Infection, Genetics and Evolution 12 (2012) 1128–1135

Contents lists available at SciVerse ScienceDirect

Infection, Genetics and Evolution

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

Genetic modulation in Be-78 and Y *Trypanosoma cruzi* strains after long-term infection in Beagle dogs revealed by molecular markers

Vanja Maria Veloso [c,e,*], Paulo Marcos da Matta Guedes [g], Marta de Lana [a,d], Helen Rodrigues Martins [f], Cláudia Martins Carneiro [a,d], Antônia Cláudia Jácome da Câmara [g], Daniella Alchaar D'Ávila [e], Ivo Santana Caldas [a], Lúcia Maria da Cunha Galvão [e], Egler Chiari [e], Maria Terezinha Bahia [a,b]

[a] *Núcleo de Pesquisas em Ciências Biológicas (NUPEB) e Instituto de Ciências Exatas e Biológicas, Universidade Federal de Ouro Preto (UFOP), Campus Universitário Morro do Cruzeiro, s/n, CEP 35400-000 Ouro Preto, MG, Brazil*

[b] *Departamento de Ciências Biológicas, Instituto de Ciências Exatas e Biológicas, Universidade Federal de Ouro Preto (UFOP), Campus Universitário Morro do Cruzeiro, s/n, CEP 35400-000 Ouro Preto, MG, Brazil*

[c] *Departamento de Farmácia, Escola de Farmácia, Universidade Federal de Ouro Preto, Rua Costa Sena 171, Centro, CEP 35400-000 Ouro Preto, MG, Brazil*

[d] *Departamento de Análises Clínicas, Escola de Farmácia, Universidade Federal de Ouro Preto, Rua Costa Sena 171, Centro, CEP 35400-000 Ouro Preto, MG, Brazil*

[e] *Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 1667, Campus Universitário, CP 486, CEP 31270-901 Belo Horizonte, MG, Brazil*

[f] *Departamento de Farmácia, Faculdade de Ciências Biológicas e da Saúde, Universidade Federal dos Vales do Jequitinhonha e Mucuri, Rodovia MGT 367 – Km 583, número 5000, Bairro Alto Jacuba, CEP 39100-000 Diamantina, MG, Brazil*

[g] *Departamento de Análises Clínicas e Toxicologicas, Centro de Ciências da Saúde, Universidade Federal do Rio Grande do Norte, Rua General Gustavo Cordeiro de Farias, Bairro de Petrópolis, s/n, CEP 59010-180 Natal, RN, Brazil*

ARTICLE INFO

Article history:
Received 17 September 2011
Received in revised form 13 March 2012
Accepted 20 March 2012
Available online 28 March 2012

Keywords:
Trypanosoma cruzi
Chagas disease
Beagle dogs
Genetic modulation

ABSTRACT

The genetic profile of *Trypanosoma cruzi* was evaluated in parasite populations isolated from Beagle dogs experimentally infected with Be-78 and Y strains that present distinct biological and genetic characteristics. Molecular characterization of the isolates obtained 30 days and 2 years after infection was carried out. For typing MLEE, sequence polymorphisms of the mitochondrial cytochrome oxidase subunit II gene (COII) and RAPD profiles were used. The profiles of MLEE were the same for the parental Be-78 strains as their respective isolates. However, changes of MLEE profile were observed in two *T. cruzi* isolates from dogs inoculated with Y strain. Changes in the mitochondrial DNA (COII) and RAPD profiles of the Y strain were also observed. The dendogram constructed by UPGMA with RAPD results indicated two major branches. Global data show that the genetic modulation in polyclonal strains during the long-term infection occurred and was strain-dependent. This study still suggests that each host (here each dog) harbors a determinate *T. cruzi* population that may change or be modulated throughout long-term infection. This might to hinder the observation of correlation between the genetics of *T. cruzi* and their biological properties and behavior in different host species due to the complexity of the parasite-host interaction in which probably the genetic background of both should be considered.

Published by Elsevier B.V.

1. Introduction

Trypanosoma cruzi, the etiological agent of Chagas disease, is heterogeneous at both the phenotypic and genetic levels. A new consensus established the existence of six genetic groups in this parasite, renamed TcI to TcVI (Zingales et al., 2009). Attempts to correlate biological and genetic characteristics of *T. cruzi* isolates with the variability of Chagas disease infection have been reported (Gonzalez et al., 1995; Montamat et al., 1996). Studies of natural populations of this parasite by isoenzymes analysis and DNA have suggested a clonal structure and evolution for this species (Tibayrenc et al., 1986; Tibayrenc and Ayala, 1987). This model predicts stability in time and space and consequently a linkage between biological diversity and *T. cruzi* genetic (Tibayrenc and Ayala, 1988) successfully demonstrated in several experimental studies (Revollo et al., 1998; Toledo et al., 2002, 2003). Moreover several studies indicate that natural *T. cruzi* populations are constituted of different natural clones (Deane et al., 1984; Tibayrenc et al., 1986; Tibayrenc and Ayala, 1988; Oliveira et al., 1998, 1999) with different virulence patterns in competitive relationships.

* Corresponding author at: Departamento de Farmácia, Escola de Farmácia, Universidade Federal de Ouro Preto, Rua Costa Sena, 171, Centro, CEP 35400-000 Ouro Preto, MG, Brazil. Tel.: +55 31 3559 1036; fax: +55 31 35591628.

E-mail address: vanjaveloso@ef.ufop.br (V.M. Veloso).

1567-1348/$ - see front matter Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.meegid.2012.03.019

The interaction between different clones of *T. cruzi* in the same host may result in parasites with new properties generally adapted to develop in different host specie (Martins et al., 2006, 2007, 2008). Thus, the particular behavior of one isolate may be the result of the interaction with the other clones present in the same host (Martins et al., 2006, 2007; Pinto et al., 2000). The coexistence of multiple parasite subpopulations in the vertebrate and invertebrate hosts has also been reported (Solari et al., 1998; Brenière et al., 1995; Torres et al., 2003). According to Andrade (1999), strains of *T. cruzi* are a complex of multiclonal populations that differ in their genetic and biological characteristics and behavior in the vertebrate host. Moreover, these populations may undergo changes as a consequence of the methods used for isolation and maintenance in the laboratory, which can promote the expansion or reduction of certain clones present in the original population (Macedo and Pena, 1998). However, it is also important to consider that the genetics of the vertebrate host may influence the selection process (Andrade et al., 2002).

