Available online at www.sciencedirect.com
SCIENCE DIRECT®
Experimental Parasitology xxx (2006) xxx–xxx
Experimental Parasitology
www.elsevier.com/locate/yexpr

Trypanosoma cruzi: Infectivity modulation of a clone after passages through different hosts

Cecilia Pérez Brandán *, Ángel Marcelo Padilla, Patricio Diosque, Miguel Ángel Basombrío

Instituto de Patología Experimental, Facultad de Ciencias de la Salud, Universidad Nacional de Salta, Avda. Bolivia 5150, CP 4400 Salta, Argentina

Received 31 August 2005; received in revised form 24 January 2006; accepted 16 February 2006

Abstract

Although *Trypanosoma cruzi* virulence can be modified through passages in vivo or long-term in vitro culture, the mechanisms involved are poorly understood. Here we report modifications in the infectivity of a *T. cruzi* clone after passages in different hosts without detectable changes in parasite genetic patterns. A clone was obtained from a *T. cruzi* IIe isolate and showed to be less virulent than the original isolate ($p < 0.05$). This clone was enzymatically similar to the original isolate as shown by multilocus enzyme electrophoresis. Infection of this clone was compared by successive passages in mice and guinea pigs. The mouse-passaged subline became more virulent for both host species compared to the guinea pig-passaged subline ($p < 0.05$). The clone line displayed similar random amplified polymorphic DNA patterns before and after passages in different hosts suggesting that alterations in virulence could be a result of a differential expression of virulence factors.

Index Descriptors and Abbreviations: *Trypanosoma cruzi*; Clones; Selection; Infectivity; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; RAPD, random amplified polymorphic DNA; MLEE, multilocus enzyme electrophoresis; LIT, liver infusion tryptose medium; LIT-HSP, LIT plus fetal bovine serum, hemin, and penicillin–streptomycin; CTA1, clone line from TolAc1 isolate; TAE, Tris-acetate EDTA; GPI, glucose-6-phosphate isomerase

1. Introduction

Trypanosoma cruzi, the etiological agent of Chagas' disease, consists of distinct parasite progenies, which circulate among humans, insect vectors, and mammalian reservoirs. In the process of geographic dispersion, *T. cruzi* has developed a great diversity of populations, which show distinct biological characteristics (Murta and Romanha, 1999). It is well established that *T. cruzi* is extremely heterogeneous in terms of host specificity, pathogenicity, virulence (Toledo et al., 2002), and molecular markers, such as isoenzyme and kinetoplastic-DNA restriction patterns (Barnabé et al., 2000; Morel et al., 1980).

Polymorphisms in ribosomal RNA genes, mini-exon genes, and microsatellite fingerprinting indicate the presence of at least two principal genetic lineages, *T. cruzi* I and *T. cruzi* II (Souto et al., 1996). Tibayrenc et al. (1986) proposed a complex multiclonal structure for *T. cruzi* and postulated that parasite propagation occurs with little or no genetic exchange (Tibayrenc and Ayala, 1987). According to this model, every clone represents a lineage that reproduces by binary fission and remains unchanged for many generations until mutation or eventual horizontal genetic exchange occur. However, recent experimental studies have reported genetic exchange among parasites of the *T. cruzi* I lineage under defined experimental conditions (Gaunt et al., 2003).

Studies based on cloned and non-cloned *T. cruzi* populations reinforce the heterogeneity hypothesis and the theory that populations are composed of subpopulations with different characteristics (Andrade, 1999; Dusanic et al., 1994). Thus, the characteristic behavior of one isolate would be the result of the interaction of several clones. The coexistence of multiple parasite strains in vertebrate and invertebrate hosts has been reported (Lana et al., 2000; Pinto et al., 1998). The time when the sample is taken and the isolation method can

* Corresponding author. Fax: +54 387 425 5333.
E-mail address: perezbrandan@yahoo.com.ar (C. Pérez Brandán).

0014-4894/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2006.02.014

act as selective factors (Deane et al., 1984). Successive passages in mice or axenic culture medium can completely eliminate one or more strains present in the original population. It has also been reported that maintenance of *T. cruzi* in culture affects gene and antigenic expression of metacyclic trypomastigotes (Contreras et al., 1998). All these mentioned factors could account for the virulence increase in *T. cruzi* populations after in vivo passages or the attenuation process after long-term in vitro culture.

