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Follow-up of experimental chronic Chagas' disease in dogs: use of polymerase chain reaction (PCR) compared with parasitological and serological methods

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Abstract

In this study, the polymerase chain reaction (PCR) was compared with parasitological and serological methods to detect the infection in dogs, 5–12 years after experimental infection with *Trypanosoma cruzi*. The ability of parasitological methods to identify a positive animal was 22 and 11% by hemoculture and xenodiagnosis/xenoculture, respectively. On the other hand, the serological tests, including conventional serology and anti-live trypomastigote antibodies (ALTA) were positive in all infected dogs. Despite its low sensitivity, if considering only one reaction, the PCR analysis showed 100% of positivity, demonstrating the presence of parasite kDNA in all infected dogs. To identify a positive dog required at least two blood samples and up to nine repeated reactions using the same sample. Serial blood sample collection, ranging from 1 to 9, revealed that the percentage of dogs with positive PCR ranged from 67 to 100%. These findings suggested that, although the PCR is useful to detect the parasite in infected hosts, it should not be used isolated for the diagnosis of Chagas' disease and warn for the necessity of serial blood collection and re-tests. Moreover, these data validate once more the dog as a model for Chagas' disease since they demonstrate the permanence of infection by PCR, parasitological and serological methods, reaching relevant requisites for an ideal model to study this disease.

Keywords: *Trypanosoma cruzi*; Dog; Chronic infection; PCR; Parasitological tests and serology

1. Introduction

Dogs have been frequently used as experimental model to study Chagas' disease. The acute phase

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of the infection has been reproduced with relative facility by different authors, especially in young animals (Andrade, 1984; Pedreira de Castro and Brener, 1985; Lana et al., 1992). However, the development of the different clinical forms of the disease is not easily to be reproduced in this model (Andrade, 1984). The great majority of publications have only recorded the indeterminate form of the disease (Andrade and Andrade, 1980; Andrade et al., 1981) with few exceptions (Laranja et al., 1948; Lana et al., 1988, 1992). Andrade (1984) suggests auto-cure in dogs. Krettli et al. (1984) suggest auto-cure in mice. Zeledon et al. (1988) suggest auto-cure in humans. For these reasons, herein is reported a long-term follow-up study of dogs, 5–12 years after acute infection with *Trypanosoma cruzi* (Lana et al., 1992). The major goal of this study was to demonstrate the permanence of *T. cruzi* in these infected dogs, using different approaches like polymerase chain reaction (PCR), parasitological and serological methods. Since the PCR reaction has been theoretically pointed out as a powerful tool for the diagnosis of Chagas' disease (Avila et al., 1991; Wincker et al., 1994; Britto et al., 1995a), it was exhaustively performed, every 3 months, during 2 years, in parallel with hemoculture, xenodiagnosis/xenoculture and serology-conventional ELISA and detection of anti-live trypomastigote antibodies (ALTA). The use of these different laboratorial approaches to detect the presence of the parasite in chronically infected dogs could fill a crucial requisite, recommended by WHO (1984), for the establishment of dogs as an experimental model for chronic Chagas' disease.

2. Material and methods

2.1. Parasite strains

T. cruzi strains were isolated from the patient Berenice, considered the first human case of Chagas' disease, in 1962 (Be-62) by Salgado et al. (1962) and in 1978 (Be-78) by Lana and Chiari (1986). Both strains were classified as *T. cruzi* II (Lana et al., 1996).

2.2. Animals

Nine out-bred dogs were acutely infected with Be-62 and Be-78 *T. cruzi* strains (Lana et al., 1992), 5–12 years before starting this study. Five were infected with Be-62 strain and four infected with Be-78 strain. Animals were inoculated with 2.0×10^3 metacyclic trypomastigotes. Two age-matched uninfected dogs were used as negative controls. All animals were evaluated every 3 months, during 2 years.

2.3. Polymerase chain reaction (PCR)

Ten milliliters of blood were collected with an equal volume of Guanidin–HCl 6 M/EDTA 0.2 M, pH 8.0 solution (Avila et al., 1991). Samples were maintained at room temperature. Seven days later, the samples were boiled during 15 min and stored at 4 °C until processing. DNA extraction was done according to Wincker et al. (1994) protocol with some modifications introduced by Gomes et al. (1998).