In this context, our group has evaluated broadly different strains and clones of *T. cruzi* and their interaction with human and different animal models. In general, data obtained have shown that long-term *T. cruzi* infection in the vertebrate host results in alterations of the biological and molecular characteristics of this parasite. Lana and Chiari (1986) demonstrated in the murine model the lower virulence of the Be-78 strain isolated from the Berenice patient in comparison to the Be-62 strain isolated from the same patient 16 years earlier. Later, Veloso et al. (2001) demonstrated that *T. cruzi* stocks obtained from chronic chagasic dogs infected with the Be-78 strain for 2–4 years of infection showed variations in benznidazole susceptibility in the murine model compared with the parental strain. All animals infected with Be-78 strain were cured after treatment. However, different cure rates (50% and 70%) were observed for the two stocks isolated from dogs after 7 and 2 years of infection, respectively. After 25 successive blood passages of these isolates in mice, Veloso et al. (2005) repeated their biological and molecular characterization and confirmed the heterogeneity of these isolates when compared to the parental strain. They suggested that the biological diversity observed among the isolates could be attributed to the capacity of each vertebrate-host to select clones of parasite better adapted to the new environment, since the parental Be-78 strain is polyclonal, including different stocks of distinct genetic profiles in its composition. Interestingly, these authors still verified that two of the four *T. cruzi* isolates showed reversibility to the parental strain genetic profile after 25 successive passages in mice.

Other studies using Be-78 isolates after long-term infection in dogs were also performed concerning their chemotherapeutic response. Thus, Dos Santos et al. (2008) showed induction and stability of benznidazole resistance for Be-78 strain after continuous *in vivo* drug pressure. These authors verified yet reversible changes in drug resistance phenotype acquired for some *T. cruzi* stocks of this strain when kept in vitro culture for a long time. In addition, Caldas et al. (2008) corroborated previous results obtained by Veloso et al. (2001, 2005) and demonstrated that the maintenance of these same stocks in mice through successive blood passages led to a decrease or stability of the drug resistance pattern and increase of the parasite virulence.

The alterations in biological behavior and molecular profile of the Be-78 strain induced by long-term infection in dog are probably related to the genetic plasticity of the parasite in the vertebrate host, but also influenced by the interaction with this host (Guedes et al., 2002). Although the *T. cruzi* isolates from dogs have been obtained of different outbreed animals of the same experimental group, infected with the same inoculum, they developed distinct clinical forms of the disease (Lana et al., 1992). These results suggest the need to use a genetically homogeneous experimental model to reduce or avoid *T. cruzi* population genetic changes.

Considering this aspect, our study was focused on the molecular characterization of *T. cruzi* isolates obtained from inbreed Beagle dogs throughout long-term infection when inoculated with two distinct parasite strains obtained from the same animal after different periods of the infection.

2. Materials and methods

2.1. *T. cruzi* strains

In this study the Berenice-78 (Be-78) *T. cruzi* strain (*T. cruzi* II) isolated by xenodiagnosis in 1978 (Lana and Chiari, 1986) from the first human case of Chagas disease, and the Y strain (*T. cruzi* II), isolated from an acute human case (Silva and Nussenzweig, 1953), were used. Despite both strains belonged to TcII group, they present very distinct biological characteristics, which result in distinct course of the infection in the vertebrate host. In addition, TcII represents an important *T. cruzi* group related to human infection, particularly in South America.

2.2. Experimental animals

Four month-old Beagle dogs of both sexes from the kennel of the Universidade Federal de Ouro Preto (UFOP), Minas Gerais (MG), Brazil were used. Animals were fed with commercial chow and water *ad libitum*. They were treated with anti-helmintic and vaccinated against the more common infectious diseases before the study.

Animals were intraperitoneally inoculated with $2.0–4.0 \times 10^3$ blood trypomastigotes (BT)/kg of body weight of Be-78 or Y strains obtained from mice. To verify the influence of the time of *T. cruzi* infection in vertebrate host on population dynamic of these strains, two distinct inoculations were employed. The first experiments were identified as Be-78/1 or Y/1. The second started after approximately 18 months of parasite maintenance in mice and identified as Be-78/2 or Y/2. In addition, Be-78/1, Be-78/2, Y/1 and Y/2 correspond to parental populations used for dogs' inoculation in two different moments and compared with the isolates obtained from animals after a long-term infection.

This project was approved by the ethics committee for animal experimentation of UFOP with protocol number 2006/69. All procedures and experimental protocols were performed according to the COBEA (Brazilian College of Animal Experimentation) instructions for the use of animals in experimental conditions.

2.3. Parasites isolation from dogs

Parasites were isolated by blood cultures using 10 ml of blood samples according to Chiari et al. (1989), with some modifications. Blood samples were collected 30 days and 24 months after inoculation, i.e. during the acute and chronic phases, respectively (Table 1). Immediately after collection, blood was centrifuged at 1000*g* 4 °C for 10 min to remove the plasma. The packed blood cells were washed by centrifugation at 4 °C in 5 ml of liver infusion tryptose (LIT) medium, resuspended in 5 ml of LIT medium, and distributed into two plastic tubes. Cultures were maintained at 28 °C, homogenized weekly, and examined monthly for 120 days. The positive blood cultures were maintained in exponential growth. Pellet of parasites were stored at −70 °C.

2.4. Multiloci Enzyme Electrophoresis (MLEE) analyses

For isoenzyme analyses, a volume of approximately 80 ml of parasites in exponential growth phase in LIT medium was used.

Table 1
Origin of the *Trypanosoma cruzi* isolates studied.

Dog	Parental strain	Experiment	Time of infection acute phase (day)	Time of infection chronic phase (months)	*T. cruzi* isolates
1	Be-78	Be-78/1	30	18	Bg1
2			30	24	Bg2
3			30	24	Bg3
4			30	24	Bg4
6		Be-78/2	30	21	Bg6
7			30	24	Bg7
8			30	24	Bg8
9			30	24	Bg9
10	Y	Y/1	–	12	Bg10
15		Y/2	30	12 and 21	Bg15
16			30	–	Bg16
17			30	–	Bg17
18			30	–	Bg18

Flagellates were washed twice with phosphate buffer solution (PBS) by centrifugation at 1000*g*, 4 °C for 20 min and the pellet stored at −70 °C until use. Enzyme extracts were cryopreserved in liquid nitrogen.

MLEE analysis was carried out on cellulose acetate plates (Helena) according to Ben Abderrazak et al. (1993). Four enzymatic systems used were: glucose-6-phosphate dehydrogenase (G6PD, E.C.1.1.1.49.), glucose-phosphate isomerase (GPI, E.C.5.3.1.9.), isocitrate dehydrogenase (IDH, E.C.1.1.1.42.), phosphoglucomutase (PGM, E.C.2.7.5.1.). The MLEE profile of the isolates were obtained from dogs after long-term infection and compared with the parental strains and standard references of *T. cruzi* groups (Zingales et al., 2009).