The main goal of this study was to determine if a particular *T. cruzi* clone could modify its virulence when submitted to different stress situations. For this purpose, we decided to analyze the genetic and biological characteristics of a single cell derived clone after successive passages in different host species. In this case, the heterogeneity of the original isolate was eliminated by cloning the initial inoculum. Furthermore, we assessed the virulence of this clone in mice compared to the original isolate.

2. Materials and methods

2.1. Parasites

The *T. cruzi* parasites used in this work derived from the *T. cruzi* isolate TolAc1 (original isolate), which comes from a *Triatoma infestans* collected in an endemic zone for Chagas disease, in the province of Chaco, Argentina. Diosque et al. (2003) classified this isolate as belonging to *T. cruzi* IIe lineage, according to multilocus enzyme electrophoresis analysis (MLEE) for 15 enzymatic loci.

Parasites were maintained at 29 °C in axenic cultures of Liver Infusion Tryptose Medium (LIT) supplemented with 10% fetal bovine serum, 1% hemin, and 100 U/ml of penicillin–streptomycin (LIT-HSP). In general, these cultures produce >95% epimastigote forms of the parasite. For enrichment of the cultures in infective trypomastigote forms, filtrates of triatomine gut homogenate were added (1%) to the cultures (Isola et al., 1986). Complement-resistant forms were selected by incubation in fresh human serum.

2.2. Cloning procedure

To derive a clone line from a single parasite, serial dilutions of the original isolate culture (TolAc1) were seeded into microplate wells and examined under an inverted microscope to confirmed well containing single parasites. Single cells were transferred into glass hemolysis tubes containing 2 ml of LIT-HSP and cultured at 29 °C. The clone used in the present study, CTA1, was derived by this procedure.

2.3. Genetic characterization of CTA1 clone

CTA1 clone and the original isolate were examined at 15 enzymatic loci by MLEE. The MLEE analysis was undertaken according to Ben Abderrazak et al. (1993) with slight modifications. We also developed random amplified polymorphic DNA assays (RAPD) to evaluate the genetic characteristics of the clone after passages through different hosts. DNA was extracted with phenol:chloroform method and used to carry out the RAPD technique. The amplification reactions were performed according to Brisse et al. (1998) using two random primers, A10 (5′GTGATCGCAT3′) and R16 (5′CTCTGCGCGT3′) (Operon Technologies). RAPD products were analyzed by electrophoresis in 1.6% agarose gels in TAE buffer (Tris-acetate 40 mM, EDTA 1 mM), stained with ethidium bromide and visualized by ultraviolet light.

2.4. Animal infections and parasitemia curves

One- or four-week-old male Balb/c mice and one- to three-month-old guinea pigs (*Cavia porcellus*) were used. Animal care guidelines of the Health Sciences Faculty of the National University of Salta were strictly followed. The propagation assays involved submitting CTA1 to successive mouse to mouse and guinea pig to guinea pig passages. The animals were inoculated by intraperitoneal route either with 1×10^3–1×10^4 *T. cruzi* bloodstreams forms per animal or 1×10^5–1×10^7 metacyclic trypomastigotes as specified. Blood was collected in heparinized glass capillary pipette by sectioning the tail tip from mice and by footpad puncture in guinea pigs. Ten microliters of blood was placed between slide and coverslip and the number of parasites per 100 fields was recorded under a microscope ($400 \times$).

2.5. Statistical analysis

The statistical significance of differences between parasitemia curves was calculated with the 2-tailed Wilcoxon signed rank test and the Mann–Whitney *U*-test. For measurement of variability within groups of animals, the standard error of the mean (SEM) was used. These tests and calculations were incorporated in the Graph Pad Prism Software (Graph Pad Software Inc., USA). Values are expressed as means ± SD from a minimum of three separate experiments.

3. Results

3.1. Genetic characterization of CTA1 clone

The phylogenetic identity of CTA1 clone obtained from the isolate TolAc1 was evaluated by MLEE. The CTA1 clone displayed the same *T. cruzi* IIe profile as compared to the original isolate for the 15 enzymatic loci examined with the MLEE technique (data not shown).

