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Predominance of lineage I among *Trypanosoma cruzi* isolates from Venezuelan patients with different clinical profiles of acute Chagas' disease

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Summary *Trypanosoma cruzi* isolates from 23 acute chagasic patients from localities of Western Venezuela (state of Barinas) where Chagas' disease is endemic were typed using ribosomal and mini-exon gene markers. Results showed that isolates of the two major phylogenetic lineages, *T. cruzi* I and *T. cruzi* II, were isolated from these patients. Six isolates (26%) were typed as *T. cruzi* II and 17 (74%) as belonging to *T. cruzi* lineage I. Analysis of random amplified polymorphic DNA (RAPD) patterns confirmed these two groups of isolates, but did not disclose significant genetic intra-lineage polymorphism. Patients infected by both *T. cruzi* I or *T. cruzi* II showed different clinical profiles presenting highly variable signs and symptoms of acute phase of Chagas' disease ranging from totally asymptomatic to severe heart failure. The predominance of *T. cruzi* I human isolates in Venezuela allied to the higher prevalence of severe symptoms of Chagas' disease (heart failure) in patients infected by this lineage do not corroborate an innocuousness of *T. cruzi* I infection to humans. To our knowledge, this is the first study describing predominance of *T. cruzi* lineage I in a large number of acute chagasic patients with distinct and well-characterized clinical profiles.

keywords *Trypanosoma cruzi*, Chagas' disease, Venezuela, genetic typing, clinical profiles, acute chagasic patients

Introduction

Trypanosoma cruzi is at present responsible for nearly 20 million chagasic people and potentially risk about 90 million more in Latin America (WHO 2000). Despite efforts aimed at interrupting domestic transmission of Chagas' disease in the last 30 years, a significant increase of acute cases and in seroprevalence of children indicate active transmission of Chagas' disease in Venezuela, with a high impact on morbidity and mortality (Añez *et al.* 1999a,b; Feliciangeli *et al.* 2002, 2003).

Trypanosoma cruzi exhibits a pattern of clonal evolution and its natural population is composed of highly heterogeneous clones. Despite their high genetic variability, *T. cruzi* isolates can be classified into two major phylogenetic lineages based on zymodemes and different genetic markers (Tibayrenc 1995; Souto *et al.* 1996; Zingales *et al.* 1998), named *T. cruzi* I and *T. cruzi* II (Anonymous 1999).

A preferential association of *T. cruzi* genotypes with sylvatic or domestic cycles of transmission has been described: *T. cruzi* II has been by far the most frequent lineage found to infect humans in regions from the American Southern Cone where Chagas' disease is endemic. Most patients with severe symptoms of Chagas' disease are from these regions, where *T. cruzi* develops a domestic cycle (Zingales *et al.* 1998, 1999). On the contrary, a low prevalence of symptomatic patients is observed in regions where *T. cruzi* circulates in the sylvatic cycle and isolates of the lineage I predominate (Zingales *et al.* 1998, 1999; Coura *et al.* 2002). These and other clinical, epidemiological, biological, biochemical and immunological data, allied to the high genetic distance between *T. cruzi* I and *T. cruzi* II lineages, supported the suggestion that human Chagas' disease results from infection with isolates belonging to *T. cruzi* II (Montamat *et al.* 1996; Brenière *et al.* 1998; Zingales *et al.* 1998, 1999; Briones *et al.* 1999; Di Noia *et al.* 2002; Buscaglia & Di Noia 2003).

Vectorial transmission is responsible for *T. cruzi* infection in Venezuela where Chagas' disease is endemic and rural, with overlapping of sylvatic and domestic cycles. The transmission of *T. cruzi* inside dwellings seems to occur very often in endemic regions of this country since many houses have palm-thatched roofs and most are surrounded by palm trees, in which *T. cruzi*-infected triatomine bugs have been usually found. In Western Venezuela, *Rhodnius prolixus* is the main domestic vector and *R. robustus* is mainly responsible for transmission inside or nearby dwellings in the absence of domiciled vectors (Miles *et al.* 1981, 2003; Feliciangeli *et al.* 2002, 2003; Añez N, Crisante G, Rojas A & Ramírez JL, in preparation).

