Revista da Sociedade Brasileira de Medicina Tropical

REFERÊNCIA

LAURIA-PIRES, L. et al. Diversity of Trypanosoma cruzi stocks and clones derived from Chagas disease patients: I-Behavioral characterization in vitro. **Revista da Sociedade Brasileira de Medicina Tropical**, Uberaba, v. 30, n. 3, p. 187-192, jun. 1997. DOI: http://dx.doi.org/10.1590/S0037-86821997000300003. Disponível em: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0037-86821997000300003&lng=en&nrm=iso. Acesso em: 31 jan. 2019.

Revista da Sociedade Brasileira de Medicina Tropical
30(3):187-192, mai-jun, 1997.

DIVERSITY OF *TRYPANOSOMA CRUZI* STOCKS AND CLONES DERIVED FROM CHAGAS DISEASE PATIENTS: I-BEHAVIORAL CHARACTERIZATION *IN VITRO*

L. Lauria-Pires, J.M. Santana, F.S. Tavares and A.R.L. Teixeira

In this study, we isolated Trypanosoma cruzi *from chronic Chagas heart disease and from megaesophagus patients. The parasite stock hSLU239 (heart disease) yielded clones h1 and h2, whereas stock mSLU142 (megaesophagus) yielded clones m1, m2, m3 and m4. The parasite growth kinetics, doubling time and differentiation in axenic liquid medium showed broad behavioral diversity. It was shown that a particular pattern of behavior for a parental stock could not necessarily be assigned for subsequent clones. This study indicates that i) each Chagas disease patient is infected with several* T. cruzi *populations; ii) clonal lines derived from patient samples may have different biological characteristics from the original isolate; and that iii) additional behavioral and/or molecular markers are required for further characterization of* Trypanosoma cruzi *stocks and clones derived from Chagas disease patients in order to identify correlations with pathology.*

Key-words: Trypanosoma cruzi. *Behavioral characterization. Genetic diversity.*

There is extensive intraspecific variation in the behavior of naturally occurring *Trypanosoma cruzi* populations. Several authors[1 3 4 5 8 9 11 14 15] have suggested that the variation in *T. cruzi* populations may be related to variation in the epidemiological and clinical aspects of Chagas disease in man. The enormous behavioral heterogeneity of this protozoan has been shown by both *in vitro* and *in vivo* studies[1 3 5 11] and by schizodeme[3 4 20 23] and RFLP profiles[14 21 27]. Variation of the parasites described in the above reports suggests that further studies should be carried out with clonal populations derived from Chagas disease patients with well-defined pathology. Examination of the *T. cruzi* isolates from heart and esophageal patients and their resulting clonal lines (each derived from a single cell yielding a genetically homogeneous population) will shed light on the role played in this scenario by a particular parasite isolate or a subpopulation thereof. This paper reports on the behavioral characteristics of *T. cruzi* isolates and their clonal lines derived from two patients with the cardiac and the digestive forms of this disease.

Laboratório Multidisciplinar de Pesquisa em Doença de Chagas, Faculdade de Ciências da Saúde, Universidade de Brasília, Brasília, DF.
This work was supported by FINEP/CNPq, Brazil.
Address to: Drª Liana Lauria-Pires. Laboratório Multidisciplinar de Pesquisa em Doença de Chagas/Faculdade de Ciências da Saúde/UnB. CP: 04-536, 70919-970 Brasília, DF..
Fax: (061) 273-4645.
Recebido para publicação em 27/05/96.