PCR conditions were the same described by Gomes et al. (1998) but using the primers: S35 (5′ - AAATAATGTACGGG(T/G)GAGATGCA-TGA-3′) and S36 (5′-GGGTTCGATTGGGG-TTGGTGT-3′) described by Avila et al. (1990) that anneal in the conserved microregion of the parasites kDNA minircicles. Briefly, 2 μl of blood DNA template was added in 10 mM Tris–HCl (pH 9.0); 75 mM KCl; 3.5 mM $MgCl_2$; 0.1% Triton X-100; 0.2 mM of dATP, dCTP, dGTP and dTTP (Sigma Company Ltd., USA); 20 pmol of each primer (S35 and S36, cited above); 1.0 unit of *Taq* DNA polymerase enzyme (Promega, Madison, WI, USA) and water up to 20 μl. The reaction mixture was overlaid with 30 μl of mineral oil and submitted to 35 cycles of amplification as followed described: an initial denaturation step at 95 °C for 5 min, two cycles with annealing at 30 °C for 2 min, 72 °C for 1 min for extension and 30 s at 95 °C for denaturation, followed by 33 cycles in which annealing was increased to 40 °C and a final extension at 72 °C for 5 min. PCR products were observed in 6% polyacrylamide gel electrophoresis and silver stained (Santos et al., 1993). Three DNA extrac-

tions and three PCR reactions using each DNA template were performed resulting on up to nine PCR for each blood sample, if necessary. Fig. 1 represents the strategy adopted for each blood sample processed.

2.4. Parasitological methods

2.4.1. Hemoculture

Twenty milliliters of heparinized blood were processed according to Luz et al. (1994). The hemocultures were maintained at 28 °C. Thirty, 60, 90 and 120 days later, each tube was examined for the detection of parasites.

2.4.2. Xenodiagnosis/xenoculture

Fifteen third star nymphs of *Triatoma infestans* were used for each procedure. After blood meal the insects were maintained at 27 °C, 70% of humidity. Forty days later, the insects were examined for the detection of parasites (xenodiagnosis). Xenoculture was performed according to Bronfen et al. (1989). The xenoculture was maintained at 28 °C and 30, 60, 90 and 120 days later it was examined for detection of parasites.

2.5. Serological methods

2.5.1. Conventional serology (ELISA)

T. cruzi specific antibodies was detected by the technique described by Voller et al. (1976) modified and adapted by Lana et al. (1991). ELISA plates were sensitized with *T. cruzi* antigen prepared by alkaline extraction (Vitor and Chiari, 1987) of Y strain obtained at exponential growth in LIT medium. Antibody binding was detected by using peroxidase-labeled *anti*-dog IgG, after reading in spectrophotometer, using 490 nm filter (BIO-RAD, 3550). The mean absorbance of 10 negative control sera plus two standard deviations was used as a cut-off to discriminate positive and negative results.