Until now, data about molecular epidemiology and transmission cycles of Chagas' disease in Venezuela were mainly provided by zymodemes, which showed the predominance of zymodeme 1 (Z1) corresponding to *T. cruzi* lineage I. The absence of megasyndromes in Venezuelan chagasic patients contrasts their occurrence in patients from countries of the American Southern Cone. This relative innocuousness of *T. cruzi* I infection to man suggested a more benign Chagas' disease in northern South America where human infection is mainly caused by *T. cruzi* I (Miles *et al.* 1981, 2003; Feliciangeli *et al.* 2002). However, despite lacking megasyndromes, many symptomatic cases of severe acute (Parada *et al.* 1997; Añez *et al.* 1999b), and chronic (Añez *et al.* 1999a) besides unapparent (Añez *et al.* 2001) Chagas' disease in endemic regions of Venezuela have been described in the last years.

Taking into account clinical data from Venezuelan patients and the knowledge on *T. cruzi* population structure, genetic typing studies of human *T. cruzi* isolates of this country could be very helpful in evaluating a role of *T. cruzi* genetic variability in the distinct manifestation of Chagas' disease. With this purpose, our aims in this study were: (a) to type *T. cruzi* isolates of 23 patients from Western Venezuela showing different clinical profiles, varying from asymptomatic to severe acute disease, in order to evaluate an association between clinical features and the lineages of the isolates; (b) to assess the intralineage polymorphism among the *T. cruzi* isolates.

Materials and methods

Origin and clinical evaluation of the patients

The 23 patients from whom *T. cruzi* isolates were characterized in this study were from 17 localities of Western Venezuelan, Barinas State, where Chagas' disease is endemic. The clinical examinations were made at the Cardiologic Unit of the University Hospital at Mérida, Venezuela and included searching for signs and symptoms of acute Chagas' disease. Clinical diagnosis of heart failure was evaluated by electrocardiogram (ECG), echocardiogram (ECHO) and chest X-rays (CHXr) according to previous criteria (Parada *et al.* 1997; Añez *et al.* 1999b). Serological diagnosis was assessed by direct agglutination test, indirect immunofluorescence test and enzyme-linked immunosorbent assay (ELISA) as previously described (Añez *et al.* 1999b). Written consent was obtained from all patients or from their representative in the case of children. The study protocol was previously approved by the Scientific Medical Committee of the Research Council of Universidad de Los Andes, Mérida, Venezuela, and the Biomedical Committee of the National Research Council of Venezuela.

Isolation and culture of *T. cruzi* human isolates

Twenty-three *T. cruzi* isolates were obtained by haemoculture from 23 acute chagasic patients, during the period from 1991 to 2000 at the Centre for Parasitological Research, Faculty of Sciences, Universidad de Los Andes, Mérida, Venezuela. Blood samples were first added to Bacto agar blood base (NNN) culture medium and the cultures were further expanded in liver infusion tryptose (LIT) medium with 10% fetal bovine serum (FBS).

Genetic typing of *T. cruzi* isolates

The DNA from cultured *T. cruzi* isolates was extracted by the classical phenol-chloroform method. For polymerase chain reaction (PCR) amplification of the divergent domain of the 24Sα-ribosomal RNA gene primers D71 and D72 were used as described by Souto *et al.* (1996) to generate 125 (*T. cruzi* II-specific) or 110 bp (*T. cruzi* I-specific) DNA bands. For PCR amplification of an intergenic region of the mini-exon gene a pool of primers, TC, TC1 and TC2, were employed as previously described (Souto *et al.* 1996), generating DNA bands of 300 or 350 bp for *T. cruzi* II and *T. cruzi* I, respectively. The PCR amplified products from both reactions were separated by electrophoresis in 2% agarose gels stained with ethidium bromide.

Random amplified polymorphic DNA analysis

Genomic DNA from cultured parasites prepared by the phenol-chloroform extraction method was used as template for random amplified polymorphic DNA (RAPD) analysis. For this study, we selected the five primers that yielded the most discriminating patterns in a previous screening using 20 primers and DNA of *T. cruzi* isolates from distinct lineages (data not shown): 601 (CCG CCC

ACT G), 606 (CGG TCG GCC A); 615 (CGT CGA GCG G), 625 (CCG CTG GAG C) and 672 (TAC CGT GGC G). DNA from the reference strains G and Y for *T. cruzi* I and *T. cruzi* II, respectively, were used for comparative purposes. Amplifications were performed according to Maia da Silva *et al.* (2004).

