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Genetic Characterization of *Trypanosoma cruzi* Directly from Tissues of Patients with Chronic Chagas Disease

Differential Distribution of Genetic Types into Diverse Organs

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We have previously shown that a low-stringency single-specific primer–polymerase chain reaction (LSSP-PCR) is a highly sensitive and reproducible technique for the genetic profiling of *Trypanosoma cruzi* parasites directly in tissues from infected animals and humans. By applying LSSP-PCR to the study of the variable region of kinetoplast minicircle from *T. cruzi*, the intraspecific polymorphism of the kinetoplast-deoxyribonucleic acid (kDNA) sequence can be translated into individual kDNA signatures. In the present article, we report on our success using the LSSP-PCR technique in profiling the *T. cruzi* parasites present in the hearts of 13 patients with chagasic cardiopathy and in the esophagi of four patients (three of them with chagasic megaesophagus). In two patients, one with the cardiodigestive clinical form of Chagas disease and the other with cardiopathy and an esophageal inflammatory process, we could study both heart and esophagus and we detected distinct kDNA signatures in the two organs. This provides evidence of a differential tissue distribution of genetically diverse *T. cruzi* populations in chronic Chagas disease, suggesting that the genetic variability of the parasite is one of the determining factors of the clinical form of the disease. *(Am J Pathol 2000, 156:1805–1809)*

Chagas disease, a parasitic infection caused by the protozoan *Trypanosoma cruzi*, affects ~20 million people throughout South and Central America.[1] Autochthonous cases are rare in the United States, where nonetheless the disease may represent a health concern because of increasing blood transmission by chronically-infected immigrants.[1] Chagas disease has a variable clinical course, ranging from symptomless infection to severe chronic disease with cardiovascular and/or gastrointestinal involvement. The heart pathology is characterized by myocarditis that frequently leads to the development of congestive heart failure and arrhythmia.[2] In the digestive forms of the disease, dilatation and muscular hypertrophy esophagus or colon (megaesophagus and megacolon, respectively) are observed in advanced stages.[3,4] Pathologically, both the heart and digestive tube exhibit focal lymphocytic infiltrates[5] along with scarring and loss of myocardial cells in the cardiopathy[6] and neuronal degeneration with denervation in megaesophagus and megacolon.[4,7] Parasites have rarely been found in tissues examined by routine staining techniques.[6,7] However, the use of sensitive molecular techniques such as PCR (polymerase chain reaction) has disclosed the presence of *T. cruzi* in involved tissues, but not healthy organs, from chronic patients.[8–10]

It remains unexplained why different patients develop cardiac, digestive, cardiodigestive, or indeterminate clinical forms. An intriguing observation is a peculiar differential geographic distribution of the organ involvement.[11] For example, in Chile the digestive form is the most frequent one, whereas in Argentina it only corresponds to 3.5% of the total. Epidemiological studies have shown variable distribution of the clinical forms even in different endemic regions inside Brazil, where overall the relative prevalence of the cardiac and digestive form are 20% to

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Table 1. Chagasic Tissue Samples Studied and Clinical Characteristics of Patients

Tissue	Patient	Age	Sex	Origin	Major clinical manifestations and pathological findings
Heart and esophagus	A29/E4	61	M	Araxá, MG	CCC, presenting heart enlargement with discreet CHF. Absence of megaesophagus; but presence of moderate myositis in the esophagus
Heart and esophagus	A36/E12	57	M	Uberaba, MG	CCC with megaesophagus grade IV
Heart	A30	66	M	Uberaba, MG	Sudden death. Asymptomatic patient with mild myocarditis. Absence of megasyndromes.
Heart	A33	47	F	Uberaba, MG	CCC with arrhythmia and CHF
Heart	A37	70	M	Bambui, MG	CCC and absence of megasyndromes
Heart	A38	42	M	Uberaba, MG	CCC with arrhythmia and discreet CHF. Absence of megasyndromes
Heart	A1	32	F	Berilo, MG	CCC with severe CHF (grade IV*). Absence of megasyndromes
Heart	A10	30	M	Três Marias, MG	CCC with severe CHF (grade IV*). Absence of megasyndromes
Heart	A11	41	F	Montes Claros, MG	CCC with severe CHF (grade IV*). Absence of megasyndromes
Heart	A48	36	M	Capelinha, MG	CCC with severe CHF (grade IV*). Absence of megasyndromes
Heart	A49	39	M	Capelinha, MG	CCC with severe CHF (grade IV*). Absence of megasyndromes
Heart	A50	26	M	Brumadinho, MG	CCC with severe CHF (grade IV*). Absence of megasyndromes
Heart	A51	33	M	Santo Antônio de Pirapitinga, MG	CCC with severe CHF (grade IV*). Absence of megasyndromes
Esophagus	E8	51	M	Araxá, MG	Megaesophagus grade III
Esophagus	E11	43	F	Iturama, MG	Megaesophagus grade IV

