

Clonal structure of *Trypanosoma cruzi* Colombian strain (biodeme Type III): biological, isoenzymic and histopathological analysis of seven isolated clones

Estrutura clonal da cepa Colombiana do *Trypanosoma cruzi* (Biodema Tipo III): análise biológica, isoenzimática e histopatológica de sete clones isolados

Edson Luiz Paes Camandaroba¹, Rozália Figueira Campos², Juracy Barbosa Magalhães¹
and Sonia G. Andrade¹

Abstract The clonal structure of the Colombian strain of *Trypanosoma cruzi*, biodeme Type III and zymodeme 1, was analyzed in order to characterize its populations and to establish its homogeneity or heterogeneity. Seven isolated clones presented the basic characteristics of Biodeme Type III, with the same patterns of parasitemic curves, tissue tropism to skeletal muscle and myocardium, high pathogenicity with extensive necrotic-inflammatory lesions from the 20th to 30th day of infection. The parental strain and its clones C1, C3, C4 and C6, determined the higher levels of parasitemia, 20 to 30 days of infection, with high mortality rate up to 30 days (79 to 100%); clones C2, C5 and C7 presented lower levels of parasitemia, with low mortality rates (7.6 to 23%). Isoenzymic patterns, characteristic of zymodeme 1, (Z1) were similar for the parental strain and its seven clones. Results point to a phenotypic homogeneity of the clones isolated from the Colombian strain and suggest the predominance of a principal clone, responsible for the biological behavior of the parental strain and clones.

Key-words: *Trypanosoma cruzi*. Colombian strain. Clonal structure. Clones. Biodemes. Zymodemes. Histopathology.

Resumo A estrutura clonal da cepa Colombiana do *Trypanosoma cruzi*, biodema Tipo III e zimodema 1, foi analisada com o objetivo de caracterizar as suas populações e estabelecer a homogeneidade ou heterogeneidade das mesmas. Foram isolados sete clones, os quais apresentaram as características básicas do biodema Tipo III, com o mesmo padrão de curvas parasitemicas, tropismo tecidual para músculo esquelético e miocárdio, alta patogenicidade, com extensas lesões necróticas-inflamatórias, do 20^o ao 30^o dia de infecção. A cepa parental e os clones C1, C3, C4 e C6 apresentaram os níveis mais elevados de parasitemia entre 20 e 30 dias pós-infecção e alto índice de mortalidade até 30 dias (79 a 100%); os clones C2, C5 e C7 apresentaram níveis mais baixos de parasitemia com baixa mortalidade até 30 dias (7,6 a 23%). Os padrões isoenzimáticos foram característicos do zimodema 1 (Z1) para a cepa parental e os sete clones. Os achados do presente trabalho indicam uma homogeneidade fenotípica entre os clones isolados da cepa Colombiana e sugerem a predominância de um clone principal, responsável pelo padrão de comportamento biológico da cepa parental e dos clones.

Palavras-chaves: *Trypanosoma cruzi*. Cepa Colombiana. Estrutura clonal. Clones. Biodemas Zimodemas. Histopatologia.

The Colombian strain of *T. cruzi* has been used in experimental investigations since the pioneering studies of Federici *et al*². They described the progressive myocarditis occurring in mice as well as the main features of chronic disease in the murine model. Comparative studies of the biological behavior and histopathological lesions produced by the Colombian, Y¹⁷ and São Felipe strains¹ showed marked differences

in tissue tropism, virulence and pathogenicity³. These strains came to represent the prototypes for three different biological types or biodemes: I, II, III. The Colombian strain was isolated from a human case originating in Colombia¹² and was typed as Biodeme Type III and identified as zymodeme Z1 by Andrade *et al*⁴, according to the isoenzymic patterns of Miles *et al*⁴. This classification corresponds to the recently

1. Laboratório de Doença de Chagas Experimental do Centro de Pesquisas Gonçalo Moniz/Fundação Instituto Oswaldo Cruz (FIOCRUZ). Salvador, BA.