Results

Diagnosis of acute chagasic patients and clinical profiles

All the 23 individuals from 17 localities of Western Venezuela, Barinas State, were acute chagasic patients according to previous reported information (Añez *et al.* 1999b, 2001). The high positivity of parasitological tests, including haemoculture (positive for all patients) and xenodiagnosis (positive for 14 patients), revealed significant levels of blood trypomastigotes circulating in these patients (Table 1). Most patients showed high levels of IgM (>1:512) (data not shown). The combination of the results obtained in the clinical examination with data from physical evaluation by ECG, ECHO and CHXr revealed nine asymptomatic cases (39.1%). In addition, 14 of the 23 patients (60.9%) showed different signs or symptoms attributable to the acute phase of chagasic infection. A range of clinical condition profiles was detected among the 14 symptomatic patients (Table 1). Four of them (28.6%) presented two to four signs/symptoms, which did not include heart failure and were defined as those with mild symptoms of acute Chagas' disease. The other 10 patients (71.4%) were clinically diagnosed as presenting severe symptoms, all showing heart failure, including a fatal case. The nine patients of this group that were also submitted to cardiac evaluation through ECG, ECHO and CHXr showed abnormal results in at least two of these examinations. All these data demonstrated that signs and symptoms of the disease were highly variable among the acute chagasic patients in Venezuela (Table 1).

Genetic typing of *T. cruzi* isolates into the lineages *T. cruzi* I or *T. cruzi* II

Genetic typing of *T. cruzi* isolates by PCR amplification of DNA using primers to analyse two markers, the 24αS-rRNA and mini-exon genes, showed concordant results for all isolates. The most frequent genotype found to infect the 23 Venezuelan chagasic patients was *T. cruzi* I (74%), corresponding to isolates from 17 patients. The other six (26%) isolates were typed as *T. cruzi* II. No patients were diagnosed as mixed infected by *T. cruzi* I and *T. cruzi* II (Figure 1; Table 1).

Analysis of relationships among *T. cruzi* lineages and clinical profiles of the respective patients

The percentage of *T. cruzi* I-infected patients showing different signs/symptoms of acute Chagas' disease was higher than the percentage of *T. cruzi* II-infected patients with similar clinical data. Heart failure evidenced by cardiac evaluation was confirmed in infections caused by both *T. cruzi* lineages (Table 1). The percentage of *T. cruzi* I-infected patients presenting severe acute disease with heart failure (nine of 17 patients, 52.9%) was higher than the percentage of *T. cruzi* II-infected patients with similar clinical picture (one of six, 16.6%). Accordingly, the percentage of *T. cruzi* I-infected patients that were asymptomatic (five of 17, 29.4%) was lower than that of *T. cruzi* II asymptomatic patients (four of six, 66%). Mild symptoms were observed in 17.6% and 16.6% of the patients infected by *T. cruzi* I (three of 17) and II (one of six), respectively (Table 1). Any significant differences between the established *T. cruzi* lineages and sex or age of the patients as well as the altitude of the localities where they lived (120–220 m.a.s.i.) were detected using the Fisher exact test (Ramsey 2002).

Genetic polymorphism between *T. cruzi* lineages and intra-lineage variability assessed by random amplified polymorphic DNA

To assess genetic polymorphism of *T. cruzi* isolates typed within the same lineage, we analysed RAPD patterns of nine isolates of lineage I and six of lineage II, selected as representatives of the different clinical profiles of Venezuelan patients. This analysis disclosed high polymorphism between isolates ascribed to lineage *T. cruzi* I or *T. cruzi* II in patterns generated with all the five primers, clearly separating the isolates into two groups according to lineages by simple visual analysis of RAPD patterns. Patterns obtained with primers 615, 601 and 672 were selected to exemplify the results obtained (Figure 2). However, low genetic variability was observed within both lineages with all primers used. Only micro-heterogeneity was detected among *T. cruzi* I isolates using primer 601 whereas the isolates typed as *T. cruzi* II showed low heterogeneity with primer 672 (Figure 2). Nevertheless, we could not associate the low polymorphism among the isolates with any specific clinical feature of patients from which they were isolated. Patterns generated using DNA from *T. cruzi* G (from an opossum of Brazilian Amazon), used as a reference strain for *T. cruzi* I, were always very similar to those generated for Venezuelan isolates of the same lineage. On the contrary, despite presenting high