MATERIAL AND METHODS

Trypanosoma cruzi stocks and clones. In this study we isolated *T. cruzi* by xenodiagnosis from two patients with chronic Chagas disease. The parasite stock *h*SLU239 was obtained from a patient with the cardiac disease, whereas stock *m*SLU142 derived from a patient with the digestive form (megaesophagus) of the disease. The metacyclic trypomastigotes passed in the feces of first instar nymphs of *Dipetalogaster maximus* used for xenodiagnosis were used to inoculate Balb/c mice. One week after inoculation the blood trypomastigotes were obtained by cardiac puncture. The red cells were lysed in 0.78% ammonium chloride and the parasite forms were used to infect primary cultures of fetal mouse skeletal muscle cells[26]. The cell cloning experiments consisted of infecting murine skeletal muscle cell culture in 24 well plates with a single trypomastigote[9 26]. The parasite suspension in culture medium was diluted to contain a single cell per 5µl. This cell dilution aliquot was placed on sterile cover slip in Petri dish and examined with an inverted microscope. Each 5µl aliquot containing a single parasite was transferred to a well of the plate. One week thereafter infected host cells in each well was observed with an inverted microscope. In a parallel control

experiment, no growth was observed in the wells receiving an aliquot of medium in which the parasite could not be seen microscopically.

Growth kinetics, doubling time and differentiation. The *T. cruzi* stocks and clones were maintained in liver infusion tryptose (LIT) medium[6]. The behavioral characterization of the parasite consisted of growth kinetics, doubling time and percent of differentiation. For the growth kinetics studies, stocks and clones of the parasite harvested from tissue culture were transferred in triplicate to LIT medium. After three passages with 5 day-intervals, the exponential growth phase parasites were used to begin the study. Each initial inoculum of 1 x 10^6 cells was seeded in fresh LIT medium and the parasite growth was determined every other day until late stationary phase. Aliquots of the parasite culture forms were counted in triplicate using a Neubauer cell counting chamber and an inverted microscope at 100X magnification. Smears of the parasite forms were fixed in methanol, stained by Giemsa's method and mounted on coverslip. The glass slides were examined under the microscope at 1000X magnification. The percent of epimastigotes and trypomastigotes were estimated from 300 flagellates counted in each smear.

RESULTS

In this study, the method used for parasite cloning ascertained that each subsequent clonal line derived from a single trypomastigote. The parasite stock *h*SLU239, isolated from a heart disease patient, yielded clones *h*1 and *h*2. In addition, the parasite stock *m*SLU142, isolated from a megaesophagus patient, yielded clones *m*1, *m*2, *m*3, and *m*4.

Figure 1 shows the means and standard deviation of the parasite growth for a period of 22 days. Figure 1A shows the growth kinetics of the *T. cruzi* stock *h*SLU239 and of its clones *h*1 and *h*2. The parental stock and clone *h*1 showed similar dividing profile with quick proliferation, and reached high cell number (≅ 2-3 x 10^7/ml) after 8 days. In contrast, clone *h*2 derived from same parental stock showed slow proliferation and substantial fewer cells (≅ 2 x 10^6/ml) after 8 days of growth.

Figure 1B shows percent of parasite differentiation from epimastigotes to trypomastigotes in the axenic medium. These observations were carried out through 22 days of parasite growth. The *T. cruzi* stock *h*SLU239 showed maximum percent differentiation of circa of 3% at days 12 and 14, whereas clone *h*1 reached 15% at day 18, and clone *h*2 reached 10% at day 20.

