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Análise da Variabilidade de Populações do Trypanosoma cruzi Isoladas de Cães Após 2 a 17 Anos de Infecção.

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Resumo dos Resultados

A variabilidade intraespecífica do Trypanosoma cruzi foi avaliada em populações isoladas de cães cronicamente infectados com as cepas Colombiana – T. cruzi I e Berenice-78 – T. cruzi II, utilizando marcadores biológicos e moleculares. Dois isolados foram obtidos de cães inoculados com a cepa Colombiana, (Col-A e Col-B) e cinco com a cepa Be-78 (Be-78 A, B, C, D e E), através de xenodiagnóstico e hemocultura, respectivamente.

A cepa Colombiana parental, as populações Col (A e B) foram consideradas, respectivamente de alta, moderada e baixa virulência e patogenicidade para camundongos, considerando o nível de parasitismo, o parasitismo e as lesões teciduais e a taxa de mortalidade, observados em camundongos albinos, inoculados com as cepas parentais e com as populações isoladas de cães. Esta diferença não foi tão acentuada entre os camundongos inoculados com a cepa Be-78 parental e com os isolados Be-78 (A, B, C, D e E). Com exceção do isolado Be-78 C, relativamente, mais virulento e patogênico, e do isolado Be-78 D, menos virulento e patogênico, os demais apresentaram grau de virulência e patogenicidade semelhantes à cepa Be-78 parental. Não foi observada diferença entre as populações isoladas e as cepas parentais com relação ao tropismo tecidual.

Estudo comparativo sobre a susceptibilidade ao Benznidazol entre camundongos inoculados com (1) a cepa Col parental e as populações Col (A e B); (2) a cepa Be-78 parental e as populações Be-78 (B, C e D), foi realizado durante a fase aguda da infecção. Nenhuma cura foi observada entre os animais inoculados com a cepa Col parental e com as Col (A e B). Animais inoculados com a cepa Be-78 parental e Be-78 B apresentaram 100% de cura. Entretanto, camundongos inoculados com as populações Be-78 C e D, apresentaram, respectivamente, 50 e 80% de cura. O perfil sorológico dos camundongos tratados mas não curados foi variável, e possivelmente dependente do grau de resistência ao Bz da população do T. cruzi estudada.

A caracterização biológica, das populações do T. cruzi isoladas de cães chagásicos crônicos, infectados com a cepa Be-78, sugeriram a ocorrência de uma alteração na dinâmica populacional destes isolados, em relação à cepa parental. Para testar esta hipótese, foi realizada a caracterização molecular da cepa Be-78 e das populações isoladas de cães, através da eletroforese de isoenzimas e do perfil de RAPD. Os parasitas provenientes de
cães foram caracterizados após a primeira e 25ª passagem em camundongos albinos, através do perfil eletroforetico de 6 enzimas (GPI, PGM, G6PD, ME, ASAT e ALAT) e dos perfis de RAPD obtidos com 5 iniciadores (3302, 3303, 3304, 3307 e λGT11R) respectivamente para cada marcador.

Foi observado o mesmo perfil isoenzimático da cepa parental em três isolados, e dois apresentaram perfis diferentes do observado pela cepa Be-78 parental. Após 25 passagens em camundongos, três dos quatro isolados avaliados, mudaram de perfil em relação à primeira passagem. Essa mudança foi gradual e reversível. Em várias ocasiões foi observada a presença de dois Zimodemas simultâneos. Um complexo perfil de RAPD foi obtido com os cinco iniciadores utilizados. Uma matriz de coeficiente de similaridade de Dice, baseada em um pareamento de bandas compartilhadas (n = 82) foi calculada para as amostras, permitindo a construção de um dendograma UPGMA. O dendograma mostrou dois grupos distintos. O Grupo 1 incluiu a cepa Be-78 parental e os isolados Be-78 (A e C) após a 1ª e 25ª passagem em camundongo, e Be-78 (B e D) após a 25ª passagem. O grupo 2 incluiu os isolados Be-78 (B e D) após a primeira passagem em camundongos, os quais foram completamente distintos da cepa parental. Curiosamente, após 25 passagens em camundongos, os perfis de isoenzimas e de RAPD, retornaram âqueles apresentados pela cepa parental.

Nossos dados mostraram (1) a presença de diferentes subpopulações na cepa Be-78 do *T. cruzi* e (2) indicaram que a permanência do parasita em cães e/ou camundongos pode levar a uma mudança na proporção relativa das subpopulações do *T. cruzi* presentes nesta cepa.
Variation in Susceptibility to Benznidazole in Isolates Derived from *Trypanosoma cruzi* Parental Strains

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In this work, the susceptibility to benznidazole of two parental *Trypanosoma cruzi* strains, Colombian and Berenice-78, was compared to isolates obtained from dogs infected with these strains for several years. In order to evaluate the susceptibility to benznidazole two groups of mice were infected with one of five distinct populations isolated from dogs as well as the two parental strains of *T. cruzi*. The first group was treated with benznidazole during the acute phase and the second remained untreated controls. The animals were considered cured when parasitological and serological tests remained persistently negative. Mice infected with the Colombian strain and its isolates Colombian (A and B) did not cure after treatment. On the other hand, all animals infected with Berenice-78 were cured by benznidazole treatment. However, 100%, 50% and 70% of cure rates were observed in animals infected with the isolates Berenice-78 B, C and D, respectively. No significant differences were observed in serological profile of infected control groups, with all animals presenting high antibody levels. However, the ELISA test showed differences in serological patterns between mice inoculated with the different *T. cruzi* isolates and treated with benznidazole. This variability was dependent on the *T. cruzi* population used and seemed to be associated with the level of resistance to benznidazole.

Key words: chemotherapy - drug susceptibility - *Trypanosoma cruzi* - benznidazole

*Trypanosoma cruzi*, the agent of Chagas disease, exhibits considerable biological variability (Carneiro et al. 1991, Andrade & Magalhães 1997). Studies using cloned or uncloned populations, reinforce the heterogeneity of the parasite and demonstrate that, in general, strains of *T. cruzi* are composed of subpopulations with distinct characteristics (Postan et al. 1986, Finley & Dvorak 1987). Several authors have extensively investigated the correlation between *T. cruzi* genetic background and the different clinical forms of Chagas disease, as well as biological characteristics, such as virulence, pathogenicity and susceptibility to drugs (Revollo et al. 1998, Andrade 1999).

Filardi and Brener (1987) and Toledo et al. (1997) described the existence of strains naturally resistant and non-resistant to benznidazole and nifurtimox. Natural resistance to nitro-derivates has been suggested as an important factor in explaining the low rates of cure detected in treated patients (Murta & Romanha 1998). Several authors have shown that resistance of *T. cruzi* strains to benznidazole and nifurtimox increased when parasites were isolated from mice previously treated with these same drugs (Marretto & Andrade 1994, Murta & Romanha 1998). The authors suggest that the initial treatment eliminated the sensitive parasites, preserving the resistant ones, which multiplied and dominated the population. In this manner, the imposition of natural and artificial pressure can result in the selection of a subset of the population.

If the strains of *T. cruzi* present clonal structure, based on the existence of clonal lines without sexual interactions (Tibayrenc & Ayala 1988), it is possible to think that clones with variable degrees of drug susceptibility presented among the whole circulating population would show different growth rates in the host. Consequently, genetic characteristics of the host could favor the development of certain clones within the host, which could reflect differences in the susceptibility to drugs. To confirm this hypothesis, the susceptibility to benznidazole of different isolates of *T. cruzi*, originated from dogs infected 2, 7, 8 and 17 years before, were comparatively studied in mice with the parental Colombian and Berenice-78 strains.

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MATERIALS AND METHODS

T. cruzi strains and populations isolated from dogs - The parental strains Colombian (Col) - T. cruzi I (Fedreri et al. 1964), resistant to benznidazole (Filardi & Brener 1987) and Berenice-78 (Be-78) - T. cruzi II (Lana & Chiari 1986), 100% susceptible to benznidazole (Toledo et al. 1995), were used as references.

T. cruzi stocks were isolated from different dogs after 8 and 17 years of infection with Col strain (Col A and B, respectively) and with Be-78 after 7 (Be-78 B and C) and 2 (Be-78 D) years of infection.