Table 1 *Trypanosoma cruzi* isolates and clinical features of respective acute chagasic patients

			Patient				Clinical picture					Cardiac evaluation					
Number	Locality	*T. cruzi* isolate	Age (years)	Sex	XD	Clinical condition	Fe	My	He	Ro	Ch	Ed	Hf	EKG	ECG	CHXr	Lineage
1	Hurtado	MHOM/Vê/91/14-91	21	F	+	A	–	–	–	–	–	–	–	N	N	N	I
2	Maporita	MHOM/Vê/92/2-92	2	F	+	S	+	–	+	–	–	+	+	A	A	ND	I
3	Canaguá	MHOM/Ve/96/23-96	38	M	+	S	+	+	+	+	–	–	+	A	A	A	I
4	Paguey	MHOM/Ve/91/1-91	46	M	+	MS	+	–	+	–	–	–	–	N	N	N	I
5	Pedraza	MHOM/Ve/95/36-95	51	F	+	S	+	+	+	+	–	+	+	A	N	A	I
6	Pedraza	MHOM/Ve/95/37-95	12	M	+	S	+	+	+	+	–	–	+	A	N	A	I
7	Santa Ines	MHOM/Ve/95/8-95	30	M	+	S	+	+	–	–	+	–	+	A	ND	A	I
8	Anaro	MHOM/Ve/94/9-94	6	F	+	MS	+	+	+	+	–	–	–	N	N	N	I
9	Quebrada Seca	MHOM/Ve/94/4-94	19	F	+	S	+	+	–	–	–	–	+	A	A	ND	I
10	Libertad	MHOM/Ve/97/MD	7	F	–	A	–	–	–	–	–	–	–	ND	N	ND	I
11	Quebrada Seca	MHOM/Ve/93/7-93	11	F	+	S	+	–	–	+	–	–	+	A	A	ND	II
12	Maporita	MHOM/Ve/93/Mon	10	F	–	A	–	–	–	–	–	–	–	ND	N	ND	II
13	Canagua	MHOM/Ve/97/1-97	47	F	+	A	–	–	–	–	–	–	–	ND	ND	ND	I
14	Santa Lucia	MHOM/Ve/98/7-98	27	M	+	S	+	+	+	+	–	–	+	A	A	A	I
15	Toruno	MHOM/Ve/98/26-98	19	M	+	S	+	+	+	+	–	–	+	A	A	A	I
16	Maporal	MHOM/Ve/99/8-99	8	M	–	A	–	–	–	–	–	–	–	ND	ND	ND	I
17	Mamonal	MHOM/Ve/99/1-99*	0.5	M	+	S	+	+	–	+	–	–	+	ND	ND	ND	I
18	Potreritos	MHOM/Ve/2000/RM	6	F	–	A	–	–	–	–	–	–	–	ND	ND	ND	II
19	Libertad	MHOM/Ve/96/27-96	20	M	–	MS	+	+	+	+	–	–	–	N	N	N	II
20	San Silvestre	MHOM/Ve/2000/AC	11	M	–	A	–	–	–	–	–	–	–	ND	ND	ND	II
21	Dolores	MHOM/Ve/97/HM	28	M	–	A	–	–	–	–	–	–	–	ND	ND	ND	I
22	Tierra Blanca	MHOM/Ve/97/JA	11	M	–	MS	–	+	+	+	–	–	–	ND	ND	ND	I
23	Dolores	MHOM/Ve/93/4-93	3	M	–	A	–	–	–	–	–	–	–	ND	ND	ND	II

XD, xenodiagnosis; A, asymptomatic; S, severe; MS, mild symptoms; Fe, fever; My, myalgia; He, headache; Ro, Romaña; Ch, chagoma; Ed, oedema; Hf, heart failure; EKG, electrocardiogram; ECG, echocardiogram; CHXr, chest X-rays; N, normal; A, abnormal; ND, not done.
* Fatal case.