CCC, chronic chagasic cardiopathy; CHF, congestive heart failure; A, heart tissue; E, esophagus tissue.
*Classification according to the New York Heart Association.

30% and 8% to 10%, respectively, and the cardiodigestive form being rarely encountered.[11] This geographical heterogeneity suggests that genetic variation of the host, the parasite, or both, is important in establishing the clinical type of the disease.

We have concentrated our efforts in studying the possible effect of parasite genetic factors. For that, we have developed a sensitive DNA profiling technique called low-stringency single-specific primer–polymerase chain reaction (LSSP-PCR) that allowed us to achieve the genetic characterization of *T. cruzi* by the direct study of infected tissues.[12] The method was based on PCR amplification of the variable region of the kinetoplast-deoxyribonucleic acid (kDNA) from *T. cruzi*, afterward translating it by LSSP-PCR into a specific and highly reproducible "kDNA signature."[12] The rationale of this approach was that genetic profiling of kDNA could be used to infer variation in nuclear genes that probably are the most relevant for tissue infectivity, because *T. cruzi* exhibits extreme levels of linkage disequilibrium between different genomic compartments,[13] including between kDNA and nuclear genome.[12,14]

Our previous study demonstrated that kDNA signatures could be easily obtained by the direct analysis of tissues from experimentally-infected animals and also from one single chronic chagasic human patient.[12] In the present article, we wish to report our success in profiling with the LSSP-PCR technique the parasites present in the hearts of 13 patients with chagasic cardiopathy and in the esophagi of four patients (three of them with chagasic megaesophagus and the other without megaesophagus but presenting an inflammatory process in this organ). In two patients, one with the cardiodigestive clinical form of Chagas disease and the other with cardiopathy and an esophageal inflammatory process, we could study both heart and esophagus and detected distinct kDNA signatures in the two organs. This provides evidence of a differential tissue distribution of genetically diverse *T. cruzi* populations in chronic Chagas disease and suggests that the genetic variability of the parasite is one of the determining factors of the clinical form of Chagas disease.

Materials and Methods

Patients

Heart Samples

We analyzed heart tissue fragments (left ventricle) obtained from thirteen patients with chronic chagasic cardiopathy (Table 1). Six of these tissue samples (A29, A30, A33, A36, A37, and A38) originated from chagasic patients living in the endemic area of Uberaba, MG, Brazil and were obtained during autopsies at the Faculdade de Medicina do Triângulo Mineiro in Uberaba. Four samples (A1, A10, A11, and A48) were also obtained at autopsy of chagasic patients followed at the Cardiological Center of the Hospital Felício Rocho, Belo Horizonte, MG, Brazil. The other three heart samples (A49, A50, and A51) were obtained from hearts removed from patients during cardiac transplants also performed at the Surgical Center of the Hospital Felício Rocho, Belo Horizonte, MG, Brazil. In all cases informed consent was obtained from the patients or their families.

