019); mortality during the incubation period, and the differential regulation of parasite populations (García et al., 2010); *T. cruzi* genotypes amplified from simple and mixed infections (García et al., 2010; Lima et al., 2014), species used in the XD (Santana et al., 2014; Ortiz et al., 2015), inhibition of a clonal genotype in mixed infection (Pinto et al., 1998) and different DTUs population densities in the vector insect (Schaub, 1989). Recently, it is described the importance of the survivorship of *M. spinolai* after feeding and fasting in the laboratory to determine the competence of this

biological vector of *T. cruzi* (Cabe et al., 2019).

With respect to the association between the different parasitological tests and presence or absence of cardiopathy, there are no statistically significant differences when XD was applied ($\chi^2_1 = 0.43$, $p = 0.51$, 95% CI). Likewise, there are no statistically significant differences in the qPCR value between cardiopaths and non cardiopaths ($F_{1,172} = 0.051$, $p = 0.82$). Nevertheless, a superior value in the qPCR of cases with positive XD in comparison with qPCR of cases with negative XD was found ($F_{1,172} = 89.76$, $p < 0.0001$). At the same time, higher values were found for qPCR-XD ($F_{1,172} = 74{,}22$, $p < 0.0001$). The statistical analysis of results, allowed to found a interaction between the XD positivity and type of technique ($F_{1,172} = 55.20$, $p < 0.0001$) by a biggest change in the qPCR values in cases with positive XD respect to cases with negative XD (Tukey test $\alpha = 0.05$, Fig. 1). Finally, differences between qPCR-B and qPCR-XD were established ($F_{1,172} = 74.21$, $p << 0.001$). The ROC curve analyses show a betters AUC for qPCR-XD (sensitivity = 94.1% and specificity = 86.3%) in comparison with qPCR-B (sensibility = 82.4% and specificity = 72.6%) (Fig. 2). In the negative XD group no differences were found with qPCRXD between cardiopaths and non cardiopaths ($\chi^2 = 2.02$, $p = 0.16$). On the contrary, when only qPCRB is applied, the differences are statistically significative ($\chi^2 = 6.18$, $p = 0.013$).

In others studies performed by our group in patients with positive XD, the concordance between the positivity of qPCR-XD and qPCR-B was 100% (Saavedra et al., 2013). In the present study, it was also detected highest parasitemia when compared qPCR-B and qPCR-XD in patients with positive XD. XD technique was useful to verify that qPCR-B can detect and to quantify *T. cruzi* when the circulating parasitemias are high. Nevertheless, a high percentage of cases with negative XD were qPCR-B negative (57.9% and 85.7%, cardiopaths and non cardiopaths groups, respectively), with the important challenge for the laboratory researchers in cases with low and no quantifiable parasitemias specially in treated and non-treated patients with tripanocidal drugs evaluated with this parasitological tools. XD was a useful parasitological tool that gives way to new methodologies. Finally, a good reason for receiving antiparasitic drugs, independent of age unless a contraindication exists, is that low parasite burdens may still causing damage to diana organs.

5. Conclusions

The statistical analysis it shows the major performance in DNA samples obtained of positive XD (gold standard for viable *T. cruzi*) detected and quantified by qPCR-XD. A high percentage of cases with XD and qPCR-XD positive (80–100%) have result concordant with qPCR-B. In studies were conventional XD is not applied, future challenges are especially related to the low parasitic load of chronic ChD patients treated with trypanocidal drugs and post-therapy parasitological evaluations by qPCR-B. Finally, we found that qPCRXD was the best and more sensitive technique to detect parasitemia differences between positive and negative XD.

CRediT authorship contribution statement

Werner Apt: Conceptualization, Formal analysis, Writing - original draft, Data curation. **Daniela Carrasco:** Formal analysis. **Cristian Fuentealba:** Formal analysis. **Mauricio Canals:** Formal analysis. **Gabriela Muñoz:** Formal analysis, Data curation. **Miguel Saavedra:** Formal analysis, Data curation. **Juan-Paul Castillo:** Formal analysis, Data curation. **Inés Zulantay:** Conceptualization, Writing - original draft, Data curation.

Declaration of Competing Interest

None.

