RESEARCH NOTE

Genetic Polymorphism Among Six *Trypanosoma cruzi* Stocks Isolated from Chronic Chagasic Patients

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Among the factors that act in the pathogenesis of Chagas disease, some are related with the parasite, such as polymorphism, tropism, antigen constituents and parasite load. Other factors are related to the host, such as genetic constitution and sex. *Trypanosoma cruzi* is not an homogeneous population but consists of different populations which circulate in nature between man, vectors, domestic animals and wild reservoirs (CD Fernandes et al. 1997 *Mem Inst Oswaldo Cruz 92*: 343-351). These populations show diverse behaviours in terms of parasitemia, virulence, pathogenicity, drug sensitivity and interaction with host cells (Z Brener 1973 *Annu Rev Microbiol 27*: 347-383).

The wide intraspecific diversity of *T. cruzi* has stimulated the study not only of populations but also of clones of the same population (M Tibayrenc et al. 1988 *Mem Inst Oswaldo Cruz 83*: 249-255).

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The random amplified polymorphic DNA reaction (RAPD) has been frequently used in the analysis of genetic polymorphism in order to clarify the real extent of *T. cruzi* variability (B Oury et al. 1997 *J Parasitol 83*: 52-57). The characterization of different stocks may contribute to the detection of possible correlations between the experimental behaviour of the stocks and their role in the pathogenicity of the disease, causing different clinical forms.

The objective of the present study was to detect genetic polymorphism among six *T. cruzi* stocks recently isolated from patients in the chronic phase of Chagas disease with different clinical presentations by the RAPD technique.

The stocks were isolated from chagasic patients at the University Hospital, Faculty of Medicine, Federal University of Goiás (A Luquetti et al. 1986 *Trans R Soc Trop Med Hyg 80*: 462-470, EC Oliveira et al. 1997 *Trans R Soc Trop Med Hyg 91*: 25-27) and the Y strain was used as control (LHP Silva & V Nussenzweig 1953 *Folia Clin Biol 20*: 191-208). The following stocks were analyzed: (1) stock MHOM/BR/93/GOCH412 from a 34-year-old male patient with the indeterminate form; (2) stock MHOM/BR/94/GOCH519 from a 25-year-old female patient with the indeterminate form; (3) stock MHOM/BR/94/GOCH491 from a 58-year-old female patient with the indeterminate form; (4) stock MHOM/BR/93/GOCH357 from a 58-year-old female patient with the digestive form (megaesophagus, Group I); (5) stock MHOM/BR/94/GOCH466 from a 53-year-old female patient with the digestive form (megacolon and megaesophagus, Group III); (6) stock MHOM/BR/93/GOCH391 from a 50-year-old male patient with the slight cardiac form. The *T. cruzi* stocks were isolated from patients by xenodiagnosis. Forty *Triatoma infestans*, *Dipetalogaster maximus* or *Rhodnius prolixus* were used and their feces were examined 30-60 days after the blood meal; the positive triatomines were separated; fecal material was diluted in isotonic saline and inoculated into isogenic Balb/C mice. The mice were examined daily until they showed positively, when blood was collected and transferred into liver infusion tryptose (LIT) medium (EP Camargo 1964 *Rev Inst Med Trop São Paulo 6*: 93-100) from which parasites were obtained for the DNA extraction.

The reaction was performed in a Perkin Elmer GeneAmp PCR System 2400 and visualized by running on 0.8% agarose gel. DNA was extracted by the method of MI Borges et al. (1990 *Fungi Gent Newsl 37*: 10). Fifteen ml of the epimastigote form culture in the exponential growth phase were used (10^6 cells/ml). Cells were washed in 20 mM Tris HCl buffer pH 8.8, 5 mM $MgCl_2$, 250 mM

sucrose by centrifugation at 10,000 g for 10 min and the process was repeated three times. Cells were incubated at 65°C with extraction buffer PVP (polyvinylpyrrolidone) 1g, H_2O 9 ml , NaCl 5 M, Tris pH 8.0 1 M, EDTA (ethylenediaminetetraacetic acid) 0.5 M, CTAB (cationic hexadecyl trimethyl ammonium bromide) 5%, β mercaptoetanol 0.1 ml, for 60 min with gentle shaking at 15 min intervals, and then cooled to room temperature and supplemented with an equal volume of chloroform-isoamylic acid (24:1). After centrifugation at 10,000 g for 10 min, the aqueous phase was transferred to another tube and an equal volume of chloroform-isoamylic acid (24:1) was added. After a new centrifugation under the same conditions as described above, the aqueous solution was again transferred to another tube and ice-cold isopropylic acid (2/3 of the volume) was added. After centrifugation at 10,000 g the precipitate was washed with 70% alcohol and dried at room temperature and the DNA was resuspended in Tris HCl pH 8.0 20 mM and EDTA 0.1 mM. The amount of DNA was determined by absorbance at 260 nm and by comparison with known amounts of DNA on agarose gel stained with ethidium bromide. Nineteen oligonucleotides were tested and nine (OPG-02/ 5'GGCACTGAGG3'; OPG-3/5'GAGCCCTCC A3'; PG-08/5'TCACGTCCAC3';OPG-10/5'AGGG CCGTCT3';OPG-13/5'CTCTCCGCCA3';OPG-14/5'GGATGAGACC3';OPG-16/5'AGCGTCC TCC3';OPG-17/5'ACGACCGACA3';OPG-18/ 5'GGCTCATGTG3' Operon Biotechnologies) of them were selected because they presented a larger number and a better definition of the amplified DNA fragments and also greater band intensity. The amplification reactions were carried out in a 25 µl system containing 5 ng/µl of DNA, buffer containing $MgCl_2$ 2.5 mM, dATP/dTTP/dGTP/ dCTP 2.5mM each, primer 10 ng/µl, 17.5 µl autoclaved milli-Q water, 2.5 U/µl Taq DNA polymerase (Cenbiot-RS). Negative controls, one of them containing all the ingredient of the mixture except DNA and the other containing no primer, were used.