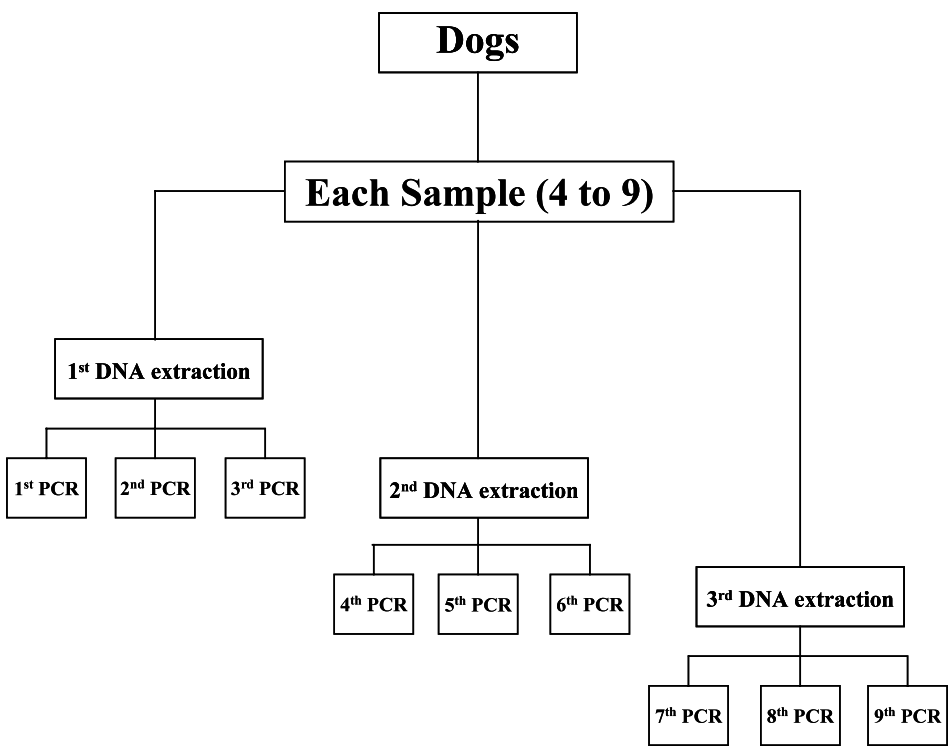

Fig. 1. Methodology used for the serial PCR. For each blood sample processed, up to three DNA extractions were performed. Three PCR reactions for each DNA extraction were carried out, resulting on up to nine PCR for each blood sample, if necessary.

Table 1
Results of PCR of serial blood collection in dogs experimentally infected with *T. cruzi* during the chronic phase of the infection

Dog	*T. cruzi* strain	Time of infection (years)	Blood sample									Total PCR+ (%)
			1st	2nd	3rd	4th	5th	6th	7th	8th	9th	
Controls												
C1	–	–	–	–	–	–	–	–	–	–	–	0
C2	–	–	–	–	–	–	–	–	–	–	–	0
Infected dogs												
1	Be-62	12	–	+	–	+	+	+	+	+	–	67
2	Be-62	11	–	+	+	+	+	+	+	N	N	86
3	Be-62	11	+	–	+	+	+	+	+	N	N	86
4	Be-62	5	+	+	+	+	+	+	+	+	+	100
5	Be-62	5	+	+	+	+	+	+	+	+	+	100
6	Be-78	10	–	+	–	+	+	–	N	N	N	50
7	Be-78	10	+	+	+	+	+	N	N	N	N	100
8	Be-78	9	+	–	–	+	N	N	N	N	N	50
9	Be-78	6	+	+	+	+	+	+	+	+	+	100
Total (%)			67	78	67	100	100	86	100	100	75	85

N, not performed due to necropsy.

Table 2
Minimal number of DNA extraction and PCR reactions necessary to detect the first positive result in dogs experimentally infected with *T. cruzi* during the chronic phase of the infection

Time of infection (years)	Dog	*T. cruzi* strain	First DNA extraction with PCR+	First PCR+
5	4	Be-62	2	5
5	5	Be-62	1	1
6	9	Be-78	2	5
9	8	Be-78	2	4
10	6	Be-78	6	16
10	7	Be-78	3	7
11	2	Be-62	6	17
11	3	Be-62	3	9
12	1	Be-62	5	14

2.5.2. Anti-live trypomastigote antibody (ALTA)

The immunofluorescence staining to detect ALTA was carried out as described by Martins-Filho et al. (1995), modified for U bottom 96 wells plate as introduced by Cordeiro et al. (2001). Briefly, after incubation with dog sera, the binding of antibodies to trypomastigotes was detected using fluorescein isothiocyanate (FITC) conjugated *anti*-dog IgG. The FITC-labeled parasites were fixed before run into cytometer. Flow-cytometric measurements were performed on a Becton Dickinson FACScalibur. The CELL-QUEST software package was used in both data storage and analysis. Trypomastigotes were first identified on the basis of their specific forward (size) and side (granularity) light-scattering properties (Fig. 2A). The relative FITC fluorescence intensity for each parasite preparation after incubation with individual sample was analyzed using a single histogram. A marker was set up on the internal control for unspecific binding (Fig. 2B) and used to determine the percentage of positive fluorescent parasites (PPFP). The samples were considered negative when PPFP $\leq 20\%$ and positive when PPFP was $> 20\%$ (Fig. 2C and D).

3. Results

3.1. Polymerase chain reaction (PCR)

PCR results obtained with up to nine reactions for each blood sample are shown in Table 1. The PCR was found to be positive in 100% of the infected dogs. The identification of a positive dog required at least two blood samples. The percentage of positive PCR, considering the total blood samples per dog, ranged from 50 to 100%. Only four dogs showed a positive PCR result for all blood samples processed with no association with the time of infection neither the *T. cruzi* strain. Serial blood sample collection, ranging from 1 to 9, revealed that the percentage of dogs with positive PCR ranged from 67 to 100%, depending on the sample processed.

The minimum number of DNA extractions necessary to detect the first positive reaction in ranged from 1 to 6 depending on the animal evaluated (Table 2). The number of PCR necessary to obtain the first positive result ranged from 1 to 17. Only one infected dog showed a positive result if only one blood sample and the first PCR was taken in account. No association was observed between the strain of *T. cruzi* and the number of DNA extraction or PCR needed to obtain the first positive result. It was interesting to observe that dogs over 10 years of infection required a higher number of PCR reactions and more DNA extractions to show the first positive result. Fig. 3 illustrates the positive PCR results for all infected dogs and one of the two uninfected animals included in this study.