Two independent Ethical Committees approved this study. It fulfilled all the criteria required by the Medical Code of Ethics and the Helsinki II statement, required by both Committees. The analysis of human tissue samples, obtained by autopsy or surgery procedures, of the patients from an endemic area for Chagas disease near Uberaba, Minas Gerais, Brazil, was approved by the Ethical Committee of the Faculdade de Medicina do Triângulo Mineiro. The Ethical Committee of the Faculdade de Medicina da Universidade Federal De Minas Gerais

also approved the work with the human samples obtained by autopsy or cardiac transplants of the patients from the Hospital Felício Rocho, Minas Gerais, Brazil.

Esophagus Samples

Esophageal samples (E4, E12) were also taken from the two patients with cardiopathy described above (A29, A36), one of whom suffered from megaesophagus (A36/E12) whereas the other, although having no esophageal dilatation, displayed prominent inflammatory infiltration (A29/E4). In addition, tissue fragments were taken from the surgically resected esophagi of two chronic chagasic patients with megaesophagus (E8 and E11; Table 1). Informed consent was obtained from the patients or their families.

Processing of Tissues

Tissue fragments were processed for both PCR and histological analysis. Immediately after autopsy, surgery samples of $\sim 2 \times 5 \times 5$ mm were frozen in liquid nitrogen and maintained at −80°C until DNA extraction for PCR, which was performed by the alkaline lysis protocol as previously described.[12] For histological studies, samples were fixed in 4% phosphate-buffered formaldehyde and routinely processed for paraffin embedding and hematoxylin and eosin stain.

Parasite Detection by PCR

Parasite detection in each tissue sample was performed using specific PCR amplification of a 330-bp fragment corresponding to the four variable regions of the *T. cruzi* kDNA minicircle. PCR was carried out in a final volume of 25 μl containing 10 mmol/L Tris-HCl, pH 8.5; 105 mmol/L KCl; 2.0 mmol/L $MgSO_4$; 200 μmol of each 2′deoxynucleotide 5′-triphosphate; 38 pmol of each primer (S35: 5′-AAATAATGTACGGGGAGATGCATGA-3′ and S36: 5′-GGGTTCGATTGGGGTTGGTGT-3′); and 1.5 units of *Taq* DNA polymerase (a gift of Cenbiot, Porto Alegre, RS, Brazil). The DNA template consisted of 3 μl of the product of the alkaline lysis diluted 10 times in double-distilled water. Thirty amplification cycles were carried out with annealing at 60°C for 1 minute, extension at 72°C for 2 minutes, and denaturation at 94°C for 1 minute, preceded by an initial denaturation at 94°C for 5 minutes. PCR products were visualized in 6% polyacrylamide gel electrophoresis and silver-stained as described elsewhere.[15]

kDNA Signatures

The production of kDNA signatures by LSSP-PCR is a two-step procedure. The first step consisted of the specific PCR amplification of the kDNA of *T. cruzi* as described above. The PCR products were loaded in a 1.5%-agarose gel (1/3 low-melting point agarose; Sigma Chemical Co., St. Louis, MO) stained by ethidium bromide, the 330-bp DNA fragments being visualized by long-wave ultraviolet radiation, and removed from the gel. These fragments were then melted, diluted ten-fold in double-distilled water and used as templates for a second step of amplification using low stringency conditions (the LSSP-PCR reaction), which was performed exactly as described, using primer S35 as driver.[12] The LSSP-PCR products were visualized by electrophoresis in 6% polyacrylamide gel and silver-stained.[15]