The amplification reaction was carried out in a thermocycler in which each cycle consisted of a defined time and temperature for DNA denaturation, oligonucleotide annealing and enzymatic reaction. An initial denaturation and annealing cycle was carried out for 2 min at 98°C, 2 min at 50°C and 1 min at 35°C. The reaction was stopped for the addition of the enzyme and a series of 35 cycles lasting 2.5 min at 72°C, 1.5 min at 92°C, 1 min at 35°C were performed, with a final extension period at 35°C. The products of DNA amplification obtained by the RAPD technique were visualized by 0.8% agarose gel electrophoresis (Sigma type I). The gel was submitted to 75 V for 4 hr. The end of electrophoresis was monitored by the migration of the bromophenol blue stain present in the sample buffer.

Analysis was performed in an ultraviolet light transluminator and the gel was photographed. Data were analyzed statistically by the Jaccard coefficient and submitted to calculation of the association coefficient, which uses binary variables to determine similarities between units. The bands or fragments were considered to be the variables and the stocks were considered to be the units. The grouping algorithm used was the unweighted pair group method, arithmetic average (UPGMA), and the program was the numerical taxonomy and multivariate analysis system (NTSYS), version 1.7, 1992.

The Jaccard coefficient was calculated by the formula $J = a/(n-d)$, where a is the number of positive agreements, i.e. of the bands or fragments present, n is the number of variables and d is the number of negative agreements, i.e., absent bands. Thus, this coefficient uses only the positive agreements as the numerator. In this analysis, the greater the similarity of two isolates, the higher will be the value of the association coefficient.

The different *T. cruzi* stocks were compared in terms of the patterns of fragments generated by genomic DNA amplification using random primers. Nine oligonucleotides were selected for the DNA amplification reactions for the various stocks. The fragments generated by PCR were analyzed on agarose gel (Fig. 1). The various oligonucleotides permitted the amplification of DNA fragments varying in size from 0.56 to 4.34 kb. The data obtained were used to construct a binary value matrix according to the presence or absence of bands among the stocks. A value of 1 was attributed to the presence of a given DNA fragment on the agarose gel, and the 0 index was attributed to its absence. A matrix with 39 lines corresponding to the genomic fragments generated by PCR and with seven columns corresponding to the Y strain and to stocks 357, 391, 412, 466, 491 and 519 was constructed.

From the data matrix the similarity of the different *T. cruzi* stocks was analyzed. Indexes varied from 0.8437 between stocks 519 and 391 to 0.3548 between stocks 466 and 357. The data were used to construct the dendrogram illustrated in Fig. 2. The *T. cruzi* stocks and control strain formed three groups: one consisting of the Y strain with similarity indexes of less than 50% in relation with the stocks, a second group consisting of a single stock (357), and a third with the remaining stocks.

Our results demonstrate the discriminative power of the RAPD technique for *T. cruzi* stocks, as reported for other pathogenic organisms (CMA

