Population Polymorphism of *Trypanosoma cruzi* in Latin America indicated by Proteome analysis and by in vitro amastigote proliferation

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Abstract: Nineteen stocks of *Trypanosoma cruzi* originating from several endemic countries for Chagas' disease in Central and South America were subjected to two-dimensional protein electrophoresis analysis. The presence or absence of a total of 492 polypeptide spots among 19 gel profiles was determined. The stocks were classified into three major distinctive groups derived from (I) Central America and the northern part of South America; (IIa) Central America and the northern part of South America; and (IIb) central and southern parts of South America, which showed perfect concordance with the previously reported classification based on isozyme and DNA sequence analyses. Late log phase of each epimastigote was inoculated to human cell lines WI-38 and Hs 224.T originating from the lung and muscle, respectively, and the number of trypomastigotes released was counted. The number of trypomastigotes from *T. cruzi* in group I released from the two cell lines was significantly higher than that in group III ($p<0.05$). The findings suggested that the phenetic distance appearing within the *T. cruzi* may, to some extent, be associated with the intracellular growth of *T. cruzi*, one of the characteristic features of growth found in the species.

keywords: *Trypanosoma cruzi*; Two-dimensional gel electrophoresis; in vitro amastigote proliferation, genetic polymorphism, Latin America

INTRODUCTION

Chagas' disease, caused by infection of the flagellate protozoan *Trypanosoma cruzi*, is an important health problem in Central and South America, since 16 to 18 million people in the endemic area suffer from this disease and an additional 90 million are exposed to the risk of acquiring the infection [1]. The illness may remain an asymptomatic infection throughout life or develop into overwhelming acute myocarditis in infants or cardiomyopathy in patients in all endemic area and/or digestive forms predominantly in the southern part of South America [2]. Although the factors influencing this variable clinical course have not been elucidated, it has been suggested that the association of clinical symptoms and the severity of the disease in certain geographic regions may be related to genetic factors in the human population and the variability within *T. cruzi* species [2-9].

The extent of genetic variability within the species *T.*

cruzi was studied in terms of its isozyme patterns (zymodemes) [2, 4-10], kinetoplast DNA restriction fragments (schizodems) [11], karyotypes [12, 13], and random amplification of polymorphic DNA (RAPD) [8, 14, 15], leading to a conclusion that *T. cruzi* exhibits by nature a high genetic variability. Recently, population-genetic approaches have shown that *T. cruzi* presents a typical clonal population structure and consists of at least two major lineages that are very ancient events [15-17], while analyses of the population of *T. cruzi* isolated from humans and insect vectors in major endemic areas of Central and South America by zymodeme [16, 18, 19] and polymorphism of several genes [20, 21] suggest the existence of three lineages.

In the present study, the proteins extracted from 19 stocks of *T. cruzi* isolated in Guatemala, Ecuador, Peru, Brazil, Paraguay, and Chile were analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The number of shared and unshared polypeptide spots on the profiles among the stocks indicated how many lineages

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Table 1. Origin of the isolates and strains of *Trypanosoma cruzi* used in this study

Name of isolates	Host	Locality	Zymodeme Classification	Reference
172, TM14, TM43, TM47, TM51, TM52,	*T. dimidiata*	Guatemala	I	19, 21
CL strain	*T. infestans*	Brazil	I	21
119	Opossum	Ecuador	I	21
H6, H18	Human	Guatemala	I	19, 21
H1, H20	Human	Guatemala	IIa	19, 21
Peru 1, Peru 2	Human	Peru	IIa	19, 20
Tulahuen	*T. infestans*	Chile	IIb	21
Y strain	Human	Brazil	IIb	21
GS, LO, RF	Human	Paraguay	IIb	18, 19

of *T.cruzi* exist in the endemic areas of Chagas' disease.

MATERIALS AND METHODS

Parasites

Nineteen stocks of *Trypanosoma cruzi* from different ecological and geographic origins were used (Table 1). Epimastigotes of individual *T. cruzi* were maintained at 26°C in liver infusion tryptose (LIT) medium supplemented with 10% heat-inactivated fetal calf serum (FCS), according to the well-established method [22].