2.5. Mitochondrial genetic typing

Based on the restriction map of Citochromo Oxidase subunit II (COII) sequences, the *AluI* restriction endonuclease was chosen for RFLP analyses of the mitochondrial COII gene. For amplification of the mitochondrial COII gene the primers TcMit21 (5′-TTGTAATAGGAGTCATGTTT-3′) and TcMit10 (5′-CCATATATTGTTGCATTATT-3′) were used. Total DNA (3 ng) were used in each PCR reaction in the following condition: 40 s denaturation at 94 °C, primer annealing for two min at 48 °C, and primer extension for two min at 72 °C, in a total of 40 cycles. After PCR amplification the amplicons were submitted to enzyme digestion for 16 h according to instructions provided by the manufacturer (Promega, Madison, Wisconsin, United States). Digested products were analyzed on polyacrylamide gel electrophoresis silver stained (Santos et al., 1993). Samples with restriction fragments of 264, 81 and 30 bp were classified as *T. cruzi* I (haplotype mitoncondrial A); fragments with 294 and 81 bp were classified as *T. cruzi* III or hybrids (haplotype mitoncondrial B); fragments with 212 and 81 bp were classified as *T. cruzi* II (haplotype mitoncondrial C) (Freitas et al., 2006).

2.6. Random amplified polymorphic DNA analysis

Parasite DNA extraction, amplification and electrophoresis were performed as described previously (Steindel et al., 1993). Four random primers, γgt11F (5′-GACTCCTGGAGCCCG-3′), γgt11R (5′-TTGACACCAGACCAACTGGTAATG-3′), 3302 (5′-CTGATGCTAC-3′) and M13F (5′-GTTTTCCCAGTCACGAC-3′) were used.

The multiband profiles of *T. cruzi* populations resulting from the RAPD analyses were scored and the data recorded with DNA-POP software (Pena and Nunes, 1990), which calculates the proportion of shared bands among samples and the standard deviations. RAPD analysis was limited to PCR-amplified products between 0.2 and 3.0 Kb. The relationships between *T. cruzi* strains were estimated by dendograms representative of the RAPD data. They were constructed based on a matrix of genetic distance that was then for unweighted pair group method analysis (UPGMA) using the Treecon software program for Windows (Van De Peer and De Wachter, 1994). The bootstrap option was used to run 1000 replicates to obtain confidence estimates for the groupings and taxonomic relatedness (Felsenstein, 1985).

3. Results

3.1. MLEE profiles

Analysis of the electrophoretic profiles of isoenzymes observed in the parental strains and isolates from Be-78/1 and Be-78/2 or Y/1 and Y/2 experiments presented similar results in all enzymatic systems correspondent to TcII (Zingales et al., 2009) [equivalent to Zimodeme A described by Romanha (1982) and Z2 of Miles et al. (1977)]. After inoculation, *T. cruzi* isolates obtained of dogs in the acute and chronic phases of infection inoculated with Be-78 strain presented the same isoenzimatic profile of the parental strain.

Regarding the animals infected with the Be-78 strain, the success of isolating the parasites of animals infected with Y strain was different in the two experiments, both in acute and chronic phases of the infection. In Y/1 experiment no parasites were obtained in the acute phase and only one isolate was obtained after 12 months of infection (chronic phase). However, in the Y/2 experiment parasites were obtained from all dogs infected in the acute phase, two isolates were obtained from one dog during chronic phase in two different occasions (12th and 21st months after inoculation).

Isoenzymatic characterization showed some differences between the isolates from dogs in relation to the parental Y strain. The isolate from dog Bg10 obtained of the Y/1 experiment in the 12th month of infection presented a variation in GPI profile equivalent to TcIV (Zingales et al., 2009) for this enzymatic system and similar to ZC profile (Romanha, 1982) or Z3 (Miles et al., 1977). Considering the other enzymes the profile detected for the same isolate was of TcVI (Zingales et al., 2009) or ZB (Romanha, 1982). The isolate obtained in the 21st month of infection presented a profile equivalent to TcVI (Zingales et al., 2009), corresponding to ZB (Romanha, 1982) for all isoenzymatic systems used, which was different from the parental strain.

3.2. DNA mit COII profiles

Fig. 1 shows the amplified products of Citochromo Oxidase subunit II (COII) gene after digestion by the *AluI* enzyme of DNA from

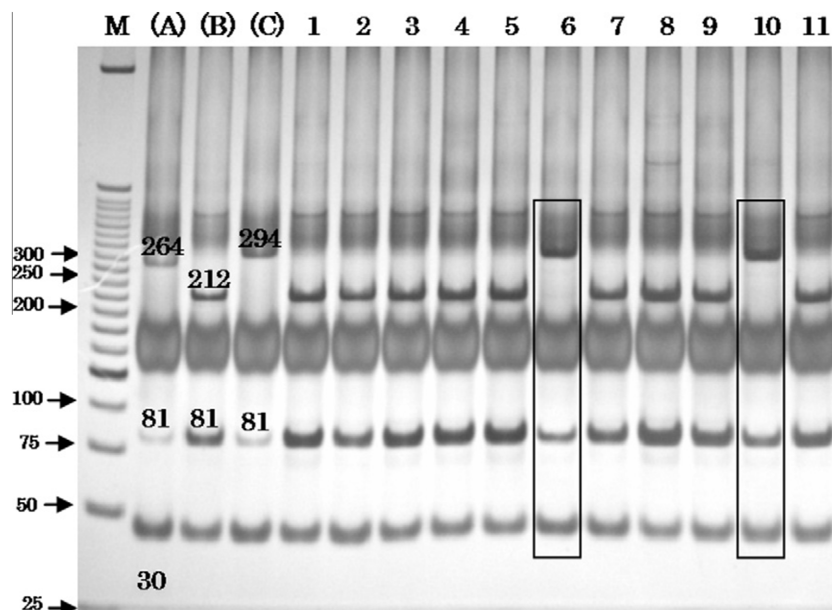

Fig. 1. Representative polyacrylamide gel of RFLP profiles of mitochondrial cytochrome oxidase II gene of different *Trypanosoma cruzi* isolates obtained from Beagle dogs (Bg) inoculated with Be-78 and Y strains 30 days (d) and 9–24 months (m) after inoculation. M: molecular size markers and the numbers on the left are the size markers in base-pairs (bp); (A): mitochondrial clade A – *T. cruzi* I; (B): clade B – *T. cruzi* III or hybrid strains; (C): clade C – *T. cruzi* II (Freitas et al., 2006). Lanes 1–11: Be-78 strain (Be-78/1 Experiment); Bg1 (18 m); Be-78 strain (Be-78/2 Experiment); Bg6 (24 m); Y strain (Y/1 Experiment); Bg10 (12 m); Y strain (Y/2 Experiment); Bg15 (30 d, 12 m and 21 m); Bg16 (30 d). In detail: Bg10 (12 m) and Bg15 (21 m) isolates – changes in restriction profiles in relation to parental strain.

the parental *T. cruzi* strains, Be-78 and Y, and of parasites isolated from Beagle dogs. The restriction fragment length profiles (RFLP) obtained showed only one type of population: the parental Be-78 and Y strains that presented bands of 81 and 212 bp, indicative of populations related to the mitochondrial C group (Freitas et al., 2006) and associated with the TcII group as defined by Zingales et al. (2009).

