Experimental and Molecular Pathology 101 (2016) 274–280

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

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

Quantitative PCR and unconventional serological methods to evaluate clomipramine treatment effectiveness in experimental *Trypanosoma cruzi* infection

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ARTICLE INFO

Article history:
Received 28 March 2016
and in revised form 21 September 2016
Accepted 22 September 2016
Available online 24 September 2016

Keywords:
Chagas disease
Trypanosoma cruzi
Chronic phase
Clomipramine treatment
qPCR
Serology

ABSTRACT

Clomipramine (CLO), a tricyclic antidepressant drug, has been used for the treatment of mice infected with *Trypanosoma cruzi*. In this work we evaluated the effectiveness of CLO treatment upon *T. cruzi*-infected mice in the chronic phase of the experimental infection using Quantitative polymerase chain reaction (qPCR) and recombinant ELISA. Sixty Swiss albino mice were inoculated with 50 trypomastigote forms of *T. cruzi* (Tulahuen strain). CLO treatment consisted of 5 mg/kg/day during 60 days by intraperitoneal injection, beginning on day 90 post infection (p.i) when the mice presented electrocardiographic (ECG) alterations compatible with the chronic phase of the disease. The evolution of experimental infection and the treatment efficacy were studied through survival, electrocardiography, serology using a mixture and individual (1, 2, 13, 30, 36 and SAPA) recombinant proteins from epimastigotes and trypomastigotes of *T. cruzi;* and qPCR on days 180 and 270 p.i. CLO treatment in the chronic phase decreased the parasite load, reduced the levels of antibodies against antigen 13 throughout 270 days p.i and reversed the ECG abnormalities in the treated animals, from 100% of the mice with alterations at the beginning of the treatment to only 20% of the mice with alterations by day 270 p.i. This study shows that qPCR and the use of recombinant antigens are more sensitive to evaluate the effectiveness of the treatment and proves that clomipramine may be considered as a new chemotherapy for the chronic phase of the disease.

1. Introduction

Chagas disease, also known as American trypanosomiasis, is a zoonosis caused by the flagellate protozoan parasite, *Trypanosoma cruzi*. Between 6 and 7 million people are estimated to be infected worldwide, mostly in Latin America. Initially, Chagas disease was confined to the region of Central and South America, but now, due to migration, it has spread to other continents (WHO, 2015).

The disease is characterized by two phases: an acute phase which appears just after infection, and a chronic phase which may have no evident pathology (stage previously known as the indeterminate phase) or evolve into irreversible cardiac and/or digestive lesions (Mitelman et al., 2011).

Chagasic cardiopathy appears to carry the worst prognosis and has become the most frequent cause of heart failure and sudden death, as well as the most common cause of cardio-embolic stroke in Latin America (Bern et al., 2007).

Chagas disease specific treatment has recently been recommended for people in either the acute or the chronic phase. To date, only two drugs have been effectively used in Chagas disease chemotherapy: Nifurtimox and Benznidazole, which present several limitations mainly due to adverse secondary effects (Apt, 2010).

Experimental studies have identified several novel targets for chemotherapy, one of them being the parasite's enzyme trypanothione reductase (TR). TR has been widely identified as a drug target for Chagas disease treatment (Meiering et al., 2005). The essential role of TR in the parasite thiol metabolism and its absence from the mammalian host render the enzyme a highly attractive target for structure based drug development against trypanosomatids (Krauth-Siegel and Inhoff, 2003).

Abbreviations: bp, base pairs; CLO, clomipramine; EA, ECG alterations; ECG, electrocardiographic; NEA, no ECG alterations; NI, non-infected; NIT, non-infected treated with CLO; OD, optical density; p.i, post infection; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; T, treated with CLO; TR, trypanothione reductase; UT, untreated.

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http://dx.doi.org/10.1016/j.yexmp.2016.09.006
0014-4800/

The structure of tricyclic neuroleptic compounds has shown them to be a promising class of TR inhibitors (Gutierrez-Correa et al., 2001). Clomipramine (CLO), a tricyclic antidepressant drug with anti-TR and anti-calmodulin effects, has been used for the treatment of mice infected with *T. cruzi* (Rivarola et al., 2005). Previous works from our laboratory demonstrated that this drug was effective in preventing cardiac damage when used either in the acute or the chronic phase with no obvious pathology (Bazán et al., 2008; Rivarola et al., 2001;) not only upon infection with the Tulahuen strain, but also upon infection with an isolate obtained from an Argentinean endemic area (Rivarola et al., 2005).