2. Departamento de Ciências Biológicas, Universidade Estadual de Feira de Santana, Feira de Santana, BA.

Financial support - PAPES II - FIOCRUZ, Rio de Janeiro, RJ.

Address to: Dra. Sonia G. Andrade. Centro de Pesquisas Gonçalo Moniz /FIOCRUZ. R. Valdemar Falcão 121, Brotas 41945-001 Salvador, BA.

Fax: 55 71 356-2155.

e-mail: sgandrade@cpqgm.fiocruz.br

Recebido para publicação em 3/7/2000.

established taxa *T. cruzi*, as referred by Momem¹⁵. Another characteristic of Biodeme Type III is its resistance to chemotherapy with benznidazole and nifurtimox⁴.

Since *T. cruzi* strains represent multiclonal populations¹⁸, investigation of their structure should contribute to define the homogeneity or heterogeneity of

its clonal constitution. This seems crucial to clarify a possible role of parasite strains in the clinical manifestations of Chagas' disease. Inasmuch the possibility of a predominance of principal clones, as representative of strains circulating in one endemic area, has been recently suggested^{2 9 10}.

MATERIALS AND METHODS

T. cruzi* strain.** The Colombian strain, maintained in our Laboratory by successive passages into mice for several years, has been used in the present investigation. ***Cloning of the strain. To obtain a single parasite, citrated blood taken from mice infected with the parental strain, on the 30th day of infection, was centrifuged at 900g and parasites in the plasma counted in a Neubauer chamber after dilution in PBS, pH 7.2. The volume of 1ml was distributed in multi-well microtiter culture plates and examined with an inverted microscope. By micromanipulation, a single trypomastigote form was isolated and intraperitoneally inoculated into an approximately 8-day-old suckling mouse. From 10 to 30 days after inoculation the peripheral blood was examined for the presence of trypanosomes. They were then defined as clones¹¹. Seven clones were isolated by this method and named as: Cl-Col-C1, C2, C3, C4, C5, C6 and C7 respectively. The blood of these mice was inoculated individually into groups of mice with 10 to 12g body weight. After 3 to 13 passages into mice, inocula (5×10^4 to 1×10^5) were obtained for the infection of the experimental groups and used for biological, isoenzymic and histopathological characterization of the clones to be compared with the parental strain. One group of mice, having the same body weight, was inoculated with the parental strain for comparison.

Biological characterization - Parasitemia was evaluated on the 7th to 50th day after infection, by microscopic examination of 5ml of fresh tail blood, between glass and coverslips, and counted in 50

microscopic fields (400X). The parasitemic levels were expressed as logarithms of the media of parasites in peripheral blood of 5 mice.

Cumulative mortality was evaluated from 30 to 50 days after infection. Morphology of trypomastigotes in peripheral blood was evaluated by the percentage of slender and broad forms, as seen in smears stained with the May Grünwald-Giemsa method.

Histopathological study. Three to five infected mice were sacrificed after 14, 20, 25 and 30 days post-infection (p.i). Fragments of the myocardium and skeletal muscle were fixed into 10% formalin and embedded in paraffin. Five-micrometer thick sections were stained with hematoxylin and eosin and microscopically analyzed.

Isoenzymic characterization. Enzymic extracts were obtained by hemoculture in axenic Warren culture medium from each clone. Parasites from culture were washed four times, with centrifugation at 2000g in buffer Tris-KRT pH 7.3 and enzymic extracts were prepared and stored in liquid nitrogen as *pearls*. The following isoenzymes were tested: 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). Electrophoresis was performed according to the method of Miles *et al*¹⁴.