The majority of the isolates obtained from infected animals presented the same profile of the parental strains, except for two isolates obtained from dogs infected with Y strain (Bg10/12th month of infection and Bg 15/21st month of infection) which showed profiles related with mitochondrial B group (associated with TcIII group or TcV or TcVI, as defined by Zingales et al., 2009), and different from the parental strain that was mitochondrial C group (TcII) (Fig. 1).

3.3. RAPD profile

Fig. 2 is representative of RAPD profiles obtained with different primers. The matrix of Dice similarity coefficients (Dice, 1945), based on pairwise band sharing, was calculated for all samples allowing the construction of a UPGMA dendrogram (Fig. 3). The dendrogram distinguished Be-78 and Y parental strains and their respective isolates in two distinct groups or clusters: (i) the first cluster includes the Be-78 (Be-78/1 and Be-78/2) strain and their respective isolates, Y(Y/1 and Y/2) strain and theirs isolates of

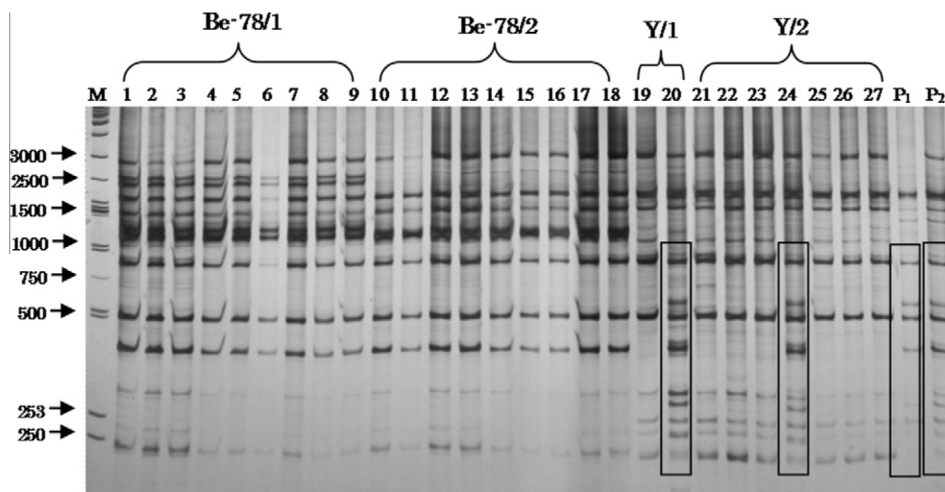

Fig. 2. Patterns of random amplified polymorphic DNA (RAPD) analysis using the primer λgt-11F for the different *Trypanosoma cruzi* isolates from Beagle dogs (Bg) inoculated with Be-78 strain (Be-78/1 and Be-78/2 Experiments) and Y strains (Y/1 and Y/2 Experiments). The isolates were obtained 30 days (d) and 9–24 months (m) after inoculation. Lanes 1–9: Be-78/1 Experiment [Be-78 strain and respective isolates: Bg1 (30 d and 18 m), Bg2 (30 d and 24 m), Bg3 (30 d and 24 m) and Bg4 (30 d and 24 m)]; lanes 10–18: Be-78/2 experiment [Be-78 strain and respective isolates: Bg6 (30 d and 21 m), Bg7 (30 d and 24 m), Bg8 (30 d and 24 m) and Bg9 (30 d and 24 m)]; lanes 19 and 20: Y/1 Experiment (Y strain and the isolate Bg10/12 m); lanes 21–27: Y/2 Experiment [Y and respective isolates: Bg15 (30 d, 12 m and 21 m), Bg16 (30 d), Bg17 (30 d) and Bg18 (30 d)]; lanes P_1 and P_2: Hybrid patterns strains. In detail: hybrid profiles.

