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Analysis of Variability of Clones and Subclones of *Trypanosoma cruzi* Derived from Mexican Strains by the Behavior in Mice and Culture Cells

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Abstract: To better understand the biological heterogeneity of behavior of *Trypanosoma cruzi* strains isolated from Mexico, 6 strains of *T. cruzi* were cloned by the Miles' method (drops of suspension with parasites were diluted in phosphate-buffered saline solution and inoculated to mice).Virulence and infectivity of 10 or 11 clones derived from each strain were determined in female Balb/c mice and Vero culture cells, respectively. Variability of clones was determined by Tukey F statistic test. Only 1 strain, T5, showed interclonal variability and its clones were subcloned by the same method; they showed similar behavior as their parental clones. Clones increased the virulence or had the same behavior after maintaining them for 1 year in mice. Seven clones were eliminated and the virulence of the 4 remaining clones was attenuated when they were maintained in LIT axenic culture for 1 year. The study showed that the strains of *T. cruzi* in Mexico are either monoclonal or polyclonal and the clones could be eliminated or selected from the environment along time; therefore it is possible to observe that the behavior of *T. cruzi* strains can change when they are maintained in laboratory for several years.

Key words: Variability, clones, *T. cruzi*, mice and culture cells

INTRODUCTION

Chagas disease is caused by the hemoflagellate protozoan *Trypanosoma cruzi* and it has a variable clinical outcome, ranging from the absence of symptoms to severe chronic phase characterized by cardiovascular and/or gastrointestinal involvement or even death (Brener, 1987; Prata, 2001). The exact causes of this variable clinical presentation are not known. However, both human and parasite factors are involved. In terms of the parasite, strains of *T. cruzi* might be composed of clones that exhibit different biological behavior, virulence, genetic characteristics and tropism for different tissues (Engel *et al*., 1982; Andrade *et al*., 1999; Buscaglia and Di Noia, 2003; Macedo *et al*., 2004). Therefore, variations in the clinical presentation of the disease might be caused by the presence of various clones (Macedo *et al*., 2004). Thus, it is demonstrated that clones isolated from strains of *T. cruzi* in South America present tropism for different tissues in infected mice (Postan *et al*., 1983; Toledo *et al*., 2002) and present different genetic and biochemical characteristics. It is proposed that the clones of parasite are dispersed in different geographic areas along American Continent (Tibayrenc and Brenier, 1988).

Chagas disease is one of the most serious public health problems and a major cause of death in Latin America, it affects nearly 16 m people and claims up to 50,000 lives every year (Weir, 2006; WHO, 2002). There are 18 endemic countries with 25% of all inhabitants at risk of contracting *T. cruzi* infection and the incidence is estimated as 700 000-800 000 new cases per year (Moncayo, 2003). Mexico is one of the endemic countries that shows evidence of intradomiciliary transmission with association between *T. cruzi* infection and electro-cardiograph alteration; however, no formal control programs have yet been established (Moncayo, 2003). Out of 31 states in Mexico, 20 states have been reported with acute and 14 states have been reported with chronic human cases; from 1982-2002, 325 cases of Chagas disease had been reported. The seroprevalence of

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1-37% was reported (Ramsey *et al*., 2003). It is believed that more than 70, 000 people are at risk of transmission by triatomines and is also estimated that 1, 768 and 376 people are infected with the parasite (Ramsey *et al*., 2003). The most important species of triatomine found in Mexico are *Triatoma dimidiata*, *Triatoma barberi* and Triatomine of phyllosoma complex, for example *Triatoma pallidipennis,* with more than 10 000 000, 2 000 000 and 48 000 000, people at risk of transmission when live with them, respectively. In Mexico, the transmission of Chagas disease due to triatomines is 96% (Ramsey *et al*., 2003). The distribution of these triatomines is very extensive here (Zarate and Zarate, 1985; Vidal *et al*., 2002). Another very important aspect is the characterization of strains of *T. cruzi*. Strains obtained from Mexico showed variability in infectivity when was determined in mice. What are the reasons of this change of behavior? Although, the variability in virulence, infectivity and molecular features have been observed, it is not known whether the strains of the parasite isolated from Mexico are monoclonal or polyclonal and whether the clones are stable for a long time.