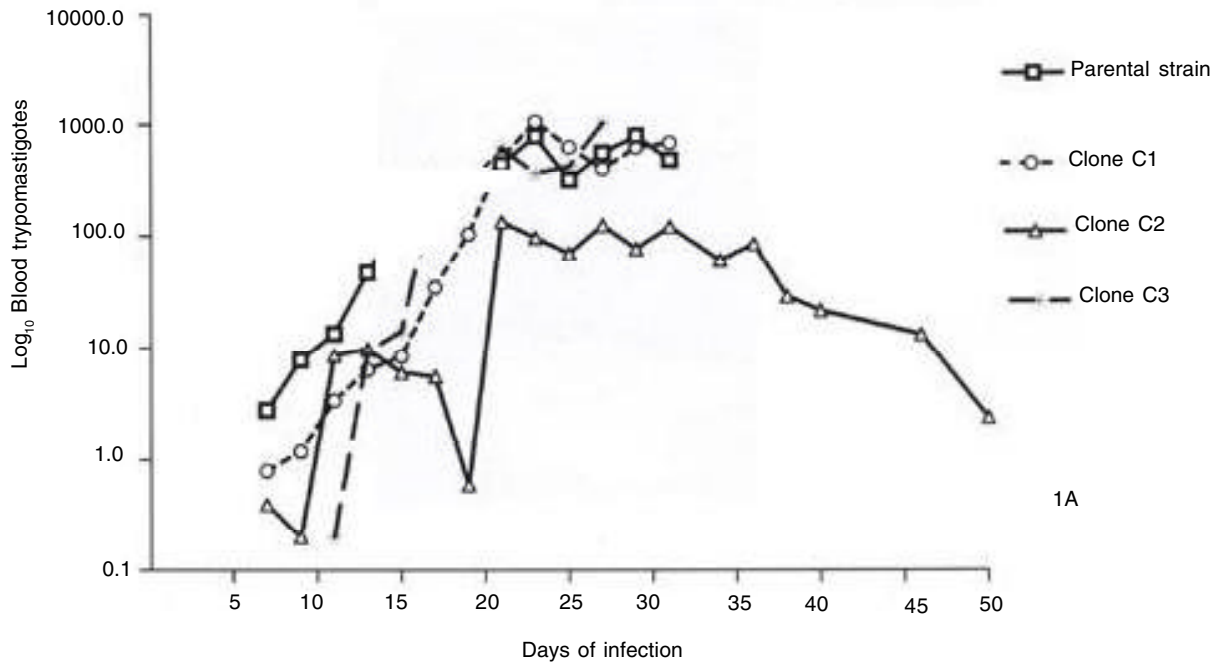
Statistical analysis. For comparative evaluation of the mortality of mice infected with parental strain and clones a non-parametric Fisher test was applied.