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Supplementary material

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References

Amato Neto, V., 2012. *Trypanosoma cruzi*: the supposed removal of the use of the xenodiagnosis and blood culture. Rev. Inst. Med. Trop. Sao Paulo 54, 235–236.

Antunes, L.C., Han, J., Pan, J., Moreira, C.J., Azambuja, P., Borchers, C.H., Carels, N., 2013. Metabolic signatures of triatomine vectors of *Trypanosoma cruzi* unveiled by metabolomics. PLoS ONE 8, e77283.

Apt, W., Arribada, A., Zulantay, I., Saavedra, M., Araya, E., Solari, A., Ortiz, S., Arriagada, K., Rodriguez, J., 2015. *Trypanosoma cruzi* burden, genotypes and clinical evaluation of Chilean patients with chronic Chagas cardiopathy. Parasitol. Res. 114, 3007–3018.

Apt, W., Arribada, A., Zulantay, I., Rodríguez, J., Saavedra, M., Muñoz, A., 2013. Treatment of Chagas' disease with itraconazole: electrocardiographic and parasitological conditions after 20 years of follow-up. J. Antimicrob. Chemother. 68, 2164–2169.

Avila, H.A., Pereira, J.B., Thiemann, O., De Paiva, E., De Grave, W., Morel, C.M., Simpson, L., 1993. Detection of *Trypanosoma cruzi* in blood specimens of chronic chagasic patients by polymerase chain reaction amplification of kinetoplast minicircle DNA: comparison with serology and xenodiagnosis. J. Clin. Microbiol. 31, 2421–2426.

Bravo, N., Muñoz, C., Nazal, N., Saavedra, M., Martínez, G., Araya, E., Apt, W., Zulantay, I., 2012. Real-time PCR in faecal samples of *Triatoma infestans* obtained by xenodiagnosis: proposal for an exogenous internal control. Parasit. Vectors 5.

Breniere, S.F., Bosseno, M.F., Tellería, 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.

Cabe, A.M, Yañez, F., Pinto, R., López, A., Ortiz, S., Martin, C.M., Botto-Mahan, C., Solari, A., 2019. Survivorship of wild caught *Mepraia spinolai* nymphs: the effect of seasonality and *Trypanosoma cruzi* infection after feeding and fasting in the laboratory. Infect. Genet. Evol. 71, 197–204.

Campos, R., Acuña-Retamar, M., Botto-Mahan, C., Ortiz, S., Cattán, P., Solari, A., 2007a. Susceptibility of *Mepraia spinolai* and *Triatoma infestans* to different *Trypanosoma cruzi* strains from naturally infected rodent hosts. Acta Trop 104, 25–29.

Campos, R., Botto-Mahan, C., Ortiz, S., Acuña, M., Cattan, P., Solari, A., 2007b. *Trypanosoma cruzi* detection in blood by xenodiagnosis and polymerase chain reaction in the wild rodent *Octodon degus*. Am. J. Trop. Med. Hyg. 76, 324–336.

Canals, M., González, C., Canals, L., Canals, A., Cáceres, D., Alvarado, S., Cattan, P.E., Saavedra, M., Zulantay, I., Apt, W., 2017. What do the numbers tell us about the temporal evolution of Chagas' disease? Rev. Chil. Infectol. 34, 120–127.

Castro, C.N., Alves, M.T., Macêdo, V.O., 1983. Importância da repetição do xenodiagnóstico para avaliação da parasitemia na fase crônica da doença de Chagas. Rev. Soc. Bras. Med. Trop. 16, 98–103.

Dorn, P.L., Flores, J., Brahney, B., Gutiérrez, A., Rosales, R., Rodas, A., Monroy, C., 2001. Comparison of polymerase chain reaction on fresh tissue samples and fecal drops on filter paper for detection of *Trypanosoma cruzi* in *Rhodnius prolixus*. Mem. Inst. Oswaldo Cruz 96, 503–505.

Duffy, T., Bisio, M., Altcheh, J., Burgos, J.M., Diez, M., Schijman, A., 2009. Accurate real-time PCR strategy for monitoring bloodstream parasitic loads in Chagas disease patients. PLoS. Negl. Trop Dis. 3, e419.