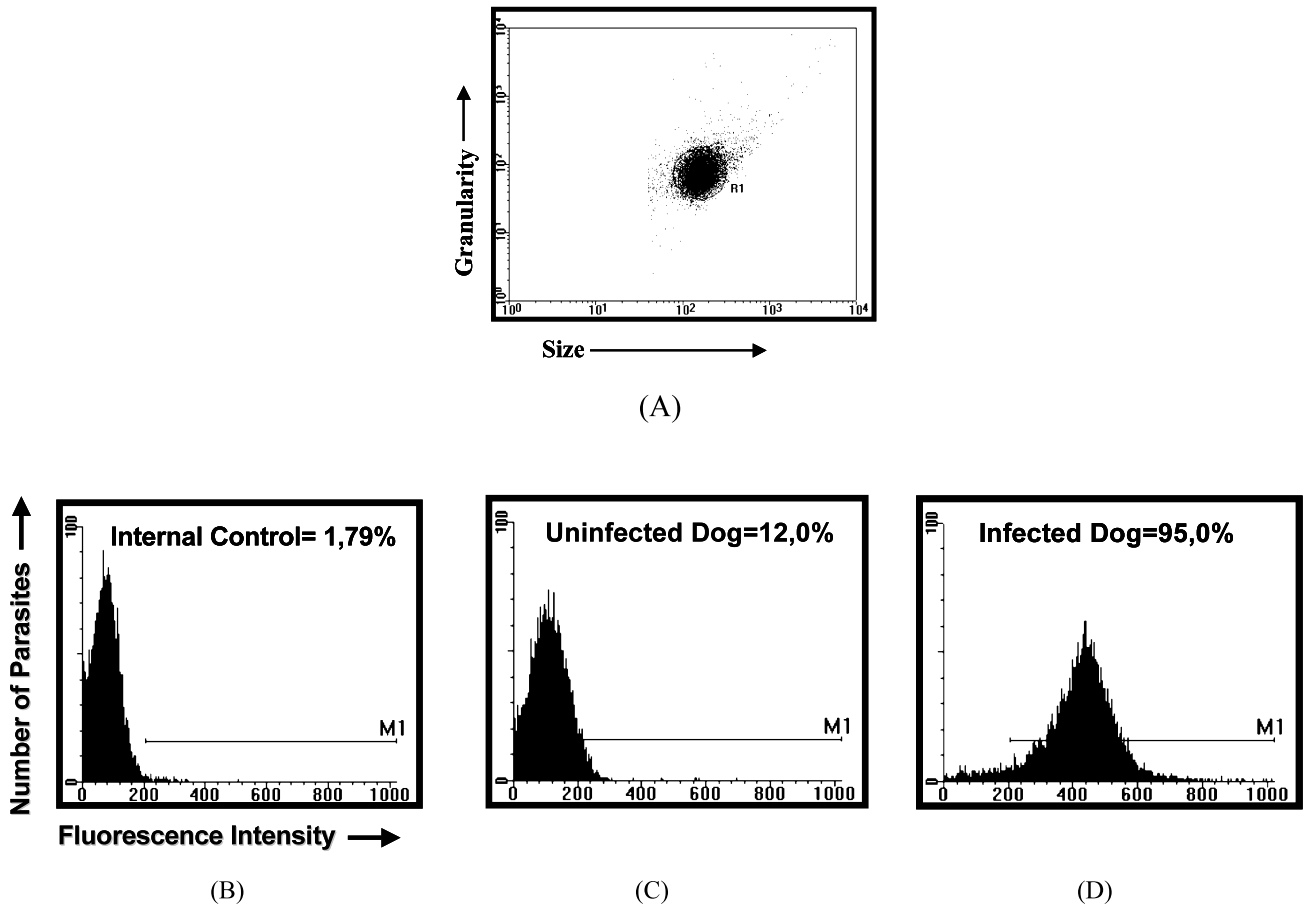

Fig. 2. Analysis of ALTA, by flow citometry. Dot Blot analysis of a representative trypomastigote distribution (R1) based on their size and granularity (A). Fluorescence single histogram representing the mean PPFP for non-specific binding uninfected control and *T. cruzi*-infected dogs (B, C and D, respectively).