RESULTS

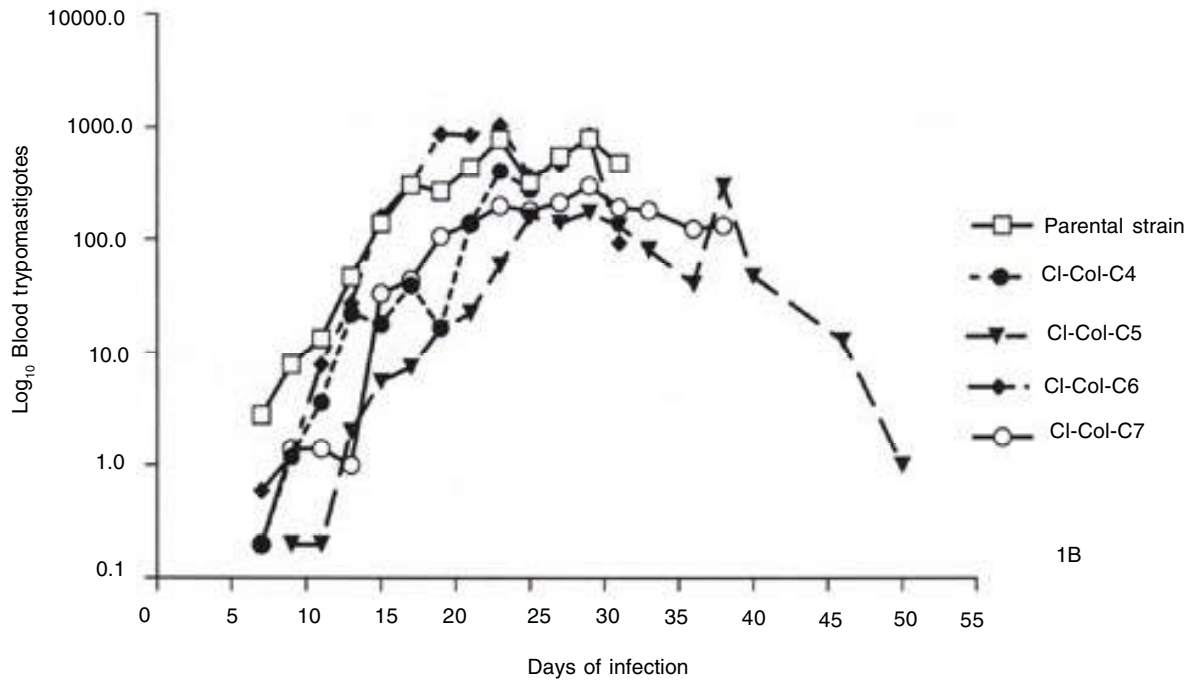
The biological behavior of the seven clones isolated from the Colombian strain were analyzed in comparison with the parental strain, taking into account several parameters, as follows: parasitemia, morphology of parasite in peripheral blood, cumulative mortality of the infected animals, tissue tropism and histopathological lesions. The parental strain and the seven clones have shown the parasitemic profiles of the Type III strain, with a slow increase in the number of parasites and with peaks of parasitemia between 20 and 30 days p.i. (Figures 1A and B). By taking the media for 5 mice, the parasitemic peaks were considered as high, above 500 trypomastigotes/50 microscopic fields (400X) or as medium, from 100 to 500 trypomastigotes. The strains with high parasitemic levels determined high mortality rates; medium parasitemic levels, coincided with low mortality. As shown in Table I, the cumulative mortality

of mice infected with the parental strain and the seven derived clones, expressed as percentage of dead animals, varied from a low (7.6 to 23%) to a high level (100%). Mortality up to 30 days of infection was low for the clones C2, C5 and C7 and high for the parental strain and the clones C1, C3, C4, C6 (100%). These findings indicate different degrees of virulence for the several clones investigated.

Morphological study of peripheral blood trypomastigotes showed a predominance of slender forms on the 10th day of infection for the parental strain and clones C3, C5 and C7, and broad forms from the 14th day onwards. The clones C1, C4 and C6 showed a predominance of slender forms up to the 14th day of infection. The parental strain and its 7 clones presented a predominance of broad forms by the 20th day of infection.



1A



1B

Figure 1A and 1B - Log mean number of blood trypomastigotes in 50 high power microscopic fields (400X) of 5 mice infected with either the parental Colombian strain or Clones CI-Col- C1, C2, C3, C4, C5, C6, C7. Parasitemic profiles are similar for the seven clones as compared with the parental strain with peaks from 20 to 30 days post-infection.

Table 1 - Cumulative mortality in mice infected with the Colombian strain of *Trypanosoma cruzi* and its clones (30 days of infection).