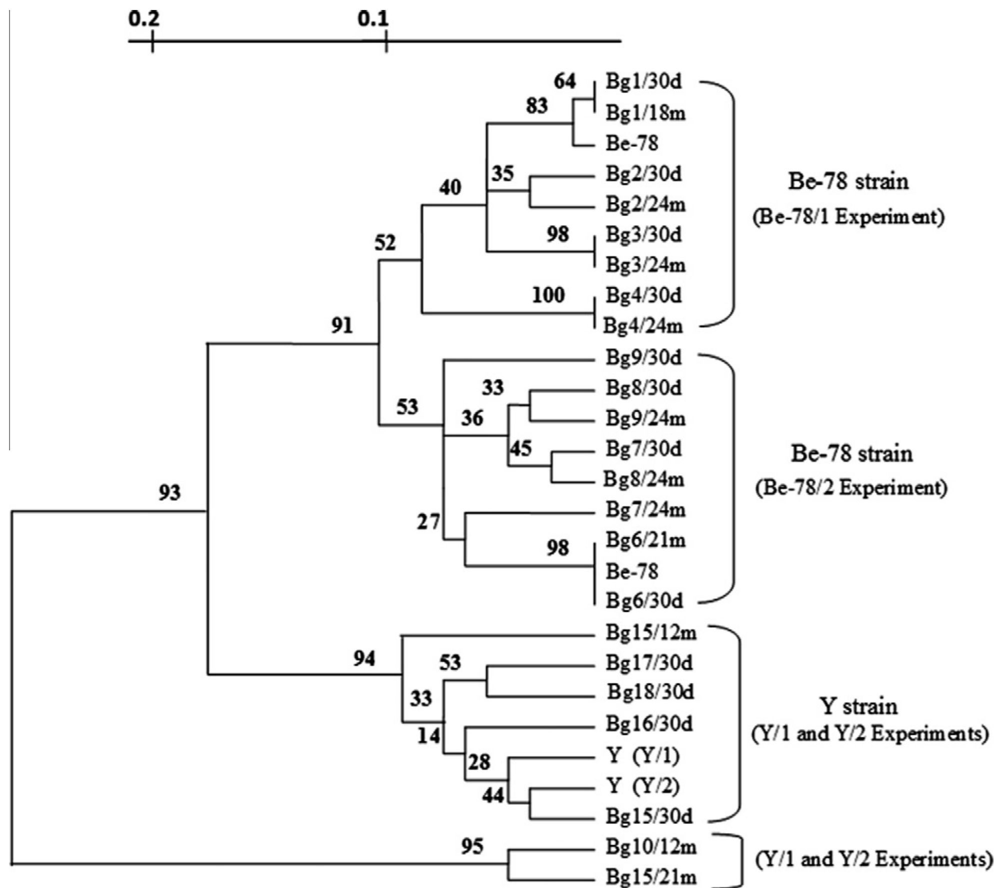

Fig. 3. Dendogram correspondent to RAPD profiles obtained with the primers (λgt-11F, λgt-11R, 3302, M13F) for Be-78 and Y *T. cruzi* strains and their respective isolates of Beagle (Bg) dogs, inoculated with these strains. The isolates were obtained 30 days (d) and 9–24 months (m) after inoculation. Populations of *Trypanosma cruzi* studied: Be-78/1 Experiment [Be-78 strain and respective isolates: Bg1 (30 d and 18 m), Bg2 (30 d and 24 m), Bg3 (30 d and 24 m) and Bg4 (30 d and 24 m)]; Be-78/2 Experiment [Be-78 strain and isolates: Bg6 (30 d and 21 m), Bg7 (30 d and 24 m), Bg8 (30 d and 24 m) and Bg9 (30 d and 24 m)]; Y/1 Experiment [Y strain and isolate: Bg10 (12 m)]; Y/2 Experiment [Y strains and respective isolates: Bg15 (30 d, 12 m and 21 m), Bg16 (30 d), Bg17 (30 d) and Bg18 (30 d)]. The number equivalent to bootstrap values to 1000 replications.

the acute phase (Bg 15; 16; 17 and 18), and the isolate of Bg15/12th, constituting a group genetically related with the parental strains; (ii) the second cluster includes two isolates of the Y strain (Bg10/12th and Bg15/21st) that were different and genetically distant from the parental strain.

In the first cluster it was verified a clear subdivision of the isolates in two branches concerned to Be-78 strain and their isolates and Y strain and their isolates. Be-78 strain showed a subdivision in two distinct clusters genetically related Be-78/1 and Be-78/2 experiments, although the bootstrap observed was low (Fig. 3).

Analysis of RAPD profiles revealed a high proportion of shared bands between parasites isolates from dogs and the respective parental strain. The primer M-13 presented less discriminating power among the isolates. However, in the evaluation intra-group, the 3302 primer showed the best discrimination among the isolates of the Y strain, while the λgt-11R primer discriminated better the isolates of the Be-78 strain.

4. Discussion

Several authors have reported changes of isoenzyme and restriction of kDNA profiles in *T. cruzi* strains after maintenance in culture or vertebrate host for long periods of time (Carneiro et al., 1990; Romanha, 1982; Veloso et al., 2005). However, the investigation of the genetic variability of this parasite during the acute and chronic infections in inbreed host was not widely explored yet to minimize the influence of the host genetics on the parasite.

In the present study, the evaluation of genetic modulation of two different *T. cruzi* strains (Be-78 and Y strains) were performed after isolation of the parasite during the acute and chronic phases of infection in Beagle dogs. Different isolates from the same strain were obtained and typed by three different molecular markers, consequently increasing the resolution (Tibayrenc and Ayala, 1999).

The phenotypic characterization of isolates from distinct animals infected with the Be-78 strain in the acute and chronic phases showed that all presented TcII profiles (Zingales et al., 2009) and similar to that of the parental strain, equivalent to ZA (Romanha, 1982) and Z2 (Miles et al., 1977). These profiles were the same observed by COII mit and rDNA markers (data not shown). This TcII profile remained the same although Be-78 strain had been submitted to successive blood passages in mice and the inoculations of the different experiments were performed with an interval of 18 months. Similar results were observed by Carneiro et al. (1990) when strains of ZA (currently denominated TcII of Zingales et al., 2009) were maintained for 18 months in C3H mice, differently from the observed with strains belonging to zimodemes ZB (TcVI of Zingales et al., 2009) and ZC (TcIV) which changed the profiles to ZA (TcII) after the same time of evaluation. These results suggest that the maintenance of the *T. cruzi* population in mice may result in selection of subpopulations.

The Be-78 strain was previously classified by De Lana et al. (1996) as Z2 (TcII of Zingales et al., 2009). However, for two isoenzymes differences were observed in the electrophoretic profile of

PGM which showed an additional band, and in the MDH enzyme, compatible with ZB (TcVI of Zingales et al., 2009) or ZC (TcIV of Zingales et al., 2009) profiles, what demonstrated genetic variability of this strain. Veloso et al. (2005) showed the existence of Z2 (TcII) and ZB (TcVI) zimodemes in isolates of Be-78 strain obtained from dogs experimentally infected for 7–12 years. Different zymodemes were observed in the same isolate, generally in higher or lower proportions among them. In some cases the presence of different profiles occurred simultaneously in similar proportions, as shown by the GPI enzyme. The authors suggested that the different subpopulations existent into Be-78 strain presented temporary fluctuations depending on the time of the infection or host-parasite relationship. However, Cruz et al. (2006) classified Be-78 strain as genotype 32 (Tibayrenc and Ayala, 1988), equivalent to Z2 of Miles et al. (1977) and to TcII group (Zingales et al., 2009). In the study of Cruz et al. (2006) the molecular typing was carried out using 22 enzymatic loci, nine primers for RAPD technique and seven microsatellite loci. Based on these results, the authors suggested that, even considering the limit of sensibility of the techniques employed, the Be-78 strain was monoclonal since the profile of the parental strain was similar to their clones obtained after microscopic micromanipulation.

Differently from MLEE and mit COII, the RAPD technique was more efficient in demonstrating any difference between isolates of the same *T. cruzi* strain. The dendogram constructed with RAPD data of Be-78 strain and its isolates were clustered in two distinct branches, but still genetically related and grouped with Be-78/1 and Be-78/2 experiments. Considering that Be-78 strain was maintained in mice for 18 months before of the infection of the second group, we could infer that the division of these populations into two different clusters in the dendogram may be due to the successive blood passages of the original strain in mice. These results suggest a possible influence of host factors in the changes observed in RAPD profiles, since the genetic profiles of both strains (Be-78 and Y) before the inoculation of the dogs was the same. These changes, although less evident, corroborated the results of Veloso et al. (2005) who demonstrated important variations in RAPD and MLEE profiles of the Be-78 strain and their isolates obtained of dogs after 2–7 years of infection. Probably the time elapsed between the inoculation and parasite isolation was insufficient for the occurrence of relevant alterations in MLEE profiles.

In animals infected with Y strain, only three isolates of *T. cruzi* during the chronic phase of infection were obtained, two from the same dog. This small number of isolates in dogs infected with the Y strain was probably due to the lower parasitemia observed in these animals when compared to those infected with the Be-78 strain. Besides, the Y strain presents predominantly slender blood trypomastigotes (Brener and Chiari, 1963; Brener, 1965) that are more sensitive to lytic antibodies (Kretlli and Brener, 1982), which could make more difficult parasite isolation by hemoculture, as demonstrated by Lana et al. (1992) and Araújo et al. (2002). Although the number of isolates of the Y strain was lower, the results of molecular typing were important. The parental Y strain presented the Z2 (TcII of Zingales et al., 2009) profile in four enzymatic systems. However, two isolates obtained from different animals and belonging to distinct experimental groups presented a MLEE profile different from the parental strain. Curiously, one isolate presented ZB (Romanha, 1982) profile for all enzymes and the other isolate presented ZC (Romanha, 1982) profile in GPI and ZB in the other enzymatic systems.