In general, control programs have focus their budgets and strategies towards the elimination of the vector insects and the treatment of infected patients in the acute stage; patients with chronic Chagas disease however, also need anti-parasitic treatment, taking into consideration that the parasite has been frequently detected during this phase (Dias et al., 2002).

The goal of *T. cruzi* specific treatment is to eliminate the parasite from the infected individual, to decrease the probability of developing the characteristics of the chronic disease (cardiac or digestive) and to break the chain of infection (Sosa-Estani et al., 2009).

A major difficulty and controversy in accurate evaluation of therapeutic efficacy depends on reliable cure criteria when blood samples are assessed by serological and parasitological techniques after drug treatment. Changes in serology, parasite load and clinical evaluation have been used as criterion of cure in clinical trials of Chagas disease treatment (Guedes et al., 2011). After etiological treatment, cure criteria relies on serological; in patients initiating therapy at the chronic phase without evident pathology however, seroconversion usually occurs several years after treatment, requiring long-term follow-up to determine effectiveness (Viotti et al., 1994).

In Latin America, a recombinant ELISA was developed using a mixture of six recombinant proteins from epimastigotes and trypomastigotes of *T. cruzi*. These antigens were: SAPA (which is reactive during the acute stage of infection), 1, 2 and 30 (which detect antibodies primarily in chronic phase) and 13 and 36 (which are reactive for both acute and chronic stages) (Rassi and Luquetti, 2003; Vergara et al., 1991). Other authors carried out ELISAs with each of the individual recombinant antigens (1, 2, 13, 30, 36 and SAPA) separately using pre and post-treatment sera, monitoring antibody levels in 18 chronic chagasic patients for three years; recombinant antigen 13 prove to be the most sensitive to treatment, since 66.6% of the patients had an early decline in this antigen and seroconversion (Sánchez Negrette et al., 2008).

Quantitative polymerase chain reaction (qPCR) has the potential to become a novel parasitological tool for prompt evaluation of trypanocidal treatment. Conventional PCR is useful to verify infection when contradictory serological results appear, and to confirm treatment failure when seropositive results persist following treatment. Quantitative PCR is considered more sensitive for parasite detection than conventional methods and therefore may be a better tool to assess treatment effectiveness (Duffy et al., 2009).

In this work we evaluated the effectiveness of CLO treatment upon *T. cruzi*-infected mice (Tulahuen strain) in the chronic phase of the experimental infection using qPCR and recombinant ELISA.

2. Materials and methods

2.1. Animals and experimental design

Sixty female and male Swiss albino mice weighing 30 ± 1 g were intraperitoneally inoculated with 50 trypomastigote forms of *T. cruzi* (Tulahuen strain). The number of parasites/mL of blood was determined in each group using a Neubauer hemocytometer. Mice were divided into the groups described in Fig. 1. Electrocardiographic (ECG) studies were performed on day 90 post infection (p.i) to determine the chronic phase of the disease.

2.2. Treatment

CLO (Sigma Chemical, St. Louis, MI, USA) treatment consisted of 5 mg/kg/day during 60 days by intraperitoneal injection, beginning on