Sample preparation

Epimastigotes were collected by centrifugation (400 x g for 15 min) followed by washing twice with phosphate-buffered saline (PBS). After measurement of the wet weight of the pellet,100 μl of lysis buffer (0.15 M NaCl, 4% Triton X-100, 10 mM Tris-HCl, pH 8.0) containing 2 mM phenyl-methylsulfonylfluoride and 20 μg/ml leupeptin was added to 100 mg pellet (approximately 1.5 x 10 parasites), mixed well by vortex, and kept on ice for 10 min. The mixture was then centrifuged at 8,000 x g for 10 min and the supernatant was collected. An aliquot of the solution was drawn off and was used for the determination of protein concentration using micro BCA protein assay reagent kit (Pierce, Rockford, IL, USA). Finally, 120 mg urea, 10 μl 2-mercaptoethanol, and 22 μl of 40% Ampholine (pH 3.5-10, Pharmacia, Uppsala, Sweden) was added to the supernatant. About 2 mg of protein, which corresponded to 50-80 μl of the final mixture, was used as a sample for 2D-PAGE.

2D-PAGE

2D-PAGE was performed according to the method of O'Farrell [23] with slight modifications. Briefly, the prepared sample was electrofocused in a tube gel containing 9.2 M urea, 2% Triton X-100, and 4% Ampholine (pH3.5-10) for a total of 6875 Vh under the condition that 0.01 M
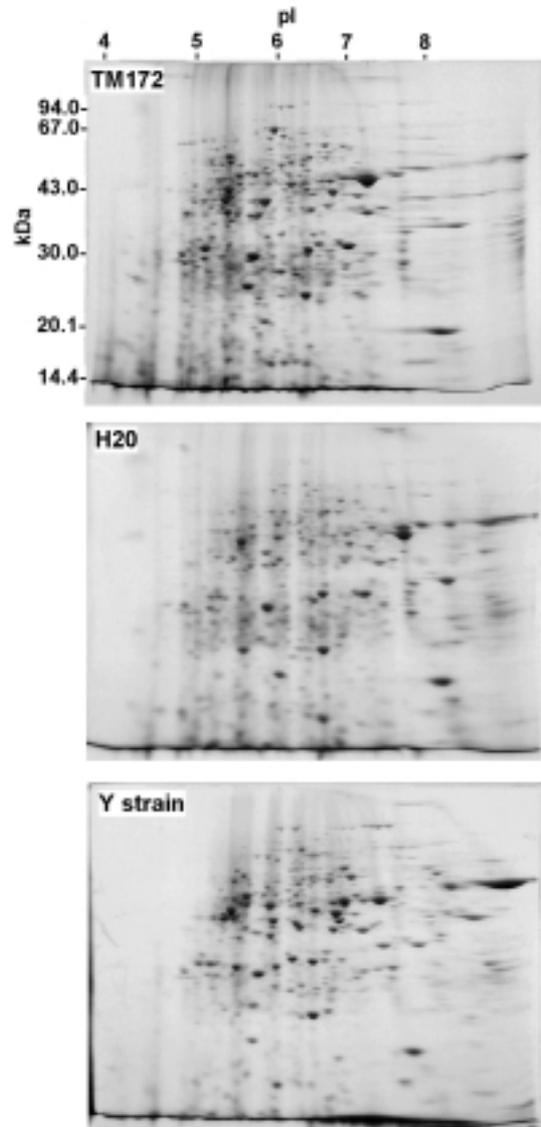

Figure 1. 2D-PAGE profiles of the proteins extracted from 6 isolates of *T. cruzi*. 2D-PAGE was performed as described under Materials and Methods. Nineteen profiles were divided broadly into 3 groups. A representative profile of each group is shown. Group 1; TM172, group IIa; H20, group IIb; Y strain.

