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STABILITY OF ISOENZYME AND KINETOPLAST DNA (k-DNA) PATTERNS IN SUCCESSIVELY CLONED *TRYPANOSOMA CRUZI* POPULATIONS

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Four Trypanosoma cruzi *strains from zymodemes A, B, C and D were successively cloned on BHI-LIT-agar-blood (BLAB). Twenty clones from the first generation (F1), 10 from the second (F2) and 4 from the third (F3) from the strains A138, B147 and C231 were isolated. The D150 strain provided 29 F1 and 23 F2 clones. The strains and clones had their isoenzyme and k-DNA patterns determined. The clones from A138, B147 and C231 strains presented isoenzyme and k-DNA patterns identical between themselves and their respective parental strains. Therefore showing the homogeneity and stability of isoenzyme and k-DNA patterns after successive cloning. The D150 strain from zymodeme D (ZD) showed heterogeneity. Twenty-eight out of 29 clones of the first generation were of zymodeme A and only one was of zymodeme C, confirming previous reports that ZD strains consisted of ZA and ZC parasite populations. The only D150 strain clone of zymodeme C showed a k-DNA pattern identical to its parental strain. The remaining clones although similar among themselves were different from the parental strain. Thus the* T. cruzi *strains had either homonogeneus or heterogeneous populations. The clones produced by successive cloning provided genetically homonogeous populations. Their experimental use will make future results more reliable and reproducible.*

Key words: isoenzyme – kinetoplast DNA – *Trypanosoma cruzi* – clones

Trypanosoma cruzi, the etiological agent of Chagas' disease, is present in nature as parasite populations circulating among men, vectors, sylvatic reservoirs and domestic animals. *T. cruzi* strains isolated from many hosts showed high intraspecific variations in blood form morphology, virulence, pathogenicity, susceptibility to chemotherapy, antigenic constitution and host cell infectivity (Brener, 1977, 1985). Since parasite behaviour can be influenced by host and enviromental conditions, intrinsic markers have been used for the characterization and identification of *T. cruzi*. Biochemical methods, such as izoenzyme and k-DNA patterns have been extensively used (Romanha et al., 1979; Morel et al., 1980; Miles et al., 1980, 1981; Miles, 1985; Gonçalves et al., 1984a, 1985; Tibayrenc & Ayala, 1988). However, the stability of these markers in *T. cruzi* strains and clones is still uncertain. Some authors working on *T. cruzi* strains and clones have detected isoenzyme instability (Romanha et al., 1979, 1987; Tanuri et al., 1984; Bahia, 1985; Marques Araújo, 1985; Bogliolo & Godfrey, 1987; Carneiro et al., 1990) although others have reported isoenzyme stability (Miles et al., 1978; Dvorak et al., 1980; Goldberg & Silva Pereira, 1983; Evangelista, 1987; Montamat et al., 1987). The stability of k-DNA restriction pattern has also been described by Morel et al. (1980) and Gonçalves et al. (1985). The utilization of *T. cruzi* clones is essential in order to understand parasite populational constitution, and to obtain reproducible and less variable results (Dvorak, 1985; Postan et al., 1986; Finley & Dvorak, 1987). Hitherto, investigations have only been made with *T. cruzi* clones from a single cloning step, making questionable their origin.

In this work, we have studied the stability of isoenzyme and k-DNA patterns on *T. cruzi* populations after successive rounds of cloning.

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MATERIALS AND METHODS

Trypanosoma cruzi *strains* – Four *T. cruzi* strains (A138, B147, C231 and D150) isolated by hemocultures on LIT (Camargo, 1964) from chagasic patients from Bambuí county, Minas Gerais State, were used and cryopreserved at −196 °C (Chiari et al., 1989). The strains comprised zymodemes A, B, C and D (Romanha, 1982).

Parasite maintenance – All strains and clones were grown and maintained on LIT medium at 28 °C in stationary phase by passage every 20-25 days. The cryopreserved strains were thawed and cultivated 2 or 3 times before cloning.