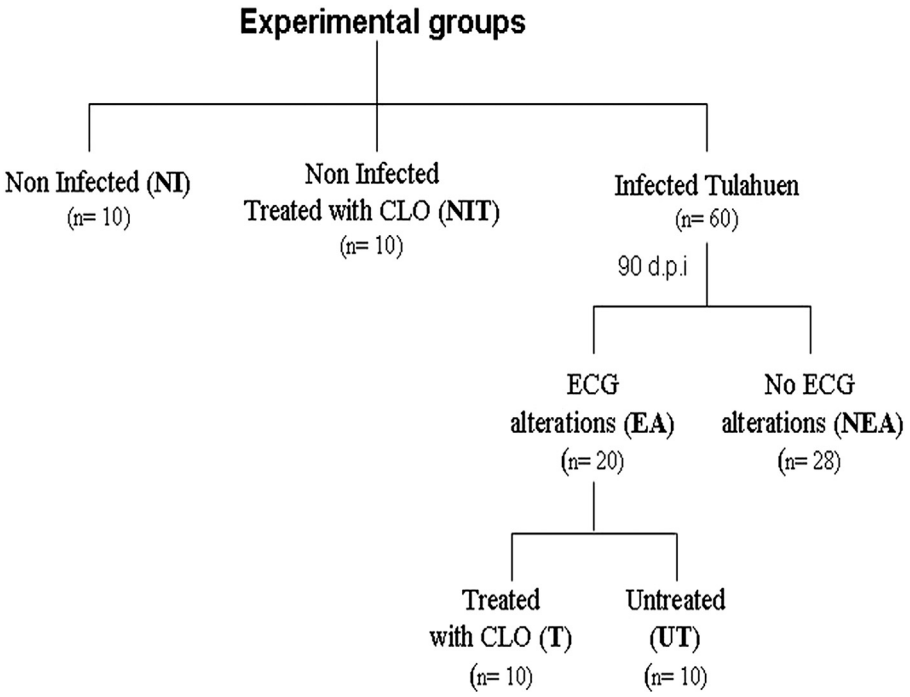

Fig. 1. Schematic representation of the experimental groups studied in the present work: non-infected mice, mice infected with *T. cruzi* (Tulahuen strain) left untreated or treated with CLO (5 mg/kg/day).

day 90 p.i when the mice presented ECG alterations compatible with the chronic phase of the disease.

The experimental procedures were carried out in accordance with the Institutional Committee for the Care and Use of Laboratory Animals from the Faculty of Medicine, National University of Córdoba, Argentina.

The evolution of experimental Chagas disease and the treatment efficacy were studied through survival, electrocardiography, serology, PCR and qPCR on days 180 and 270 p.i.

2.3. Survival

Survival was monitored daily.

2.4. ECG studies

Electrocardiograms were obtained with a Fukuda Denshi ECG unit (FD 16 Model) under Ketamine ClH (Ketalar®, Parke Davis, Warner Lambert Co, USA) anesthesia (10 mg/kg), before the infection and on days 90 and 360 p.i. The electrocardiographic tracings were obtained with six standard leads (dipolar leads DI, DII, DIII and unipolar leads aVR, aVL, aVF), recording at 50 mm/s with amplitude set to give 1 mV/10 mm.

In order to follow the progress of the cardiopathy, ECG parameters evaluated were: heart rate (beats per minute), modifications in atrioventricular conduction (prolonged PR segment) and ventricular conduction (prolonged QT interval) in milliseconds.

The mice were considered altered when one or more of the following were present: increased or decreased heart rate (control values for comparison: 555.54 ± 13.99), prolonged PR segment (control: 0.0247 ± 0.0009) and prolonged QT interval (control: 0.0298 ± 0.0008). The records were analyzed manually.

2.5. Serological assay

First, the control used for ELISA was performed using a mixture of six recombinant proteins from epimastigote and trypomastigote forms of *T. cruzi* (SAPA, 1, 2, 13, 30 and 36). Specifications from the Wiener recombinant ELISA Kit 3.0 were followed.

Second, ELISAs with each of the individual recombinant antigens separately were performed using pre and post treatment sera. The antigens in both assays were used to monitor the antibody variations at two times points after the treatment (180 and 270 p.i), with the sera diluted 1:20 according to the specifications of the recombinant (version 3) Wiener Chagatest-ELISA (Gariglio et al., 2000).

Elisa with the recombinant antigen mixture and each of the individual recombinant antigens separately were quantitated by optical density (OD) measurements.