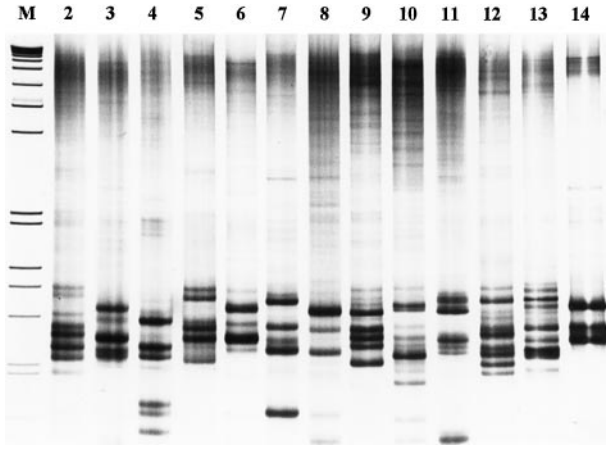

Figure 1. kDNA signatures of the 330-bp minicircle fragment of *T. cruzi* obtained from heart of chronic patients with chagasic cardiopathy. Five μl of the LSSP-PCR reaction products were loaded in each lane in a 6% polyacrylamide gel and silver stained: **lane 2,** A29; **lane 3,** A30; **lane 4,** A33); **lane 5,** A36; **lane 6,** A37; **lane 7,** A38; **lane 8,** A1; **lane 9,** A10; **lane 10,** A11; **lane 11,** A48; **lane 12,** A49; **lane 13,** A50; **lane 14,** A51. Migration of the markers of the 1-kb ladder (Life Technologies, Inc., Gaithersburg, MD) is shown in **lane M** with the following molecular sizes (from the bottom up): 134, 154, 201, 220, 298, 344, 396, 506, 517, 1018, and 1636 bp.

Results

kDNA Signatures of Heart Tissues

The first step of our study was to genetically profile the parasites present in heart samples obtained from 13 chronic patients with chagasic cardiopathy (Table 1). In each patient we obtained a different kDNA signature (Figure 1, lanes 2–14). However, some LSSP-PCR profiles had noticeable similarities as seen in Figure 1 (eg, A1 and A11, lanes 8 and 10; and A30 and A37, lanes 3 and 6). The histological analysis of the heart samples showed, as expected, the presence of a predominantly mononuclear inflammatory infiltrate and fibrosis (data not shown).

kDNA Signatures of Esophageal Tissues

We also analyzed the *T. cruzi* parasites present in esophageal tissues from four chagasic patients. Although LSSP-PCR was also able to genetically distinguish the parasites in each patient (Figure 2A, lanes 1–8), two of them, E8 and E11, showed quite similar kDNA signatures (Figure 2A, lanes 3 and 4). The esophageal samples of the patients with advanced megaesophagus (E8, E11, and E12) exhibited moderate (E8) to intense (E11 and E12) inflammatory processes in the muscle layer and

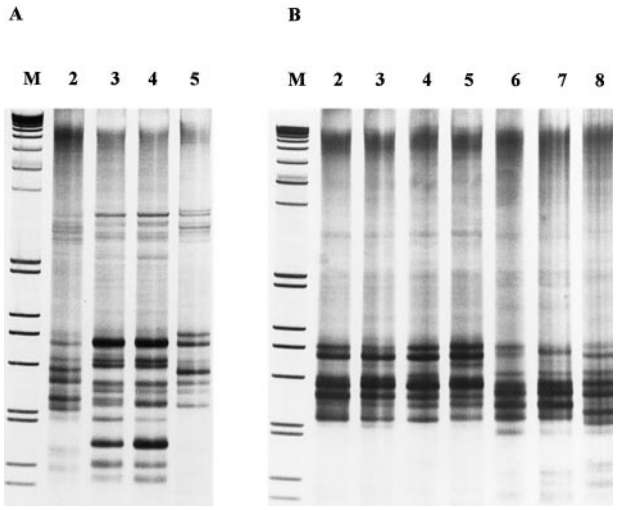

Figure 2. A: kDNA signatures of the 330-bp minicircle fragment of *T. cruzi* obtained from esophagi of chronic chagasic patients. E4: **lanes 1** and **2**, duplicates; E8: **lanes 3** and **4,** duplicates; E11: **lanes 5** and **6,** duplicates; E12: **lanes 7** and **8,** duplicates. Five μl of the LSSP-PCR reaction products were loaded in each lane in a 6% polyacrylamide gel and silver stained. **B:** kDNA signatures obtained from heart and esophagus of the same patients (pairs, A36/E12; A29/E12) and reproducibility studies thereof. **Lanes 2** and **3,** A36; **lanes 4** and **5,** E12; **lanes 6** and **7,** A29; and **lane 8,** E4. Each of the two replicates of A36, E12, and A29 represent two different fragments of the tissue submitted independently to LSSP-PCR of kDNA. **Lane M** shows the migration of the markers of the 1-kb ladder (Life Technologies, Inc.) with the following molecular sizes (from the bottom up): 134, 154, 201, 220, 298, 344, 396, 506, 517, 1018, and 1636 bp.