Parasite cloning – The strains were cloned in BHI-LIT-agar-blood (BLAB) (Gomes et al., 1991). Forty parasites, suspended in 100 µl of LIT, were homogeneously spreaded on the BLAB surface in petry dishes of 8.5 cm diameter. The petry dishes were incubated at 28 °C during 25-30 days. After this time, the colonies were picked and transferred to LIT medium. The clones were grown and analyzed by isoenzyme and k-DNA electrophoresis. Clones with similar and distinct profiles were respectively sorted out and selected for the next round of cloning. The second (F2) and third (F3) clone generations were analized in the same way. From each strain 34 clones were obtained, 20 from F1, 10 from F2 and 4 from F3, except for the strain D150 which gave 52 clones (29 on F1 and 23 on F2). For A138, B147 and C231 strains F2 clones were obtained from 5 different F1 clones and F3 from 4 different F2 clones. For D150 strain F2 clones were obtained from 4 F1 clones A and the unique clone C. The strains and clones were grown in LIT medium to a final concentration of 10^9 parasites, harvested and washed 3 times in KRT (Krebs Ringer Tris, pH 7.3) buffer by centrifugation at 2,000 g for 10 min at 4 °C. The parasite pellets were stored at −20 °C until used for isoenzyme and k-DNA analysis.

Isoenzyme patterns – The parasite pellet was thawed and submitted to an osmotic lysis in enzymatic stabilizer buffer (2.0 mM dithiothreitol, 2.0 mM epsilon-amino-caproic acid and 2.0 mM Na_2-EDTA, pH 7.0). The lysate was centrifuged at 4 °C at 25,000 g for 60 min and the supernatant (enzymatic extract) was stored in 15 µl samples at −196 °C. Isoenzyme electrophoresis was performed in refrigerated thin layer starch gels. The following enzymes were studied alanine aminotransferase (ALAT) [E.C.2.6.1.2]; aspartate aminotransferase (ASAT) [E.C.2.6.1.1]; glicose phosphate isomerase (GPI) [E.C.5.3.1.9] and phosphoglucomutase (PGM) [E.C.2.7.5.1]. The runing and developing conditions were performed according to Romanha et al. (1979).

k-DNA patterns – The parasite pellet stored at −20 °C was thawed and the k-DNA extracted according to Gonçalves et al. (1984b). The k-DNA was digested with EcoR1, the fragments separated by electrophoresis on slab polyacrilamide gels and silver stained (Gonçalves et al., 1990).

RESULTS

Isoenzyme patterns – The results shown in Table and Fig. 1a, b demonstrate that after 3 successive clonings the clones of the strains A138, B147 and C231 presented identical zymodemes to their parental strain independent of cloning generation. On the other hand, (D150) presented on the first cloning 28 clones of zymodeme A and one clone of zymodeme C, that were stable after further cloning (Table and Fig. 2a, b). In parallel to the cloning procedure, the D150 parental strain was maintained on LIT medium in stationary phase. After approximately 8 months maintenance it changed from zymodeme D to zymodeme C (data not shown).

TABLE

Zymodemes of *Trypanosoma cruzi* clones after three successive rounds of cloning

Parental strain	Rounds of cloning		
	F1	F2	F3
A 138	20 A	10 A	4 A
B 147	20 B	10 B	4 B
C 231	20 C	10 C	4 C
D 150	28 A	18 A	ND
	1 C	5 C	

A, B, C and D are the zymodemes. The figures before zymodeme mean the number of clones analyzed. ND = not done.