The aim of the present study is to determine the variability of the clones derived from a *T. cruzi* strain isolated from Mexico by behavior in mice and culture cells to answer these questions and to focus on the understanding of Chagas disease in Mexico and on the characterization of strains.

MATERIALS AND METHODS

Isolation of *T. cruzi*: All strains, except *Teques* were isolated from feces of triatomines, *Queretaro* and *Purisima* from *Triatoma barberi*, *Oaxtepec* from *T. pallidipennis, T5* and *BS* from *T. dimidiata*; only *Teques* was isolated from a pig. Triatomines were collected from human dwellings. Oaxtepec was collected near the door of house, whereas the other strains were collected from inside the dwelling. These houses were not divided into living room, bedroom, or kitchen; all the rooms were in the same space. In case of Purísima strain isolated from *T. barberi*, prevalence of *T. cruzi* infection in the triatomines collected was less than 9% (3 out of 26 collected bugs, all of them collected from the same dwelling, a house without an internal division). For BS and T5, the prevalence of *T. cruzi* infection for *T. dimidiata* was 89% intradomiciliary and 11% peridomiciliary. For Queretaro, data about prevalence of *T. cruzi* infection is not known, it was collected form inside the house. For Oaxtepec, the insect was collected when a person observed the bug into the house and sent it to the laboratory for examination. The result was positive when *T. cruzi* was isolated. Parasites of each strain were isolated from feces of triatomine and were intraperitoneally (i.p.) inoculated into a CD-1 male mouse of 20-30 g of weight. Blood of each mouse was observed in every 72 h and the presence of *T. cruzi* was identified by its morphological features using giemsa stain (Souza, 1999; Schmidt and Roberts, 2000). After death of the infected mice, *T. cruzi* was also identified by presence of amastigote nests in histological sections of heart (Souza, 1999; Schmidt and Roberts, 2000). To increase the quantity of parasites of each strain, trypanosomes were reinoculated intraperitoneally in the other 5 male CD-1 mice of 15-20 g and 6-8 weeks old with 1×10^3-1×10^4 flagellates in 200-300 μL of infected blood per mouse. When mice showed parasitemia, *T.* cruzi was cloned. The animals were from the School of Medicine, UNAM.

Cloning and subcloning: Mice with parasitemia were bled and the bloodstream trypomastigotes (BTs) of *T. cruzi* were separated from red cells, plasma and platelets by a centrifugation gradient with lymphoprep solution. Parasites were washed thrice with a 4% PBS-albumin solution, centrifuged at 900 *g* for 5 min, counted in a Neubauer chamber and diluted with PBS until 1 parasite was observed for each 50 μL, because this volume is equal to a drop of solution, approximately. Cloning technique was performed according to the method proposed by Miles (1975). Briefly, drop to drop of suspension was colocated into the wells of polyestirene which were observed under inverted microscope until each well had 1 BT and then were completed to 300 μL of PBS at 35°C in a sterile conditions. Each strain was cloned using 30 wells. The whole suspension of a well with 1 trypanosome was inoculated intraperitoneally into a male CD-1 mouse as mentioned above. This manipulation was performed 30 times, 1 mouse for each well. After several weeks, only the mice with parasitemia were selected to continue the experiments because they were considered as infected with only 1 clone. Thus, to increase the parasites, other 5 CD-1 male mice were infected with each clone as described above and when animals showed parasitemia, they were bled by cardiac puncture. BTs were separated as mentioned above and were manipulated to determine virulence of clones in 2 (all strains except T5) or 3 directions: 1st, trypanosomes were diluted in PBS to have 1×10^6 BTs per mL and were used to determine the virulence in mice; second, they were used to determine the infectivity in culture cells; and 3rd, they were subcloned again as mentioned above (only clones from T5 strain because it was the only strain that showed interclonal variability).