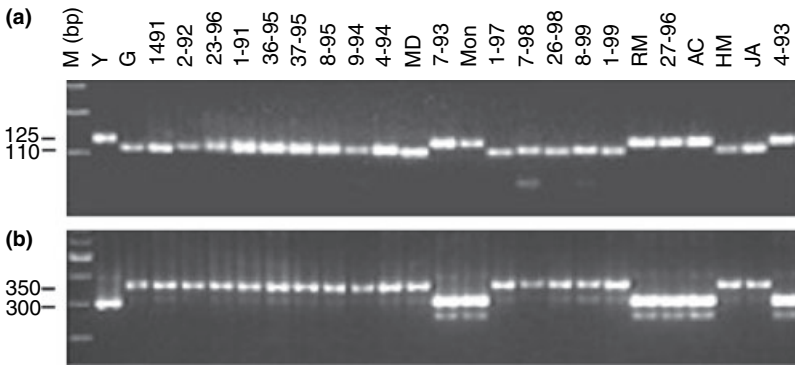

Figure 1 Lineage typing through agarose gel (2%) electrophoresis of polymerase chain reaction (PCR) products generated by amplification of DNA from isolates of *Trypanosoma cruzi* from Venezuelan acute patients: (a) amplification of 24Sα-rDNA sequences and (b) amplification of mini-exon gene sequences. DNA from the reference strains G and Y for *T. cruzi* I and *T. cruzi* II, respectively, were used as positive controls.

similarity, RAPD patterns of the Brazilian human isolate *T. cruzi* Y, used as reference for *T. cruzi* II, always could be distinguished from those generated for the Venezuelan isolates (Figure 2).

Discussion

To evaluate whether there is any relationship between the clinical profile of acute chagasic patients and the molecular