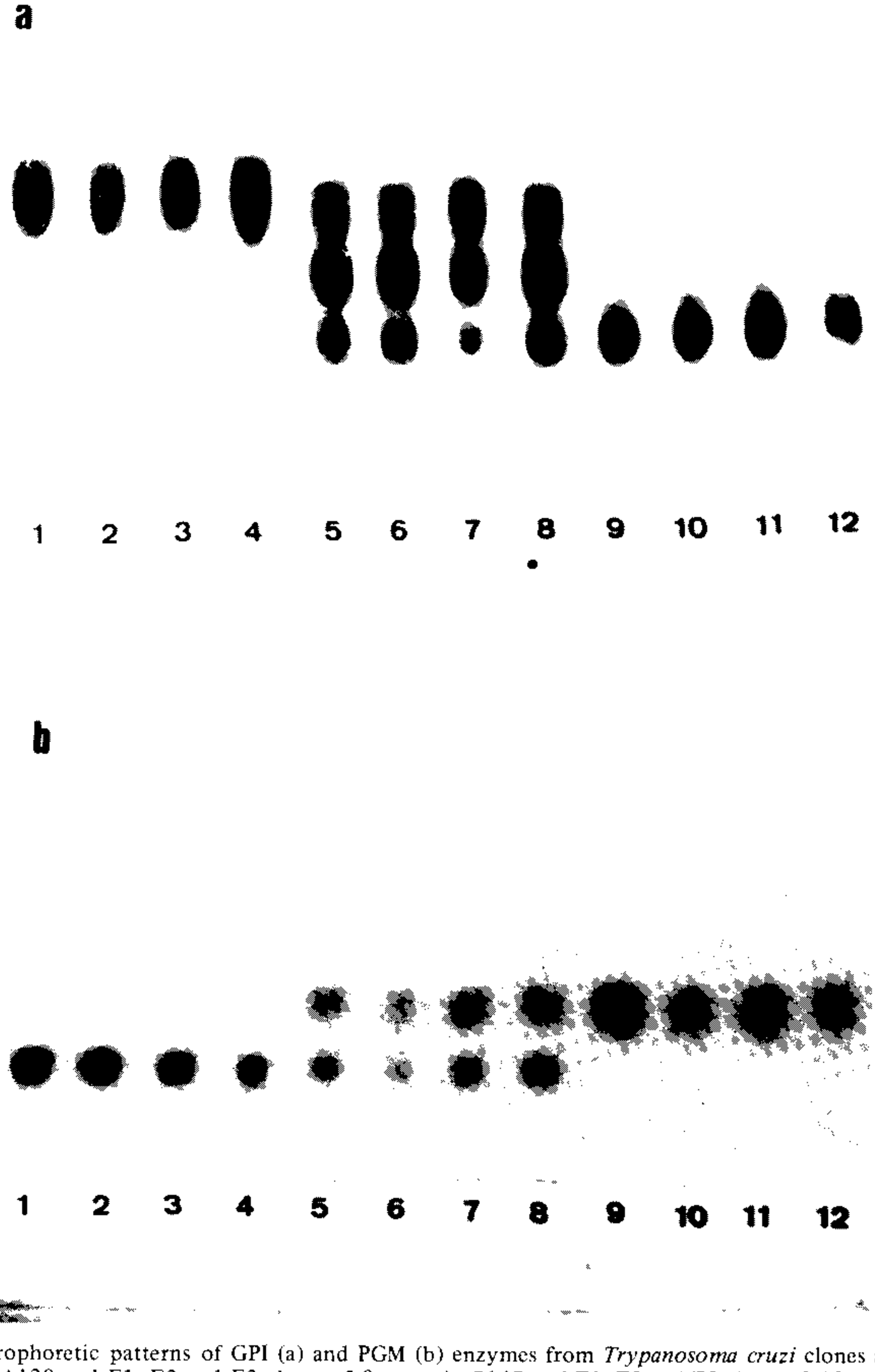

Fig. 1: electrophoretic patterns of GPI (a) and PGM (b) enzymes from *Trypanosoma cruzi* clones and strains. 1-4 – strain A138 and F1, F2 and F3 clones; 5-8 – strain B147 and F1, F2 and F3 clones; 9-12 – strain C231 and F1, F2 and F3 clones.

k-DNA patterns – All clones from A138, B147 and C231 strains presented k-DNA patterns identical to their parental strains, independent of cloning generation. Fig. 3 shows k-DNA patterns of parental strains and one representant of the F1 and F2 generation clones of each strain. Eight clones of D150 strain were analyzed and presented 2 distinct patterns. One clone presented a pattern identical to the parental strain and the other 7 although mutually identical presented a pattern different from that of the parental strain.