around or inside the esophageal ganglia (data not shown). Patient E4, although without megaesophagus, had moderate and focal inflammatory exudation in the muscular layer, mainly near the myenteric plexus (data not shown).

kDNA Signatures of Distinct Tissues from the Same Patient

Two of the 15 patients in our study had both cardiac and esophageal involvement and we were able to study both organs (A36/E12 and A29/E4). The remarkable finding was that in both patients the kDNA signatures obtained from the distinct tissues (heart and esophagus) of the same patient were different (Figure 2B, lanes 2–8). Independent LSSP-PCR reproducibility experiments were done with two different tissue fragments of the pathological hearts (A36, A29) and esophagus (E12; unfortunately we did not have enough material to allow a second experiment of E4). As can be seen in Figure 2B (A36, lanes 2 and 3; E12, lanes 4 and 5; A29, lanes 6 and 7) there is excellent reproducibility of the LSSP-PCR kDNA signatures in replicate samples of the same diseased organ in the same patient.

Discussion

Chagas disease is a parasitic infection characterized by an evident clinical pleomorphism that many authors, including Carlos Chagas himself,[16] attributed to differential tissue tropism by diverse strains of *T. cruzi*. Most, if not all, studies dedicated to the demonstration of specific histotropism of *T. cruzi* have used only microscopy for determining the distribution of parasites and inflammatory processes in experimental animals, mainly during the acute phase of the infection.[17–20] Our study represents the first direct evidence of differential tissue distribution of diverse *T. cruzi* populations in chronic human Chagas disease.

We had previously shown that kDNA signatures obtained from animals experimentally infected with different *T. cruzi* clones were highly reproducible and identical to those produced from the originating cultures.[12] Moreover, we showed that the degree of similarity displayed by the different kDNA signatures of two strains reflected, at least in part, the genetic distance (as established by DNA fingerprinting with multilocal probes) between the strains.[12] This was a key finding that highlighted the existence of strong linkage disequilibrium between genetic markers in the kDNA and in the nuclear genome as a consequence of the clonal structure and absence of sexual reproduction in *T. cruzi.*[21,22] Tibayrenc and Ayala[14] had already demonstrated the existence of strong linkage disequilibrium between isoenzyme loci and kDNA restriction fragment length polymorphisms. In this fashion, genetic variation in kDNA sequence profiled by LSSP-PCR is correlated with nuclear polymorphisms, establishing the theoretical rationale for the present study. More recently we have demonstrated in animals infected with two different *T. cruzi* clones that the LSSP-PCR patterns of double-infected organs represent the sum of the kDNA signatures of each separate clone.[23] Thus, we can expect that the correlation of kDNA signature similarity with genetic similarity applies not only to clones or monoclonal strains, but also to polyclonal strains, which seem to be the majority in nature.[22,24–26]

Our results with 13 heart and four esophagus samples clearly show a different kDNA signature in each case. This reflects our previous observation of great genetic variability in *T. cruzi* (reviewed by Macedo and Pena[27]). This variability originates not only from enormous clonal diversity, but also the fact that most infecting strains probably represent different combinations of genetically different clones. However, similarities of kDNA profiles were still observed between different patients coming from the same geographic region (see Figure 2A, E8 and E11, lanes 3 and 4). This suggests that genetically-related populations of *T. cruzi*, possibly existing in the same or adjoining geographical areas, might be involved in determining the development of the same clinical form of the disease.