Experimental conditions

Mice infection - For each strain or stock of T. cruzi, 12 Swiss outbred mice (30 days old, 18-20 g weight) were infected by intraperitoneal route with 5 x 10^3 blood trypanosomates, obtained from infected mice. Six mice were submitted to treatment with benznidazole (N-benzyl-2-nitro-1-imidazo-lacetamide - Roche Company), and six remained as untreated infected controls. The experiments were repeated when the percentage of cure was different between the parental strains and their isolates.

Treatment schedules - Animals were treated after the detection of parasitaemia, approximately four days post-inoculation. Benznidazole was administered in a suspension made with 4% arabic gum (Sigma) by oral doses of 100 mg of benznidazole/kg, for 20 consecutive days. Animals were considered cured when both parasitological (fresh blood examination, hemoculture and PCR) and serological (ELISA) tests were negative.

Parameters evaluated

Parasitaemia and mortality - The parasitaemia was followed from the 4th day of infection until the negativation of the parasitaemia by fresh blood collected from the mouse's tail and the number of parasites was estimated as described by Brener (1962). Curves were plotted using the mean of the parasitaemia from six mice. Mortality rate was expressed as a percentage of accumulated deaths within the period of 180 days after the inoculation.

Hemoculture - Mice were bled from the orbital venous sinus 30 and 180 days after the end of treatment. The blood was split into two tubes containing 5 ml of Liver Infusion Tryptose medium (Filardi & Brener 1987). The tubes were incubated at 28°C for 30-90 days and microscopically examined for the presence of parasites 30, 60 and 90 days later.

PCR assay - PCR were performed only in samples from animals with positive hemoculture. Mice were bled from the orbital venous sinus and 200 µl of blood were collected. The sample were immediately mixed with 400 µl of 6 M guanidine HCL/0.2 M EDTA solution (Avila et al. 1991) and stored at room temperature for one week. This mixture was boiled for 3 min to break the minicircles (Britto et al. 1993). DNA extraction was performed according to Wincker et al. (1994), but using 40 µg of Glycogen (Boeringer Mannheim) to precipitate the DNA. The pellets obtained were resuspended in 40 µl of distilled sterile water and stored at 60°C for 1 h. PCR conditions were described by Gomes et al. (1998). The PCR mixture contained 2 µl of DNA solution, 10 mM Tris-HCl (pH 9.0), 75 mM KCl, 3.5 mM MgCl2, 0.1% Triton X-100, 0.2 mM each dATP, dCTP, dGTP and dUTP (Sigma Co. Ltd.), 20 pmol of S 35 (5' AA A A T A A AT G T A C G G G G T G T G A T G C A T G A 3') and S36 (5'GGGTTCCATTGGGTTGGTG3') primers (Avila et al. 1990), 1.0 unit of Taq DNA polymerase (GIBCO), and 40 µl with water. The reaction mixtures were overlaid with 30 µl of mineral oil and subjected to 35 cycles of amplification in a Research Programmable Thermal Controller (MinCycler TM). The temperature profile were 95°C for 1 min for denaturation with a longer initial time of 5 min at 95°C, 65°C for 1 min for primer annealing, and 72°C for 10 min to extend the annealed primers. Five microliters of PCR products were analyzed by electrophoresis on a 6% polyacrylamide gel and visualized by staining with silver salts (Santos et al. 1993).

Serological profile - Ten microliters of blood were collected from mice tails every two weeks for three months and monthly thereafter until seven months post-infection in 90 µl de PBS, pH 7.2. The sera were obtained were stored at -20°C, and samples from treated and untreated mice were tested in parallel. ELISA test were performed according to Vollet et al. (1976). Peroxidase conjugated goat anti-mouse IgG (Sigma) was used. The cut-off was determined by using the mean absorbance from ten uninfected animals plus two standard deviations.

RESULTS

Parasitological cure - The Table shows the percentage of cure induced by benznidazole after long-term treatment of different groups of mice infected with each parental strains and their isolates from dogs. Animals infected with Col strain and isolates Col A and Col B did not respond to treatment with benznidazole. On other hand, mice infected with Be-78 strain showed 100% of cure after treatment, whereas mice infected with their isolates Be-78B, C and D) displayed different spectra of susceptibility to benznidazole, with 100%, 50% and 70% of cure rates, respectively. The evaluation of the presence of specific antibodies in the sera of these animals after treatment correlated with the parasitological findings.

The effect of benznidazole on the reduction of parasitaemia - Mice infected with Col strain and
Col-A isolated showed suppression of parasitaemia during treatment, whereas in animals infected with Col-B isolate this parameter was reduced but not completely suppressed. All mice inoculated with Col strain and Col (A and B) isolates showed patent parasitaemia on the 7th day post treatment. Treated animals showed a decrease in parasitaemia levels of 2.5 and 5 times the levels of untreated control (Fig. 1). All mice infected with Be-78 strain and Be-78 (B, C and D) showed a suppression of parasitaemia on the first day of treatment onset, which persisted throughout and after the treatment period.

The mortality rate - At 180 days post treatment the mortality rate was 100% in the control group infected with Col strain and 40% in the treated group. There was no mortality in treated and untreated mice infected with Col (A and B) isolates and Be-78 strain as well as their isolates (Be-78 B, C and D).

Serological profile - T. cruzi specific IgG antibodies were detected 20-30 days after infection in sera of infected control groups and all animals displayed high levels of antibodies regardless of the strain or isolate used for inoculation (Figs 2, 3). However differences in the serological pattern among mice infected with different populations of T. cruzi after treatment was detected.

In all animals infected with Col strain, IgG antibodies were detected from one to 10 days post treatment. In animals infected with Col-A the antibody levels were under the cut-off value (Abs = 0.233) between 30 and 60 days of infection, raising to control levels thereafter. Antibody levels in treated and untreated animals infected with Col B were similar throughout the infection period (Fig. 2).

Similar serological profiles were observed in mice infected with Be-78, and isolates Be-78 that responded to treatment with antibodies levels always under the cut-off value. In all animals infected with Be-78 (C and D), that remained infected post treatment, the antibody levels remained under the cut-off (0.233) until 90 and 120 days after treatment slowly raising afterwards but without reaching the same level of untreated controls (Fig. 3).

DISCUSSION

Natural resistance of T. cruzi to nitro derivates has been described as an important factor to explain the low rates of cure detected in treated chagasic patients (Filardi & Brener 1987). The authors described the existence of strains that are naturally resistant and non-resistant to benzimidazole and nifurtimox.

Relatively few reports have been published concerning the in vivo induction of drug resistance to T. cruzi. Andrade et al. (1977) showed that the resistance to drugs benzimidazole and nifurtimox increased when the parasites were isolated from mice previously treated with the same drugs. Regarding this aspect, it is important to mention that the isolates used in this work were obtained from dogs that were not treated, eliminating, in this way, the possibility of influence of the drug on induction of parasite resistance to treatment.
groups inoculated with Col strain and Col-A. The anti-*T. cruzi* antibody production was delayed in mice infected with the parental Col and Col-A while, in mice infected with Col-B, IgG antibodies were detected also during treatment, similar to observed in control untreated groups. Camandaroba (1999) demonstrated the high resistance of Col strain to benznidazole and verified also similar resistance in seven clones of this strain.

In this study we observed stability of the resistance to benznidazole in the populations from the Colombian strain isolated of dogs 8 and 17 years after infection. However, the fact of the animals infected with the Col-B isolate presented patent parasitaemia during treatment suggests the presence of a higher proportion of resistant subpopulation to benznidazole in this isolate in relation to the parental strain and Col-A. This supposition is corroborated by analysis of the *T. cruzi* specific IgG profile observed in these mice in relation to the
Andrade et al. (1977) suggested the selection of resistant parasites to explain the parasite persistence in vertebrate hosts after prolonged treatment. To test this hypothesis Murta and Romanha (1998) investigated the development of drug-resistance of *Trypanosoma cruzi* Y strain isolated from mice not cured after treatment with benznidazole or nitifurtox. The authors demonstrate the in vivo selection of a population and clones of *T. cruzi* resistant to benznidazole from the Y strain (50% resistant to benznidazole) and suggest that the resistance of the strain seems to be related to the sensitive/resistant clone ratio in the population.

The present work, however, shows a different situation since we observed a change of susceptibility to benznidazole in isolates of the Be-78 strain considered 100% susceptible (Toledo et al. 1995). Mice inoculated with the isolates (Be-78 C and D) from dogs infected for seven and two years with Be-78 showed susceptibility of 50 and 70%, respectively. These results are difficult to be explained based on the selection of a subset of the population. If within the parental population a clone resistant to benznidazole was to be present, the parental Be-78 strain should present some degree of resistance to treatment. Moreover, correlations between the sensibility to benznidazole and nitifurtox and the genetic distance between *T. cruzi* stocks have been described in vitro (Revollo et al. 1988) and in vivo (Toledo et al. 2000). Also, Andrade et al. (1992) revealed 82% of coincidence between treatment results in patients and mice infected with same strains.