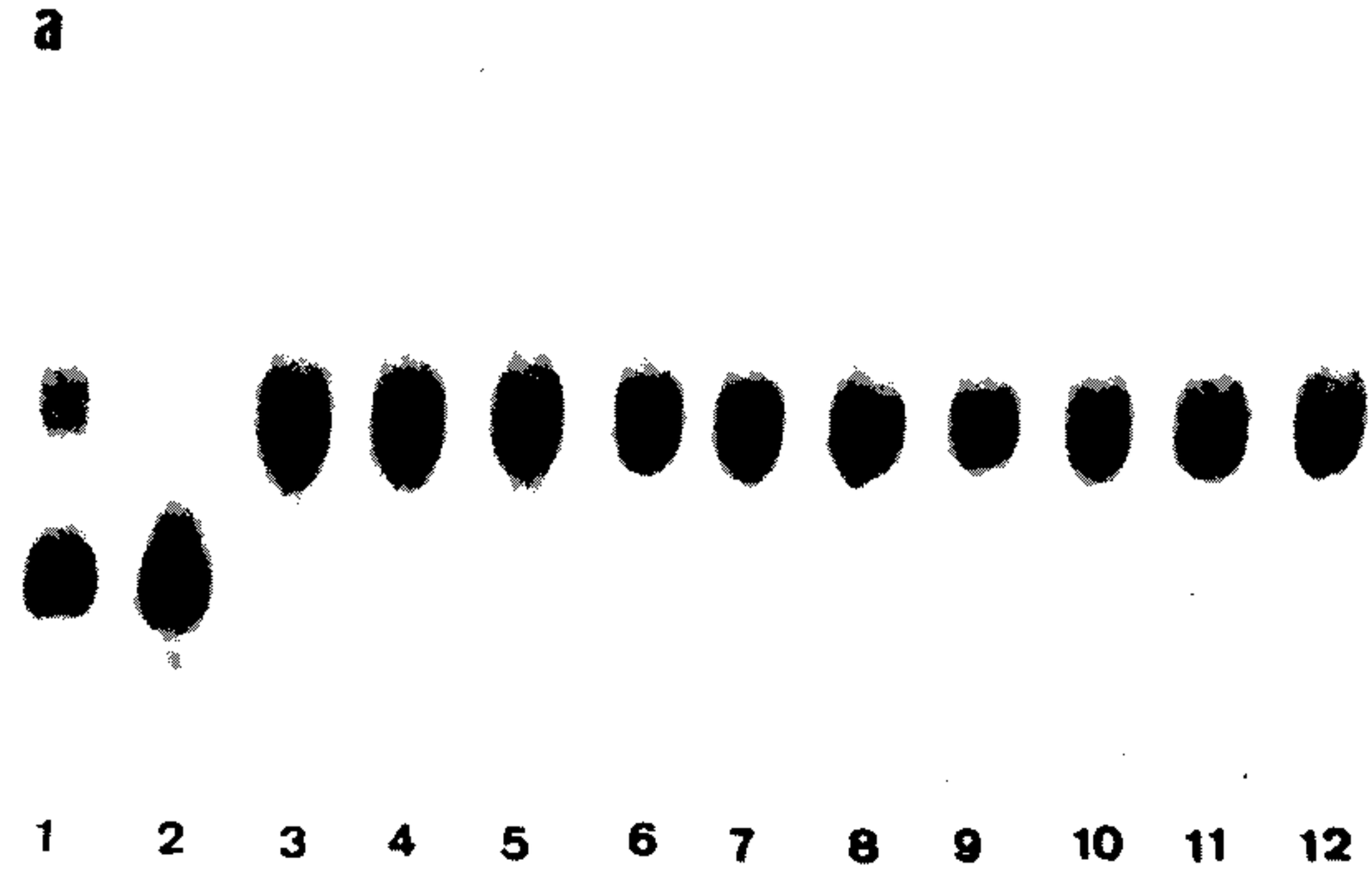

Fig. 2: electrophoretic patterns of enzymes GPI (a) and PGM (b) of *Trypanosoma cruzi* D150 strain and F1 clones. 1 – parental strain D150 (ZD); 2 – F1 clone (ZC); 3-12 – ten F1 clones (ZA).