The Y strain is a reference strain in the study of Chagas disease and probably has been one of the more often mentioned in the literature. Therefore, their biological and molecular characterization was already widely explored (Romanha et al., 1979; Oliveira et al., 1998; Barnabé and Tibayrenc, 2004). However, several contradictions remain.

Barnabé and Tibayrenc (2004) suggested that the Y strain is found in several laboratories presenting at least two distinct genotypes (A and B), equivalents to *T. cruzi* IIb and *T. cruzi* IId DTUs (Discrete Typing Units defined by Brisse et al., 2000), recently denominated TcII and TcV groups, respectively (Zingales et al., 2009). According to these authors, these subdivisions would be highly stable in space and time. However, fluctuations among these genotypes were verified by other authors, as clearly reported in the present study. For example, Romanha (1982) registered the transition between A, B and C zymodemes detected by GPI enzyme, intermediary stages between one zymodeme and another. Later, Alves et al. (1993) verified changes in isoenzyme profile of ZA to ZB or ZC after sub-cultures of clones originated from Y strain in different culture media, or after successive passages in newborn mice. These authors demonstrated that TcII (ZA) profile was predominant in culture media with blood and TcVI (ZB) and TcIV (ZC) in cultures less rich in nutrients and blood. Barnabé and Tibayrenc (2004) tried to reproduce these results with two subclones of the Y strain belonging to genotypes A and B using isoenzymes and RAPD markers, but without success. The authors attributed the discrepancy of the results to differences among the resolution power of the cloning methods.

Although we have used only four enzymatic systems in this study, what could lead to some bias, they were the enzymes that revealed differences in several studies previously carried out by tour team (De Lana et al., 1996; Veloso et al., 2005; Cruz et al., 2006) with Be-78 strain. Nevertheless, the studies with the Y strain also give support for the choice of the enzymatic systems used (Romanha, 1982; Alves et al., 1993). Additionally, these are the more polymorphic systems in *T. cruzi* (Barnabé and Tibayrenc, 2004, which corroborated RAPD data, a molecular marker also employed in this study.

The results obtained in present study reconfirmed the polyclonal character for the Y strain and also for Be-78 strain as demonstrated later for Valadares (2007) and that the dynamic of subpopulations can to change and be dependent on the pressures to which they were submitted during the permanence for long-term in the vertebrate host. Similar results were observed in other experimental models where the parental strains with populations isolated by different methods of maintenance and handling were compared (Carneiro et al., 1990; Lauria-Pires et al., 1996; Veloso et al., 2005).

However, the impact of the predominance of one or other population on the course of the infection remains to be more elucidated. Our group has evaluated the Be-78 and Y strains concerning several aspects in different experimental models. Several studies revealed changes in virulence, pathogenicity and drug susceptibility when the parental strains were compared with theirs isolates obtained after long-term infection in dogs (Veloso et al., 2001; Caldas et al., 2008) or when these isolates were submitted to drug pressures in murine model (Dos Santos et al., 2008). In general, these authors demonstrated that after long-term infection in dogs the strains developed increase resistance to Benznidazole (Veloso et al., 2001), and that a continuous *in vivo* drug pressure induced resistance (Dos Santos et al., 2008). Nevertheless, after long maintenance in mice (Caldas et al., 2008) or culture (Dos Santos et al., 2008), this phenotype can revert to the parental strain profile.

However, only Veloso et al. (2005) performed the molecular characterization of parasites during infection and showed correlation between these data with the genetic variability of the strain. Similar to Veloso et al. (2005), this work confirmed the modulation of population in *T. cruzi* strains during the infection in vertebrate host, now employing a more genetic homogeneous model (Beagle dogs) that theoretically minimizes the host influence in this process. However, the results that we obtained were not the same in all animals. Herein the molecular typing using three different

molecular markers revealed that only two populations isolated from Beagle dogs infected with Y strain changed its genetic profile during long time of infection.

The detection of *T. cruzi* genetic changes by the same molecular markers in parasites of the same *T. cruzi* strain (Be-78) and of Y strain, both of the same *T. cruzi* genetic lineage, now maintained in inbreed dogs (genetically homogenous) versus outbreed dogs (genetically heterogeneous) previously studied by our group illustrates the great plasticity of *T. cruzi* specie.

In conclusion, this study leads to the idea that each host (here each dog) harbors a determinate population of *T. cruzi* that probably may change or be modulated throughout the long-term infection. Thus, it is reasonable to presume that each animal may establish a particular relationship with the parasite which may result in genetic alterations, especially when infected with polyclonal strains. These changes may hinder the observation of possible correlations between the parasite genetic and the clinical forms of Chagas disease due to the complexity of the parasite-host interaction in which probably the genetic background of both should be considered in addition to the environmental conditions (Tibayrenc, 2010).

Acknowledgments

We thank Lívia de Figueiredo Diniz and Geovam Crepalde for the care with Beagle dogs. This study was supported by grants from Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Universidade Federal de Ouro Preto (UFOP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). We would like also to thank the fellowship of technical support and REDE Mineira de Bioterismo – 2824/05, FAPEMIG.

References

Alves, A.M., Tanuri, A., De Almeida, D.F., Von Kruger, W.M., 1993. Reversible changes in the isoenzyme electrophoretic mobility pattern and infectivity in clones of *Trypanosoma cruzi*. Exp. Parasitol. 77, 246–253.

Andrade, S.G., 1999. *Trypanosoma cruzi*: clonal structure of parasite strains and the importance of principal clones. Mem. Inst. Oswaldo Cruz 94, 185–187.

Andrade, L.O., Machado, C.R.S., Chiari, E., Pena, S.D.J., Macedo, A.M., 2002. *Trypanosoma cruzi*: role of host genetic background in the differential tissue distribution of parasite clonal populations. Exp. Parasitol. 100, 269–275.

Araújo, F.M., Bahia, M.T., Magalhães, N.M., Martins-Filho, O.A., Veloso, V.M., Carneiro, C.M., Tafuri, W.L., Lana, M., 2002. Follow-up of experimental chronic Chagas' disease in dogs: use of polymerase chain reaction (PCR) compared with parasitological and serological methods. Acta Trop. 81, 21–31.

Barnabé, C., Tibayrenc, M., 2004. *Trypanosoma cruzi*: long-term sub-cultures in two different culture media do not confirm the existence of highly versatile multilocus genotypes. Int. J. Parasitol. 34, 779–784.

Ben Abderrazak, S., Guerrini, F., Mathieu-Daude, F., Truc, P., Neubauer, K., Lewicka, K., Barnabé, C., Tibayrenc, M., 1993. Isoenzyme electrophoresis for parasite characterization. Methods Mol. Biol. 21, 361–382.