Considering that the genetic characteristic may be one of the factors that modulate the benznidazole resistance/sensibility process, the variation of benznidazole susceptibility observed, specially with Be-78 C, may reflect the genetic plasticity observed in *T. cruzi* demonstrated by several authors. Pacheco and Brito (1999) studying parasite population in mice revealed evidence that polymorphism in minicircles sequences can emerge during infection with a single clone of *T. cruzi*. MacDaniel and Dvorak (1993) also showed that under stress conditions, or selective pressures, chromosomes and minicircles shown evidences of genetic plasticity.
in *T. cruzi* and *Leishmania*. The authors postulate that errors in DNA synthesis or replication may occur. These errors could be lethal or produce organisms with either reduced or enhanced survival potential depending upon the influence of external modulating variables such as the environment. In *Plasmodium falciparum* the chromosome size polymorphism among different strains is caused by the acquisition and deletion of mainly repetitive elements in a subtelomeric position (Lanzer et al. 1994).

An alternative hypothesis to explain the variability in drug resistance in Be-78 C and D isolates is that drug-resistant mutants arise for genetic exchange. Two studies of multiple *T. cruzi* isolates from single localities have found some evidence of genetic exchange (Bogliolo et al. 1996, Carassco et al. 1996). Although clonal propagation may predominate in transmission cycles involving humans, genetic exchange in natural populations might emerge *T. cruzi* strains with new resultant biological properties, such as virulence and drug resistance.

In this study we also demonstrated variability of specific antibody production profiles in mice infected with *T. cruzi* populations with different patterns of resistance to benznidazole. The variation of antibody levels observed between mice treated and not cured, apparently related to the parasite resistance to benznidazole, seems to correlate with the decrease of parasitaemia. According to Marretto and Andrade (1994) initial treatment would eliminate the sensitive parasites, leading to a predominance of a resistant population. Our data suggest that the serological profile is related to the sensitive/resistant clone ratio in the population. In mice infected with a *T. cruzi* resistant strain, the suppression of the parasitaemia after treatment is slower and consequently the antigenic stimulation of antibody production is higher.

In this work we demonstrate the alteration in the benznidazole resistance/sensitivity characteristic in the populations isolated of dogs infected with Be-78 strains. However, the biochemical mechanism that underlines drug resistance in isolates of this parasite remains to be elucidated.

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Biological and Genetic variability of the Berenice Trypanosoma cruzi strain maintained for up to 7 years in dogs and after successive passages in mice

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Abstract
In our study, Trypanosoma cruzi populations isolated from dogs chronically infected during two and seven years were compared with the Berenice-78 T. cruzi parental strain to verify the occurrence of genetic variation during the long-term chronic
infection of the vertebrate-host and also, the factors that would influence the patterns of experimental infection in mice. Our results demonstrated significant differences even the Be-78 parental strain and T. cruzi subpopulations isolated from chronic dogs in relation to virulence and pathogenicity for mice. Parasites were submitted to molecular characterization using two strategies: isoenzyme and Randomly Amplified Polymorphic DNA profiles. This analysis showed two distinct groups that included: (1) the parental Berenice-78 and the isolates Berenice-78 A and Berenice-78 C, after 1 and 25 passages in mice, and Berenice-78 B and Berenice-78 D after 25 passages, and (2) the Berenice-78 B and Berenice-78 D isolates, after a single passage in mice. Our findings showed the presence of different subpopulations in Berenice-78 strain of T. cruzi and that maintenance of the parasite in the dog or mouse may or may not select T. cruzi subpopulations.

Index Descriptors and Abbreviations: Trypanosoma cruzi; dogs; biological characterization; molecular characterization; isoenzyme; randomly amplified polymorphic DNA (RAPD); Liver Infusion Triptose (LIT); Phosphate Buffered Saline (PBS).
1. Introduction

The diverse clinical outcome of human *T. cruzi* infection has been attributed to the genetically heterogeneity of parasite populations. Studies using cloned and uncloned *T. cruzi* stocks reinforce the genetic and biological heterogeneity of this parasite. Also they led to the hypothesis that *T. cruzi*, in nature, exhibit a complex and heterogeneous population (Morel et al., 1986; Finley and Dvorak, 1987; Marques de Araújo and Chiari, 1988; Lauria-Pires et al., 1996; Macedo and Pena, 1998). New concepts have been introduced to elucidate the complexity of the dynamics of *T. cruzi* populations. Tibayrenc and Ayala (1988) proposed that *T. cruzi* natural populations have a complex multiclonal structure. Thus, the characteristics of one strain maintained in laboratory after a long period would be the result of the interaction of behaviors of several clones (Lambrecht, 1965). In this case, if one considers that amastigotes and epimastigotes derived from clonal lines exhibit differences in the pre-replicative lag period, amastigote doubling time and duration of the complete intracellular cycle *in vitro* (Engel et al., 1985), the experimental conditions used to maintain *T. cruzi* strains may result in a selective advantage; in addition, it can benefit the best adapted clones and their expression would be perpetuated in relation to the other subpopulations present.

In this context, the long interaction of multiclonal populations with the vertebrate host can determine alterations in the patterns of interactions within the swarms of clones, especially by the action of host defense mechanisms. In addition, experimental evidences have demonstrated that strains isolated from patients in the acute phase of Chagas disease showed more complex genetic composition than those obtained during chronic phase (Macedo and Pena, 1998).

Lana and Chiari (1986) using the murine model demonstrated a lower virulence pattern for the Berenice-78 strain, isolated from the patient Berenice, in relation to the Berenice-62 strain, isolated from the same patient 16 years before. In addition, Veloso et al. (2001) observed alterations in the benznidazole susceptibility among *T. cruzi* subpopulations isolated from dogs infected with the Berenice-78 strain during the chronic phase of the disease. These changes observed in biological behavior may reflect changes in the population dynamics of the parental strain or the genetic plasticity observed in *T. cruzi*. To confirm this hypothesis, we characterized in the present work four *T. cruzi* isolates obtained from the Berenice-78 *T. cruzi* strain maintained during two and seven years in dogs and after the 1st and 25th passages in mice. Parasites isolated from mice were submitted to biological and molecular characterization.
2. Materials and Methods

2.1. T. cruzi stocks

Berenice-78 parental strain (Be-78) - T. cruzi II (Lana and Chiari, 1986) and four isolates obtained from chronic chagasic outbred dogs infected with the parental strain during 2 (Be-78D) and 7 (Be-78 A, B and C) years were analyzed. Information on these stocks is presented in table 1. Immediately after isolation from the dog, parasites were inoculated in mice and maintained through successive passages. After the 1st and 25th passages, mice were submitted to hemoculture (Filardi and Brener, 1987). All T. cruzi samples were grown in LIT medium (Camargo, 1964) at 28°C, washed 3 times in PBS, pH 7.4, by centrifugation for 10 minutes at 1,500 x g, at 4°C and the parasite pellets were stored at -70°C until needed for preparation of enzymatic extracts and DNA.

2.2. Mice infections and parasitemia curves

Groups of six outbred male Swiss albino mice of 18-21g, were inoculated intraperitoneally with 5 x 10^3 bloodstream trypomastigotes from the Be-78 T. cruzi strain and with each T. cruzi subpopulation (Be-78 A, B, C and D) isolated from dogs that had been maintained for 1, 15 and 25 blood passages in mice. Parasitemia was followed from the 4th day of infection and then every other day until negativation was observed. Fresh blood was collected from the mouse’s tail and the number of parasites was estimated as described by Brener (1962). Curves were plotted using the mean of the parasitemia of six mice. Mortality rate was expressed as a percentage of accumulated deaths within the period of 100 days after inoculation.