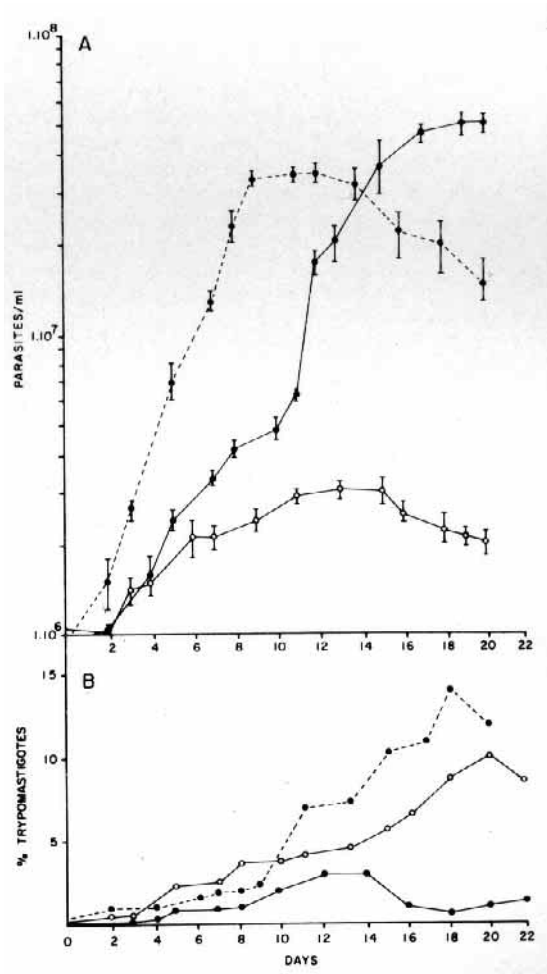

Figure 1 - A) Profiles of Trypanosoma cruzi *growth in LIT medium; B) Patterns of the parasite differentiation from epimastigotes to trypomastigotes in the axenic culture: Stock* h*SLU239 (●—● and its clones* h*1 (●···● and* h*2 (○—○. Each finding represents the means ± standard deviation in triplicate countings of at least 300 parasites.*

Figure 2A shows the growth kinetics of the *T. cruzi* stock *m*SLU142 and of its four clones. The parental stock and clone *m*2 showed moderate growth: stock *m*SLU142 reached approximately 1 x 10^7 cell culture forms/ml after 10 days in axenic culture, whereas clone

*m*2 reached ≅ 3 x 10^6 cells/ml at day 12. In marked contrast, clones *m*3 and *m*4 multiplied very quickly and reached approximatelly 7 x 10^7 cells/ml after 12 days of growth. Figure 2B shows that *m*SLU142 had moderate differentiation reaching a maximum of 5 to 6 per cent between days 14 to 20. Clones *m*2 and *m*4 showed the lowest differentiation (1%) from days 12 to 16, whereas clone *m*3 epimastigotes differentiated to circa 15% trypomastigotes, at day 16 in LIT medium.

The *T. cruzi* population doubling time (DT) was indicated by the number of hours required for two-fold increase of the parasite population.

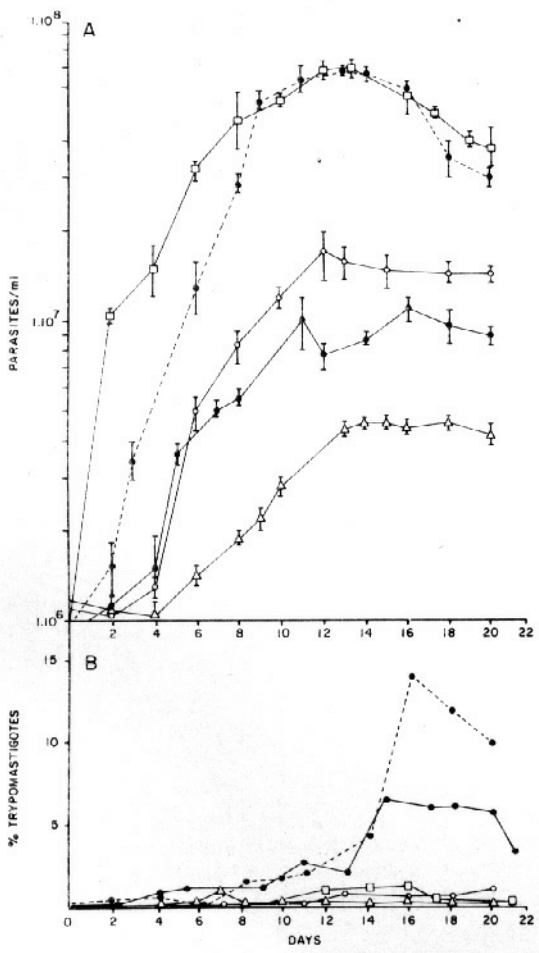

Figure 2 - A) Profiles of Trypanosoma cruzi *growth in LIT medium; B) Patterns of the parasite differentiation from epimastigotes to trypomastigotes in the axenic culture: Stock* m*SLU142 (●—●) and its clones* m1 (▵···▵), m2 (○—○), m3 (●···●) *and* m4 (□···□). *Each finding represents the means standard deviation in triplicate countings of 300 parasites.*