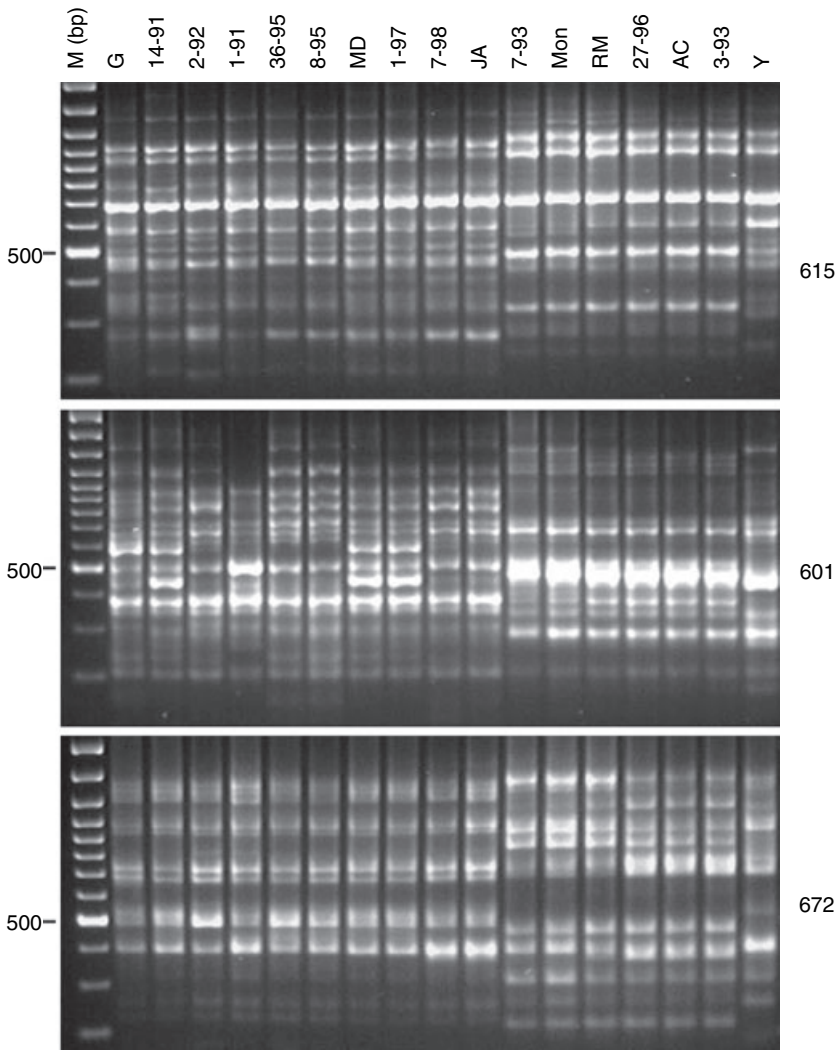

Figure 2 Agarose gels (2%) stained with ethidium bromide showing random amplified polymorphic DNA (RAPD) patterns generated by primers 615, 601 and 672 selected to illustrate the grouping patterns and genetic polymorphisms of Venezuelan *Trypanosoma cruzi* isolates from acute patients. DNA from the reference strains G and Y for lineages I and II, respectively, was used for comparative purposes.

characterization of their respective *T. cruzi* isolates, in the present paper we reported 23 clinically and sero-parasitologically positive acute cases from which the parasites were isolated. The genetic typing of the 23 human *T. cruzi* isolates using rDNA and mini-exon gene markers that identify the two major phylogenetic lineages (Souto *et al.* 1996) resulted in a high number of *T. cruzi* I isolates (17), with consequent clear predominance of this lineage (74%) among chagasic patients in Venezuela. This picture contrasts to that of the American Southern Cone, where most human isolates from acute and chronic cases were ascribed to *T. cruzi* II (Zingales *et al.* 1998, 1999). Although a high predominance of human Z1/*T. cruzi* I isolates was also found in Mexico (Bosseno *et al.* 2002) and Colombia (Jaramillo *et al.* 1999; Montilla *et al.* 2002) clinical data of the patients were not analysed in these studies.

In our study, both *T. cruzi* I and *T. cruzi* II were found to circulate in the same regions and were able to infect humans. Both lineages resulted in acute infections with different clinical profiles and severity irrespective of their sex and age. *Trypanosoma cruzi* I was isolated from a very young child (6 months old) that died because of this infection, suggesting that parasites from this lineage can be congenitally transmitted and can be fatal for children in acute phase. We never found both *T. cruzi* I and *T. cruzi* II lineages in a same culture from any patient. Similarly, most of the cultured human isolates showed to belong to a single lineage in other studies (Barnabé *et al.* 2001; Bosseno *et al.* 2002; Montilla *et al.* 2002; Diosque *et al.* 2003). We could not discard the possibility that some patients of our study could be infected by parasites of more than one *T. cruzi* lineage. Actually, mixed

infections by *T. cruzi* I and *T. cruzi* II are being increasingly diagnosed by analyses of *T. cruzi* populations directly from blood samples of chagasic patients (Solari *et al.* 2001; Brenière *et al.* 2002). Thus, it is not impossible that the procedures employed for parasite isolation (haemoculture) and posterior cultivation have favoured isolation of *T. cruzi* I parasites from mixed infected patients. However, the percentage of the less frequent *T. cruzi* II (26%) in this study does not allow suggesting that these *in vitro* procedures result in an obvious tendency of one specific genotype to eliminate the other since both lineages were isolated from patients living in a same area. Moreover, there is no consensus about the lineage that would be favoured during the isolation procedures (Bosseno *et al.* 2000; Solari *et al.* 2001).