2.3. Histopathological evaluation

Groups of six mice inoculated with Be-78 parental T. cruzi strain or with the four T. cruzi subpopulations (Be-78 A, B, C and D) isolated from dogs after 25 passages in mice were sacrificed. Three mice were sacrificed during the acute (20 days of infection) and three in the chronic (100 days of infection) phase. Fragments of brain, heart, spleen, liver and skeletal muscle were fixed in 10% formalin, embedded in paraffin, sectioned (5 µm thick), stained with hematoxylin-eosin and examined by light microscopy. Sections were evaluated with regard to the presence and intensity of the inflammatory process, according to the following criteria: + = discrete, inflammatory cells isolated or in small foci; ++ = moderate, multifocal inflammatory cell infiltrates (6-15) or diffuse infiltrates in small areas; +++ = accentuated, multifocal inflammatory cell infiltrates (15-20) or diffuse infiltrates in most of the tissue section; ++++ = intense, multifocal inflammatory cell infiltrates (more than 20) or diffuse infiltrates in most of the tissue section. Tissue parasitism was examined in all microscopic fields and characterized as: + = discreet, single parasite nests; ++ = moderate, 5-15 nests; +++ = accentuated, 16-50 nests; ++++ = intense, more than 50 nests.

2.4. Isoenzyme patterns

Isoenzyme patterns were obtained as described by Carneiro et al. (1990). The following 6 enzymes were analyzed: alanine aminotransferase (ALAT) [E.C.2.6.1.2]; aspartate aminotransferase (ASAT) [E.C.2.6.1.1]; glucose phosphate isomerase (GPI) [E.C.5.3.1.9]; phosphoglucomutase (PGM) [E.C.2.7.5.1]; glucose-6-phosphate dehydrogenase (G6PD) [E.C.1.1.1.49] and malic enzyme (ME)
Standard *T. cruzi* zymodemes Z1, Z2, ZB and ZC were used for reference (Miles et al., 1977; Romanha et al., 1979).

2.5. DNA preparation and Randomly amplified polymorphic DNA patterns

Isolation, amplification and electrophoresis of the parasite DNA was essentially as described previously (Steindel et al., 1993). Briefly, parasite pellet was resuspended in 50 mM Tris-HCl/ 50 mM EDTA/ 100 mM NaCl/ 0.5% SDS, pH 8.0 (extraction buffer) and incubated with 20 µg ml⁻¹ proteinase K for 2 hr at 45°C. After phenol/chloroform extraction and ethanol precipitation DNA was resuspended in 10 mM Tris-HCl/ 1 mM EDTA pH 8.0 (TE buffer). DNA concentration was determined after agarose gel electrophoresis, followed by staining with ethidium bromide by comparison with known standards.

Amplification reactions were done in a thermocycler (MJR Research Inc. - PTC 100), in a final volume of 10 µl containing 0.5 Units of *Taq* DNA polymerase (Phoneutria, Belo Horizonte, Brazil)/ 200 µM of each dNTP/ 1.5 µM MgCl2/ 50 mM KCl/ 10 mM Tris-HCl, pH 8.5 and 12.8 pmol each primer and 1.0 ng of template DNA. Five random primers, 3302 (5′-CTGATGCTAC 3′), 3303 (5′-TCACGATGCA 3′), 3304 (5′-GACTGTCA-3′), 3307 (5′-AGTGCTACCC 3′) and λgt111F (5′-GGTGGCGACGACTCTGGAGCCCG 3′), were arbitrarily selected from the laboratory stocks. The reaction mixture was overlaid with 20 µl mineral oil and, following an initial denaturation step at 95°C for 5 min, it was subjected to: 2 cycles of 2 min at 30°C for annealing, 1 min at 72°C for extension and 30 sec at 95°C for denaturation, followed by 33 cycles in which the annealing temperature was altered to 40°C. In the final cycle, the extension step was for 5 min. After amplification, 3 µl of each reaction were mixed with 0.6 µl of 6x DNA sample buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) and subjected to electrophoresis through a 6% non-denaturing polyacrylamide gel. Gels were fixed with 10% ethanol/ 0.5% acetic acid for 20 min and DNA bands revealed by staining with 0.2% silver nitrate for 30 min and reduction with 0.75 M NaOH/ 0.1 M formaldehyde for 10 min as previously described (Santos et al., 1993).

2.6. Statistical analysis

Eight biological parameters were analyzed between different experimental groups: infectivity, mortality, pre-patent period, patent period, maximum parasitemia, parasitism and tissue lesions. Infectivity and mortality, determined by mouse survival rates, were compared using the chi-square test. Mean of prepatent, patent period, maximum parasitemia and day of maximum parasitemia were compared using Scheffe’s Test (Snedecor and Cochran, 1989). In order to compare levels of parasitemia for the animals in different groups Kolmogorov and Smirnov Test (Conover, 1980) were used. Chi-square analysis was used to compare mouse survival rates between different groups (Snedecor and Cochran, 1989). Differences were considered statistically significant when the *P* value was smaller than 0.05.

RAPD analysis was limited to PCR-amplified products between 0.3 and 2.5 Kb. A phenetic tree based on band sharing between all possible pairs in an analysis group was constructed using the Dice similarity coefficient (Dice, 1945). Data derived from this coefficient were collated into a matrix of similarity which was then used for Unweighted Pair Group Method Analysis (UPGMA) (Sneath and Sokal, 1962).
3. Results

3.1. Biological characterization

We have characterized biologically the Be-78 T. cruzi strain maintained for 2 and 7 years in dogs and after the 1st, 15th and 25th passages in mice, in relation to infectivity, parasitemia, mortality rate and histopathology in mice. Fig. 1 shows the representative parasitemia curves performed in mice inoculated with blood trypomastigote from the 25th passage in mice of Be-78 T. cruzi strain and the four subpopulations isolated from dogs infected with this strain during 2 (Be-78D) and 7 years (Be-78 A, B and C). Two parasitemia profiles were registered: the Be-78 parental strain and Be-78 C showed parasitemia curves with two distinct peaks and very high parasitemia levels in relation to the other subpopulations studied. The Be-78 A, B and D produced curves showing a slow increase in parasitemia, with irregular and not very high peaks. Comparative analysis between the areas of the curves showed that mice infected with Be-78C exhibited very high parasitemia levels, similar to the parental strain (Be-78). In contrast, all animals infected with the parental strain exhibited parasitemia levels significantly higher in relation to the ones inoculated with the other subpopulations. Be-78 A and Be-78 B showed intermediary parasitemia levels and Be-78 D, the lowest parasitemia levels, significantly lower than the parental strain (P<0.01). All mice infected with isolate Be-78 D showed levels of circulating trypomastigotes significantly lower in relation to the ones inoculated with the other T. cruzi subpopulations studied.

No changes in the pattern of parasitemia occurred during 25 successive passages in mice carried out during approximately 12 months. However, a discreet tendency for increase in the parasitemia was observed in mice inoculated with blood trypomastigotes during passage in mice, for all isolates (data not shown).

Table 2 summarizes the means and the standard deviations of biological parameters analyzed in mice inoculated with blood trypomastigotes in the 1st, 15th and 25th passages in mice of the parental T. cruzi strain and the four isolates. All mice infected with Be-78 and Be-78 C showed a short pre-patent period (6 to 7 days), and similar and higher parasitemia levels. On the other hand, a significantly larger pre-patent period and a shorter parasitemia levels were observed in mice infected with the isolates Be-78 A, B and D (P<0.001). No mortality was observed in animals inoculated with Be-78 and Be-78 D. Animals inoculated with the other isolates showed mortality rates varying from 3 (Be-78 A and D) to 11% (Be-78 C).

Histopathological studies revealed mononuclear inflammatory infiltrates and amastigotes forms of T. cruzi associated or not with the inflammatory infiltrate in acutely infected animals. Both inflammatory processes and parasites occur mainly in the heart and skeletal muscle, being absent in the brain, liver and spleen. A similar tissular tropism was observed for the parental strain and its isolates. On the other hand, variation in the intensity of the inflammatory process and parasitism was observed in animals inoculated with different populations of T. cruzi. Infiltrate and parasitism were found to be intense in animals infected with Be-78 C, accentuated among the ones infected with parental strain and isolates Be-78 A and Be-78 B, and moderated in those inoculated with Be-78 D (Table 2, Fig. 2).

During the chronic phase a discreet inflammatory process and parasitism were found, being more evident among animals infected with Be-78 C, followed by those inoculated with the parental strain and isolates Be-78 A and Be-78 B. In animals inoculated with Be-78 D inflammatory process and parasitism were extremely rare or
absentee (Table 2, Fig. 3). Discrete foci of fibrosis were observed in heart muscle of animals infected with the parental strain and isolate Be-78 C.