Experimental groups	Inoculum	Mice number	Mortality	Mortality (%)
Parental strain	1 x 10 ⁵	25	9/90	100.0
Cl-Col-C1	5 x 10 ⁴	18	11/11	100.0
Cl-Col-C6	1 x 10 ⁵	25	14/14	100.0
Cl-Col-C3	5 x 10 ⁴	25	12/13	92.3
Cl-Col-C4	1 x 10 ⁵	25	15/19	78.9
Cl-Col-C7	1 x 10 ⁵	25	3/13	23.0
Cl-Col-C2	1 x 10 ⁵	25	1/13	7.6
Cl-Col-C5	1 x 10 ⁵	25	1/13	7.6

Comparison of percentage of mortality between clones and parental strain has shown significant differences for clones C7, C2, C5 (p < 0.01 Fischer test).

Isoenzymic characterization. The electrophoretic patterns obtained for the parental strain and its clones, regarding the enzymes PGM, GPI, ALAT and ASAT, reproduced the profiles already described for the

Biodeme Type III⁷, corresponding to zymodeme Z1¹⁴ (Figures 2, 3 and 4). Clone C1 revealed 3 bands with the enzyme ALAT (Figure 4A); clone C3 revealed 2 bands with the enzyme ASAT (Figure 4B).

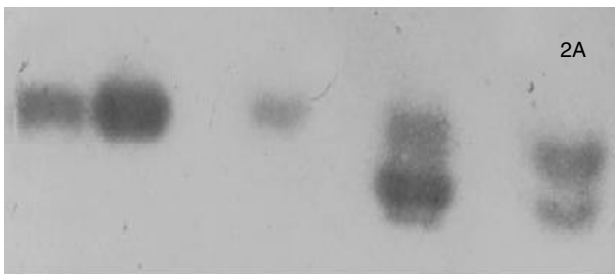


Figure 2A and 2B - Isoenzymic profiles for PGM of the Colombian parental strain and the clones Cl-Col-C3 and C4 in comparison with prototypes of Biodemes I (Per), II (21SF), III (Col).

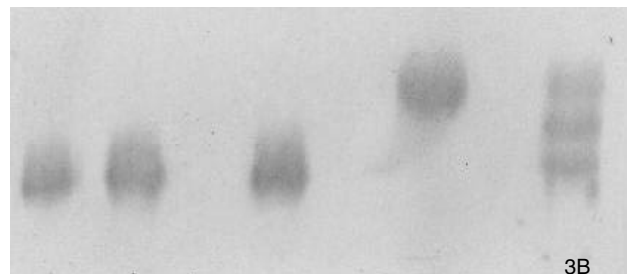
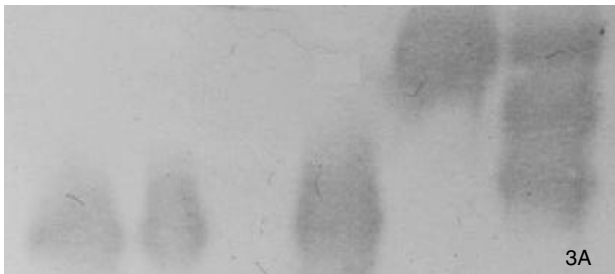


Figure 3A and 3B - Electrophoretic isoenzymic patterns for GPI of the Colombian parental strain and the clones Cl-Col-C2 and C7 as compared with the Biodemes I, II, III respectively: Per, 21SF, Col.

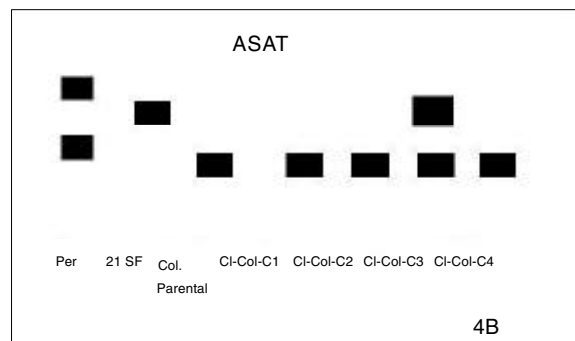
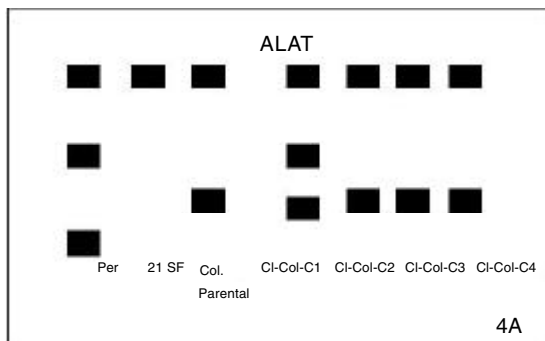