Determination of virulence *in vivo*: Ten female Balb/c mice of 20 g and 6-8 weeks old were inoculated intraperitoneally with 1×10^5 BTs of each clone or with the

parental strain in a final volume of 200 μL. Kinetics of parasitemia, accumulative mortality and histotropism were recorded during the infection time. Parasitemia was measured on each 3rd or 4th day during 60 days as follows: 10 μL of blood samples were taken from the tails of infected mice and were mixed with 90 μL of 0.87% ammonium chloride to lyse the red cells; parasites were counted in a Neubauer chamber using a microscope. Accumulative mortality was recorded during this period and the mice were then sacrificed to determine histotropism as follows: for each mouse, 9 organs were dissected: brain, heart, lung, spleen, liver, esophagus, large intestine, skeletal muscle and kidney. The tissues were fixed in 10% formalin-PBS embedded in paraffin and cut 10 times to obtain 5 μm thick sections; the sections were performed at 50 μm intervals and were stained with hematoxylin-eosin. To determine the intensity of infection, each section was observed under microscope at 1000×magnification, 100 fields were reviewed and the number of amastigote nests was counted.

Determination of infectivity in culture cells: Vero cells were allowed to adhere to square glass slides of 1 cm^2 covered with 0.1% nutritive gelatin medium at 37°C in 5% CO_2, in moist air, in a volume of 50 μL of Medium Eagle's Minimum essential (MEM) supplemented with 10% inactivated fetal bovine serum (FBS), 100 UI of penicillin, 100 μg of streptomycin, under sterile conditions. Nine hours after plating, Vero cells formed a monolayer, 1×10^5 BTs were then added to obtain a parasites/Vero cells ratio of 10: 1. Glass slides were kept at 37°C for 8 h to allow internalization of parasite. Infected Vero cells were then thoroughly washed with warm MEM solution without FBS to remove free parasites and were then left for incubation in FBS-supplemented MEM medium at 37°C for 72-96 h. Slides were then washed thrice with fresh MEM medium without FBS and were fixed with methanol for 3 min, dried, stained with Giemsa and mounted (covered with a coverslips). Two parameters of infectivity in culture cells were determined: percentage of infected cells and infection index. To determine infectivity of clones, subclones or parental strains of *T. cruzi*, slides were observed under microscope at 1000×magnification. To determine the percentage of infected cells and the infection index, the number of infected Vero cells and mean number of parasites per Vero cell were recorded by counting 200 Vero cells in each triplicate slides.

From the behavior of *T. cruzi*, clones derived from a strain of the parasite are considered homogeneous if their virulence and infectivity values are similar and it can be said that the strain is monoclonal. If they show variability, the strain is polyclonal. Statistical significance of interclonal variability among clones derived from each strain was evaluated by a one-way analysis of variance (ANOVA) with Tukey's test. In all cases, $p<0.05$ was considered significant. Data of parameters of virulence in mice (peak of maximum parasitemia, day of maximum parasitemia, mortality and number of infected organs) and infectivity in culture Vero cells (percentage of infected cells and the infection index) were taken into account. ANOVA statistics of Fisher was used to determine variability among subclones derived from the clones of T5 strain, with a significance level p-value of 0.05. The True Significance Difference Value (TSDV) was calculated by comparing all the subclones for each parameter of virulence and infectivity (Daniel, 1999).

RESULTS

Clonal variability for strains of *T. cruzi*: To know whether strains of *T. cruzi* are monoclonal or polyclonal and to determine variability among clones that belong to a single strain, 10 clones of each strain except T5 with 11 clones of *T. cruzi*, were obtained. Variance obtained for accumulative mortality is higher in clones of T5 strain than in the remaining strains (Table 1). According to the variance in all the parameters in both virulence and infectivity and according to the F Tukey values observed, only T5 strain had statistical significant ($p<0.05$) clonal variability (Table 1).