DISCUSSION

Isoenzyme and k-DNA patterns were stable after successive cloning of 4 *T. cruzi* strains. The clones of strains A138, B147 and C231 presented identical isoenzyme patterns between themselves and their respective parental strains. Our results are in accordance with Goldberg & Silva Pereira (1983) who showed that the isoenzyme patterns of 18 subclone of Y and CL *T. cruzi* strains were identical to their parental strains and, Dvorak et al. (1980) who detected constant isoenzyme patterns among 9 *T. cruzi* clones, after maintenance in LIT medium and

Fig. 3: k-DNA restriction patterns of *Trypanosoma cruzi* strains and clones generated using EcoR1. 1-2 – B147 strain and clone; 3-4 – C231 strain and clone; 5-6 – A138 strain and clone; 7-9 – D150 strain and F1 clones.

tissue culture for 6 months. Montamat et al. (1987) also observed that 6 enzymes studied in different samples of the same *T. cruzi* clone kept constant after successive collections every 3 months for more than a year. In addition, isoenzyme stability was observed both by Miles et al. (1978) and by Evangelista (1987) in *T. cruzi* strains.

In contrast to our results, Tanuri et al. (1984) observed isoenzyme variations of 3 consecutive clonings of *T. cruzi* Y strain. F1 clones presenting zymodeme A did not have an altered pattern after different maintenance conditions and long term culture. However, after a second and a third cloning, the subclones were classified as B and C, respectively. This isoenzyme variation from zymodeme A to C was observed by Romanha et al. (1979), when the Y strain was maintained in exponential growth phase in LIT medium. The solid medium used by Tanuri et al. (1984) has a different composition which might contribute to isoenzyme variation.

The instability of isoenzyme patterns has also been observed on *T. cruzi* clones after passages through vertebrate hosts (Marques Araujo, 1985), in strains after serial subculture in LIT medium (Romanha et al., 1979) and after sucessives passages in vertebrate hosts (Romanha et al., 1987; Carneiro et al., 1990) and in invertebrate hosts (Bahia, 1985). These isoenzyme variations might be explained on the basis of mixed *T. cruzi* and clones. The finding of only one clone of zymodeme C and 28 of zymodeme A in strain D150, is surprising. Romanha (1982) observed the inverse rate, 32 clones from zymodeme C and only one from the zymodeme A, after cloning of a zymodeme D strain on agar-LIT. We believe that the BLAB medium used for cloning in our experiments favored the

growth of zymodeme A parasite clones. The D150 strain, initially classified as zymodeme D, after maintenance in stationary phase for 8 months on LIT medium changed its isoenzyme pattern to zymodeme C. Romanha et al. (1979) observed also that the *T. cruzi* Y and CL strains classified as zymodemes A and B, respectively, changed to zymodeme C. However this change occurred when these strains were maintained in an exponential growth phase. A comparative analysis among zymodemes and schizodemes from this strain showed that the unique F1 clone with zymodeme C presented a restriction pattern identical to that of the parental strain. This strain and the F1 clone presented different zymodemes even though they had the same restriction patterns. However, data from other authors have shown that a *T. cruzi* strain from a single zymodeme can present different schizodemes (Gonçalves et al., 1984a; Carneiro et al., 1990).

The electrophoretical analysis of k-DNA of the F1 and F2 clones from A138, B147 and C231 showed identical restriction patterns both between themselves and their parental strains after 2 consecutive clonings, showing the stability of this character. Our results confirmed the data presented by Morel et al. (1980); Gonçalves et al. (1985) and Garcia et al. (1986) who observed no alteration in k-DNA patterns in parasite populations maintained in a long term culture or after serial passage through vertebrate and invertebrate hosts. In contrast, Carneiro et al. (1990) showed alterations in the restriction patterns of 5 out of 13 strains maintained in mice for 18 months. The authors reported the possibility that these apparent alterations had occurred due to the fact that the original populations were composed of 2 or 3 subpopulations with different genotypes which were then selected by maintenance in mice over a long term period.

Our results show that the *T. cruzi* strains studied presented a variable level of heterogeneity. Other authors studying biological and/or biochemical characters of *T. cruzi* strains have also demonstrated the existence of homogeneous and heterogeneous strains (Morel et al., 1980; Romanha, 1982; Montamat et al., 1987; Marques Araujo & Chiari, 1988).

Although one out of 4 *T. cruzi* strains was heterogeneous in constitution the isoenzyme and k-DNA patterns were stable after successive cloning. This fact allows us to recommend the use of these biochemical characters as adequate intrinsic markers of *T. cruzi* characterization. Furthermore the clones produced by successive cloning provided genetically homogeneous populations. Their experimental use will make future results more reliable and reproducible.