Figure 4A and 4B - Isoenzymic profiles for ALAT and ASAT of the Colombian parental strain and the clones Cl-Col-C1, C2, C3, C4 in comparison with the prototypes of Biodemes I, II, III. A 3-band profile of ALAT in clone C1 and a 2-band profile for ASAT in clone C3 represent alternative patterns for these enzymes.

Histopathological study. Study of the tissue lesions and parasite tropism was comparatively performed for the parental strain and its clones. The parental strain determined progressive skeletal muscle and myocardium lesions, from the 14th day of infection, with slight mononuclear infiltration and low parasitism of myocytes. By the 20th day of infection a progressive parasitism of myocardiocytes was seen, with focal myocyte necrosis and diffuse mononuclear-cell infiltration (Figure 5A). Tissue parasitism and destruction of myocells with dense inflammatory infiltration were prominent in skeletal muscles and progressive until 30 days of infection (Figure 5B).

Histopathological lesions determined by each one of the seven clones, from 14 to 30 days of infection, showed the same evolution and histopathological aspects of the parental strain. For the seven clones, parasitism and inflammatory infiltration were present at 14 and 20 days p.i., varying from slight to moderate in keeping with the virulence of the clone. Necro-inflammatory lesions were more prominent in skeletal muscles and intensified up to the 30th day of infection. In general, this late phase exhibited parasite and myocell disintegration, together with severe mononuclear-cell infiltration and rare preserved parasites (Figures 5C,D, E and F).