Variability in clones isolated from parental T5 strain: Table 2 presents the data of virulence and infectivity of each clone derived from the parental T5 strain. It shows that the clones caused considerable variability in all the studied parameters. Mortality data varied from 0-100%; the parental strain caused neither the highest nor the lowest mortality in comparison with the rest of the clones. F of Tukey value indicated that in all studied parameters, the clones showed variability at significance level ($p<0.05$). The clones could be clustered in minimum of 2 groups; one with low values and the other with high values for each parameter in accordance with TSDV (see material and methods), when results were compared among them (data not shown). For example, in peak of maximum parasitemia, the clones T5.5, T5.1, T5.4, T5.6 and T5.3 could be grouped in a cluster with less than 2×10^6 and the others in the second or more groups with more than 6×10^6 Bts mL^{-1} of blood. With other parameters of virulence and infectivity, clones may also be clustered in low or high infectivity; however, the grouping of clones is different for each parameter. Considering the mean of infected organs, only T5.5, T5.1 and T5.4 could be clustered; on the basis of the percentage of infected Vero cells incubated for 72 h, T5.5 and T5.2 form a group and

Table 1: Interclonal variability among clones derived of each strain of *Trypanosoma cruzi*

	Virulence *in vivo*					Infectivity *in vitro*	
Strain	Mortality (%)	PMP	DMP	IO	MIO	Percentage of infected vero cells	Infection index
Queretaro	40.0±8.2	4.8±0.2*	20.0±2.1*	5	3.9±0.9*	35.5±4.3*	1.8±0.5*
Purisima	0.0	2.3±0.4*	26.8±3.32*	1	0.6±0.51*	0.0*	0.0*
Oaxtepec	8.0±4.2	0.4±0.2*	34.6±4.3*	1	0.8±0.9*	3.8±1.0*	0.9±0.3*
Teques	33.0±8.2	3.4±0.9*	32.5±2.4*	2	1.8±0.5*	2.8±0.9*	1.3±0.7*
BS	0.0	0.3±0.1*	36.0±2.6*	0	0.0*	0.0*	0.0*
T5	57.3±44.3	8.5±9.1†	33.5±9.3†	9	4.7±3.1†	29.0±23.9†	2.1±0.3†

For all parameters the data represent the mean±standard deviation among clones derived from each strain. PMP: Peak of Maximum Parasitemia (1×10^6 BT mL^{-1} of blood); DMP: Day of Maximum Parasitemia; IO: maximum number of infected organs for any clones; MIO: Mean of Infected Organs); Data of infectivity in culture cells was determined at 72 h of incubation. * = data in which $F < F_{p0.05}$ and $p>0.05$ (there is not statistically significant variability); † = data in which $F > F_{p0.05}$ and $p<0.05$ (there is statistically significant variability)

Table 2: Infectivity and virulence of clones and parental T5 strain in mice and vero cells

	Virulence *in vivo*					Infectivity *in vitro*			
						Percentage of infected Vero cells		Infection index	
Clones	Mortality (%)	PMP	DMP	IO	MIO	72 h	96 h	72 h	96 h
T5.5	0	0.1±0.1	49.6±1.6	2	0.9±0.7	2.3±0.8	4.0±0.5	1.6±0.4	1.8±0.2
T5.1	30	0.3±0.1	36.9±1.2	1	0.5±0.5	5.4±1.3	11.0±1.5	1.9±0.3	2.0±0.1
T5.4	0	0.4±0.1	38.4±1.3	1	0.4±0.5	6.2±0.8	8.2±0.3	1.9±0.6	1.6±0.2
T5.6	10	1.8±0.2	48.5±3.0	6	4.3±1.2	4.0±1.3	4.3±0.8	1.8±0.5	2.2±0.4
T5.3	20	0.7±0.1	32.4±2.1	4	3.3±0.8	2.3±0.3	3.3±0.6	2.1±0.3	2.1±0.3
T5.2	90	21.5±1.9	29.5±1.3	5	4.2±0.8	23.0±2.0	41.0±1.8	1.7±0.4	2.0±0.2
T5.7	90	15.2±1.7	25.6±1.5	8	6.3±1.2	83.2±2.0	71.2±2.4	3.1±0.4	2.1±0.3
T5.11	90	6.6±0.9	31.6±2.5	9	7.5±1.0	27.0±2.2	41.2±2.0	1.6±0.2	1.8±0.4
T5.8	100	15.1±1.5	23.4±1.9	9	8.3±0.8	44.7±1.3	41.7±1.0	2.9±0.4	2.7±0.3
T5.10	100	24.6±2.0	20.6±2.2	9	8.8±0.4	40.7±1.3	36.7±1.3	1.5±0.3	2.5±0.1
T5.9	100	7.6±0.8	31.9±2.5	9	6.9±1.0	41.3±2.6	57.0±1.5	2.0±0.3	2.4±0.2
T5 parental	80	4.5±3.6	24.8±5.1	9	6.4±1.3	31.8±4.6	34.8±1.5	1.6±1.2	2.7±1.0