REFERENCES

BAHIA, M. T., 1985. *Comportamento biológico de cepas de* Trypanosoma cruzi *no invertebrado.* Thesis, UFMG, 100 p.

BOGLIOLO, A. R. & GODFREY, D. G., 1987. Isoenzyme changes during the life cycle of *Trypanosoma cruzi. Trans. R. Soc. Trop. Med. Hyg., 81:* 222-229.

BRENER, Z., 1977. Intraespecific variations in *Trypanosoma cruzi:* two types of parasite populations presenting distinct characteristics. *PAHO, Sc. Pub., 347:* 11-21.

BRENER, Z., 1985. General review on *Trypanosoma cruzi* classification and taxonomy. *Rev. Soc. Bras. Med. Trop., 18:* 1-8.

CAMARGO, E. P., 1964. Growth and differentiation in *Trypanosoma cruzi.* I. Origin of metacyclic trypanosome in liquid media. *Rev. Inst. Med. Trop. São Paulo, 6:* 93-100.

CARNEIRO, M.; CHIARI, E.; GONÇALVES, A. M.; SILVA PEREIRA, A. A.; MOREL, C. M. & ROMANHA, A. J., 1990. Changes in the isoenzyme and kinetoplast DNA patterns of *Trypanosoma cruzi* strains induced by maintenance in mice. *Acta Tropica, 47:* 35-45.

CHIARI, E.; DIAS, J. C. P.; LANA, M. & CHIARI, C. A., 1989. Hemocultures for the parasitological diagnosis of human Chaga's disease in the chronic phase. *Rev. Soc. Bras. Med. Trop., 22:* 19-23.

DVORAK, J. A., 1985. Single cell isolates of *Trypanosoma cruzi.* How and why? *Rev. Soc. Bras. Med. Trop., 18:* 29-38.

DVORAK, J. A.; HARTMAN, D. L. & MILES, M. A., 1980. *Trypanosoma cruzi:* Correlation of growth kinetics to zymodeme type in clones derived from various sources. *J. Protozool., 27:* 472-474.

EVANGELISTA, E. A., 1987. Tripomastigota sanguineos do *Trypanosoma cruzi:* Características de eluição em colunas de DEAE-celulose e perfis de isoenzimas. Thesis, UFMG, 130 p.

FINLEY, R. W. & DVORAK, J. A., 1987. *Trypanosoma cruzi:* Analysis of the population dynamics of heterogeneous mixtures. *J. Protozool., 34:* 409-415.

GARCIA, E. S.; VIEIRA, E.; GONÇALVES, A. M. & MOREL, C. M., 1986. A strain of *Trypanosoma cruzi* and its biochemical characterization after passage through different invertebrate hosts. *Ann. Trop. Med. Parasitol., 80:* 361-363.

GOLDBERG, S. S. & SILVA PEREIRA, A. A., 1983. Enzyme variation among clones of *Trypanosoma cruzi. J. Protozool., 69:* 91-96.

GOMES, M. L.; MARQUES DE ARAUJO, S. & CHIARI, E., 1991. *Trypanosoma cruzi:* Growth of clones on solid medium using culture and blood forms. *Mem. Inst. Oswaldo Cruz, 86:* 131-132.

GONÇALVES, A. M.; CHIARI, E.; DEANE, M. P.; CARNEIRO, M.; ROMANHA, A. J. & MOREL, C. M.; 1984a. Schizodeme characterization of natural and artificial populations of *Trypanosoma cruzi* as a tool in the study of Chaga's disease, p. 235-275. In B. A. Newton, *Application of biochemical and molecular biology techniques to problems of parasite and vector identification.* Proceedings of and International Symposium, World Health Organization, Geneva, 1982.

GONÇALVES, A. M.; NEHME, N. S. & MOREL, C. M., 1984b. Trypanosomatid characterization by schizodeme analysis, p. 95-109. In C. M. Morel, *Genes and antigens of parasites. A laboratory manual.* 2nd ed., Fundação Oswaldo Cruz, Rio de Janeiro.