3.2. Molecular characterization

The genetic variability of the Be-78 T. cruzi strain maintained for 2 and 7 years in dogs and afterwards, for 1 (Be-78A1, B1, C1 and D1) and 25 (Be-78 A25, B25, C25 and D25) passages in mice was evaluated by isoenzyme and RAPD analyses. Fig. 4 shows the results of one representative enzyme (GPI) and Table 3 summarizes the results for all of the enzymes. Immediately after the isolation from dogs two isolates (Be-78A1 and Be-78C1) showed the same isoenzyme profile and two, (Be-78 B1 and Be-78 D1), had isoenzyme profile different from that of the parental strain. However, after 25 passages in mice isolates Be-78 A25, Be-78 B25, and Be-78 D25 had their isoenzyme profiles changed. Two of them showed isozyme profile similar to that of the parental strain. The isoenzyme profile changes were gradual and reversible. In several occasions the simultaneous presence of two zymodemes could be observed. Zymodemes 22, 2C and 2Z were identified in most of the isolates, in higher or lower proportion, in different periods of maintenance and by type of host.

Complex RAPD profiles were obtained for the five different primers (3302, 3303, 3304, 3307 and λgt11R). Fig. 5 shows the RAPD profiles of Be-78 T. cruzi strain and four subpopulations maintained in dogs and after 1 and 25 passages in mice, amplified with primer 3303. Eighty-two bands obtained with the five primers were used for band sharing analysis. The bands were chosen based on their resolution, reproducibility in separate amplification reactions and intensity. A matrix of Dice similarity coefficients, based on a pairwise band sharing was calculated for the samples allowing the construction of an UPGMA dendrogram (Fig. 6). The dendrogram shows two distinct groups. Group I includes the Be-78 parental strain and the isolates Be-78 A1, B1 and Be-78 C1, 25, after the 1st and 25th passage in mice and Be-78 B25 and Be-78 D25, after 25 successive passages in mice. Group II includes the isolates Be-78 B1 and Be-78 D1, after a single passage in mice, being these isolated quite distinct from the parental strain. Interestingly, the 25 passages in mice, reverted their isoenzyme and RAPD profiles to that of the parental strain. There was a direct relationship between RAPD analysis and isoenzyme patterns.
4. Discussion

The existence of biological and genetic variability in T. cruzi populations have been clearly demonstrated. Several studies have attempted to establish a relationship between different sources of T. cruzi isolates and the clinical manifestations of Chagas disease (Oliveira et al., 1997; Espinoza et al., 1998). Despite these studies, no clear correlation between a parasite strain, defined by stable biochemical and physiological markers, and a particular feature of the disease, has been demonstrated (Carneiro et al., 1991; Lauria-Pires and Teixeira, 1996). On the other hand, studies using cloned T. cruzi populations have reported correlations between genetic characteristic and in vitro biological behavior as well as in vertebrate models (Revollo et al., 1998; Lana et al., 1998; Toledo et al., 2002). However, Fernandes et al. (1998) demonstrated that a clear correlation could be made with the morbidity of the Chagas disease: areas with high morbidity present the circulation of T. cruzi II while T. cruzi I is evidenced in areas where Chagas disease is infrequent, and morbidity, evaluated by the level of abnormal electrocardiograms, is low.

In our study, T. cruzi populations isolated from dogs chronically infected during 2 and 7 years were compared with the T. cruzi parental strain to verify the occurrence of genetic variation during the long-term chronic infection of the vertebrate-host and also, the factors that would influence the patterns of experimental infection in mice.

Our results demonstrated significant differences between the Be-78 parental strain and T. cruzi subpopulations isolated from chronic dogs in relation to parasitemia, virulence and pathogenicity. Differences were constant in the majority of the parameters examined: (a) Be-78 C showed the highest virulence, similar to the parental strain; (b) Be-78 A and Be-78 B, moderate virulence, (c) Be-78 D, low virulence. On the other hand, animals infected with isolates Be-78 A and B exhibited tissue parasitism and lesions similar to parental strain, being observed lesions lightly larger in the animals infected with the isolated Be-78 C and smaller in those infected with the isolated Be-78 D. These results can be related with the evaluation period, because whereas the animals infected with the parental strain showed maximum of parasitemia 13 day after inoculation, the mice infected with all isolated exhibited maximum parasitemia around the 20th day of infection, period in which the histopathological evaluation was performed. Furthermore, the genetic diversity reported here can not be related with behavioral profiles (virulence and pathogenicity in mice). Moreover, mice infected with isolates Be-78 B, Be-78 C and Be-78 D displayed different spectra of susceptibility to benzidazole, with of cure rates of 100%, 50% and 70%, respectively (Veloso et al., 2001). Similar results were obtained by Lauria-Pires et al. (1996; 1997), in T. cruzi stocks isolated from a chagasic patient with heart disease and patients with megaesophagus. These authors demonstrated a great biological and genetic diversity among the clones derived from these isolates, and concluded that each chagasic patient was infected with several T. cruzi subpopulations and that clonal lineages derived from each stock can have different biological characteristics from the original one.

The correlation between the T. cruzi genetic population and their biological characteristics, such as virulence and pathogenicity, has been investigated. Andrade and Magalhães (1997) studying 138 T. cruzi stocks isolated from various hosts and several geographic areas of Latin America classified the stocks into three groups called "biodemes" which were correlated with "zymodemes". In this study, this correlation was not verified probably because the subpopulations studied were originated from the same T. cruzi strain. The biological variability observed could be interpreted as a consequence of the phenotypic alterations induced by maintenance of the parasite in
different host species. In addition, the four T. cruzi populations used in this study were isolated from dogs with different clinical forms of the disease (indeterminate and cardiac), although inoculated with the same T. cruzi strain. In two out of four experiments, dogs were from the same litter (dogs A and B) and were inoculated with the same inoculum (Table 1). Furthermore, subpopulation Be-78 A, isolated from a dog with chronic chagasic cardiopathy was significantly less virulent for mice compared to Be-78 C, isolated from the animal with indeterminate form of the disease. These results reinforce the influence of specific genetic host factors in determining the host immune response and susceptibility to T. cruzi infection and the degrees of tissue lesions.

The question that emerges is related to the host-parasite relationship. According to Tibayrenc and Ayala (1988), T. cruzi has a clonal population structure; infecting strains are often multiclonal and natural selection favors only certain genetic patterns or combinations. Several authors observed a correlation between biological and phylogenetic divergence of T. cruzi using several in vitro and in vivo parameters (Revollo et al., 1998; Lana et al., 1998; Toledo et al., 2002). These findings indicate that circulating populations of T. cruzi can be composed of clones with different biological behaviors, sharing different virulence factors or striving for selective advantages such as distinct tropisms and pathogenicity. Consequently, genetic characteristics of such clones are relevant to the pathogenesis of Chagas disease. In addition, when Andrade et al. (1999), inoculated mice simultaneously with two different T. cruzi populations they observed a clear differential tissue distribution for the two populations, indicating a significant influence of genetic polymorphism of infecting T. cruzi populations in the pathogenesis of chronic Chagas disease. Interestingly, Gomes et al. (1998) discuss that the human host selects specific varieties of T. cruzi clones from presumably mixed infective parasite populations. Our data agree with this hypothesis, demonstrating that T. cruzi populations isolated from different dogs chronically infected with the Be-78 parental strain showed distinct biological behavior and genetic characteristics.

Our results suggest that changes in zymodeme and RAPD profiles may be a consequence of the parasite persistence in dogs, because a genetic profile distinct from that of the parental strain emerged in two populations (Be-78 B and Be-78 D). Interestingly, after 25 passages in mice populations Be-78 (A, B, C and D) and the parental strain became in a group very genetically close, in spite of the differences in virulence and pathogenicity along the successive passage in mice. These results could reflect the dynamic populational changes of this strain, or the emergence of a mutant population, less or more pathogenic, that eventually becomes dominant in the population, since Pacheco and Brito (1999) suggested the evidence of genetic plasticity in T. cruzi. Besides, RAPD profiles proved to be stable even after prolonged parasitic cultivation, up to 100 generations of a T. cruzi clone, indicating that, under rigidly controlled conditions, RAPD can have good reproducibility (Zingales et al., 1997).