For all parameters the data represent the mean±standard deviation among clones derived from each strain. Abbreviations in each parameter are the same that in table I. $F > F_{p0.05}$ and $p<0.05$ for all parameters (there is statistically significant variability)

Table 3: Infectivity of subclones derived from clones of parental T5 strain in mice and vero cells

		Virulence *in vivo*					Infectivity *in vitro*	
Clones	Subclones	Mortality (%)	PMP	DMP	IO	MIO	Percentage of infected vero cells	Infection index
	T5.5.a	10	1.4±0.2	49.9±1.5	2	1.0±0.8	1.7±0.3	1.6±0.1
T5.5	T5.5.b	0	1.5±0.2	50.5±2.1	2	1.0±0.8	1.8±0.3	1.8±0.1
	T5.5.c	0	1.5±0.3	50.5±1.3	2	1.0±0.8	1.8±0.6	1.7±0.1
	T5.4.a	10	0.7±0.2	37.0±1.2	2	1.1±0.9	4.0±0.5	1.5±0.3
T5.4	T5.4.b	0	0.5±0.2	37.7±1.2	2	1.0±0.8	4.3±0.3	1.7±0.2
	T5.4.c	10	0.4±0.1	37.1±1.4	2	1.3±0.7	3.7±0.3	1.7±0.2
	T5.10.a	90	24.0±1.5	21.4±2.5	9	8.5±0.7	24.2±1.3	2.2±0.1
T5.10	T5.10.b	90	23.2±1.4	21.5±1.5	9	8.5±0.5	24.7±1.1	2.3±0.2
	T5.10.c	100	23.8±1.2	21.0±1.4	9	8.7±0.5	26.8±0.8	2.5±0.1
	T5.8.a	100	24.9±0.9	24.2±1.5	9	8.2±0.4	30.8±1.3	2.3±0.1
T5.8	T5.8.b	100	24.9±1.2	24.7±1.2	9	8.3±0.5	30.7±1.3	2.5±0.2
	T5.8.c	100	24.1±1.0	25.1±0.7	9	8.4±0.5	31.3±2.5	2.4±0.1

For all parameters the data represent the mean±standard deviation among subclones derived from each clone. Abbreviations in each parameter are the same that in Table 1. Only data of PMP for subclones of T5.4 and percentage of infected Vero cells for subclones of T5.10 had $F > F_{p0.05}$ and $p<0.05$

T5.1, T5.4 and T5.6 could be grouped in a second group, the remaining clones are clustered in one or more groups. On the basis of this result, it was interesting know whether clones of T5 showed intraclonal variability. Therefore, clones of T5 strain were subcloned. In this case, on the one hand, T5.5 and T5.4 as clones of low virulence and on the other hand, T5.10 and T5.8 as clones of high virulence were selected to compare intraclonal virulence.

Infectivity and virulence of subclones: Subclones derived from their corresponding clone did not have intraclonal variability, except to T5.4 and T5.10 in peak of maximum parasitemia and percentage of infected Vero cells, respectively (Table 3). For the remaining parameters, F-values were $p> 0.05$, it means that the variance was similar for subclones derived from a clone and therefore sub cloning was not necessary again. The behavior of subclones was the same as their parental clone with