Duschak, V.G., 2019. Major kinds of drug targets in Chagas disease or American Trypanosomiasis. Curr. Drug. Targets Apr. 23https://doi.org/10.2174/1389450120666190423160804. [Epub ahead of print].

Egaña, C., Vergara, F., Campos, R., Ortiz, S., Botto, C., Solari, A., 2014. *Trypanosoma cruzi* infection in *Mepraia gajardoi* and *Mepraia spinolai*: the effect of feeding nymphs from the field. Am. J. Trop. Med. Hyg. 91, 534–536.

Elias, M.C., Vargas, N.S., Tomazi, L., Zingales, B., Schenkman, S., Briones, M.R., 2003. Organization of satellite DNA in the genome of *Trypanosoma cruzi*. Mol. Biochem. Parasitol. 129, 1–9.

Fernández, M.D.P., Gaspe, M.S., Gürtler, R.E., 2019. Inequalities in the social determinants of health and Chagas disease transmission risk in indigenous and creole households in the Argentine Chaco. Parasit. Vectors 12, 184.

Frías-Laserre, D., González, C.R., Reyes, C., de Carvalho, D.B., Oliveira, J., Canals, M., da

Rosa, J.A., 2017. Wing polymorphism and Trypanosoma cruzi infection in wild, peridomestic and domestic collections of Mepraia spinolai (Hemiptera: Reduviidae) from Chile. J. Med. Entomol. 54, 1061–1066.
García, E.S., Genta, F.A., De Azambuja, P., Schaub, G.A., 2010. Interactions between intestinal compounds of triatomines and *Trypanosoma cruzi*. Trends Parasitol. 26, 499–505.
Gumiel, M., Da Mota, F.F., Rizzo, V., de, S., Sarquis, O., De Castro, D.P., Lima, M.M., García, Ede, S., Carels, N., Azambuja, P., 2015. Characterization of the microbiota in the guts of *Triatoma brasiliensis* and *Triatoma pseudomaculata* infected by *Trypanosoma cruzi* in natural conditions using culture independent methods. Parasit. Vectors 8, 245.
Kollien, A.H., Schaub, G.A., 1998. The development of *Trypanosoma* cruzi (Trypanosomatidae) in the reduviid bug *Triatoma infestans* (Insecta): influence of starvation. J. Eukaryot. Microbiol. 45, 59–63.
Kollien, A.H., Schaub, G.A., 2000. The development of *Trypanosoma cruzi* in Triatomine. Parasitol. Today 16, 381–387.
Kooy, R.F., Ashall, F., Van der Ploeg, M., Overdulve, J.P., 1989. On the DNA content of *Trypanosoma cruzi*. Mol. Biochem. Parasitol. 36, 73–76.
Lima, V.S., Jansen, A.M., Messenger, L.A., Miles, M.A., Llewellyn, M.S., 2014. Wild *Trypanosoma cruzi* I genetic diversity in Brazil suggests admixture and disturbance in parasite populations from the Atlantic Forest region. Parasit. Vectors 7, 263.
Maguire, J., Mott, K., Souza, J., Almeida, E., Ramos, N., Guimaraes, A., 1982. Electrocardiographic classification and abbreviated lead system for population-based studies of Chagas' disease. Bol. PAHO 16, 47–58.
MINSAL, Ministerio de Salud. Gobierno de Chile, 2014. Norma General Técnica. Control y Prevención Nacional de la Enfermedad de Chagas. https://diprece.minsal.cl/wrdprss_minsal/wp-content/uploads/2016/03/NORMA-TECNICA_CHAGAS_edici%C3%B3n-definitiva-140514.pdf Acces on May, 30, 2019.
MINSAL, Ministerio de Salud. Gobierno de Chile, 2017. Manual de Procedimiento para la Atención de Pacientes con Enfermedad de Chagas. https://diprece.minsal.cl/wrdprss_minsal/wp-content/uploads/2018/01/2018.01.18_ENFERMEDAD-DE-CHAGAS-2017.pdf Accessed on July 28, 2019.