2.5.1. ELISA procedure

The antigens were coated with carbonate buffer (100 mM, pH 9.6) for 18 h at 4 °C and the optimal concentrations in 96-well microwell plates. Following dilution, the serum samples were added to the microwell plates and the plates were incubated at 37 °C for 30 min. After five washes (NaCl [1.4 M] in phosphate buffer [concentrated to 100 mM]) to remove the unbound immunoglobulin (Ig), the samples were incubated at 37 °C for 30 min with a 1:40,000-diluted anti-human IgG conjugate labeled with peroxidase. Unbound conjugate was removed by another wash step. The microwell plates were then revealed with hydrogen peroxide (60 mM in citrate buffer [50 mM], pH 3.2) and tetramethybenzidine (0.01 mM in chlorhydric acid [0.1 N]). The solution develops a blue color with intensity proportional to the concentration and affinity of the anti-*T. cruzi* antibodies in the sample. The reaction was stopped by the addition of sulfuric acid (2 N), and the color of the solution turned to yellow. Within 20 min the strips were read at a wavelength of 450 nm. A sample is considered nonreactive if the absorbance is lower than the cutoff value. On the other hand, a sample is considered reactive if the absorbance is equal to or greater than the cutoff value: 0.371. (Sáenz-Alquézar et al., 2004).

2.6. DNA extraction from blood samples

Blood samples from each infected mice (1 mL) were mixed with an equal volume of guanidine hydrochloride 6 M/EDTA 0.2 M (Avila et al., 1991); DNA was extracted from 200 μL of the blood/guanidine mixture, using conventional phenol: chlorophorm:isoamylic techniques (Lachaud et al., 2001), precipitated with ethanol and re-suspended in sterile nuclease free water. The samples were conserved at −20 °C (Wincker et al., 1994) until used for the amplification of the parasite DNA by qPCR.

2.7. Parasite detection

2.7.1. PCR conventional

The detection of parasites in each sample was determined by amplification of a 188 base pairs (bp) nuclear fragment of the parasite DNA using two specific primers: **TCZ-1** (5′ CGA GCT CTT GCC CAC ACG GGT GCT 3′) and **TCZ-2** (5′ CCT CCA AGC AGC GGA TAG TTC AGG 3′) (Virreira et al., 2003)

PCR amplifications were performed in a final volume of 25 μL, containing 10 mM of PCR buffer, 25 pmol of each primer, 200 μM of dNTPs and 0.5 Units of platinum Taq DNA polymerase (Invitrogen). Finally, 5 μL of isolated DNA sample were added. The amplification was performed on DNA Thermal Cycler (Ivema-T-18). PCR conditions used were: initial heating step of 95 °C for 4 min, followed by 40 cycles of 95 °C for 30 s (denaturation), 60 °C for 30 s (annealing) and 72 °C for 30 s (extension); a final elongation step was allowed at 72 °C for 5 min.

5 μL of the PCR products were subjected to electrophoretic fractionation on 1·6% agarose gels and then stained with ethidium bromide. The samples were considered positive when they presented a 200 bp band.

The quality of the DNA samples was verified by the amplification of a 289 bp constitutive gene from the host (β-actin), using the corresponding primers: **β-act-F** (5′ CGG AAC CGC TCA TTG CC 3′) and **β-act-R** (5′ AAC CAC ACT GTG CCC ATC TA 3′).

2.7.2. qPCR

Was performed using a Stratagene Mx 3005 thermocycler (Angilent Technologies Inc., Santa Clara, USA). Each amplification tube containing 10 μL of mix master SYBR Green qRT-PCR 2× (Stratagene), 1 μL of each primer (10 pmol, TCZ-1 and TCZ-2) and 10 ngr of sample. PCR conditions used were: initial heating step of 95 °C for 15 min, followed by 40 cycles of 95 °C for 30 s (denaturation), 60 °C for 1 min (annealing) and 72 °C for 30 s (extension), the final step consisted of: 95 °C for 15 s, 60 °C for 1 min and 95 °C for 30 s. The software to evaluate data quality and amplification reactions was the program MxPro™ Q-PCR version 3.20 (Stratagene Agilent Technologies, Santa Clara, CA, USA).

2.7.3. T. cruzi standard calibration curve

The standard curve was generated from nine serial dilutions in water (1:10) of DNA extracted from culture containing 10^8 epimastigotes of *T. cruzi* (Y strain). qPCR reactions performed in triplicate showed a linear curve from 10^8 to 0.1 parasite equivalent/mL.

2.8. Statistical analysis

Data were analyzed and compared using: ANOVA and multiple comparisons by Fisher test, and Chi square test. Differences were considered significant when $P < 0.05$. The software used was InfoStat (Faculty of Agricultural Sciences. National University of Córdoba).