Table 1 shows the DT observed for each parasite stock and clone. Interestingly, the DT of stock *h*SLU239 (65.4 ± 0.9h) was similar to that of stock *m*SLU142 (65.6 ± 4.9h). The DT of both *T. cruzi* stocks, however, differed significantly from their clones. Clone *h*1 DT reached 37.5 ± 0.9, which was much lower than that of clone *h*2 that required 87.2 ± 4.3h. The parasite clones derived from the parental *m*SLU142 also showed marked divergence. Clone *m*2 showed DT of 56.4 ± 3.6h, whereas clones *m*3 and *m*4 showed 38.4 ± 1h. In marked contrast, clone *m*1 showed DT of 124.8 ± 3.6h.

Table 1 - Doubling time of Trypanosoma cruzi *stocks and clones in LIT medium at 28ºC.*

Stocks and clones	Doubling time (hours)*	Mean ± SD
*h*SLU239	64.3, 65.4, 66.5	65.4 ± 0.9
*h*1	39.4, 42.5, 41.7	37.5 ± 0.9
*h*2	83.9, 92.3, 85.4	87.2 ± 4.3
*m*SLU142	72.4, 61.3, 63.1	65.6 ± 4.9
*m*1	128.9, 120.1, 125.4	124.8 ± 3.6
*m*2	58.3, 59.5, 51.4	56.4 ± 3.6
*m*3	37.4, 39.5, 38.5	38.6 ± 0.9
*m*4	36.7, 38.4, 39.5	38.2 ± 1.1

* The doubling time in *h* was indicated by the parasite growth in LIT medium at the exponential growth phase. Cell countings were made every 48h from triplicate culture tubes.

An attempt to correlate the *T. cruzi* growth kinetics with the parasite population doubling time is shown in Figure 3. It was observed that clones *h*2 and *m*1 that had very long DT showed slow growth. Furthermore, clones *m*3 and *m*4 that had very short DT presented very

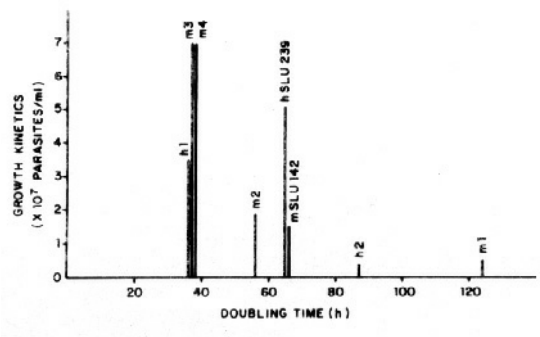

Figure 3 - Patterns of Trypanosoma cruzi *growth kinetics and population doubling time in axenic culture. Doubling time is shown on the axis; vertical bars represent maximum yield of cells. Notice that stock* m*SLU142 that had moderate doubling time presented low growth, whereas clones* m3 *and* m4 *that had short doubling time showed very high growth yield.*

high growth yields reaching high cell countings after 10 days in culture (Figure 2). However, clone *h*1 that had very short DT presented moderate growth (Figure 1). In addition, stock *h*SLU239 that had moderate DT showed high growth kinetics similar to those shown by clones *m*3 and *m*4.

In brief, DT in LIT medium allowed classification of the populations of *T. cruzi* stocks and clones into three groups: I- long DT (from 87.4 to 124h) composed of clones *h*2 and *m*1; II- moderate DT (from 56.4 to 65.6), consisted of stocks *h*SLU239, *m*SLU142 and clone *m*2; III- short DT (from 37.5 to 38.2h), which comprised clones *h*1, *m*3 and *m*4.