Our most significant finding, however, was the observation that in two instances the kDNA signatures obtained from the heart and esophagus of the same patient differed significantly in the two organs. As demonstrated in Figure 2B, the reproducibility of the kDNA profiles was excellent. Our recent microsatellite studies have clearly demonstrated that *T. cruzi* populations are frequently multiclonal.[22,28] Presumably, each of these two patients was infected with a multiclonal strain, one or more clone(s) successfully chronically infecting the heart and the other(s) lodging in the esophagus. We only had these two cases for study because postmortem examinations are

not common medical practice in Brazil, and it is difficult to obtain heart and esophagus tissue from the same patient with the cardiodigestive form of Chagas disease (the least common of the chronic forms of the disease). Thus, with the caveat of being based on the study of only two patients, this represents the first direct evidence of probable human differential tissue tropism of *T. cruzi* clones and provides support to the "clonal-histotropic" model of Chagas disease pathogenesis proposed by us.[27] We have recently obtained strong experimental support for this model, studying BALB/c mice during the acute or chronic phase of infection with two genetically different clonal populations.[23] Double-infected animals showed clear differential tissue tropism for the two *T. cruzi* populations during the chronic phase. The extent of the pathological changes observed in the histological analysis of the infected organs of the mice was also dependent on the *T. cruzi* population present in each organ.[23]

In conclusion, we have provided in the present work evidence that the genetic polymorphism of infecting *T. cruzi* populations might exert an influence on the pathogenesis of chronic Chagas disease. The kDNA signatures obtained by LSSP-PCR from chronic chagasic patients thus constitute an important new clinical tool to study the molecular epidemiology of Chagas disease.