3. Results

3.1. Parasitemias and survival

Infected animals presented a peak in parasitemia levels on day 14 p.i (108.81 ± 31.14 parasites per milliliter of blood), which became negative from day 49 p.i until the end of the experiments.

No differences were found between the groups infected when comparing the survival rates observed by day 270 p.i (group UT: 63% and group T: 67%) (data not shown).

3.2. Electrocardiography

Table 1 shows percentages of ECG alterations found in UT or T mice. As can be observed, in the UT mice, the percentage of animals with intra-ventricular block decreased while the arrhythmias increased with the evolution of the infection (270 days p.i). In T mice, on the other hand, the percentage of mice with either intra-ventricular block or arrhythmias decreased with the evolution of infection ($P < 0.05$), based on the surviving animals at that moment of the infection.

3.3. Serology

Serologic follow-up included data from 90 to 270 days p.i. Fig. 2, shows the variations in ODs by sera from all groups infected were tested with the recombinant antigen mixture. T mice group, no decrease in the absorbance values was observed at any time post treatment.

For some antigens presented absorbance levels after treatment less than those for the pretreatment samples. Antigen 13 displayed the best ability to reveal this effect (Fig. 3).

3.4. PCR conventional

Fig. 4 shows the amplification products from blood samples obtained from mice infected with *T. cruzi* NEA and EA, UT and T. As can be observed, the PCR was positive for all groups throughout infection. At 270 days p.i, T group presented higher percentage of positive PCR that UT group.

3.5. qPCR

For quantify parasitemia by DNA amplification, a standard curve was plotted based on Ct values obtained from samples containing 10^8–0.1 epimastigotes/mL against the log of the estimated DNA copy number in the sample. The coefficient of determination (R^2) was 0.9841. The slope of the standard curves was −3.1 (Fig. 5).

All animals from the infected groups exhibited amplification products and an increase in the number of parasites throughout the infection. However, the amount of parasites detected on days 90 and 180 p.i is below the detection limit of the qPCR standard curve. Despite the fact that the amount of parasites increased with the evolution of the infection (270 days p.i.), the values from the T mice were smaller than the ones from the UT group (Fig. 6).

Table 1
Percentages of electrocardiographic alterations in mice infected with *T. cruzi* Tulahuen strain and left untreated or treated with clomipramine 5 mg/kg/day.

Groups	Days post infection	AVB	IVB	Arrhythmias
Untreated (n = 10)	90	NF[a]	67[a]	36[a]
	180	10[b]	NF[b]	33[a]
	270	NF[a]	NF[b]	50[b]
Treated (n = 10)	90	NF[a]	33[c]	64[b]
	180	NF[a]	NF[b]	57[b]
	270	NF[a]	NF[b]	20[c]

AVB: atrio-ventricular block (prolonged PR segment); IVB: intra-ventricular block (prolonged QT interval); N.F.: not found. Within each column, values with different superscript symbols differ significantly: $P < 0.05$.

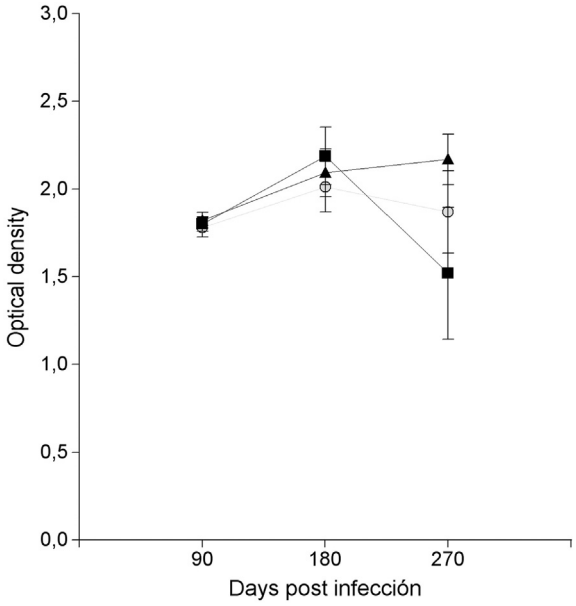

Fig. 2. Evolution of antibodies levels (optical density) against total recombinant antigen mixture in sera from all infected groups: NEA (●); EA, T (▲) and EA, UT (■).