Our results demonstrate the presence of different T. cruzi subpopulations in Be-78 strain. The simultaneous presence of two Zymodemes, in similar proportions or not, corroborates for this hypothesis. These results could be explained by the oscillation in the proportion of the two subpopulations, the amplitude of the oscillation decrease and an eventual replacement by the faster growing parasite occur. One possible explanation for the coexistence of the diverse growth rate is that different selective pressure exists during the chronic phase of the infection in dogs or during the acute infection in mice. This fact can be explained by changes in isoenzyme profiles of Be-78 B and D isolates after 25 passages in mice, when a distinct profile, although similar to the parental strain, emerged during maintenance in mice. Andrews et al. (1999) using isoenzyme profile
also distinguished clones in a multiclonal parasite population. In addition, it has been demonstrated by Lauria-Pires et al. (1996) that isoenzyme characterization indicates mixed infection for *T. cruzi*. Curiously, although changes of isoenzymatic and RAPD profiles were observed during maintenance of these subpopulations after 25 successive blood passages in mice, biological characteristics remained the same. Similar results were shown by Carneiro et al. (1991) who have demonstrated that parasitemia curves were not altered throughout passages in mice carried out during about eighteen months.

In our study, differences observed in the biological behavior of *T. cruzi* populations isolated from different dogs infected with the *T. cruzi* Be-78 parental strain are probably related to the interaction of the host with its *T. cruzi* population. This interaction could induce a new populational dynamics or for an output of alternative proteins or virulence factor, which could explain the alterations in virulence, pathogenicity or drug resistance in *T. cruzi* populations lated from different dogs chronically infected with the same *T. cruzi* parental strain.
Acknowledgments

This work was supported by grants from FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) and UFOP (Universidade Federal de Ouro Preto), Brasil.
B-U-21 - TRYPANOSOMA CRUZI - BIOLOGICAL AND HISTOPATHOLOGICAL VARIABILITY OF POPULATIONS ISOLATED FROM VERTEBRATE HOSTS AFTER 2 AND 7 YEARS OF INFECTION

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T. cruzi presents a considerable biological and biochemical variability, as verified by various experimental studies. Clonal structure has been proposed to explain these differences (Tibayrenc et al., 1988). The passage of the multiclone population of T. cruzi through the vertebrate host may cause the elimination of some clones due to their inability to compete and propagate in the host environment (Machado & Pena, 1988). To verify this phenomenon, comparative studies were performed using Be-78 strain and four isolates from dogs infected with this strain seven (Be-78-A, B and C) and two (Be-78-D) years ago. Biologic parameters (infectivity, parasitemia, mortality and histopathology) were comparatively evaluated in Swiss mice inoculated through the intraperitoneal route with 5000 blood trypomastigotes of the 15th, 20th and 25th passages in mice of the isolates as well as the original Be-78 strain. Parasitemia was evaluated daily according to Brener (1962). Histopathological studies were carried out during the peak of parasitemia (acute phase) and 120th day of infection (chronic phase). Necropsy was complete with systematic collection of the heart, brain and digestive and genito-urinary tracts. During the acute phase, inflammatory infiltrates composed predominantly of mononuclear cells associated or not with intact or disrupted amastigote nests (pseudocysts) were observed. These findings were most striking in the heart, followed by smooth and skeletal muscles, digestive and genito-urinary tracts. Parasitemia and inflammation were not observed in the brain. There was no difference in tissue tropism between Be-78 strain and the isolates (Be-78 A, B, C and D). All lesions observed during acute and chronic phase were more intense in animals infected with Be-78C isolate, compared to Be-78 strain. The Be-78 A and B isolates caused lesions with intermediate intensity, similar to Be-78 strain. All animals infected with Be-78D showed more discrete lesions than animals infected with Be-78 strain. The data agree with parasitologic results displayed in the following table. These results suggest that T. cruzi undergoes changes in or selection throughout the infection in vertebrate hosts. This selection could depend on the host-parasite relationship. Further biochemical and genetic characterization of the four populations will be done.

<table>
<thead>
<tr>
<th>strain</th>
<th>pre-patent period (day after inoculation)</th>
<th>peak of parasitemia (trypanomastigotes/0.1 ml de blood)</th>
<th>mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Be-78</td>
<td>50</td>
<td>80249</td>
<td>0%</td>
</tr>
<tr>
<td>Be-78A</td>
<td>10</td>
<td>40608</td>
<td>3.03%</td>
</tr>
<tr>
<td>Be-78B</td>
<td>8</td>
<td>47694</td>
<td>3.33%</td>
</tr>
<tr>
<td>Be-78C</td>
<td>6</td>
<td>168910</td>
<td>13.8%</td>
</tr>
<tr>
<td>Be-78D</td>
<td>9</td>
<td>27944</td>
<td>0%</td>
</tr>
</tbody>
</table>

Supported by FAPESP and UFOP

B-U-22 - ECOLOGY OF TRYPANOSOMA CRUZI TRANSMISSION CYCLE IN THE SYLVATIC ENVIRONMENT: STUDY OF INFECTION IN LEONTOPITHECUS ROSALIA AND CALLITHRIX JACCHUS (PRIMATA: CALLITHRICIDAE)

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Previous studies on the ecology of the Trypanosoma cruzi transmission in different ecosystems fragments, have shown that distinct and simultaneous transmission cycles may occur. It's worthy emphasizing that the barriers which separate these cycles are not determined by the edge of the forest layer that the reservoir or vector occupy. These findings show that the wild transmission cycle of the T. cruzi is much more complex than it has been described and also they call attention to the mistakes that may be caused by epidemiological conclusions based on secondary data. The studies at the Poço das Antas Biological Reserve, Silva Jardim, put the question very clearly: in this fragment of the Rain Forest, the opossum (Didelphis marsupialis), widely considered as the most important reservoir for the T. cruzi was the mammal that presented the lowest prevalence, even lower than the one of the rodents, animals that are not normally considered to be reservoirs. In this area, the principal reservoir for the T. cruzi is the golden lion tamarin (Leontopithecus rosalia). This species shows high infection rates (50%) and still is infected with a subpopulation of the parasite associated to human cases of the Chagas' disease, T. cruzi H. Golden lion tamarins infected by T. cruzi have also been observed in the farms that surround the reserve and in one of them we found a particularly intriguing situation: in the Rio Vermelho farm, where many groups of golden lion tamarins live associated with Callithrix jacobini, the infection of the T. cruzi has been observed in six specimens of the later species and in none specimens of the L. rosalia. It's known that C. jacobini live in sympatry with the golden lion and behaviour data show that these animals share, in some cases, almost half of their time together. Is it that some behavior patterns of these animals are influencing the transmission and maintenance of the parasite in this area? What are the associations between these two sympatrical species? These aspects should be taken into account not only in relation to T. cruzi, but also in relation to other parasitic diseases in general, principally in species that are subordinate to any type of handling.

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occupation, demographic pressure and increase in the poverty level, as people move outwards to previously endemic area for L. chagasi. Finally, the ecological aspects determined in this current study are extremely relevant for the design of control measures for leishmaniasis, although there is still a lack of a complete comprehension of the multiple and complex factors involved in the transmission of Leishmania in Rio Grande do Norte.

Support: Conselho Nacional de Pesquisa (CNPq) and National Institutes of Health (NIH)

BU70 - GENETIC VARIABILITY OF THE BERENICE TRYPANOSOMA CRUZI STRAIN MAINTAINED FOR UP TO 10 YEARS IN DOGS AND AFTERWARDS FOR 25 PASSAGES IN MICE

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The genetic variability of the Berenice T. cruzi strain maintained for 2 to 10 years in dogs and afterwards for 25 passages in mice was evaluated. The Berenice T. cruzi strain (Be78) and five isolates obtained from outbred dogs infected with this strain during 2 (Be78-D), 7 (Be78 A, B and C) and 10 (Be78-E) years were analyzed. Immediately after isolation from dog, the parasites were inoculated in mice and analyzed after 1 and 25 passages. The parasites were isolated and used for the preparation of enzymatic extract and DNA. The parasite samples were characterized using the isoenzyme profiles of 5 different enzymes (GPI, PGII, G6PD, ASAT and ALAT) and the Randomly Amplified Polymorphic DNA (RAPD) profiles obtained with 5 different primers (3302, 3303, 3304, 3307 and LGT11R). The maintenance in dog showed three isolates with the same and two with a different isoenzyme profile than that of the parental strain. The further 25 passages in mice changed the isoenzyme profile of 3 out of 4 isolates. The isoenzyme changes were gradual and reversible. In several occasions the simultaneous presence of two zymodemes could be observed. Complex RAPD profiles were obtained for the five primers. A matrix of Dice similarity coefficients, based on a pairwise band sharing (n=110 bands) was calculated for the samples allowing the construction of an UPGMA dendrogram. The dendrogram showed two distinct groups. Group I included the parental Be78 and the isolates Be78-A and Be78-C, after 1 and 25 passages in mice and Be78-B and Be78-D after 25 passages. Group II included the Be78-B and Be78-D isolates, after a single passage in mice. These isolates were quite distinct from the parental strain. Interestingly, the 25 passages in mice, returned their isoenzyme and the RAPD profiles to that of the parental strain. No relationship was observed with change in isoenzyme or RAPD profiles with either the time of maintenance or the kind of host. Our findings showed the presence of different subpopulations in Be78 strain of T. cruzi and that the maintenance of the parasite in dog or mouse may or may not select T. cruzi subpopulation.