Table 4: Infectivity of clones of T5 strain maintained in mice for one year

	Virulence *in vivo*					Infectivity *in vitro*	
Clones	Mortality (%)	PMP	DMP	IO	MIO	Percentage of infected vero cells	Infection index
T5.5	0	1.1±0.1	52.6±1.6	2	1.6±0.5	3.2±0.3	2.1±0.1
T5.1	10	1.7±0.5	41.9±3.1	1	1.0±0.0	10.8±0.8	2.5±0.1
T5.4	10	2.0±0.5	41.0±2.7	2	1.4±0.5	9.2±0.3	2.6±0.0
T5.6	10	2.1±0.2	51.1±2.3	7	5.5±1.0	5.0±0.5	2.7±0.1
T5.3	10	1.6±0.3	28.5±1.8	6	4.2±0.9	4.5±0.5	2.5±0.4
T5.2	100	23.2±2.1	27.3±1.6	5	4.2±0.4	45.7±1.0	2.4±0.1
T5.7	90	17.2±1.9	24.2±1.6	8	7.3±0.8	75.2±2.2	2.3±0.1
T5.11	90	7.3±0.5	29.0±2.8	9	8.4±1.0	46.2±2.7	2.1±0.3
T5.8	90	16.7±1.4	21.5±2.7	9	8.5±0.5	43.3±0.8	3.1±0.1
T5.10	100	26.3±1.2	20.0±1.6	9	8.9±0.3	37.7±1.0	3.1±0.1
T5.9	100	9.8±1.5	29.2±1.7	9	7.5±1.2	51.7±1.3	2.8±0.1
T5 parental	70	5.7±1.0	24.7±1.9	8	5.9±1.2	38.2±1.5	2.3±0.3

For all parameters the data represent the mean±standard deviation among clones. Abbreviations in each parameter are the same that in table I. Infectivity was determined at 72 h of incubation. In all cases $F > F_{p0.05}$ and $p<0.05$. Thus, there is statistically significant variability

Table 5. Infectivity of clones maintained in LIT culture medium for one year

	Virulence *in vivo*					Infectivity *in vitro*	
Clones	Mortality (%)	PMP	DMP	IO	MIO	*Percentage of infected Vero cells	*Infection index
T5.5	Nd	Nd	Nd	Nd	Nd	Nd	Nd
T5.1	0	0.1±0.1	50.7±2.1	1	0.4±0.5	6.5±0.5	2.0±0.1
T5.4	Nd	Nd	Nd	Nd	Nd	Nd	Nd
T5.6	Nd	Nd	Nd	Nd	Nd	Nd	Nd
T5.3	Nd	Nd	Nd	Nd	Nd	Nd	Nd
T5.2	0	3.8±0.6	51.3±3.4	3	1.4±1.1	40.0±1.3	1.9±0.1
T5.7	Nd	Nd	Nd	Nd	Nd	Nd	Nd
T5.11	Nd	Nd	Nd	Nd	Nd	Nd	Nd
T5.8	0	4.0±0.3	44.5±6.6	3	1.3±1.1	38.3±1.0	2.7±0.1
T5.10	0	4.1±0.3	50.8±4.7	3	1.5±1.2	27.3±2.0	2.7±0.2
T5.9	Nd	Nd	Nd	Nd	Nd	Nd	Nd
T5 parental	10	4.0±2.0	40.1±11.9	4	2.1±1.4	33.5±2.6	1.9±0.1

For all parameters the data represent the mean±standard deviation among clones. Abbreviations in each parameter are the same that in table I. Infectivity was determined at 72 h of incubation. In all cases $F > F_{p0.05}$ and $p<0.05$. Thus, there is statistically significant variability. Nd: no determined because the clones did not produce infection neither mice nor Vero culture cells

respect to low or high infectivity and virulence. Table 3 shows that subclones derived from T5.5 and T5.4 showed low and T5.10 and T5.8 showed high virulence.

Behavior of clones 1 year after maintaining them in mice: To know whether clones remain stable along the time, clones were maintained for 1 year both in mice and axenic culture LIT medium. The behavior of clones in mice was nearly same for each one. Clones showed variability according to Tukey F values ($p<0.05$). Five out of 11 clones were of low virulence (see mortality, peak of parasitemia, infected organs and percentage of infected Vero cells). It is important to notice that the clones whose mortality was between 90 and 100% showed peaks of maximum parasitemia up to 7×10^6 Bts mL^{-1} of blood and most of them infected between 4 and 9 organs per mouse. T5.3 and T5.2 presented intermediate virulence for several parameters, but T5.2 caused high and T5.3 caused low mortality in mice. Although, similar to virulent clones, T5 strain also showed virulence in the midst of the clones. Clones showed variability ($p<0.05$) with respect to the infectivity *in vitro*. However, it was more difficult to group the clones according to infectivity because in percentage of infected Vero cells, a cluster could be formed with T5.5, T5.1, T5.4, T5.6 and T5.3, whose percentages ranked from 3 to approximately 5% and the other groups varied in more than 37%. However, in infection index parameter, the clustering should be different because all of them showed infection index close among themselves (Table 4).