The genetic variability of CTA1 clone and TolAc1 isolate was examined by RAPD after passage in mice or guinea pigs. No differences were observed between the clone and the original isolate for the two primers used. The data presented in Fig. 1 indicates no differences between the culture-derived clone and the clone maintained in different

hosts. TolAc1 and CTA1 clone displayed a similar RAPD before and after in vivo passages suggesting that parasites maintained an identical genetic background.

3.2. Compared infectivity of the original isolate TolAc1 and the clone CTA1 in mice

TolAc1 isolate is maintained in our laboratory by successive in vivo passages allowing us to compare it with CTA1 clone maintained in mice. Cloned parasites were shown to be significantly less virulent in mice than the original isolate from which they were derived ($p < 0.05$), as shown by using blood trypomastigotes (Fig. 2). The peak in the parasitemia curve for mice infected with TolAc1 isolate was in average 60 parasites per 100 fields at day 15 postinfection compared with only 10 parasites per 100 fields for CTA1. These differences were also observed in 1-week-old mice inoculated with culture-derived forms of both the clone and the original strain (data not shown). No significant differences were observed between the clone and the original isolate in mice regarding prepatent period, patent period, and mortality.

3.3. CTA1 clone infectivity in mice and guinea pigs

The behavior of CTA1 clone varied significantly in the different hosts regarding various parameters. Parasitemia curves generated from different successive passages through mice are shown in Fig. 3. Higher parasitemia levels were observed in mice with successive passages. The clone CTA1 became more virulent with every passage even though older animals and lower inocula were given in later passages. The differences among parasitemia curves of different passages were statistically significant ($p < 0.05$).

The first attempts at infecting guinea pigs with the culture-derived clone showed negative results with the parasitological test used. Successful primary infection and sequential passage were achieved by immunosuppressing the animals with cyclophosphamide (350 mg/kg) 24 h before infection. During the successive passages, no substantial increases (<15 parasites/100 fields) were achieved in parasitemia levels. The prepatent period was significantly shorter in mice compared to guinea pigs ($p < 0.001$); however, mortality was very low and did not differ among species.

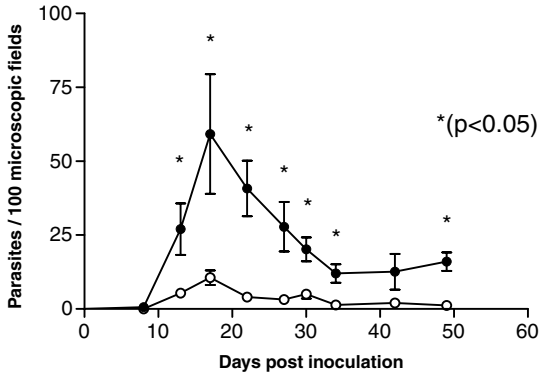

Fig. 2. Comparison of clone and original isolate infectivity in mice. Parasitemia curves in 45-days-old mice infected with 3×10^4 blood trypomastigotes of the original TolAc1 isolate maintained in mice (●) and the mouse-passaged CTA1 clone (○).