Supported by: FAPEMIG, FIOCRUZ, CNPq and UFOP.

BU71 - DOUBLE INFECTION WITH DIFFERENT POPULATIONS OF TRYPANOSOMA CRUZI IN RAT—GENETIC CHARACTERIZATION OF PARASITES AND HISTOPATHOLOGICAL ANALYSIS IN DISTINCT ORGANS

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Previous studies have shown that in rats infected with JG strain (10,000 trypomastigotes) the mortality is null, the parasitemia very low and the myocarditis diffuse and discrete with intense foci. In contrast, the CL-Brener clone (1,000 trypomastigotes) provokes high mortality (70%), higher parasitemia and more severe myocarditis. Now we tested the effect of double infection in male Holtzman rats inoculated with: 10,000 trypomastigotes of JG strain + 10,000 trypomastigotes of CL-Brener clone; or 10,000 parasites of JG strain + 1,000 parasites of CL-Brener clone. The inoculation of 10,000 trypomastigotes of both isolates induced three patterns of parasitemia (low, moderate and high) and caused the death of all infected animals during the acute phase, as observed in the single CL-Brener infection. However the 10,000 + 1,000 inocula provoked parasitemic patterns (low and moderate) characteristic of the CL-Brener with 1,000 parasites, but the mortality was null, as described for the JG infection. The genetic characterization of parasites present in different organs during the acute phase showed LSSP-PCR profiles characteristic of JG strain in the hearts of all double-infected rats (10,000 of both isolates or 10,000 JG + 1,000 CL-Brener). The hearts of the rats infected with 10,000 parasites of both isolates displayed inflammatory process similar to that observed in animals infected only with the CL-Brener clone. In contrast, the double infection with 10,000 + 1000 parasites induced cardiac inflammation characteristic of JG strain. The LSSP-PCR showed a multiband pattern similar to the
and heart toxicity by the later. The absence of toxicity of oral Kp was confirmed in mice receiving a total of 480 mg Kp (dry weight) in 30 days in contrast to significantly high enzyme serum levels in animals receiving a total of 120 mg.i.p. Glucantime (32.4 mg Sb). This case report suggests for the first time that the effectiveness of oral Kp seen in mice may be extended to man with acute cutaneous leishmaniasis.

**CT35 - EXPERIMENTAL CHEMOTHERAPY IN DOGS: SEROLOGICAL EVOLUTION OF ANIMALS AFTER LONG-TERM FOLLOW-UP**

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The difficulty to shown the *Trypanosoma cruzi* in the organism associated with the lack of a gold standard laboratory method and/or a clinical parameter which would assure the presence or absence of the parasite, have hampered the evaluation of the efficacy of therapeutical agents. The purpose of this work was to evaluate dogs as experimental model in the therapeutics of Chagas’ disease as well the methodology of criterion of cure used in humans. A group of 22 dogs of indeterminate breed were inoculated with 2000 blood trypanosomes/k.w. and treated with Benznidazole (Bz) during the acute (AP) and chronic (CP) phases. The drug was administered during 45 consecutive days with dose of 7mg/kg b.w/day, fractional in two doses daily. Six dogs were inoculated with the Colombian strain (*T. cruzi* I), six with Be-78 strain (*T. cruzi* II) of these eight were treated (AP), and 4 left as untreated controls. Ten dogs were inoculated with Be-78 strain, eight of them were treated 120 days after inoculation and two left as controls non-treated. Evaluation of therapeutic results was performed using parasitological tests (fresh blood examination, hemoculture and PCR) and serological tests (ELISA and LMCo). Animals were considered cured when both serological and parasitological tests remained persistently negative. The percentage of cure induced by Bz during the AP was 75% and 0% for dogs infected with Be-78 and Colombian strains, respectively. Treatment during CP induced a percentage of cure of 37.5% in dogs inoculated with Be-78. Hemoculture were positive 37.5% (AP) and 0% (CP) of the animals treated non-cured (TNC). The PCR reaction was persistently positive in all TNC dogs. The results of ELISA and LMCo were in agreement with the parasitological tests. In animals treated during AP the antibody levels were similar between TC and TNC animals three months after treatment. Afterwards the levels of antibodies in the TNC animals were similar to those observed in the infected controls. In contrast, TC dogs during CP presented serological tests positive (ELISA and LMCo) until the 18th and 25th month after treatment. All animals that presented higher antibodies levels before treatment showed an abrupt decrease after treatment. Three months after treatment the absorbancy (ELISA) was 0.300, 0.400 and 0.900nm in TC, TNC and C, respectively. This difference persisted until the 24th month after treatment with absorbancy 0.200, 0.300 and 0.600nm in TC, TNC and C, respectively. Stimulation indexes from proliferation of *T. cruzi* antigens stimulated peripheral blood mononuclear cells evaluated 2 years after treatment were also different in TC, TNC and control animals. These data indicate that the dogs can be useful as experimental model in the chemotherapy of the Chagas' disease and can be important for the evaluation of the methods used in control of cure of this disease.

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**CT36 - SUSCEPTIBILITY TO BENZNIDAZOLE OF POPULATIONS OF TRYPAansomA CRUZI ISOLATED AFTER 2 TO 17 YEARS OF INFECTION IN DOGS**

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The susceptibility to Benznidazole (Bz) has been used by different authors for the characterization of *T. cruzi* populations. In the present report, we investigated the susceptibility to Bz among: (i) the Be-78 strain (*T. cruzi* II) of *T. cruzi* and three populations isolated from dogs, infected with this strain after 7 (Be-78 B and C) and 2 (Be-78 D) years of infection; (ii) the Colombian strain (*T. cruzi* I) and two population isolated from dogs, previously inoculated with Col strain, after 8 and 17 years of infection (Col A and B). For the evaluation of the susceptibility to Bz groups of 12 Swiss mice were inoculated through the intraperitoneal route with 5000 blood trypanosomes. The population of *T. cruzi* used to infect each group were respectively: Be-78 strain, Be-78 (B, C and D), Col strain and Col (A and B). Each experimental group was divided into two subgroups of 6 animals; the first was treated with Bz and the second left untreated controls. Treatment started when the animals showed positive parasitaemia (between the 5th and 7th day of infection). The drug was administered during 20 consecutive days with doses of 100mg/kg b.w./day. 
The animals were considered cured when both parasitological (fresh blood examination haemoculture and PCR) and serological tests (ELISA) remained persistently negative. The parasitological tests were performed 3 - 6 months after the treatment and serum levels of IgG were determined by ELISA monthly until 200 days after treatment. Mice infected with Be-78 strain showed 100% cure rate after treatment with Bz. On the other hand 100%, 50% and 80% cure rates were observed in animals infected with the populations Be-78 B, C and D, respectively. All animals showed suppression of parasitaemia. In contrast, mice infected with Col strain and Col (A and B) presented 0% cure. In mice inoculated with Col strain and Col-A suppression of parasitaemia was observed whereas no suppression was detected for the Col-B isolate. All mice inoculated with Col strain and Col (A and B) presented patent parasitaemia after treatment. No significant differences were observed in serological response in infected controls, all animals presented higher antibodies levels. However, the ELISA test revealed differences in the serological pattern in mice inoculated with different population of *T. cruzi* and treated with Bz. The antibody levels remained permanently bellow the cut-off in treated cured animals infected with Be-78, and Be-78 (A, B and D). When *T. cruzi* specific IgG levels were measured in the sera of infected treated non-cured animals, three different profiles were observed when compared to untreated controls: i) in animals infected with Col-B, antibody levels were similar between treated and untreated animals throughout the infection; ii) antibody levels of animals infected with Col-A were bellow the cut-off limit between days 30 and 60 of treatment, raising to the control levels thereafter; iii) antibody levels of animals infected remained bellow the cut-off until day 40 of treatment and raised slowly afterwards but not reaching the levels of their untreated controls (Be-78 C and Be-78 D). These observations show that susceptibility to Bz varied among the parental Be-78 strain and its different isolates. They also suggest that the serological profile can vary in treated and untreated animals depending on the parasite inoculated and this profile may be associated with the level of resistance to Bz treatment.