4. Discussion

In this paper we evaluated, through unconventional immunological and molecular methods, the effect of clomipramine for the treatment of the chronic phase of Chagas disease in mice infected with the Tulahuen strain.

PCR has been proposed as an alternative tool for the detection and quantification of *T. cruzi* in numerous researches as it is more sensitive than traditional parasitological methods (microscopic examination of fresh blood samples, xenodiagnoses and hemoculture) (Caldas et al., 2012; Moser et al., 1989). Considering that the present work was

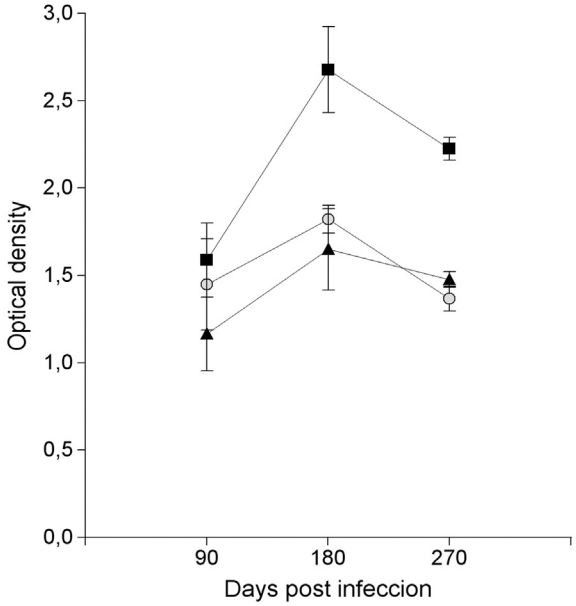

Fig. 3. Evolution of antibodies levels (optical density) against antigen 13 in sera from all infected groups: NEA (●); EA, T (▲) and EA, UT (■).

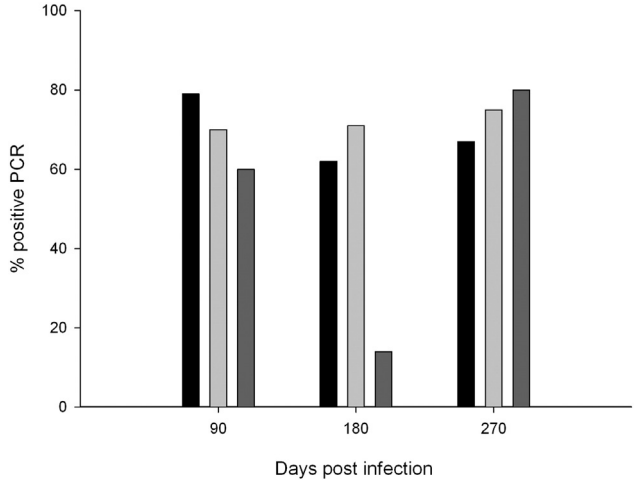

Fig. 4. Percentage of mice with positive PCR results for the presence of *T. cruzi* (Tulahuen strain). NEA (■); EA, UT (■) and EA, T (■) on days 90, 180 and 270 p.i.

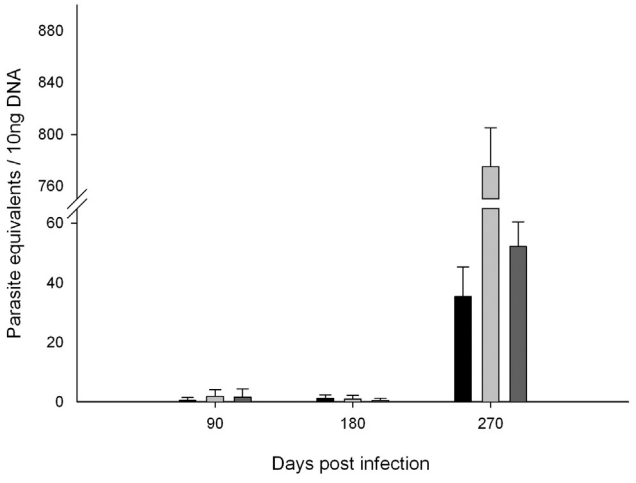

Fig. 6. Estimated parasite equivalents/10 ng DNA detected by qPCR in blood samples from mice infected with *T. cruzi* (Tulahuen strain). NEA (■); EA, UT (■) and EA, T (■) on days 90, 180 and 270 p.i.

performed in the experimental chronic stage, such a sensitive technique was necessary for the detection and quantification of the scarce circulating parasites, non-detectable by traditional techniques. Both conventional and quantitative PCR results confirmed the presence of *T. cruzi* in the chronic phase of the infection, even almost a year after the mice were infected with *T. cruzi* (Tulahuen strain).