Virulence and infectivity of clones maintained in LIT culture medium: The maintaining of clones in LIT culture during 1 year caused the elimination of 7 out of 11 clones (Table 5). Furthermore, virulent clones that were not eliminated showed a slight change in their virulence and infectivity and all of them were less virulent. The peak of maximum parasitemia was less than 4×10^6 BTs and they infected 1-3 organs in mice. Results showed that the infection index were similar to the data before maintaining 1 year in LIT medium. T5 strain showed less virulence in mice but up to the survivor clones, meanwhile its infectivity was not affected *in vitro*.

DISCUSSION

One interesting outcome of Chagas disease is the variable clinical presentation in human, ranging from asymptomatic cases to severe chonic cardiovascular and/or gastrointestinal involvement in which the course of infection is an unpredictable result. On the other hand, geographical variations in the prevalence of clinical forms of infection have been reported. What are the reasons for this clinical heterogeneity? It is an important aspect that remains unexplained yet. Although, biological considerations on the host genetic factors cannot be discarded (Marinho *et al*., 2004), this variation is primarily determined by biological characteristics of *T. cruzi*. Many strains of the parasite have been characterized and these strains showed differences in their behavior either *in vitro* cultures or in laboratory animals and demonstrated that they are composed of many clones (Engel *et al*., 1982; Andrade *et al*., 1999; Buscaglia and Di Noia, 2003). These are selected and can be considered as a strain in a host; but the strain can change its clonal composition when is transferred to a new environment (Deane *et al*., 1984; Devera *et al*., 2003). Thus, a single host can be infected with numerous clones and thereby can act as a filter; that is, it can contain a parasite community; however, it is possible that selection pressures imposed by these new conditions influence the survival of parasites (Deane *et al*., 1984). Clones can be selected when strains of *T. cruzi* are transmitted to humans by the feces of hematophagus triatomine bugs, each species of triatomine is then infected by different populations of *T. cruzi*. In regions where there are many species of triatomines, as Mexico with 31 species, situation of variability of strains could be an interesting subject to study the variability of *T. cruzi* because it is possible to find monoclonal or polyclonal strains. In this study, *T. cruzi* strains were isolated from triatomines collected from inside human dwellings. But it is known that triatomines from Mexico can inhabit both indoor and outdoor and the bugs could be present outdoor before collection. Thus, the insects could have got infected in sylvatic cycle when they fed on several vertebrate hosts. Thus, the probability to obtain multiple clones or a single clone of *T. cruzi* depends on the host or number of hosts that the insect feed. On the other hand, it is possible that T5 strain was enzootic because was collected from *T. dimidiata* which inhabit in sylvatic cycle. The others were collected from triatomines which inhabit indoor.

It is possible that the initial isolation of *T. cruzi* caused strong selective pressures; the clones studied in this work are the result of that selection. There is a limited number of principal clones, which could be present in different geographical areas (Campos and Andrade 1996; Tibayrenc and Breniere 1988). However, we think that strains of *T. cruzi* represent swarms of clones that may present symbiotic relationships and they compete for available host resources because if a clone predominates in an environment, when that clone is transferred to other media, it could be eliminated as observed in this study and reported by others (Macedo *et al*., 2004). We could then obtain an evidence of either monoclonal or polyclonal strains, but we could not find the existence of representative clones because if they are representative in a medium, they could not be representative in other environment.

This work represents the first comparative study of virulence in mice and infectivity of culture cells with single-cell-isolate clones and subclones derived from a *T. cruzi* strain isolated from Mexico. It also shows that strains of *T. cruzi* isolated recently can be composed of either monoclonal or polyclonal populations. Only T5 strain is polyclonal. Triatomine species was not a factor that influenced polyclonality of parasite. The probability to find multiclonal population in an invertebrate vector is high if the triatomine feed on many vertebrate host because triatomines have to obtain blood from several hosts, at least 5 hosts to complete their biological cycle. That is the explanation why in this study triatomines were chosen to isolate strains of *T. cruzi*. Among triatomines that inhabit in sylvatic cycles, *T. dimidiata* is found in an extensive sylvatic and intradomiciliar zone. It is another explanation which increased the probability to find T5 strain as a polyclonal strain. However, it appears that the outcome is at random event in nature because only 1 out of 6 strains was polyclonal.