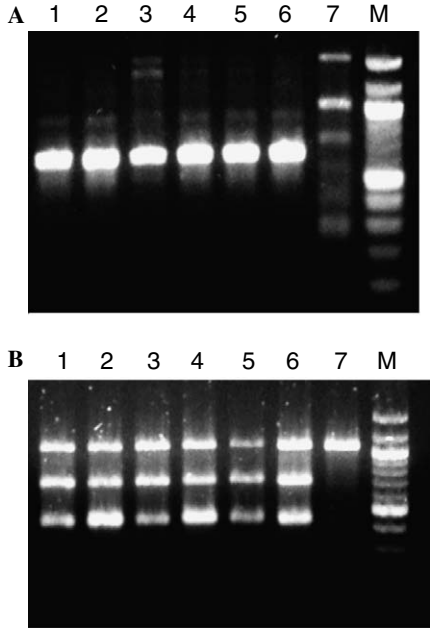

Fig. 1. Patterns obtained by random amplified polymorphic DNA analysis for the different parasite lines. (A) Primer A10. Lane 1, original TolAc1 culture; lane 2, mouse-passaged TolAc1 isolate; lane 3, CTA1 clone from culture; lane 4, CTA1 clone from mice; lane 5, guinea pig-passaged CTA1 clone; lane 6, *T. cruzi* IIe reference strain; lane 7, *T. cruzi* I reference strain; lane M, 100 bp molecular weight marker. (B) Primer R16. Lane 1, original TolAc1 culture; lane 2, mouse-passaged TolAc1 isolate; lane 3, CTA1 clone from culture; lane 4, CTA1 clone from mice; lane 5, guinea pig-passaged CTA1 clone; lane 6, *T. cruzi* IIe reference strain; lane 7, *T. cruzi* I reference strain; lane M, 100 bp molecular weight marker.

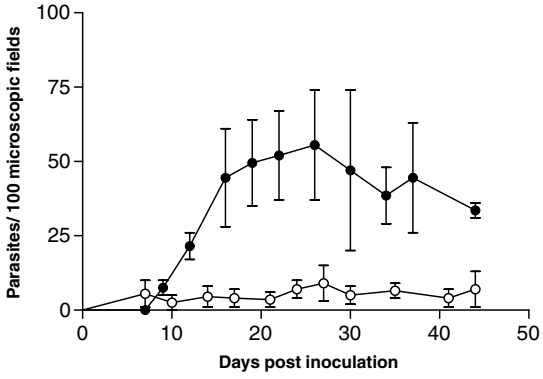

Fig. 3. Effect of previous passages on the virulence of the CTA1 clone in Balb/c mice. Long-term passaged parasites were significantly more virulent ($p < 0.05$), even at lower dose and for older mice. (●) Parasitemia generated by CTA1 clone maintained for 7 months through mouse-passages and inoculated into 31-day-old mice (3×10^4 trypomastigotes). (○) Parasitemia generated by CTA1 clone maintained for 1 month in mouse-passages and inoculated into 8-day-old mice (9×10^4 trypomastigotes).

3.4. Differences in CTA1 infectivity in cross passages

Cross passages from mouse to guinea pig and guinea pigs to mice were conducted to test for host-specific adaptations of the parasite. Mouse-passaged parasites were more infective than guinea pig-passaged parasites when tested in mice ($p < 0.05$) (Fig. 4A). However, the reciprocal effect was not observed in guinea pigs. Mouse-passaged parasites were more infective than guinea pig-passaged parasites when tested in guinea pigs (Fig. 4B). These results suggest that the increase in virulence is associated with host genetic characteristics. The parasite load was higher in guinea pigs compared to mice with the mouse-passaged parasites probably due to the immunosuppressive regimen to which guinea pigs were subjected.

4. Discussion

Several studies indicate that natural *T. cruzi* populations are composed of different biological clones with different virulence factors, which could confer competitive advantages. This interaction could result in parasites with new properties more adapted to develop in different host species (Macedo and Pena, 1998) or in defined culture conditions (Finley and Dvorak, 1987). Several studies show interactions among different genotypes of parasites, illustrating the influence of multiclonality at both, individual host and epidemiological levels (Read and Taylor, 2001). This heterogeneity could also affect the biological properties of the parasite during the process of attenuation by long-term culture and the increase in virulence after reintroduction to animal hosts. Another important mechanism involved in these processes could be the differential expression of virulence factors by parasite clones.