Quantitative PCR assays to determine the load of parasites in the bloodstream and follow its evolution during or after the treatment however, are more useful in this case than qualitative PCR, as an indicator of the result of prolonged treatment schedules (Urbina, 2001). This is required in the management of patients with certain viral infections. Moreover, the parasitic load can be a useful epidemiological tool to estimate infectivity of patients regarding risk of transmission (Schijman et al., 2003). In our results we can observe that qualitative PCR results remain positive for about 70% of animals (either treated or not treated with clomipramine) by day 270 p.i; quantitative PCR results however show that untreated mice presented parasitic load values higher than the treated animals. These results represent an interesting finding: although most conventional methods account for the persistence of *T. cruzi* after the treatment, qPCR shows the real and current levels of circulating parasites, which should be considered as the main indicator of chemotherapeutic effectiveness in chronic Chagas disease.

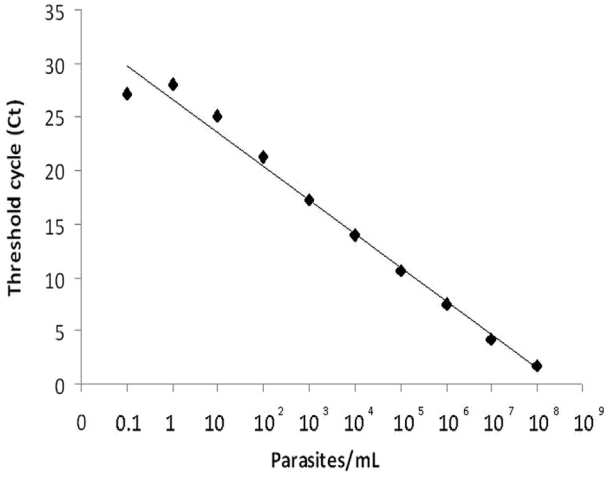

Fig. 5. Standard curve generated from Ct values obtained from samples containing 10^8–0.1 epimastigotes/mL against the log of the estimated DNA copy number in the sample. Slope = −3.1. R square: 0.9841.

The characterization of the effectiveness of specific antiparasitic treatment in adult patients with chronic *T. cruzi* infection is not currently defined. Conventional serology remains reactive in the vast majority of patients, even many years after the end of the trypanocide treatment, and traditional parasitological methods (xenodiagnosis, blood culture) have low sensitivity at this stage of the infection. Additionally, Chagas disease has a gradually progressive clinical evolution and a high percentage of infected patients remain in the indeterminate period (without electrocardiographic and/or radiological alterations) even without treatment (Fabbro et al., 2007).

Present serology results were similar to those reported by other researchers. On the one hand, total antibodies remained elevated throughout the experiment, in a similar way as with the treatment in the chronic phase in humans. For some researchers, seroconversion is an indication of cure; however, this only happens in 70–75% of acute cases and in 100% of congenital acute cases. In chronic cases, seroconversion often happens 20 to 30 years after the end of the therapy (Apt and Zulantay, 2011). This probably means that chronic cases may have parasitological cure but positive conventional serology. In this paper, in order to measure the levels of anti-*T. cruzi* antibodies, we used a mixture of six recombinant proteins from epimastigotes and trypomastigotes and each of the individual recombinant antigens (1, 2, 13, 30, 36 and SAPA) separately. The results indicated an increased sensitivity of the response to some of these antigens to treatment. Particularly, antibodies against antigen 13 showed similar levels to those found in mice without ECG alterations in mice infected with the Tulahuen strain and treated by day 90 p.i. In untreated animals however, the levels of antibodies against this antigen were significantly augmented. Similar results were found by Sanchez Negrette et al., who showed that levels of antibodies against antigen 13 were higher in pre-treatment than in post-treatment samples: 6 out of 9 patients that were originally positive for this antigen became negative after the treatment (Sánchez Negrette et al., 2008).