3.2. Hemoculture, xenodiagnosis and xenoculture

Table 3 summarizes the results of all parasitological methods. Serial hemoculture (5–9) was negative in all dogs infected with Be-62 *T. cruzi* strain and two dogs infected with Be-78 strain. The total percentage of dogs with positive hemoculture was 22%. Both, serial xenodiagnosis (5–9) and xenoculture were negative in all dogs infected with Be-62 strain and positive in only one dog infected with Be-78 strain. The total percentage of dogs with positive xenodiagnosis/xenoculture was 11%.

3.3. Serology

Immunoenzimatic tests to detect circulating anti-*T. cruzi* antibodies were performed using 4–9 blood samples per animal (Table 4). Results of conventional ELISA are shown in Table 4. Despite three negative results out of nine reactions observed for the dog #1, all infected animals displayed positive ELISA. Repetition of ELISA confirmed these results. Analysis of ALTA demonstrated positive results for all samples tested during the 2 years of evaluation. Control animals were always negative in all serological examinations.

4. Discussion

The goal of this work was to demonstrate the permanency of *T. cruzi* in dogs during long-term chronic infection. These dogs were experimentally infected 5–12 years prior the beginning of this study. All animals were exhaustively examined during the acute phase, showing positive parasitological (fresh blood examination, hemoculture, xenodiagnosis) and serological examinations (Indirect Immunofluorescence Test (IIT) and ELISA), commonly used for the diagnosis of Chagas' disease (Lana et al., 1991, 1992). Many symptoms of the acute phase of the disease and electrocardiographic alterations compatible with Chagas' disease were also recorded (Lana et al., 1992). Herein they were re-submitted to laboratorial investigations including well-established parasitological and serological methods as well as new tools for diagnosing Chagas' disease, such as ALTA and PCR analysis.

The low performance of parasitological methods observed here is not totally unexpected, since longitudinal evaluation of these same dogs showed a lowering positivity of these test during the first 3 years after infection (Lana et al., 1992). The scarcity of positive parasitological examinations observed here, mainly in dogs infected with

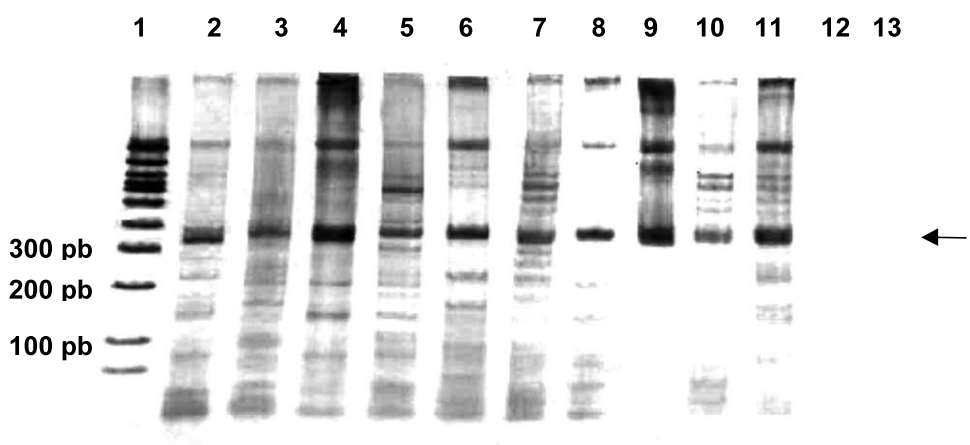

Fig. 3. Representative silver-stained gel showing the specific amplification of 330–bp fragments (arrow) of kDNA minicircles of *T. cruzi* isolated from chronically infected dogs (lanes 2–10), control infected dog (11), one uninfected dog (lane 12). Lane 1 represents the molecular size markers (100-pb ladder, Life Technologies, Gaithersburg, MD) and lane 13 is the PCR internal control where no DNA was added to PCR mixture.