GONÇALVES, A. M.; NEHME, N. S. & MOREL, C. M., 1985. Schizodeme analysis of *Trypanosoma cruzi. Rev. Soc. Bras. Med. Trop., 18:* 67-73.

GONÇALVES, A. M.; NEHME, N. S. & MOREL, C. M., 1990. An improved silver staining procedure for schizodeme analysis in polyacrylamide gradiente gels. *Mem. Inst. Oswaldo Cruz, 85:* 101-106.

MARQUES ARAUJO, S., 1985. *Caracterização biológica de clones e cepas parentais do* Trypanosoma cruzi *em camundongos C3H isogenicos.* Thesis, UFMG, 119 p.

MARQUES ARAUJO, S. & CHIARI, E., 1988. Caracterização biológica de clones das cepas Y, CL e MR de *Trypanosoma cruzi* em camundongos C9H isogênicos. *Mem. Inst. Oswaldo Cruz, 83:* 175-181.

MILES, M. A., 1985. Isoenzyme characterization. *Rev. Soc. Bras. Med. Trop., 18:* 53-59.

MILES, M. A.; CEDILLOS, R. A.; POVOA, M. M.; SOUZA, A. A.; PRATA, A. & MACEDO, V., 1981. Do radically dissimilar *Trypanosoma cruzi* strains (zymodemes) cause Venezuelan and Brazilian forms of Chagas' disease. *Lancet, 20:* 1338-1340.

MILES, M. A.; LANHAM, S. M.; SOUZA, A. A. & POVOA, M., 1980. Further enzymic characters of *Trypanosoma cruzi* and their evaluation for strain identification. *Trans. R. Soc. Trop. Med. Hyg., 74:* 221-237.

MILES, M. A.; SOUZA, A.; POVOA, M.; BARRETT, T. V. & HOFF, R. H., 1978. Epidemiological studies of three *Trypanosoma cruzi* zymodemes in northern and eastern Brazil. V Reunião Anual sobre Pesquisa Básica em doença de Chagas, Caxambu, MG, p. 10-13.

MONTAMAT, E. E.; ARAUJO, S.; CAZZULO, J. J. & SUBIAS, E., 1987. Characterization by electrophoretic zymograms of 19 *Trypanosoma cruzi* clones derived from two chronic chagasis patients. *Comp. Biochem. Physiol., 87B:* 417-422.

MOREL, C. M.; CHIARI, E.; MATTEI, D. M.; ROMANHA, A. J. & SIMPSON, L., 1980. Strains and clones of *Trypanosoma cruzi* can be characterized by pattern of restriction endonuclease products of kinetoplastic DNA minicircles. *Proc. Nat. Acad. Sci. USA, 77:* 6810-6814.

POSTAN, M.; Mc DANIEL, J. P. & DVORAK, J. A., 1986. *Trypanosoma cruzi:* constancy of clone pathogenicity for inbred mice during long-term *in vitro* maintenance. *Trans. R. Soc. Trop. Med. Hyg., 80:* 659-662.

ROMANHA, A. J., 1982. *Heterogeneidade isoenzimatica em Trypanosoma cruzi.* PhD Thesis, UFMG, 110 p.

ROMANHA, A. J.; CARDOSO, J. E. & BRENER, Z., 1987. Instability of *Trypanosoma cruzi* isoenzyme characters during experimental chagasic infection. *Mem. Inst. Oswaldo Cruz, 82:* 63.

ROMANHA, A. J.; SILVA PEREIRA, A. A.; CHIARI, E. & KILGOUR, V., 1979. Isoenzyme patterns of cultured *Trypanosoma cruzi:* changes after prolonged subculture. *Comp. Biochem. Physiol., 62B:* 139-142.

TANURI, A.; VON KRUGER, W. & ALMEIDA, D. F., 1984. Intraspecific isoenzyme diversity in *Trypanosoma cruzi.* XV Reunião Anual sobre Pesquisa Básica em Doença de Chagas, Caxambu, MG, Abstract BQ 43.

TIBAYRENC, M. & AYALA, F. J., 1988. Isoenzyme variability in *Trypanosoma cruzi,* the agent of Chagas' disease: genetical, taxonomical and epidemiological significance. *Evolution, 42:* 277-292.