Moreira, O.C., Ramírez, J.D., Velázquez, E., Melo, M.F., Lima-Ferreira, C., Guhl, F., Sosa-Estani, S., Marín-Neto, J.A., Morillo, C.A., Britto, C., 2013. Towards the establishment of a consensus real-time qPCR to monitor *Trypanosoma cruzi* parasitemia in patients with chronic chagas disease cardiomyopathy: a substudy from the benefit trial. Acta Trop. 125, 23–31.
Muñoz, C., Zulantay, I., Apt, W., Ortiz, S., Schijman, A.G., Bisio, M., Ferrada, V., Herrera, C., Martínez, G., Solari, A., 2013. Evaluation of nifurtimox treatment of chronic Chagas disease by means of several parasitological methods. Antimicrob. Agents Chemother. 57, 4518–4523.
Muñoz, G., Vergara, C., Martínez, G., Apt, W., Zulantay, I., 2019. Quantification of immunoglobulin G against *Trypanosoma cruzi* in individuals with chronic Chagas disease treated with nifurtimox and evaluated in prolonged follow-up. Korean J. Parasitol. 57, 39–41.
Muñoz-San Martín, C., Apt, W., Zulantay, I., 2017. Real-time PCR strategy for the identification of *Trypanosoma cruzi* discrete typing units directly in chronically infected human blood. Infect. Genet. Evol. 49, 300–308.
Oda, E., Solari, A., Botto-Mahan, C., 2014. Effects of mammal host diversity and density on the infection level of *Trypanosoma cruzi* in sylvatic kissing bugs. Med. Vet. Entomol. 28, 384–390.
Ortiz, S., Zulantay, I., Apt, W., Saavedra, M., Solari, A., 2015. Transferability of *Trypanosoma cruzi* from mixed human host infection to *Triatoma infestans* and from insects to axenic culture. Parasitol. Int. 64, 33–36.
Pinto, A.S., De Lana, M., Bastrenta, B., Barnabé, C., Quesney, V., Noël, S., Tibayrenc, M., 1998. Compared vectorial transmissibility of pure and mixed clonal genotypes of *Trypanosoma cruzi* in *Triatoma infestans*. Parasitol. Res. 84, 348–353.
Piron, M., Fisa, R., Casamitjana, N., López-Chejade, P., Puig, L., Vergés, M., Gascón, J., Gómez i Prat, J., Portús, M., Sauleda, S., 2007. Development of a real-time PCR assay for *Trypanosoma cruzi* detection in blood samples. Acta Trop. 103, 195–200.
Pizarro, J.C., Lucero, D., Stevens, L., 2007. A method for the identification of guinea pig blood meal in the Chagas disease vector *Triatoma infestans*. Kinetoplastid Biol. Dis. 6, 1.
Qvarnstrom, Y., Schijman, A.G., Veron, V., Aznar, C., Steurer, F., Da Silva, A.J., 2012. Sensitive and specific detection of *trypanosoma cruzi* dna in clinical specimens using a multi-target real-time PCR approach. PLoS. Negl. Trop. Dis. 6, e1689101371.
Ramírez, J.C., Cura, C.I., da Cruz Moreira, O., Lages-Silva, E., Juiz, N., Velázquez, E., Ramírez, J.D., Alberti, A., Pavia, P., Flores-Chávez, M.D., Muñoz-Calderón, A., Pérez-Morales, D., Santalla, J., Marcos da Matta Guedes, P., Peneau, J., Marcet, P., Padilla, C., Cruz-Robles, D., Valencia, E., Crisante, G.E., Greif, G., Zulantay, I., Costales, J.A., Alvarez-Martínez, M., Martínez, N.E., Villarroel, R., Villarroel, S., Sánchez, Z., Bisio, M., Parrado, R., Maria da Cunha Galvão, L., Jácome da Câmara, A.C., Espinoza, B., Alarcón de Noya, B., Puerta, C., Riarte, A., Diosque, P., Sosa-Estani, S., Guhl, F., Ribeiro, I., Aznar, C., Britto, C., Yadón, Z.E., Schijman, A.G., 2015. Analytical validation of quantitative real-time PCR methods for quantification of *trypanosoma cruzi* DNA in blood samples from CHAGAS disease patients. J. Mol. Diagn. 17, 605–615.