Table 3
Results of hemoculture, xenodiagnosis and xenoculture in dogs experimentally infected with *T. cruzi* during the chronic phase of the infection

Dog	*T. cruzi* strain	Hc+/Hc total	Xd+/Xd total	Xc+/Xc total
Controls				
C1	–	N	N	N
C2	–	N	N	N
Infected dogs				
1	Be-62	0/9	0/9	0/9
3	Be-62	0/7	0/7	0/7
4	Be-62	0/7	0/7	0/7
5	Be-62	0/9	0/9	0/9
6	Be-62	0/9	0/9	0/9
7	Be-78	2/6	1/6	1/6
9	Be-78	1/5	0/5	0/5
10	Be-78	0/5	0/5	0/5
11	Be-78	0/9	0/9	0/9
Total (%)		22	11	11

N, not preformed.

the Be-62 strain, was previously reported by Lana et al. (1992). Although the time of infection seems to decrease the parasitemia (Castro et al., 1983, 1999) the positivity of hemoculture and xenodiagnosis also seems to be dependent on the type of *T. cruzi* strain. Lana et al. (1988), Veloso et al. (2001) easily detected positive hemoculture and xenodiagnosis in dogs experimentally infected with Colombian strain 8 and 15 years after infection which presents a predominance of stout forms in the blood and consequently more resistant to lysis mediated by complement (Krettli et al., 1984). Herein, the Be-62 strain, differently from Be-78 strain, which presents a predominance of slender trypomastigotes in blood (Lana and Chiari, 1986) that are more sensitive to complement mediated lysis, resulting on lesser permanency on circulating blood, also showed a lower positivity on parasitological methods.

The conventional serological method (ELISA) was positive in all dogs, despite an oscillating result in one of them. Oscillating results in serological tests are also reported in humans (Rassi et al., 1969), and can be the reason why some patients with positive xenodiagnosis/hemoculture and/or PCR present negative serology (Brenière et al., 1984; Gomes et al., 1999). However, using the flow cytometry assay to detect ALTA, all infected dogs were tagged with positive results thoroughly all blood samples. This result confirms the permanency of the *T. cruzi* in all chronically infected dogs, since ALTA has been indicated as a good marker for active infection and this method is

Table 4
Serological status of dogs experimentally infected with *T. cruzi* during the chronic phase of the infection

Dog	*T. cruzi* Strain	+ELISA/total	+ALTA/total
Controls			
C1	–	0/9	0/9
C2	–	0/9	0/9
Infected dogs			
1	Be-62	6/9	9/9
3	Be-62	7/7	7/7
4	Be-62	7/7	7/7
5	Be-62	9/9	9/9
6	Be-62	9/9	9/9
7	Be-78	6/6	6/6
9	Be-78	5/5	5/5
10	Be-78	4/4	4/4
11	Be-78	9/9	9/9
Total (%)		100	100

proposed as an new approach to detect cure in Chagas' disease (Martins-Filho et al., 1995).

Although the PCR was found to be positive in 100% of the infected dogs, to identify a positive dog it was required at least two blood samples and up to nine repeated reactions using the same sample, depending on the blood sample analyzed. Further investigations, including other PCR protocols, followed by slot-blot hybridization and analysis of different organs and tissues of these dogs are currently been investigated to improve the detection of *T. cruzi* during chronic infection.

The number of PCR necessary to obtain the first positive result ranged from 1 to 17. Only one infected dog showed a positive result if only one blood sample and the first PCR was taken in account. It was interesting to observe that dogs over 10 years of infection required a higher number of PCR reactions and more DNA extractions to show the first positive result.

Britto et al. (1995b), Junqueira et al. (1996) showed only 44.7% and 59.4% of positive PCR, respectively in patients. The most acceptable reason to explain the low performance of the PCR to detect DNA in biological material (blood, serum and tissue) is the level of parasitemia presented in the host when the sample is collected (Moser et al., 1989; Gomes et al., 1998). The results presented here do not discard this hypothesis, since a low percentage of positivity was observed when parasitological methods were conduced in parallel to PCR.

However, as the ability of PCR to detect a positive host increases from 67% when one sample was evaluated to 100% when a second sample was taking in account. The percentage of positive PCR, considering the total blood samples per dog, ranged from 50 to 100%. Then, we do believe that besides considering the low parasitemia of chronic infection as the major restraining feature leading to a negative PCR, the necessity of serial blood collection and re-tests should also be considered. As there are no available methods to test dogs samples for the presence of inhibitor elements during the DNA extraction procedure, we cannot discard that all these repeated negative results of PCR were due to phenol and chloroform, which can inhibits the *Taq* DNA polymerase enzyme generating false negative results.

In conclusion, the hallmark of this work was: (1) the evidence of *T. cruzi* presence in all dogs, even after a long-chronic period of infection. Then, these results validate the dog as a good experimental model to study the chronic phase of Chagas' disease and (2) the establishment of the necessity of repeated PCR procedures to assure the diagnosis of the chronic *T. cruzi* infection indicating that this method should not be used isolated as a conclusive tool for diagnosis of Chagas' disease.

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