DISCUSSION

A stable behavioral marker for each single parasite population used to infect a singeneic host has been sought in order to determine whether the outcome of lesions in Chagas disease may be related to *Trypanosoma cruzi* superinfections. In this paper we describe several parasite populations (*h*1, *h*2, *m*1-*m*4) in two stocks derived from patients with chronic Chagas disease, which showed different kinetics of growth, doubling time and differentiation in axenic culture. This study suggests that a particular pattern of *in vitro* behavior of a parental *T. cruzi* stock can not necessarily be extrapolated to its clones. For example, the growth kinetics of clone *h*2 were much slower than those observed for the parental *h*SLU239. Also, clones *m*3 and *m*4 demonstrated more rapid growth kinetics than the parental *m*SLU142. However, clone *m*1 showed significantly slower growth than the parental *m*SLU142. Moreover, the parasite population doubling time varied from one clone to another regardless of stock origin. In this regard, clones *h*2 and *m*1 (group I) showed the longest doubling time, in contrast with clones *h*1, *m*3 and *m*4 (group III) that showed from one-half to one-third of the doubling time. A correlation between the kinetics of growth and the population doubling time was not seen in all cases. This observation is hard to explain because the factors associated with the regulation of *T. cruzi* population growth in axenic culture are basically unknown. Furthermore, variability between stock and derivative clonal lines is also found for the parasite differentiation in culture. Clones *h*1 and *h*2 differentiated more extensively than their parental stock *h*SLU239, whereas clones *m*2 and *m*4 showed minimal differentiation.

It has been postulated that a particular feature of a *T. cruzi* population might be correlated to the pathology of Chagas disease[1 3 8 10 11]. The behavioral characteristics of the parasite stocks and derived clones reported here, however, are in keeping with the idea that each Chagas disease patient is infected with several *T. cruzi* populations having broad biological diversity[8 22 24]. Some behavioral aspects of a particular parasite population may change from time to time[7 12 25]. In addition, it has been shown that selection of some *T. cruzi* populations may take place in the intestine of the insect-vector[13]. Sampling (xeno) with *D. maximus* may not have isolated all the biological types. In this respect, the parasite populations described in this paper will be used in the future to determine if the *T. cruzi* stocks and clones maintained *in vitro* and *in vivo* can change their phenotypic profiles.

The results of this study highlight the dificulty of understanding the role that parasite variation plays in the highly variable pathogenesis of disease. In order to circumvent this difficulty, an attempt has been made to determine stable isoenzyme and DNA marker of these clones, such as polymorphisms of DNA sequences encoding the glycolytic enzymes piruvate kynase[2], fructose bi-phosphate aldolase[19], glucose phosphate isomerase[18] and glyceraldehyde-3-phosphate dehydrogenase[16] for each parasite population[17]. In conclusion, we believe that additional behavioral and molecular markers are required for further characterization of the various *T. cruzi* clones present in the parasite stocks derived from Chagas disease patients.

RESUMO

Neste estudo, foram obtidos estoques de Trypanosoma cruzi *de pacientes chagásicos com a doença cardíaca ou com megaesôfago. O estoque* h*SLU239 (doença cardíaca) forneceu os clones* h*1 e* h*2, enquanto o estoque* m*SLU142 (megaesôfago) forneceu os clones* m*1,* m*2,* m*3 e* m*4. A cinética de crescimento do parasito, tempo de duplicação e diferenciação em meio líquido axênico mostraram ampla diversidade comportamental. Observou-se que um padrão particular de comportamento de um estoque parental podia não ser necessariamente encontrado na linhagem subclonal subseqüente.*

Este estudo indica que i) cada paciente chagásico é infectado com várias subpopulações de T. cruzi*; ii) linhagens clonais derivadas de cada estoque do parasito podem ter características biológicas diferentes do isolado original de paciente chagásico; e que iii) marcadores comportamentais e/ou moleculares adicionais são necessários para melhor caracterização de estoques de* T. cruzi *e seus clones derivados de pacientes com doença de Chagas, a fim de identificar as possíveis correlações com a patologia.*

Palavras-chaves: Trypanosoma cruzi. *Caracterização do comportamento. Diversidade genética.*

ACKNOWLEGMENTS

We would like to thank Ana Rosa Bogliolo for technical assistance and to Nancy R. Sturm and David A Campbell for the careful reading of the manuscript.