Brener, Z., 1965. Comparative studies of different strains of *Trypanosoma cruzi*. Ann. Trop. Med. Parasitol. 59, 19–26.

Brener, Z., Chiari, E., 1963. Morphological variations observed in different strains of *Trypanosoma cruzi*. Rev. Inst. Med. Trop. São Paulo 19, 220–224.

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

Brisse, S., Dujardin, J.C., Tibayrenc, M., 2000. Identification of six *Trypanosoma cruzi* lineages by sequence-characterised amplified region markers. Mol. Biochem. Parasitol. 111 (1), 95–105.

Caldas, S., Santos, F.M., Lana, M., Diniz, L.F., Machado-Coelho, G.L.L., Veloso, V.M., Bahia, M.T., 2008. *Trypanosoma cruzi*: acute and long-term infection in the vertebrate host can modify the response to benznidazole. Exp. Parasitol. 118, 315–323.

Carneiro, M., Chiari, E., Gonçalves, A.M., Pereira, A.A.S., Morel, C.M., Romanha, A.J., 1990. Changes in the isoenzyme and kinetoplast DNA patterns of *Trypanosoma cruzi* strains induced by maintenance in mice. Acta Trop. 47, 35–45.

Chiari, E., Dias, J.C.P., Lana, M., Chiari, C.A., 1989. Hemocultures for the parasitological diagnosis of human chronic Chagas' disease. Rev. Soc. Bras. Med. Trop. 22, 19–23.

Cruz, R.E., Macedo, A.M., Barnabé, C., Freitas, J.M., Chiari, E., Veloso, V.M., Carneiro, C.M., Bahia, M.T., Tafuri, W.L., Lana, M., 2006. Further genetic characterization of the two *Trypanosoma cruzi* Berenice strains (Be-62 and Be-78) isolated from the first human case of Chagas disease (Chagas, 1909). Acta Trop. 97, 239–246.

De Lana, M., Chiari, C.A., Chiari, E., Morel, C.M., Gonçalves, A.M., Romanha, A.J., 1996. Characterization of two isolates of *Trypanosoma cruzi* obtained from the patient Berenice, the first human case of Chagas' disease described by Carlos Chagas in 1909. Parasitol. Res. 82 (3), 257–260.

Deane, M.P., Jansen, A.M., Mangia, R.H.R., Gonçalves, A.M., Morel, C., 1984. Are our laboratory strains representative samples of *Trypanosoma cruzi* populations that circulate in nature? Mem. Inst. Oswaldo Cruz 72, 19–24.

Dice, L.R., 1945. Measures of the amount of ecological association between species. Ecology 26, 297–302.

Dos Santos, F.M., Caldas, S., de Assis Cáu, S.B., Crepalde, G.P., de Lana, M., Machado-Coelho, G.L., Veloso, V.M., Bahia, M.T., 2008. *Trypanosoma cruzi*: induction of benznidazole resistance in vivo and its modulation by in vitro culturing and mice infection. Exp. Parasitol. 120 (4), 385–390.

Felsenstein, J., 1985. Phylogenies and the comparative method. Am. Nat. 125, 1–15.

Freitas, J.M., Augusto-Pinto, L., Pimenta, J.R., Bastos-Rodrigues, L., Goncalves, V.F., Teixeira, S.M., Chiari, E., Junqueira, A.C., Fernandes, O., Macedo, A.M., Machado, C.R., Pena, S.D., 2006. Ancestral genomes, sex, and the population structure of *Trypanosoma cruzi*. PLoS Pathog. 2 (3), 226–235 (e24).

Gonzalez, J., Muñoz, S., Ortiz, S., Anacona, D., Salgado, S., Galleguillos, M., Neira, I., Sagua, H., Solari, A., 1995. Biochemical, immunological, and biological characterization of *Trypanosoma cruzi* populations of the Andean north of Chile. Exp. Parasitol. 81, 125–135.

Guedes, P.M., Veloso, V.M., Tafuri, W.L., Galvão, L.M.C., Carneiro, C.M., Lana, M., Chiari, E., Soares, K.A., Bahia, M.T., 2002. The dog as model for chemotherapy of the Chagas' disease. Acta Trop. 84 (1), 9–17.

Kretlli, A.U., Brener, Z., 1982. Resistance against *Trypanosoma cruzi* associated to anti-living trypomastigote antibodies. J. Immunol. 128 (5), 2009–2012.

Lana, M., Chiari, C.A., 1986. Caracterização biológica comparativa das cepas Berenice e Berenece-78 de *Trypanosoma cruzi*, isoladas da mesma paciente em diferentes períodos. Mem. Inst. Oswaldo Cruz 81, 247–253.

Lana, M., Chiari, E., Tafuri, W.L., 1992. Experimental Chagas'disease in dogs. Mem. Inst. Oswaldo Cruz 87 (1), 59–71.

Lauria-Pires, L., Bogliolo, A.R., Teixeira, A.R.L., 1996. Diversity of *Trypanosoma cruzi* stocks and clones derived from Chagas disease patients II. Isozyme and RFLP characterizations. Exp. Parasitol. 82, 182–190.

Macedo, A.M., Pena, S.D.J., 1998. Genetic variability of *Trypanosoma cruzi*: implications for the pathogenesis of Chagas disease. Parasitol. Today 14, 119–124.

Martins, H.R., Toledo, M.J.O., Veloso, V.M., Carneiro, C.M., Machado-Coelho, G.L., Tafuri, W.L., Bahia, M.T., Valadares, H.M., Macedo, A.M., Lana, M., 2006. *Trypanosoma cruzi*: impact of dual-clone infections on parasite biological properties in BALB/c mice. Exp. Parasitol. 112, 237–246.

Martins, H.R., Silva, R.M., Valadares, H.M., Toledo, M.J., Veloso, V.M., Vitelli-Avelar, D.M., Carneiro, C.M., Machado-Coelho, G.L., Bahia, M.T., Martins-Filho, O.A., Macedo, A.M., Lana, M., 2007. Impact of dual infections on chemotherapeutic efficacy in BALB/c mice infected with major genotypes of *Trypanosoma cruzi*. Antimicrob. Agents Chemother. 51 (9), 3282–3289.

Martins, H.R., Figueiredo, L.M., Valamiel-Silva, J.C., Carneiro, C.M., Machado-Coelho, G.L., Vitelli-Avelar, D.M., Bahia, M.T., Martins-Filho, O.A., Macedo, A.M., Lana, M., 2008. Persistence of PCR-positive tissue in benznidazole-treated mice with negative blood parasitological and serological tests in dual infections with *Trypanosoma cruzi* stocks from different genotypes. J. Antimicrob. Chemother. 61 (6), 1319–1327.