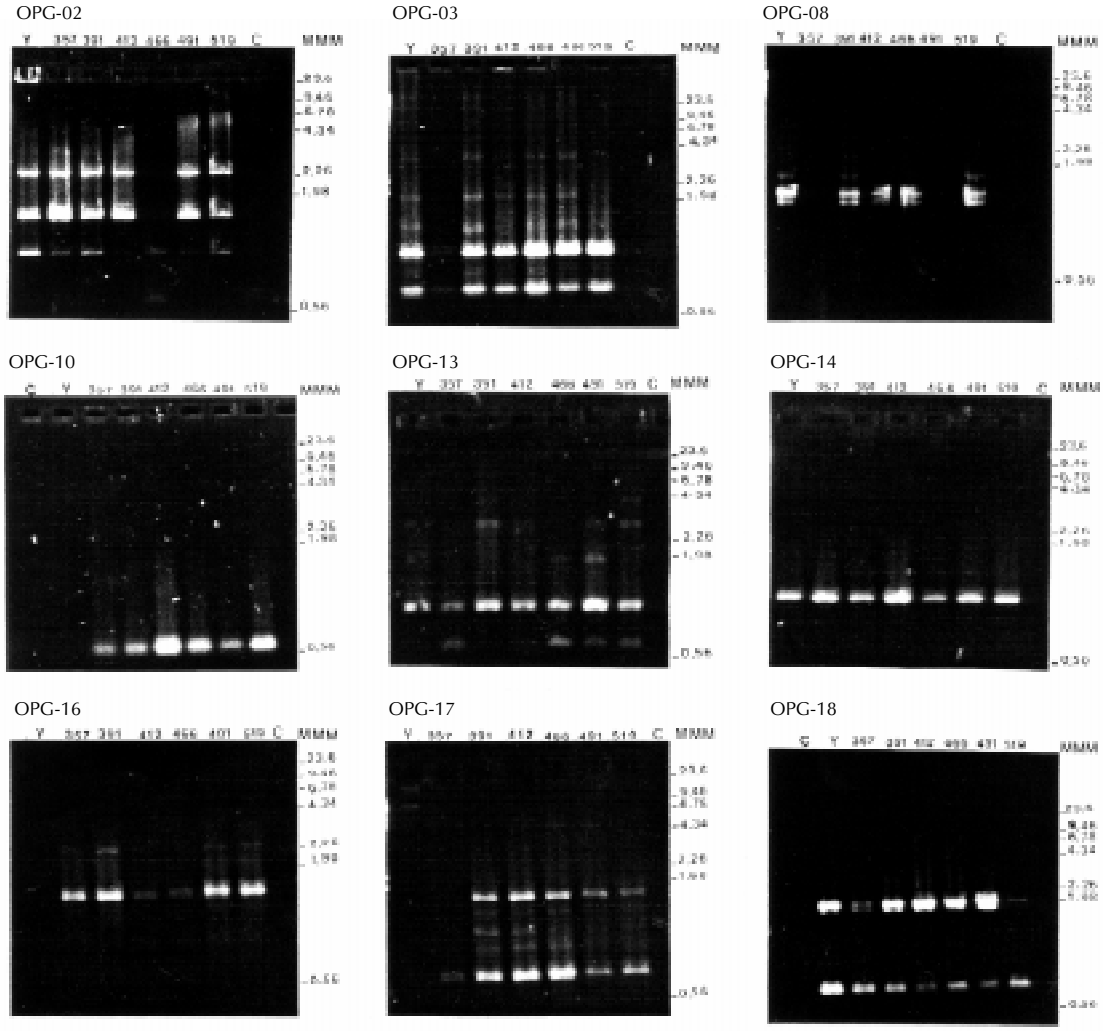

Fig.1: genomic fragments originated by polymerase chain reaction with random primers. OPG refers to the identification of the nine oligonucleotides used. The numbers above the figure refer to the different stocks of *Trypanosoma cruzi*. Y: control strain; C: negative control; MMM: molecular mass marker of DNA of the λ phage digested with Eco RI and Hind III.

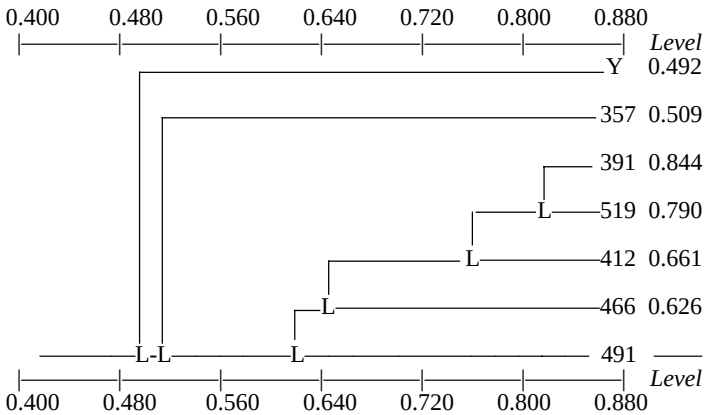

Fig.2: dendrogram from six stocks of *Trypanosoma cruzi* and Y strain, constructed on the basis of fragments generated by random amplified polymorphic DNA.

Soares et al. 1995 *J Clin Microbiol 33*: 505-507, AC Hilton et al. 1997 *Lett Appl Microbiol 24*: 243-248). ML Gomes et al. (1998 *Acta Trop 69*: 99-109) reported the use of the RAPD technique in the investigation of genetic differences between *T. cruzi* stocks isolated from chronic chagasic patients. RP Oliveira et al. (1997 *Parasitol Today 13*: 196-200), demonstrated that strains isolated from chronic chagasic patients show lower variability (similarity indexes higher than 64%) in RAPD profiles than those isolated from patients in the acute phase of Chagas disease or from sylvatic hosts. Both groups did find, as in this study, differences between stocks, detected by RAPD, but, as also described in the present work, no clear correlation between RAPD subgroups and the clinical form was observed. For example, our results demonstrate that the RAPD profile of stock 357 was different from the other five stocks, but the clinical presentation of the patient from which parasites were isolated (megaesophagus group I) was not different from the other digestive form included (stock 466), suggesting that there was no relation between clinical forms and differences in stocks, by RAPD.

We conclude that patterns of DNA fragments generated by RAPD permitted the discrimination between *T. cruzi* stocks, but were not correlated with the different clinical forms studied.

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