REFERENCES

1. Allert S, Ernest J, Poliszczak A, Opperdoes FR, Michels PAM. Molecular cloning of two tandemly linked genes for pyruvate kinase of *Trypanosoma brucei*. European Journal of Biochemistry 200: 19-27, 1991.
2. Andrade SG. Caracterização de cepas de *Trypanosoma cruzi* isoladas no Recôncavo Baiano. Contribuição ao estudo da patologia geral da doença de Chagas. Revista Patologia Tropical 3: 65-121, 1974.
3. Apt W, Aguilera X, Arribada A, Gomez L, Miles M, Widmer G. Epidemiology of Chagas' disease in northern Chile: Isozyme profiles in *Trypanosoma cruzi* from domestic and sylvatic transmission cycles and their association with cardiopathy. The American Journal of Tropical Medicine and Hygiene 37: 302-307, 1987.
4. Bogliolo AR, Chiari E, Silva-Pereira RO, Silva-Pereira AA. A comparative study of *Trypanosoma cruzi* enzyme polymorphisms in South America. Brazilian Journal of Medical and Biological Research 19: 673-683, 1986.
5. Brener Z. Intraspecific variations in *Trypanosoma cruzi*. Two types of parasite populations presenting distinct characteristics. *In:* Chagas' disease. Pan American Health Organization Scientific Publication 347: 11-21. Washington DC, 1977.
6. Camargo EP. Growth and differentiation in *Trypanosoma cruzi*. I. Origin of metacyclic trypanosomes in liquid media. Revista do Instituto de Medicina Tropical de São Paulo 6: 93-100, 1964.
7. Carneiro M, Chiari E, Gonçalves AM, Da Silva AA, Pereira CM, Morel CM, Romanha AJ. Changes in the isoenzyme and kinetoplast DNA patterns of *Trypanosoma cruzi* strains induced by maintenance in mice. Acta Tropica 47: 35-45, 1990.
8. Deane MP, Brito T, Deane LM. Pathogenicity to mice of some strains of *Trypanosoma cruzi* isolated from wild animals of Brazil. Revista do Instituto de Medicina Tropical de São Paulo 5:225-285, 1963.
9. Dvorak JA. Single cell isolates of *Trypanosoma cruzi*: How and Why? Revista da Sociedade Brasileira de Medicina Tropical 18:29-38, 1985.
10. Dvorak JA, Hartman DL, Miles MA. *Trypanosoma cruzi*: correlation of growth kinetics to zymodeme types in clones derived from various sources. Journal of Protozoology 27:472-474, 1980.
11. Engel JC, Dvorak JA, Segura E, Crane MJ. *Trypanosoma cruzi*: Biological characterization of 19 clones derived from two chronic chagasic patients. I. Growth kinetics in liquid medium. Journal of Protozoology 29: 555-560, 1982.
12. Garcia ES, Vieira E, Gomes JEPL, Gonçalves AM. Molecular biology of the interaction *Trypanosoma cruzi* invertebrate host. Memórias do Instituto Oswaldo Cruz 79: 33-37, 1984.
13. Garcia ES, Vieira E, Gonçalves AM, Morel CM, Alves MM, Colli W. A strain of *Trypanosoma cruzi*, and its biochemical characterization after passage through different invertebrate hosts. Annals of Tropical Medicine and Parasitology 80: 361-363, 1986.
14. Gibson WC, Miles MA. The karyotype and ploidy of *Trypanosoma cruzi*. EMBO Journal 5:1299-1305, 1986.
15. Gonzalez-Cappa SM, Chiale P, Del Prado GE, Katzin AM, De Martini GW, Isola ED, Orrego LA, Segura EL. Aislamiento de una cepa de *Trypanosoma cruzi* de un paciente com miocardiopatia chagasica cronica y su caracterizacion biologica. Medicina (Buenos Aires) 40: 63-68, 1980.
16. Kendall G, Wilderspin AF, Ashal F, Miles MA, Kelly JM. *Trypanosoma cruzi* glycosomal glyceraldehyde-3-phosphate dehydrogenase does not conform to the 'hotspot' topogenic signal model. EMBO Journal 9:2751-2758, 1990.