References

1. Moncayo A: Chagas disease. UNDP World Bank WHO Special Programme for Research and Training in Tropical Diseases (TDR). Tropical Disease. Progress in International Research. Geneva, World Health Organization, 1993, 11:67–75
2. Tanowitz HB: Chagas disease. Clin Microbiol Rev 1992, 5:400–419
3. Lopes ER, Rocha A, Meneses AC, Lopes MAB, Fatureto MC, Lopes GP, Chapadeiro E: Prevalência de megas em necrópsias realizadas no Triângulo Mineiro no período de a 1988. Rev Soc Bras Med Trop 1989, 22:211–215
4. Adad SJ, Andrade DCS, Lopes ER: Contribuição ao estudo da anatomia patológica do megaesôfago chagásico. Rev Inst Med Trop São Paulo 1991, 33:443–450
5. Brener Z: Immune response and immunopathology in *Trypanosoma cruzi* infection. Chagas Disease (American Trypanosomiasis): Its Impact on Transfusion and Clinical Medicine. Edited by Brener Z, Wendel S, Camargo ME, Rassi A. São Paolo, ISBT Brazil, 1992, pp 31–47
6. Palacius-Pru E, Carrasco H, Scorza C, Espinoza R: Ultrastructural characteristics of different stages of human chagasic myocarditis. Am J Trop Med Hyg 1989, 41:29–40
7. Köeberle F: Chagas disease and Chagas syndromes: the pathology of American trypanosomiasis. Adv Parasitol 1968, 6:63–116
8. Jones EM, Colley DJ, Tostes S, Lopes ER, Vnencak-Jones CL, McCurley TL: Amplification of a *Trypanosoma cruzi* DNA sequence from inflammatory lesions in human chagasic cardiomyopathy. Am J Trop Med Hyg 1993, 48:348–357
9. Brandariz S, Schjman A, Vigiliano C, Arteman P, Viotti R, Beldjord C, Levin MJ: Detection of parasite DNA in Chagas heart disease. Lancet 1996, 347:914
10. Vago AR, Macedo AM, Adad SJ, Reis DA, Corrêa-Oliveira R: PCR detection of *Trypanosoma cruzi* DNA in esophageal tissues of patients with chronic Chagas disease. Lancet 1996, 348:891–892
11. Dias JCP: Epidemiology of Chagas disease. Chagas Disease (American Trypanosomiasis): its Impact on Transfusion and Clinical Medicine. Edited by Brener Z, Wendel S, Camargo ME, Rassi A. São Paolo, ISBT Brazil 1992, pp 49–80
12. Vago AR, Macedo AM, Oliveira RP, Andrade LO, Chiari E, Galvão LMC, Simpson AJG, Tostes S, Pena SDJ: Kinetoplast DNA signatures of *Trypanosoma cruzi* strains obtained directly from infected tissues. Am J Pathol 1996, 149:2153–2159
13. Zhang Q, Tibayrenc M, Ayala FJ: Linkage disequilibrium in natural populations of *Trypanosoma cruzi* (Flagellate), the agent of Chagas' disease. J Parasitol 1988, 35:81–85
14. Tibayrenc M, Ayala FJ: Forte corrélation entre classification isoenzymatique et variabilité de l'ADN kinétoplastique chez *Trypanosoma cruzi*. C R Acad Sci Paris 1987, 304:89–92
15. Santos FR, Pena SDJ, Epplen JT: Genetic and population study of a Y-linked tetranucleotide repeat DNA polymorphism with a simple non-isotopic technique. Hum Genet 1993, 90:655–656
16. Chagas C: Nova entidade morbida do homem. Resumo geral de estudos etiológicos e clínicos. Mem Inst Oswaldo Cruz 1911, 3:219–275
17. Tagliaferro WH, Pizzi T: Connective tissue reactions in normal and immunized mice to a reticulotropic strain of *Trypanosoma cruzi*. J Infect Dis 1955, 96:199–226
18. Bice DE, Zeledon R: Comparison of infectivity of strains of *Trypanosoma cruzi* (Chagas). J Parasitol 1909, 1970, 56:663–670
19. Melo RC, Brener C: Tissue tropism of different *Trypanosoma cruzi* strains. J Parasitol 1978, 64:475–482
20. Schempler BR, Ávila CM, Coura JR, Brener Z: Course of infection and histopathological lesions in mice infected with seventeen *Trypanosoma cruzi* strains isolated from chronic patients. Rev Soc Bras Med Trop 1983, 16:23–30
21. Tibayrenc M, Ward P, Moya A, Ayala FJ: Natural populations of *Trypanosoma cruzi*, the agent of Chagas disease, have a complex multiclonal structure. Proc Natl Acad Sci USA 1986, 83:115–119
22. Oliveira RP, Broude NE, Macedo AM, Cantor CR, Smith CL, Pena SDJ: Probing the genetic population structure of *Trypanosoma cruzi* with polymorphic microsatellites. Proc Natl Acad Sci USA 1998, 95:3776–3780
23. Andrade LO, Machado CRS, Chiari E, Pena SDJ, Macedo AM: Genetic variability of *Trypanosoma cruzi*: differential tissue distribution of diverse clones of Trypanosoma cruzi in infected mice. Mol Biochem Parasitol 1999, 100:163–172
24. Tibayrenc M, Ayala FJ: Towards a population genetics of microorganisms: the clonal theory of parasitic protozoa. Parasitol Today 1991, 7:228–232
25. Deane MP, Sousa MA, Pereira NP, Gonçalves AM, Momen H, Morel C: *Trypanosoma cruzi*: inoculation schedules and re-isolation methods selected individual strains from doubly infected mice, as demonstrated by schizodeme and zymodeme analyses. J Protozool 1984, 31:276–280
26. Carneiro M, Chiari E, Gonçalves AM, Pereira AAS, Morel C, Romanha AJ: Changes in the isoenzyme and kinetoplast DNA patterns of *Trypanosoma cruzi* strains induced by maintenance in mice. Acta Tropica 1990, 47:35–45
27. Macedo AM, Pena SDJ: Genetic variability of *Trypanosoma cruzi*: implications for the pathogenesis of Chagas disease. Parasitol Today 1998, 14:119–123
28. Oliveira RP, Melo AIR, Macedo AM, Chiari E, Pena SDJ: The population structure of *Trypanosoma cruzi*: expanded analysis of 54 strains using eight polymorphic CA-repeat microsatellites. Mem Inst Oswaldo Cruz 1999, 94(suppl. I): 65–70