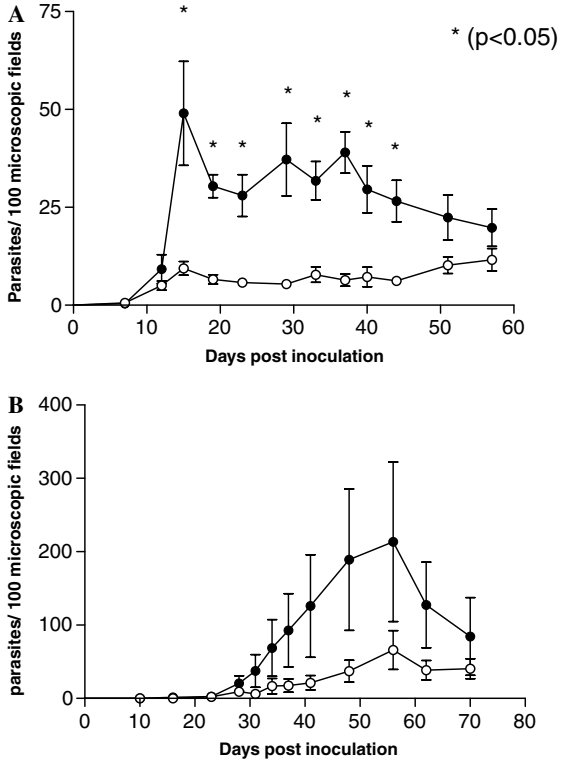

Fig. 4. Cross passages of CTA1 clone in mice and guinea pigs. (A) Parasites maintained by successive passages in mice (●) and in guinea pigs (○) were inoculated into 35-day-old Balb/c mice (3.6×10^3 mouse-derived trypomastigotes). (B) Parasites maintained by successive passages in mice (●) and in guinea pigs (○) were inoculated into guinea pigs (3.6×10^3 guinea pig-derived trypomastigotes).

The goal of the present work was to determine if a *T. cruzi* clone could modify its infectivity when submitted to different environmental conditions. Our findings demonstrate significant differences in the infectivity of the CTA1 clone in mice and guinea pigs. The selection of a more infective subpopulation at the beginning of the study was avoided, since the experiments were conducted with a single cell clone. Therefore, the variation observed in infectivity during the study could only be attributed to changes in the individual clone. MLEE analyses showed the stability of isoenzyme profile of the TolAc1 isolate and CTA1 clone. Our results show that the maintenance of CTA1 clone in two different hosts leads to alterations in its virulence but not in its genetic pattern as shown by RAPD assays. CTA1 clone passaged through mice was more virulent to mice and guinea pigs, whereas the virulence of guinea pigs-passaged CTA1 clone was unchanged. This phenomenon seems to be host-dependent since the clone's behavior in both cases was different. Host characteristics, such as immune responses probably influenced the changes observed in virulence. We only detected an increase in virulence in mice, which were relatively easier to infect than guinea pigs. In contrast, no virulence changes were observed in guinea pigs, which had to be immunosuppressed to set infection. These results suggest a strong influence of host genetic factors on the behavior of *T. cruzi* populations. Therefore, our experimental studies confirm the importance of both, host and parasite genetic background in *T. cruzi* infections, as previously reported by other authors (Andrade et al., 2002).

It is reasonable to think that parasite subpopulations with different genotypes are present within natural isolates, and that subpopulations can be selected after long-term culture or vertebrate host–parasite interaction. The fact that the CTA1 clone used in our work displayed a lower infectivity than the TolAc1 original isolate suggests the original population contains clones with different infective capacities, which are effectively transmitted as a whole. Martins et al. (2006) have recently demonstrated interactions among clonal *T. cruzi* populations and the impact in their biological properties. In this context, host and parasite genetics, multiclonal interactions, and *T. cruzi* phenotypic plasticity (modifications of infective capacity within clonal lines), should be considered for a better understanding of the diverse clinical forms of Chagas' disease.

Acknowledgments

We thank Rubén M. Cardozo, Alejandro Uncos, Federico Ramos, Clara Romero, and Ricardo Rossi for their technical assistance and Charles S. Rosenberg for critical advice and English revision of the manuscript. We also thank Howard Hughes Medical Institute for financial support.

References

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

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

Barnabé, C., Brisse, S., Tibayrenc, M., 2000. Population structure and genetic epidemiology of *Trypanosoma cruzi*, the agent of Chagas disease: a multilocus enzyme electrophoresis approach. Parasitology 120, 513–526.

Ben Abderrazak, S., Guerrini, S., Mathieu-Daudé, F., Truc, P., Neubauer, K., Lewika, K., Barnabé, C., Tibayrenc, M., 1993. Isoenzyme electrophoresis for parasite characterization. In: Hyde, J.E. (Ed.), Methods in Molecular Biology. Humana, New York, pp. 361–382.

Brisse, S., Barnabè, C., Bañuls, A.L., Sidibè, I., Noèl, S., Tibayrenc, M.A., 1998. Phylogenetic analysis of the *Trypanosoma cruzi* genome project CL Brener reference strain by multilocus enzyme electrophoresis and random amplified polymorphic DNA fingerprinting. Molecular and Biochemical Parasitology 92, 253–263.