17. Lauria-Pires L, Bogliolo AR, Teixeira ARL. Diversity of *Trypanosoma cruzi* stocks and clones derived from Chagas disease patients: II- Isoenzyme and RFLP characterizations. Experimental Parasitology 82: 182-190, 1996.
18. Marchand M, Kooystra U, Wieranga RK, Lambier AM, Beeumen JV, Opperdoes FR, Michels PAM. Glucose phosphate isomerase from *Trypanosoma brucei*. Cloning and characterization of the gene and analysis of the enzyme. European Journal of Biochemistry 184: 455-464, 1989.
19. Marchand M, Poliszczak A, Gibson WC, Wierenga RK, Opperdoes FR, Michels, PAM. Characterization of the genes for fructose bi-phosphate aldolase in Trypanosoma brucei. Molecular Biochemical Parasitology 29: 65-76, 1988.
20. Miles MA, Toyé PJ, Oswald SC, Godfrey DG. The identification by isoenzyme patterns of two distinct strain-groups of *Trypanosoma cruzi* circulating independently in a rural area of Brazil. Transactions of the Royal Society of Tropical Medicine and Hygiene 71:217-225, 1977.
21. Morel CM, Chiari E, Camargo EP, Mattei DM, Romanha AJ, Simpson L. Strains and clones of *Trypanosoma cruzi* can be characterized by patterns of restriction endonuclease products of kinetoplast DNA minicircles. Proceedings of the National Academy of Sciences 77:6810-6814, 1980.
22. Postan M, Dvorak JA, McDaniel JP. Studies of *Trypanosoma cruzi* clones in inbred mice. I. A comparison of the course of infection of C3H/HEN mice with two clones isolated from a common source. The American Journal of Tropical Medicine and Hygiene 32: 497-506, 1983.
23. Romanha AJ, Silva Pereira AA, Chiari E, Kilgour V. Isoenzyme patterns of cultured *Trypanosoma cruzi*: changes after prolonged subculture. Comparative Biochemical Physiology 62: 139-142, 1979.
24. Sanchez G, Wallace A, Olivares N, Aguilera X, Apt W, Solari A. A biological characterization of *Trypanosoma cruzi* zymodemes: *in vitro* differentiation of epimastigotes and infectivity of culture metacyclic trypomastigotes to mice. Experimental Parasitology 71: 125-133, 1990.
25. Solari A, Munoz S, Venegas J, Wallace A, Aguilera X, Apt W, Brenière FS, Tibayrenc M. Characterization of Chilean, Bolivean and Argentinian *Trypanosoma cruzi* populations by restriction endonuclease and isoenzyme analysis. Experimental Parasitology 75: 187-195, 1992.
26. Teixeira ARL, Junqueira Jr LF, Solórzano E, Zappalá M. Doença de Chagas experimental em coelhos isogênicos III/J. I. Fisiologia das Arritmias e da morte súbita do chagásico. Revista da Associação Médica Brasileira 29: 77-83, 1983.
27. Zavalla-Castro JE, Vescasco-Casteron O, Hernandez R. Molecular characterization of Mexican stocks of *Trypanosoma cruzi* using total DNA. The American Journal of Tropical Medicine and Hygiene 47:201-209, 1992.