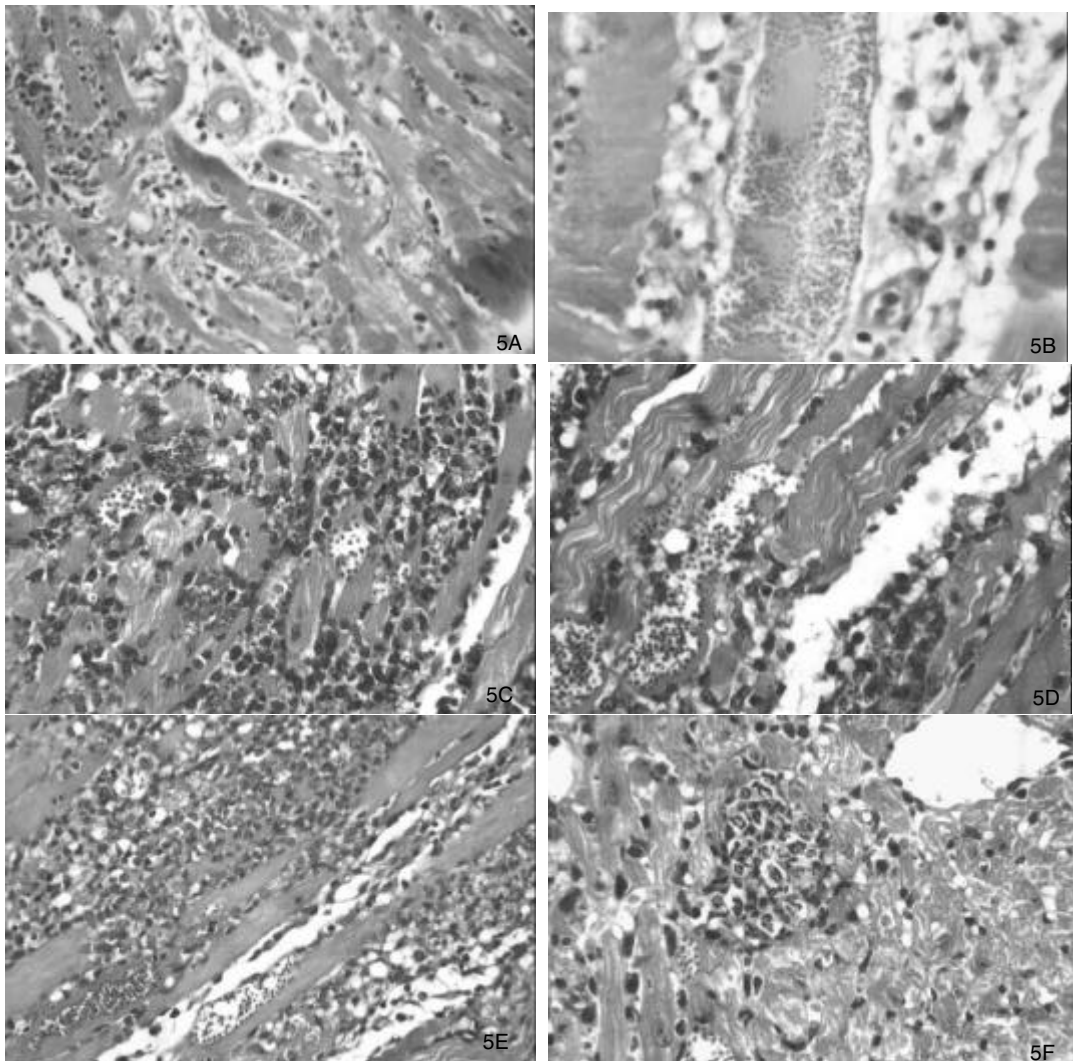


Figure 5A and 5B - Cardiac and skeletal muscle lesions in mice infected with the Colombian parental strain: **A** - Diffuse and moderate myocarditis and presence of parasites in the cardiac myocytes (250X); **B** - necrosis of skeletal muscle cells with mononuclear infiltrate; presence of a large nest of amastigotes in a damaged skeletal muscle cell (400 X); **Figure C and D** - Clone Cl-Col-C4: **C** -Intense myocarditis and presence of intracellular parasites in cardiac myocells (250X); **D** - Skeletal muscle cell with intracellular parasites associated with myocell disintegration and the presence of polymorphonuclear and mononuclear inflammatory cells infiltration (400X); **Figure E and F** - Clone Cl-Col-C6: **E** - Intense destructive lesions of the skeletal muscle with parasite debris and intracellular amastigotes; intense polymorphonuclear and mononuclear inflammatory infiltration (250X); **F** - myocardium showing intense mononuclear infiltration and the presence of focal disintegration of parasitized myocell with intense polymorphonuclear infiltration (250X).

DISCUSSION

T. cruzi strains represent multiclonal populations, as described by Tibayrenc *et al*²⁰. based on the multiloci isoenzymic profile of 121 strains from different geographical areas. Macedo and Pena¹³ suggest that multiclonal strains are formed by clonally propagating organisms with different tissue tropisms, correlated with complementary molecular interactions between the invading clones of *T. cruzi* and the host tissues. Clonal populations can be homogeneous or heterogeneous. It has been admitted that a limited number of *principal* clones could be circulating within different geographical areas¹⁹. The present study has demonstrated a homogeneity of the clones obtained from the Colombian strain, both considering its biological behavior and isoenzymic profiles. On analyzing the behavior of the Colombian strain after long period of maintenance in laboratory, by successive passages into mice, an increased virulence was detected, which was revealed by high mortality up to 30 days of infection. The Colombian strain, as initially described³, was characterized by a slow progression of parasitemia, that reached high levels by the 20th to 30th day of infection, with predominance of broad parasite forms from the initial phase of infection and low mortality up to 30 days p.i. Activation of parasite virulence, evaluated in infected mice, either after macrophage blockade with Indian Ink or treatment with corticoid, results in earlier increasing of parasitemia and the presence of slender parasitic forms⁶.