H PO was used for upper reservoir solution and 0.02 M NaOH for lower reservoir solution. After electrofocusing, gels were removed from each glass tube and were soaked in sample buffer for SDS-PAGE for 1 h. One of the gels was cut into small pieces, soaked in water, and measured pH of the solution. Next, SDS-PAGE containing 11% acrylamide was performed. The gels were soaked in ethanol/acetic acid/water (4:1:5) overnight to remove Ampholine and then was stained with coomassie brilliant blue R-250 for 1 h. After destaining, the gel was dried using Gel Drying film (Promega, Madison, WI, USA). The molecular mass of the
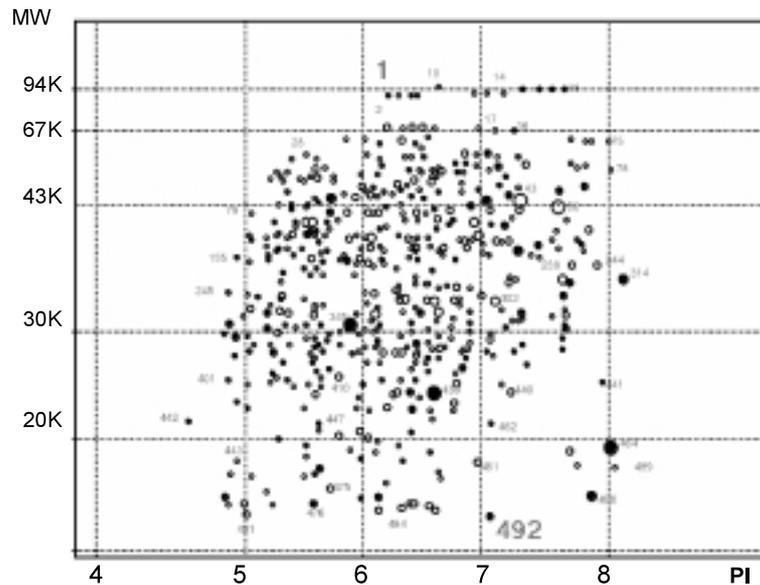
Figure 2. A schematic representation of 492 polypeptide spots. The selected 492 spots were used to judge the presence or absence of the spots among 19 stocks of *T. cruzi*. Part of the spot numbers is shown. Among them, 160 spots (closed circle) were shared among the stocks and 332 spots (open circle) were present in limited stocks.

spots on the gel was estimated using the following standards: phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20 kDa; and α-lactalbumin, 14.4 kDa.

Growth of epimatigotes in medium and release of trypomastigotes from human cell lines

Epimastigotes of *T. cruzi* were adjusted to 1 x 10 /ml in LIT medium containing 10% FCS and cultured in 24-well culture plates (Corning, Corning, NY, USA) for 8 days. The number of epimastigotes in the medium was counted every day at 24 h intervals during culture. The doubling time of each stock was calculated from the number of epimastogotes on day 2-6 showing exponential growth.

Human lung fibroblast-like cell line WI-38 and human muscle rhabdomyocarcinoma cell line Hs 224.T were obtained from American Type Culture Collection (Rockville, MD, USA). The cell lines were maintained in Minimum Essential Medium Eagle (MEME) medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% FCS (complete medium) at 37℃ in a humidified atmosphere containing 5% CO2.

A suspension of the cells was obtained from the confluent monolayer culture of the cells after treatment of trypsin/EDTA. The cells (1 x 10) were cultured with 1 ml complete medium in 24-well culture plate for 24 h. Late log phase of epimastigotes cultured for 8 days was transferred to a 15 ml tube, washed twice with PBS, and finally suspended in MEME medium. One ml of suspension of parasites (1 x 10) was inoculated to the monolayer culture of

WI-38 or Hs 224.T cells (parasites: cells=10:1) [24]. After 24 h incubation of the co-culture, the *T. cruzi* in the medium and attached to the bottom of the plate were removed completely by several changes of the MEME medium and finally resuspened in 2 ml of MEME medium containing 5% FCS added to each well. The cultures were further continued for 8 days. An aliquot of the medium from the culture was taken off every day at 24 h intervals and the trypomastigotes that appeared in the medium were counted. All cultures were set up in triplicate.

Data analyses

The similarity coefficient (S) defined by Dice [25] was calculated from the number of shared and unshared spots between two stocks among all combinations of *T. cruzi*. The distance (D) was estimated by the following formula [14,26]:

D=1-S=1-2 a/ (2 a+b+c), where
a=the number of spots shared between profile 1 and 2;
b=the number of spots present in profile 1 but absent in 2;
c=the number of spots absent in profile 1 but present in 2.

The unpaired Student's *t*-test was used to determine the significance of differences in the cumulative number of trypomastigotes among the groups. *P* values less than 0.05 were considered statistically significant.