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IM38 - EFFECTS OF CYCLOOXYGENASE INHIBITORS TREATMENT IN MURINE MODEL OF TREPONEMA CRIWI INFECTION

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Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely employed for the treatment of acute and chronic inflammatory diseases. The mechanism of action of NSAIDs has been shown to result from blockade of prostaglandin production by inhibition of cyclooxygenase (COX). In these studies we examined the effect of different NSAIDs on the course of infection in mice with Treponema cruzi, the etiologic agent of Chagas' disease. C57BL/6 and Swiss mice were infected intraperitoneally with 5 x 10^7 blood trypomastigotes of T. cruzi (Y strain). Since the 5th day of infection, parasitemic and survival levels were observed. At days 7 and 14 post infection, 5 animals of each group were sacrificed for hematological analysis. Treatment with classes II NSAIDs (indomethacin, L2mg/kg) or class III NSAIDs (aspirin, 15 mg/kg) enhanced mortality rates of C57BL/6 and Swiss mice. Aspirin unlike indomethacin provoked a reduction in the levels of parasitemia of the infected mice, but all the animals succumbed to infection. Furthermore, the treatment with the nonsteroidal anti-inflammatory drugs was associated with the worsening of the anemia observed in acute phase of T. cruzi infection. These results suggest that NSAIDs don't have a potential therapeutic application in the control of parasite replication in experimental Chagas' disease and their use in humans infected should be taken with caution.

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IM39 - PRELIMINARY EVIDENCE THAT WORMTHANN AFFECTS THE SURVIVAL OF TREPONEMA GONDI IN SHRINE ACTIVATED PERITONEAL MACROPHAGES BUT DOES NOT REGULATES INHIBITION OF NITRIC OXIDE PRODUCTION CAUSED BY THE PARASITE

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It has been shown that T. cruzi infection causes phosphorylation/deamination 3-kinase (PKA) activation and treatment of macrophages with wormthann strongly blocked parasite infection (Todorov et al, J. Biol. Chem. 275: 31826-2, 2000). Thus, we decided to investigate wormthann's effect on NO production in T. gondii and the effect of Trepontasma gondii within activated macrophages in relation to the nitric oxide (NO) production. For this, murine peritoneal macrophages were cultured over coverslips for 24 h in Dulbecco's Modified Eagle's Medium (DMEM) with 5% fetal bovine serum (FBS) supplemented with interferon-gamma (IFN) and Epoxydroacyclic (LPS) in the presence or absence of 20 nM of wormthann. Tachyzoitos, RH strain, were obtained by peritoneal washes of infected mice. Macrophages were infected with 10 to 1 T. gondii/macrophage ratio for 2 h, washed and cultured for 24 h with IFN and LPS in the presence or absence of wormthann was added again. After 24 and 48 h of interaction, the supernatants were collected for nitrate evaluation. Coverslips containing cells were collected after 2, 24 and 48 h of infection and an association index determined. Wormthann did not influence the invasion index of T. gondii but decreased survival of the parasite after 24 and 48 h. The inhibition of NO production by T. gondii infected macrophages (Seabaa et al, Exp Parasitol 100: 62-70, 2002) was not affected by wormthann. However, wormthann decreased NO production after 48 h of macrophage activation. These results suggest that the survival of T. gondii in activated macrophage depend of PKA.

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IM340 - ANTI-TREPONEMA GONDI IgG AVIDITY IN EXPERIMENTALLY INFECTED DOGS

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Trepontasma is a highly prevalent disease that affects a great variety of warm-blooded animal, including man. In dogs, the acute disease causes erythema, lymphadenectomy and fever, with systemic and ocular involvement. As dogs could be used as an environmental indicator of Trepontasma prevalence, we decide to study the evolution of IgG avidity antibodies in this species, allowing a better estimation of environmental risk of trepontasemia. We screened 6 dogs with approximately 6 months of age, without defined race, vaccinated and parasite free, by negative specific IgG Indirect Immunofluorescence Assays (IFA). These animals were inoculated with 2X10^8 cells of Y strain Trepontasma gondii (half I and half II), with one sham inoculated animal as control. The animals were maintained at the kennel of Veterinary Hospital/USP and observed by 6 months after infection. Injected animals presented characteristic symptoms in the first weeks, resolving the infection, without ocular involvement. Blood was collected weekly and used for anti-T. gondii IgG detection for IIF and blood count. All samples were also analyzed by ELISA and avidity determination, using a 8M urea washing. Antibodies were detected, for both methods, after the 30th day of infection. IIF gave ELISA was attempted but was negative in ICB and avidity normal, in the 10th and 180th days of infection. Blood monocytosis occurred at the 10th day of infection, similar to human disease, immune suppression with Azoospermia at the 10th 30th days of infection do not influence the reactivation of eye disease. Antibody titre by the assay was increased during the first 2 weeks of infection achieving a plateau at the 45th day of infection, but antibody maturation (100% avidity) was only achieved at day 60th of infection, presenting a linear correlation with infection time until this time. As expected, the avidity measurement of IgG antibodies in dogs was similar to that of humans and this test could be used to discriminate acute infections in those animals, allowing the determination of incidence of this disease in free living dogs, a good indicator of environmental contamination by Trepontasma gondii.

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UNIVERSIDADE FEDERAL DE OURO PRETO
NÚCLEO DE PESQUISAS EM CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

ANÁLISE DA VARIABILIDADE DE POPULAÇÕES DO Trypanosoma cruzi
ISOLADAS DE CÃES APÓS 2 A 17 ANOS DE INFECÇÃO

AUTORA: VANJA MARIA VELOSO

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Dissertação apresentada ao Programa de Pós-Graduação do Núcleo de Pesquisas em Ciências Biológicas do Instituto de Ciências Exatas e Biológicas da Universidade Federal de Ouro Preto, como parte integrante dos requisitos para obtenção do título de Mestre em Ciências Biológicas, área de concentração: Protozoologia Parasitária Humana.

Ouro Preto, outubro de 2000
Ata da Banca Examinadora de Dissertação de Mestrado Intitulada:

"Análise da Variabilidade de Populações do Trypanosoma cruzi Isoladas de Cães Após 2 a 17 anos de Infeção"

Aos 26 dias do mês de outubro de 2000, às 13:30h, na sala 10 da Diretoria do Instituto de Ciências Exatas e Biológicas da Universidade Federal de Ouro Preto, reuniu-se a Comissão Examinadora da Dissertação de Mestrado da aluna Vanja Maria Veloso. A defesa da dissertação iniciou-se pela apresentação oral feita pela candidata e, em seguida, arguição pelos membros da banca. Ao final, os membros da banca examinadora reuniram-se e decidiram por aprovar a candidata.

Membros da Banca Examinadora:

Prof. Drª. Maria Terezinha Bahia
presidente/Oríentadora (UFOP)

Prof. Dr. Egler Chiari
Examinador (UFMG)

Prof. Dr. Luís Carlos Crocco Afons
Examinador (UFOP)

DATA DA DEFESA: 26/10/2000
CARACTERIZAÇÃO DAS LESÕES TECIDUAIS E DO PERFIL DE CITOCINAS DO TIPO 1 INDUZIDAS POR DIFERENTES ISOLADOS DA CEPA BERENICE-78 DO *TRYPANOSOMA CRUZI*, DURANTE A FASE AGUDA DA INFECÇÃO

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Monografia apresentada ao Departamento de Ciências Biológicas do Instituto de Ciências Exatas e Biológicas da Universidade Federal de Ouro Preto como parte integrante dos requisitos para obtenção do título de Bacharel em Ciências Biológicas.

Ouro Preto, Setembro 2002
Universidade Federal de Ouro Preto
Instituto de Ciências Exatas e Biológicas
Departamento de Ciências Biológicas

CARACTERIZAÇÃO DAS LESÕES TECIDUAIS E DO PERFIL DE CITOCINAS DO TIPO 1 INDUZIDAS POR DIFERENTES ISOLADOS DA CEPA BERENICE-78 DO TRYPANOSOMA CRUZI, DURANTE A FASE AGUDA DA INFECÇÃO

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