Miles, M.A., Toyé, P.J., Oswald, S.C., Godfrey, D.G., 1977. The identification by isoenzyme patters of two distinct strain-groups of *Trypanosoma cruzi*, circulating independently in a rural area of Brasil. Trans. Roy. Soc. Trop. Med. Hyg. 71, 217–225.

Montamat, E.E., De Luca D'Oro, G.M., Gallerano, R.H., Sosa, R., Blanco, A., 1996. Characterization of *Trypanosoma cruzi* populations by zymodemes: correlation with clinical picture. Am. J. Trop. Med. Hyg. 55, 625–628.

Oliveira, R.P., Broude, N.E., Macedo, A.M., Cantor, C.R., Smith, C.L., Pena, S.D.J., 1998. Probing the genetic population structure of *Typanosoma cruzi* with microsatélites. Proc. Natl. Acad. Sci. USA 95, 3776–3780.

Oliveira, R.P., Melo, A.I.R., Macedo, A.M., Chiari, E., Pena, S.D.J., 1999. The population structure of *Trypanosoma cruzi*: expanded analyses of 54 strains using eight polymorphic microsatélites. Mem. Inst. Oswaldo Cruz 94, 65–70.

Pena, S.D.J., Nunes, A., 1990. DNA-POP and PATER two simple computer programs for population studies and paternity analyses with DNA fingerprintings. Fingerp. News 2, 7–8.

Pinto, A.S., de Lana, M., Britto, C., Bastrenta, B., Tibayrenc, M., 2000. Experimental *Trypanosoma cruzi* biclonal infection in *Triatoma infestans*: detection of distinct clonal genotypes using kinetoplast DNA probes. Int. J. Parasitol. 30, 843–848.

Revollo, S., Oury, B., Laurent, J.P., Barnabé, C., Quesney, V., Carriere, V., Noel, S., Tibayrenc, M., 1998. *Trypanosoma cruzi*: impact of clonal evolution of the parasite on its biological and medical properties. Exp. Parasitol. 89, 30–39.

Romanha, A.J., 1982. Heterogeneidade enzimática em *Trypanosoma cruzi*. PhD Thesis, Universidade Federal de Minas Gerais.

Romanha, A.J., Pereira, A.A.S., Chiari, E., Kilgour, V., 1979. Isoenzyme patterns of cultured *Trypanosoma cruzi*: changes after prolonged subculture. Comp. Biochem. Physiol. 62, 139–142.

Santos, F.R., Pena, S.D., Epplen, J.T., 1993. Genetic and population study of a Y-linked tetranucleotide repeat DNA polymorphism with a simple non-isotopic technique. Human Genet. 90, 655–656.

Silva, L.H.P., Nussenzweig, V., 1953. Sobre uma cepa de *Trypanosoma cruzi* altamente virulenta para o camundongo branco. Folia Clin. Biol. 20, 191–203.

Solari, A., Wallace, A., Ortiz, S., Venegas, J., Sànchez, G., 1998. Biological characterization of *Trypanosoma cruzi* stocks from Chilean insect vectors. Exp. Parasitol. 89, 312–322.

Steindel, M., Dias-Neto, E., Menezes, C.L.P., Romanha, A.J., Simpson, A.J.G., 1993. Random amplified polymorphic DNA analysis of *Trypanosoma cruzi* strains. Mol. Biochem. Parasitol. 60, 71–80.

Tibayrenc, M., 2010. Modelling the transmission of *Trypanosoma cruzi*: the need for an integrated genetic epidemiological and population genomics approach. Adv. Exp. Med. Biol. 673, 200–211.

Tibayrenc, M., Ayala, F.J., 1987. *Trypanosoma cruzi* populations: more clonal than sexual. Parasitol. Today 3, 189–190.

Tibayrenc, M., Ayala, F.J., 1988. Isozyme variability in *Trypanosoma cruzi*, the agent of Chagas disease: genetical, taxonomical, and epidemiological significance. Evolution 42, 277–292.

Tibayrenc, M., Ayala, F.J., 1999. Evolutionary genetics of *Trypanosoma* and Leishmania. Microbes Infect. 1, 465–472.

Tibayrenc, M., Ward, P., Moya, A., Ayala, F.J., 1986. Natural populations of *Trypanosoma cruzi*, the agent of Chagas disease, have a complex multiclonal structure. Proc. Natl. Acad. Sci. USA 83, 115–119.

Toledo, M.J.O., Lana, M., Carneiro, C.M., Bahia, M.T., Machado-Coelho, G.L.L., Veloso, V.M., Barnabé, C., Tibayrenc, M., Tafuri, W.L., 2002. Impact of *Trypanosoma cruzi* clonal evolution on its biological properties in mice. Exp. Parasitol. 100, 161–172.

Toledo, M.J., Bahia, M.T., Carneiro, C.M., Martins-Filho, O.A., Tibayrenc, M., Barnabé, C., Tafuri, W.L., Lana, M., 2003. Chemotherapy with benznidazole and itraconazole for mice infected with different *Trypanosoma cruzi* clonal genotypes. Antimicrob. Agents Chemother. 47, 223–230.

Torres, J.P., Ortiz, S., Solari, A., 2003. *Trypanosoma cruzi* isolate from Chile are heterogeneous & composed of mixed populations when characterization by Schizodeme & Southern analyses. Parasitology 128, 161–168.

Valadares, H.M., 2007. Análises populacionais em *Trypanosoma cruzi* baseada em microssatélites polimórficos de DNA. PhD Thesis, Universidade Federal de Minas Gerais.

Van De Peer, Y., De Wachter, R., 1994. TREECON for windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows Environment. Comput. Appl. Biosci. 10, 569–570.

Veloso, V.M., Carneiro, C.M., Toledo, M.J.O., Lana, M., Chiari, E., Tafuri, W.L., Bahia, M.T., 2001. Variation in susceptibility to benznidazole in isolates derived from *Trypanosoma cruzi* parental strains. Mem. Inst. Oswaldo Cruz 96 (7), 1005–1011.

Veloso, V.M., Romanha, A.J., Lana, M., Murta, S.M.F., Carneiro, C.M., Alves, C.F., Borges, E.C., Tafuri, W.L., Machado Coelho, G.L.L., Chiari, E., Bahia, M.T., 2005. Influence of the long-term *Trypanosoma cruzi* infection in vertebrate host on the genetic and biological diversity of the parasite. Parasitol. Res. 96, 383–389.

Zingales, B., Andrade, S.G., Briones, M.R.S., Campbel, L.D.A., Chiari, E., Fernandes, O., Guhl, F., Lages-Silva, E., Macedo, A.M., Machado, C.R., Miles, M.A., Romanha, A.J., Sturm, N.R., Tibayrenc, M., Schijman, A.J., 2009. A new consensus for *Trypanosoma cruzi* intraspecific nomenclature: second revision meeting recommends TcI to TcVI. Mem. Inst. Oswaldo Cruz 104 (7), 1051–1054.