The fact in which 7 eliminated clones have survived in nature were isolated is explained in the following way: a strain might be composed of several clones, but when they were transferred in a new environment, they were 11 or more. In this case, we could observe at least 11 clones. When the T5 strain was cloned and was passed into new host, several clones of *T. cruzi* were eliminated and only 7 clones were recovered.

The behavior of T5 strain before cloning is the result of predominant clones as described by Campos and Andrade (1996) and Campos *et al*. (1999) with the 21 SF strain and the behavior of clones determine the behavior of the parental strain as described by others (Andrade *et al*., 1999). In this case, virulent clones predominated in the parental T5 strain.

It is important to notice that the clones with increased number of tissues were those that caused higher parasitemia and the time of maximum peak of parasitemia was longer. This is a result of the intracellular

reproduction of biological cycle of *T. cruzi* in vertebrate host (Brener, 1973; Burleigh and Andrews, 1995). Although, the virulence in mice and infectivity in Vero culture cells were similar for every parental strains and their clones, that is another random event, because in other study, it was demonstrated that the behavior *in vitro* and *in vivo* has not be related; however it was studied with other strains without cloning (Toma *et al*., 2000).

According to Campos *et al*. (1999), stability of *T. cruzi* strains can be considered as the possibility for maintaining its populations in equilibrium after several laboratory passages and it is reached when clones have been selected after passage by different environmental conditions, which act as filters (Tibayrenc and Ayala, 1988; Bosseno *et al*., 2000; Brandan *et al*., 2006). In our study, the fact that subclones did not show variability in comparison with their respective clones showed that the new environment was acted as a filter after cloning; therefore, it was not necessary to carry out new cloning in the T5 strain. It is assumed that the characteristics are stable after the cloning when the parasites are maintained under the same conditions as it was described in other studies (Brener *et al*., 1974) because the behavior for each cll was similar during maintaining them in mice along 1 year. Several clones maintained in mice for 1 year increased their virulence, whereas other clones were eliminated or their virulence was attenuated. This has been observed in other studies. For example, clones derived from Colombian strain increased their virulence along passages in mice (Camandaroba *et al*., 2001); Chia-Tung Pan also demonstrated an increase in virulence in clones after maintaining them during 8 passages in CD-1 mice and the virulence was low when they were maintained in culture medium (Chia-Tung, 1982). On the other hand, the maintenance of parasites in long-term cultures contributed to the attenuation of virulence (Menezes, 1968) and some of the parasites are eliminated. In this study, the maintenance of clones in LIT culture medium during 1 year caused the elimination of 7 out of 11 clones (more than 50%). In other investigations, there are elimination of parasites when they are cloned (Miles, 1975; Goldberg and Chiari, 1980; Wittner *et al*., 1982). All these facts yet remain unexplained. However, another explanation is that the expression of several proteins is involved in attenuation or virulence of the parasite (Duschak *et al*., 2001).

Characterization of strains and isolates of *T. cruzi* obtained from different regions of Mexico have been studied without cloning and the results showed high variability in mice. Perhaps they are composed of more than one clone and their final data are the result of predominant clones (Lopez *et al*., 1998; Espinoza *et al*., 1998; Cardenas *et al*., 1975; Zavala *et al*., 1992).

The findings of this study support the idea that strains of *T. cruzi* might be polyclonal or strains might be composed of clones with a few differences in their biological behavior. It cannot be said that principal clones from Mexico can be responsible for the clinical manifestation because in other media, those clones could be eliminated or could be avirulent.

Although, variability in behavior of clones of *T. cruzi* influence in the variable clinical presentation of Chagas disease, it is very important to consider that genetic background (Andrade *et al*., 2002), environmental, nutritional and immunological factors of the host are also involved (Macedo, 2004).

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