RESULTS

The polypeptide mapping by 2D-PAGE

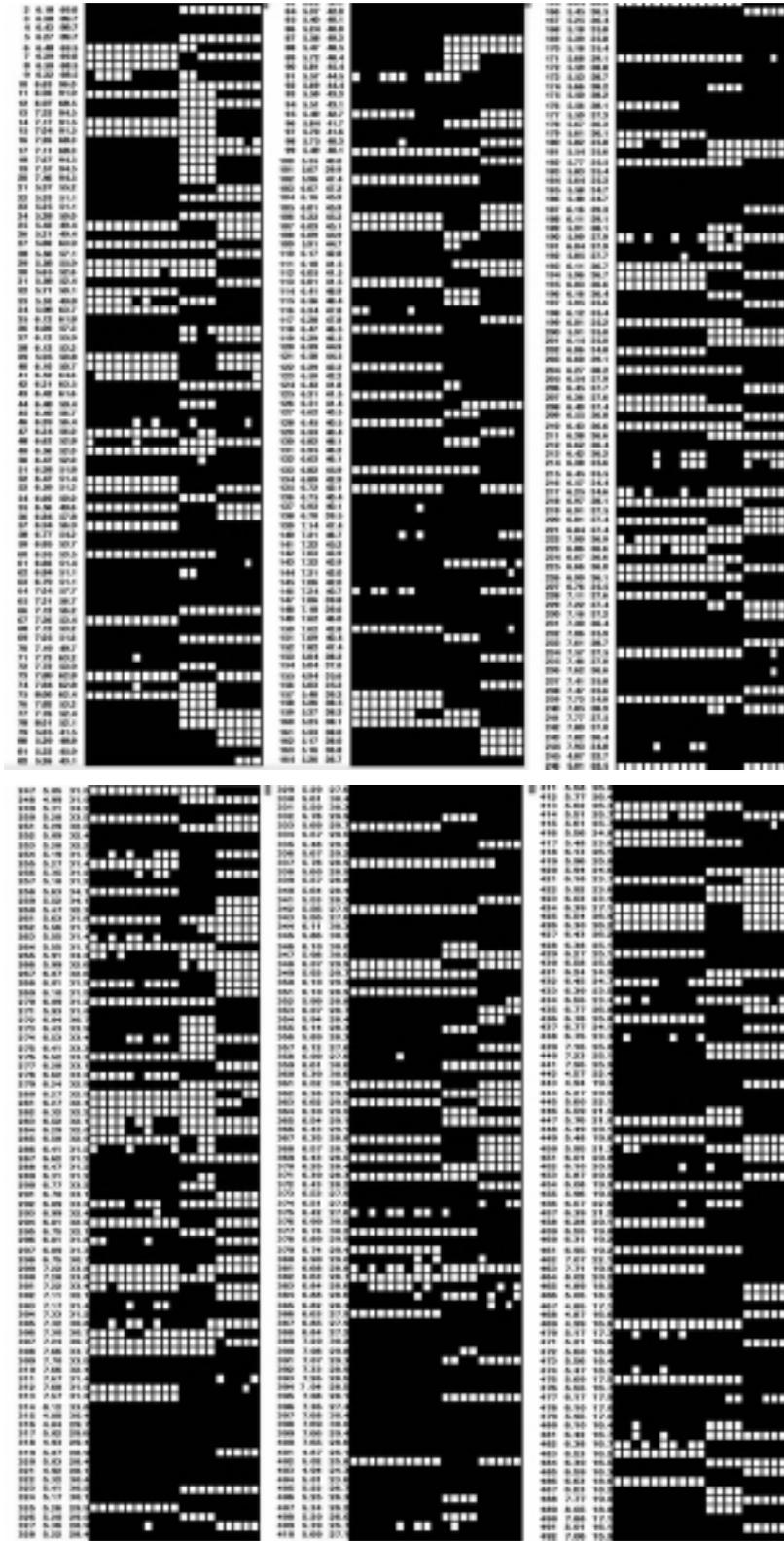2D-PAGE analyses of the proteins extracted with 4%

Figure 3. Diagram of the presence (black square) and absence (white square) of 492 spots in individual stocks of *T. cruzi*. Polypeptide spots are shown in order of number. Estimated values of p*I* and molecular mass (*M*) are indicated besides each spot number. The isolates are aligned from the left to the right as follows, TM17, TM43, TM47, TM51, TM52, 119, 172, CL strain, H6, H18, H1, H20, Peru 1, Peru 2, Y strain, Tulahuen, GE, RF, LO
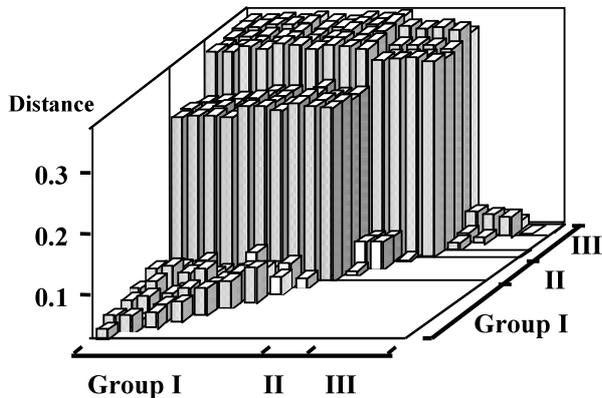
Figure 4. Matrix display of each distance between the two strain combinations from 19 isolates examined.