The relative importance of the elimination or reduction of the parasite in the infected organism, and the consequent decrease in antibody titers, is associated with the pending controversy about the pathogenesis of Chagas disease.

No definite data proves beyond doubt that the disease results from an autoimmune process (Benoist and Mathis, 2001). In recent years, increasing importance has been assigned to the presence and persistence of the parasite for the development of the disease.

Some researchers postulate the presence of a "trigger" that initiates the pathologic process. This "trigger" activates certain specific T cells (specific for autoantigens and / or cross-reacting antigens of the

parasite). When these T cells are activated, they secrete inflammatory cytokines that would be responsible for the heart damage. The released autoantigens (for example, myosin) are recognized by another group of autoreactive T cells and autoantibodies, further damaging the heart tissue. This heart damage would simultaneously favor the induction of co-stimulatory molecules, necessary for the specific activation of autoreactive T cells (Gironès and Fresno, 2003).

Regardless of the controversy over the pathogenic mechanisms of the disease, patients get a real benefit with the reduction of the parasite load (Tarleton, 2003). Electrocardiography is one of the most sensitive diagnostic methods for chronic chagasic myocarditis, especially in patients with positive serology and epidemiological history compatible with Chagas disease. Early cardiac manifestations of Chagas disease are detected by an electrocardiogram, since conduction abnormalities precede myocyte and microcirculation injuries (Prata, 1990). The detection of intraventricular conduction disorders, such as right bundle branch block, is a highly sensitive diagnostic marker. If they are combined with the presence of bradycardia, nonspecific repolarization alterations and ventricular arrhythmia, the diagnosis of chronic chagasic cardiomyopathy is virtually certain, especially if it is a young person (Elizari, 1999).

In our experimental model, we observed that the percentage of mice with electrocardiographic alterations increased with the evolution of the infection. Studies with chagasic untreated patients showed that parasitemia is not always associated with the evolution or the clinical condition of the patient (Castro et al., 2005); others however have established a close correlation between the persistence of *T. cruzi* and the disease progression (Zulantay et al., 2005).

Mice infected and treated on day 90 p.i, presented a progressive decrease in ECG abnormalities: by day 270 p.i only 20% of these mice had ECG abnormalities (arrhythmias) while 50% of the untreated animals were altered.

Our results agree with those obtained by Viotti et al., who observed a marked reduction in the development of electrocardiographic changes and a lower frequency of deteriorated clinical condition despite the persistence of positive parasitological tests, in individuals treated with benznidazole and evaluated during eight years after the treatment (Viotti et al., 1994). Similar results have been reported in chronic chagasic patients treated with itraconazole and allopurinol (Apt et al., 2003).

Some researchers argue that regression is possible if electrocardiographic abnormalities are detected early. Conversions of minor alterations, such as prolonged QT interval and 1st and 2nd grade atrioventricular blocks, can be observed even without etiologic treatment. Chemotherapeutic intervention may be responsible for the reversion of more severe alterations, such as fascicular and bifascicular blocks, thus modifying the natural history of the disease (Arribada et al., 1993). Even though the BENEFIT study (Morillo et al., 2015) demonstrated that trypanocidal therapy with benznidazole in patients with established chagasic cardiomyopathy significantly reduced serum parasite detection, the treatment did not significantly reduce cardiac clinical deterioration through 5 years of follow-up.

5. Conclusions

In summary, this study shows that qPCR and the use of recombinant antigens are more sensitive to evaluate the effectiveness of the treatment and expands the available data on clomipramine. Further studies are necessary to know its role as a new drug in chronic phase of Chagas disease in monotherapy or multidrug combination.

Acknowledgements

This work was supported by grants from the Secretaría de Ciencia Técnica (SECyT (203/14)) from Universidad Nacional de Córdoba and from Universidad Nacional de La Rioja, FONCYT (PICT-2007-01620), Ministerio de Ciencia y Tecnología de la Provincia de Córdoba and CONICET.

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