Rassi Jr., A., Rassi, A., Marcondes de Rezende, J., 2012. American trypanosomiasis (Chagas disease). Infect. Dis. Clin. North. Am. 26, 275–291.
Requena, J.M., López, M.C., Alonso, C., 1996. Genomic repetitive DNA elements of *Trypanosoma cruzi*. Parasitol. Today 12, 279–283.
Saavedra, M., Zulantay, I., Apt, W., Castillo, J., Araya, E., Martínez, G., Rodríguez, J., 2016. Quantification by real-time PCR of *Trypanosoma cruzi* DNA in samples of *Triatoma infestans* used in xenodiagnosis of chronic Chagas disease patients. Parasit. Vectors 9, 382.
Saavedra, M., Zulantay, I., Apt, W., Martínez, G., Rojas, A., Rodríguez, J., 2013. Chronic Chagas disease: PCR-xenodiagnosis without previous microscopic observation is a useful tool to detect viable *Trypanosoma cruzi*. Biol. Res. 46, 295–298.
Santana, R.A., Magalhães, L.K., Prestes, S.R., Maciel, M.G., Da Silva, G.A., 2014. *Trypanosoma cruzi* strain TCI is associated with chronic Chagas disease in the Brazilian Amazon. Parasit. Vectors 7, 267.
Schenone, H., 1999. Xenodiagnosis. Mem. Inst. Oswaldo Cruz. 94, 289–294.
Schijman, A.G., Bisio, M., Orellana, L., Sued, M., Duffy, T., Mejia Jaramillo, A.M., Cura, C., Auter, F., Veron, V., Qvarnstrom, Y., Deborggraeve, S., Hijar, G., Zulantay, I., Lucero, R.H., Velazquez, E., Tellez, T., Sanchez Leon, Z., Galvão, L., Nolder, D., Monje Rumi, M., Levi, J.E., Ramirez, J.D., Zorrilla, P., Flores, M., Jercic, M.I., Crisante, G., Añez, N., De Castro, A.M., Gonzalez, C.I., Acosta Viana, K., Yachelini, P., Torrico, F., Robello, C., Diosque, P., Triana Chavez, O., Aznar, C., Russomando, G., Büscher, P., Assal, A., Guhl, F., Sosa Estani, S., Dasilva, A., Britto, C., Luquetti, A., Ladzins, J., 2011. International study to evaluate PCR methods for detection of *Trypanosoma cruzi* DNA in blood samples from Chagas disease patients. PLoS. Negl. Trop. Dis. 5, e931.
Schaub, G.A., 1989. *Trypanosoma cruzi*: quantitative studies of development of two strains in small intestine and rectum of the vector *Triatoma infestans*. Exp. Parasitol. 68, 260–273.
Silber, A., Búa, J., Porcel, B., Segura, E., Ruiz, A., 1997. *Trypanosoma cruzi*: specific detection of parasites by PCR in infected humans and vectors using a set of primers (BP1/BP2) targeted to a nuclear DNA sequence. Exp. Parasitol. 85, 225–232.
Sosa-Estani, S., Segura, E., 2015. Integrated control of Chagas disease for its elimination as public health problem. A review. Mem. Inst. Oswaldo Cruz 110, 289–298.
Sturm, N.R., Degrave, W., Morel, C., Simpson, L., 1989. Sensitive detection and schizodeme classification of *Trypanosoma cruzi* cells by amplification of kinetoplast minicircle DNA sequences: use in diagnosis of Chagas' disease. Mol. Biochem. Parasitol. 33, 205–214.
Tarleton, R.L., Curran, J.W., 2012. Is Chagas disease really the "new HIV/AIDS of the Americas" PLoS. Negl. Trop. Dis. 6, e1861.
Zulantay, I., Apt, W., Valencia, C., Torres, A., Saavedra, M., Rodríguez, J., Sandoval, L., Martínez, G., Thieme, P., Sepúlveda, E., 2011. Detection of *Trypanosoma cruzi* in untreated chronic chagasic patients is improved by using three parasitological methods simultaneously. J. Antim. Chemother. 66, 2224–2226.