Triton X-100 from 19 stocks of epimastigotes (Table 1) were examined to evaluate the variability within *T. cruzi* species. Polypeptide spots in the range from 604 to 798 could be detected on individual profiles. Some of the profiles resembled each other, while others were dissimilar. All profiles were then roughly divided into three groups (completely corresponding to I, IIa, and IIb reported by using isozyme profiles [19]) when the gels showing a similar pattern were collected together in one group. Representative profiles of group I (TM172), IIa (H20), and IIb (Y strain) are shown in Figure 1.

Detection of shared and unshared spots of all the stocks

Since it was difficult to determine whether the spots on each profile of two stocks from intergroups were shared or unshared, two of three extracts; TM-43 (group I), H1 (IIa), and Y strain (IIb) or a total of three combinations were mixed and analyzed by 2D-PAGE. By comparison among the three gels, one being a mixture of two stocks and the others were the two individual stocks, it was possible to determine the presence or absence of the spots between stocks (data not shown).

A total of 492 polypeptide spots out of 604 to 798 spots from 19 profiles were selected when the spot could be detected in more than two strains and the presence or absence could be judged among the 19 profiles. A schematic representation of the numbered spots is presented in Figure 2. Among them, 160 spots were shared among the 19 stocks, while 332 were found in limited stocks. The physicochemical properties (relative molecular mass and pI) and the presence or absence of individual spots on the 19 profiles are summarized (Figure 3). The sum of the spots detected on the profiles in each stock ranged from 320 (RF) to 358 (H 18).

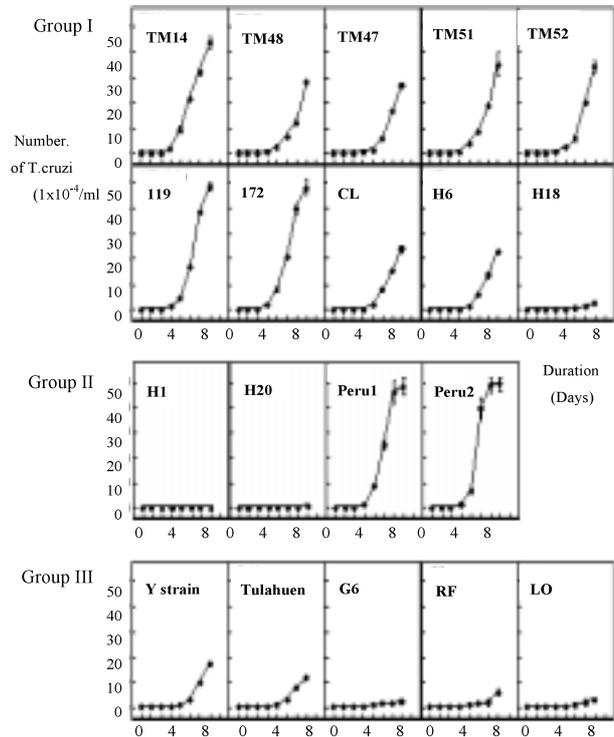Based on the presence or absence of the spots, the number of shared and unshared spots between two stocks

Figure 5. Release of trypomastigotes from human cell line WI-38. Human cell line WI-38 (1 x 10 /well) cultured in 24-well culture plate was co-cultured with the late log phase of epimastigotes (1 x 10) of *T. cruzi* for 24 h. After removal of *T. cruzi* with several changes of culture medium, the cells were cultured for the indicated number of days. The trypomastigotes released from WI-38 cells were counted every day at 24 h intervals.

among all combinations of the 19 stocks was counted. The number of unshared spots was calculated between the stocks and summarized in Figure 4. The results indicate that the 19 stocks of *T. cruzi* can be divided into 3 groups: group I; TM14, TM43, TM47, TM51, TM52, 119, 172, CL strain, H6, and H18: group IIa; H1, H20, Peru 1, and Peru 2: group IIb; Y strain, Tulahuen, GS, RF, and LO. Group I consisted of 10 *T. cruzi*; 8 were isolated in Guatemala, 1 in Brazil and 1 in Ecuador: group IIa 4 *T. cruzi*; 2 in Guatemala and 2 in Peru: group IIb 5 *T. cruzi*; 3 in Paraguay, 1 in Brazil, and 1 in Chile.