Until now, most of the genetic typed *T. cruzi* I human isolates were obtained from patients without severe symptoms of Chagas' disease (Zingales *et al.* 1998, 1999; Coura *et al.* 2002). The few symptomatic patients found to be infected by this lineage were often suggested as probably having a mixed infection by *T. cruzi* II (Montamat *et al.* 1996; Brenière *et al.* 1998, 2002; Barnabé *et al.* 2001; Solari *et al.* 2001). The Venezuelan *T. cruzi* I and *T. cruzi* II isolates were obtained from asymptomatic patients, as well as from symptomatic patients showing severe (heart failure) or mild signs and/or symptoms of acute Chagas' disease. These data indicate that, at least in the geographic region studied, typing of the parasite lineage does not have a strong clinical prognostic value for *T. cruzi* infection. However, we cannot disregard that the percentage of *T. cruzi* I-infected patients presenting heart failure was three times higher than that of *T. cruzi* II-infected patients. Moreover, our data revealed an unexpected twice-higher percentage of asymptomatic *T. cruzi* II-infected patients when compared with *T. cruzi* I-infected patients. Therefore, despite the low (six) number of patients harbouring *T. cruzi* II, these analyses point to the existence of a higher severity of acute Chagas' disease in the Venezuelan patients that were infected by *T. cruzi* I. Accordingly, data from patients analysed in this study revealed positive xenodiagnosis in 13 of 17 patients infected by *T. cruzi* I (76.4%) and only in one of the six patients infected by *T. cruzi* II (16.6%) (Table 1), suggesting that parasitaemia is higher in patients infected by *T. cruzi* I. In Southern Cone countries, the predominance of *T. cruzi* II parasites in xenodiagnosis was suggested to be associated with a high myocardial damage of *T. cruzi* II-infected patients (Zingales *et al.* 1998). In these countries, molecular and immunological markers able to differentiate *T. cruzi* I and *T. cruzi* II corroborated that human chagasic infection is due principally to *T. cruzi* II and that this lineage is responsible for a more severe disease than lineage *T. cruzi* I (Brenière *et al.* 1998; Zingales *et al.* 1998, 1999; Di Noia *et al.* 2002; Buscaglia & Di Noia 2003; Miles *et al.* 2003).

Despite these data, we are not suggesting that *T. cruzi* I isolates from Venezuela could be more pathogenic to man than *T. cruzi* I from the Southern Cone countries. Neither are we proposing that *T. cruzi* II from Venezuela could be less harmful to humans than *T. cruzi* II from these countries. Instead, in our opinion, our results confirmed that several other variable features from both parasite and man have important influence in the clinical picture of Chagas' disease (Tarleton 2003), besides the phylogenetic origin of *T. cruzi* isolates as defined by their lineages.

Results obtained in this study allied to a high prevalence of *T. cruzi* in children and dogs (Añez *et al.* 1999b) and in triatomines found inside or in palms around houses (N. Añez *et al.*, in preparation, 2004) suggest that both *T. cruzi* I and *T. cruzi* II lineages are being transmitted in peridomestic and domestic environment in Barinas State. We are currently isolating and typing *T. cruzi* isolates from triatomines collected in palm trees and inside houses aiming to better understand the transmission cycles of both *T. cruzi* lineages in Venezuela.

Against high polymorphism within *T. cruzi* II, presenting at least five phylogenetic sublineages of isolates from distinct geographic regions, lineage I has been considered homogeneous (Brisse *et al.* 2000, 2001). However, a recent study disclosed a small genetic heterogeneity within lineage I (Diosque *et al.* 2003). Seeking to assess genetic polymorphism of *T. cruzi* isolates typed within the same lineage, we analysed RAPD patterns from isolates of lineages I and II representatives of the different clinical pictures of acute Venezuelan patients. This method has been used for fine level detection of *T. cruzi* polymorphism (Brisse *et al.* 2000). In this study, all attempts to differentiate isolates of the two lineages using RAPD method were successful, confirming the high genetic distance separating the two major *T. cruzi* lineages. However, our RAPD analysis did not show high intra-lineages polymorphism and the micro-heterogeneity detected within both lineages was not associated with any specific clinical feature presented by the respective patients. Despite the low number of isolates investigated and the lack of statistical data analysis (both sample size and genetic variability are quite limited for this purpose) our preliminary RAPD analysis showed small genetic polymorphism among *T. cruzi* I isolates from Venezuela. The real significance of this variability will be investigated using a large number of isolates from chagasic patients showing different clinical pictures and from sylvatic and domestic triatomine bugs, by characterizing different molecular markers.

Concluding, our data show that *T. cruzi* I and *T. cruzi* II circulate in the same endemic localities of Western Venezuela and that both are responsible for human acute infection with highly variable clinical profiles, ranging from totally asymptomatic to severe heart failure. The higher prevalence of severe Chagas' disease in patients infected by *T. cruzi* I allied to the occurrence of severe disease in chronic patients of the same region (Añez *et al.* 1999a), do not corroborate neither a innocuousness nor a more benign disease in humans infected by this lineage.

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