Contreras, V.T., De Lima, A.R., Zorrilla, G., 1998. *Trypanosoma cruzi*: maintenance in culture modify gene and antigenic expression of metacyclic trypomastigotes. Memorias do Instituto Oswaldo Cruz 93, 753–760.

Deane, M.P., Sousa, M.A., Pereira, N.M., Goncalves, A.M., Momen, H., Morel, C.M., 1984. *Trypanosoma cruzi*: inoculation schedules and reisolation methods select individual strains from doubly infected mice, as demonstrated by schyzodeme and symodeme analyses. Memorias do Instituto Oswaldo Cruz 79, 513–515.

Diosque, P., Barnabé, C., Padilla, A.M., Cardozo, R.M., Tibayrenc, M., Basombrío, M.A., 2003. Multilocus enzyme electrophoresis (MLEE) análisis of *Trypanosoma cruzi* isolates from a geographically restricted endemic area for Chagas' disease of Argentina. International Journal for Parasitology 33, 997–1003.

Dusanic, D.G., Testa, J.M., Chao, D., 1994. Biological and immunological studies on a low virulence isolate of the Tulahuen strain of *Trypanosoma cruzi*. Chinese Journal of Microbiology and Immunology 27, 25–37.

Finley, R.W., Dvorak, J.A., 1987. *Trypanosoma cruzi*: analysis of the population dynamics of heterogeneous mixtures. Journal of Protozoology 34, 409–415.

Gaunt, M.W., Yeo, M., Frame, I.A., Stothard, J.R., Carrasco, H.J., Taylor, M.C., Mena, S.S., Veazey, P., Miles, G.A.J., Acosta, N., Rojas de Arias, A., Miles, M.A., 2003. Mechanism of genetic exchange in American trypanosomes. Nature 421, 936–939.

Isola, E.L.D., Lammel, E.M., Gonzalez Cappa, S.M., 1986. *Trypanosoma cruzi*: Differentiation after interaction of epimastigotes and *Triatoma infestans* intestinal homogenate. Experimental Parasitology 62, 329–335.

Lana, M., Pinto, A.S, Bastentra, B., Barnabé, C., Noel, S., Tibayrenc, C., 2000. *Trypanosoma cruzi*: infectivity of clonal genotypes infections in acute and chronic phases in mice. Experimental Parasitology 96, 61–66.

Martins, H.R., Toledo, M.J., Veloso, V.M., Carneiro, C.M., Machado-Coelho, G.L., Tafuri, W.L., Bahia, M.T., Valadares, H.M., Macedo, A.M., Lana, M., 2006. *Trypanosoma cruzi*: Impact of dual-clone infections on parasite biological properties in BALB/c mice. Experimental Parasitology, in press.

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

Morel, C., Chiari, E., Plessman Camargo, E., Marrei, D.M., Romanha, A.J., Simpson, L., 1980. Strains and clones of *Trypanosoma cruzi* can be characterized by pattern of restriction endonuclease products of kinetoplast DNA. Proceedings of the National Academy of Sciences of the United States of America 77, 6810–6814.

Murta, S.M.F., Romanha, A.J., 1999. Characterization of *Trypanosoma cruzi*. Memorias do Instituto Oswaldo Cruz 94, 177–180.

Pinto, A.S., Lana, M., Bastrenta, B., Barnabé, C., Quesney, V., Noel, S., Tibayrenc, M., 1998. Compared vectorial transmissibility of pure and biclonal infections of *Trypanosoma cruzi* in *Triatoma infestans*. Parasitology Research 84, 348–353.

Read, A.F., Taylor, L.H., 2001. The ecology of genetically diverse infections. Science 292, 1099–1102.

Souto, R.P., Fernandes, O., Macedo, A.M., Campbell, D.A., Zingales, B., 1996. DNA markers define two major phylogenetic lineages of *Trypanosoma cruzi*. Molecular and Biochemical Parasitology 20, 141–152.

Tibayrenc, M., Ward, P., Moya, A., Ayala, F.J., 1986. Natural populations of *Trypanosoma cruzi*, the agent of Chagas disease, have a complex multiclonal structure. Proceedings of the National Academy of Sciences of the United States of America 83, 115–119.

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

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