However, considering the profiles of parasitemia, the tissue tropism and pathogenicity pattern, the biological behavior of the Colombian strain was maintained. The seven clones, with the same biological patterns of the parental strain, exhibited different degrees of virulence, as expressed by high levels of parasitemia and elevated mortality; however as has been previously demonstrated¹, virulence is not a stable character, and therefore cannot independently be used for strain characterization. The same type of strain can present different degrees of virulence, evaluated by the levels of parasitemia and the rate of mortality of infected animals. The several strains isolated from São Felipe, BA classified as Biodeme Type II, could either exhibit low virulence, without mortality by the 20th day of infection; medium virulence, with 50% mortality or high virulence, with 100% mortality by 20 days of infection. However, comparative histopathological study revealed that lesions determined by the parental strain and its clones disclosed the same pattern of pathogenicity: same tissue tropism, with predominant involvement of skeletal muscles, intense myocarditis and high tissue parasitism. These findings suggest that when a predominant principal clone is present the histotropism of the strain and of the clones is similar, confirming the stability of strain behavior. The isoenzymic profile of Biodeme Type III represents the same pattern of zymodeme 1 as described by Miles *et al*¹⁴. The

isoenzymic patterns for GPI and PGM represent the same profile for the parental strain and clones, representing a homozygosis for these enzymes. However, the presence of a profile of three bands for ALAT in the clone C1 and two bands for ASAT in the clone C3 represents alternative patterns, as previously demonstrated^{7,14}. According to Miles *et al*¹⁴, of the 11 enzymes which did contribute to the separation of the zymodemes, four gave alternative patterns within individual zymodemes; as for ALAT, up to four ancillary, prominent bands were seen in zymodeme 1 (Z1)¹⁴; and ASAT has been described with one or two bands, for Z1^{7,14}.

The present results, indicative of homogeneity for the clones isolated from the Colombian strain, support the suggestion that the presence of a *principal clone* may be influencing the behavior of Type III strains that circulate in a given endemic area. The predominance of the same type of strain has been recently confirmed by Rodriguez *et al*¹⁶, studying 13 strains from Colombia, and showing that all of them belong to zymodeme Z1 and conform only one cluster by analysis of the dendogram.

The zymodeme Z1 is recognized as representing a sylvatic origin of the parasite⁸ but with presence in the domestic environments. Humans infected with this type of strain have already been identified⁵, and this can influence clinico-pathological manifestations and therapeutic responses in Chagas' disease patients, in endemic areas where sylvatic reservoirs or vectors occur. Resistance to chemotherapy observed with the parental strain is shared by each of its clones, as seen in recent studies (in publication). This seems to confirm the clonal homogeneity of the Colombian strain and its influence in the response to treatment, at least when the isolation of clones is performed around the 30th day p.i. The possibility of monoclonality could not be disproved, but the isolation of a limited number of clones could not confirm the absence of different clones not revealed with the technical approaches used in this study.

This does not preclude that different results could be obtained if the isolation of the clones was performed during an earlier phase of the infection. In this sense, new investigations are in course, with the objective of analyzing clones of Type III strains, obtained in the initial phase of infection and to test their sensibility to chemotherapeutics.

The findings of the present study point to an important role of the parasite strain in the course of infection in the vertebrate host, taking into account the predominance of a principal clone with a marked tissular tropism and high pathogenicity and resistance to chemotherapy. This could be responsible for severity of acute illness or explain chronic disease manifestations in patients infected with this type of strain, identified as of sylvatic origin, and corresponding to the proposed taxa *T. cruzi* I¹⁶.

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