Growth of epimatigotes in medium and release of trypomastigotes from human cell lines

The doubling time of epimastigotes of the 10 stocks in group I, 4 in group IIa, and 5 in Group IIb was calculated to be 24.0± 0.5 h, 23.7± 0.5 h, and 24.0± 0.5 h, respectively. The values were not significantly different among the three groups.

The time course of the release of trypomastigotes from WI-38 cells after infection of epimastigotes in the late log

Table 2. Number of released trypomastigotes (day 7) from different cell lines infected by *T. cruzi*

T. cruzi isolates	WI-38 cells (x 10 /ml)	Hs 224 T cells (x 10 /ml)
Group I	39.0+23.0	71.2+24.4
Group IIa	47.7+47.4	47.2+47.2
Group IIb	9.3+6.9	26.9+27.0

*Group I vs IIb p<0.02

phase is shown in Figure 5. Trypomastigotes appeared in the medium after four to seven days' culture and thereafter the number increased as a function of the culture day. Similar results were obtained when Hs 224.T cells were used. The number of trypomastigotes released from WI-38 and Hs 224.T cells on day 7 after infection of epimastigotes is shown in Table 2. The release of trypomastigotes in group I was significantly higher than that in group IIb (p<0.05). However, within group IIa, two showed a high number of released trypomastigotes while the other two were very low, making it difficult to compare between Group IIa and the other groups.

DISCUSSION

2D-PAGE is widely utilized to detect genetic variations in the population of a species, genus, or family. This method was applied to identify the surface proteins and monitor the change of expression of these surface proteins in the differentiation of epimastigotes to amastigotes and trypomastigotes in *T. cruzi* [27-30]. To our knowledge, this is the first report on an attempt to determine the extent of variabilities of proteins within the *T. cruzi* species.

Among the 492 spots analyzed in this study, 160 spots (32.5%) were shared among the 19 stocks of *T. cruzi* and 332 (67.5%) were present in several limited stocks (Fig.3). The number of shared and unshared spots between two profiles in all combinations of *T. cruzi* was used to characterize the groups (Fig.3). At present, it is difficult to determine whether the extent of variability observed in *T. cruzi* species is high or low, since there have only been reports on the estimation of variabilities by 2D-PAGE in populations in *plasmodium falciparum* (49%, the value being altered to spot unsharing) [31, 32] and *Cryptosporidium pavum* species (3%) [33] in protozoa, *Drosophila* (23%) [34] in insects and the cat family (15%) [35] bear (37%) [36], hominoid primates (40%) [37], and humans (3-6%) [38].

There have been a number of reports concerning the genetic variabilities of *T. cruzi* species analyzed mainly by zymodemes [4-10, 19] and DNA polymorphisms [11-19, 20], suggesting the existence of several lineages of *T. cruzi* within the *T. cruzi* species [reviews: 39-41]. Our observation is essentially consistent with both previous reports and our previous observations [16-21, 41].

As indicated in Figure 3 and 4, there are several identifiable intra-group variations, although the level of the variability is much smaller than that observed between groups. Thus, the prototype of the genome of each group has been well conserved during geographical expansion. It seems there were very few genetic exchange events between groups which might have occurred in the overlapping geographical areas, although the genetic exchange could be detected by the population study [42] as well as in vitro study [43].

The doubling time of epimastigotes of the tested isolates showed no difference, while the number of trypomastigotes released from two human cell lines, WI-38 and Hs 224.T, varied. The number of released trypomastigotes of H 18, H1, H20, GS, LO, and RF was significantly lower than that of the other strains, and the number of trypomastigotes released in group I was significantly higher than that in group IIb (p<0.05) (Table 2).

The digestive form of this disease is rare in Central America but frequently observed in South America. It has been suggested that the association of clinical symptoms and severity of the disease with certain geographic regions is related to genetic factors within the species [2-10]. The fact that the isolates of group IIb showed low proliferative activity of amastigotes in vitro might be directly or indirectly related to the pathogenicity of magacolon in South America.

ACKNOWLEDGMENTS

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