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CLINICAL ANALYSIS AND PARASITE GENETIC DIVERSITY IN HUMAN IMMUNODEFICIENCY VIRUS/CHAGAS' DISEASE COINFECTIONS IN BRAZIL

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Abstract. To evaluate the possible role of parasitemia on Chagas' disease reactivation in Chagas' disease/human immunodeficiency virus (HIV) coinfection cases and the impact of HIV coinfection on *Trypanosoma cruzi* genetic diversity, 71 patients with Chagas' disease (34 HIV+ and 37 HIV-) were surveyed. Moreover, 92 *T. cruzi* stocks from 47 chronic chagasic patients (29 HIV+ and 18 HIV-) were isolated and analyzed by multilocus enzyme electrophoresis and a random amplified polymorphic DNA procedure. High parasitemia appeared to play a major role in cases of Chagas' disease reactivation. In HIV+ patients, the genetic diversity and population structure (clonality) of *T. cruzi* was similar to that previously observed in HIV- patients, which indicates that immunodepression does not modify drastically genotype repartition of the parasite. There was no apparent association between given *T. cruzi* genotypes and specific clinical forms of Chagas' disease/HIV associations.

Chagas' disease, the American trypanosomiasis, is widespread from the southern United States (where the indigenous cases are rare) to northern Argentina. It is characterized by two successive phases, an acute one, with high parasitemia, and a chronic one, with low parasitemia. Although efficient measures of transmission control have been implemented in some countries (by eliminating the insect vector), Chagas' disease still is a priority health problem in Latin America. Chagas' disease/human immunodeficiency virus (HIV) associations appear to be a growing threat in large Latin American cities, and can be extremely pathogenic, with severe cases involving the central nervous system (CNS) with either tumoral lesions or meningoencephalitis.[1–5]

The causative agent of Chagas' disease is *Trypanosoma cruzi,* a flagellate protozoan. This parasite exhibits considerable genetic diversity,[6,7] and it has been proposed that specific clinical forms of Chagas' disease were due to this diversity.[8] Genetic diversity of *T. cruzi* appears to be governed by a predominant clonal evolution.[6] Clonality in *T. cruzi* and in other pathogens has important medical implications.[9] First, if clonal evolution is predominant, the multilocus genotypes of the pathogen propagate themselves unchanged as genetic photocopies, which makes them relevant as powerful markers for epidemiologic surveys. However, if genetic recombination is predominant, the multilocus genotypes of the pathogen are unstable and cannot be used as epidemiologic markers. Second, in case of clonal evolution, the natural clones correspond to distinct evolutionary units that tend to accumulate divergent mutations, including those genes that control properties such as virulence or resistance to drugs. A challenging hypothesis to clonality is that the immune defenses of the host eliminate most of the genotypes of the pathogen, which generates pseudoclones and apparent linkage disequilibrium (nonrandom association between genotypes occurring at different loci) even if the pathogen is not clonal.

In the present study, we have explored the clinical diversity of Chagas' disease/HIV associations in Brazil and have tested the following hypotheses: 1) that reactivation of Chagas' disease in HIV+ patients is due to high parasitemia independent of specific *T. cruzi* genotypes; 2) that these genotypes play a specific role in reactivation of Chagas' disease in HIV+ patients; and 3) that apparent *T. cruzi* clonal population structure is due to immune defenses of the host rather than to true clonal evolution and therefore disappears in HIV+ immunocompromised patients. It is worth noting that these patients probably acquired Chagas' disease prior to the HIV infection.

MATERIALS AND METHODS

Patients. Patients were recruited in four Brazilian hospitals: Services of Infectious Diseases of the University Hospitals of Sao Paulo, Campinas, and Uberlândia, and The Hospital for Hemophilic Patients in Rio de Janeiro. They were included in the study if they showed positive results in at least two of three serologic assays for Chagas' disease (indirect hemagglutination, indirect immunofluorescence, and ELISA). Seventy-two chronic chagasic patients were selected according to this criterion (Table 1). Within this group of 72 patients, an HIV+ and an HIV- group was defined according to positivity or negativity for HIV serologic reactions (ELISA and Western blot). We were able to isolate and characterize the parasite stocks from 47 (29 HIV+ and 18 HIV-) of 72 patients. The criterion for reactivation of Chagas' disease was the presence of infective trypomastigote forms of the parasite detected by microscopic examination of peripheral blood. The Medical Ethics Committee of each participating center approved the study protocol. Informed consent was obtained from all study patients.

Xenodiagnosis. This technique uses *T. cruzi*-free triatomine vectors (reared in the laboratory). Thirty third-stage

TABLE 1

Characteristics of the 72 patients: age, geographic origin, clinical forms, parasitemia, and code of *Trypanosoma cruzi* corresponding stocks*

HIV-positive patients							HIV-negative patients						
Patient code	Age (years)	Birth locality†	Clinical form	Parasitemia‡	No. of stocks	Code§	Patient code	Age (years)	Birth locality†	Clinical form	Parasitemia‡	No. of stocks	Code§
HC1	37	MG	Indeterminate	61.9%	3	T10-T6-T7	U1	79	MG	Cardiac	9.1%	1	T1
HC2	29	BA	Cardiodigestive	27.7%	2	N7-T3	U2	58	MG	Cardiac	26.3%	1	T2
HC3	47	SP	Cardiodigestive	24%	1	T2	U3	40	MG	Indeterminate	4.5%	1	T1
HC4	27	BA	Cardiac	53.8%	4	T3-T4-T9-T11	U8	53	CE	Digestive	0		NS
HC5	33	PE	Cardiodigestive	6.7%	1	T1	U10	59	SP	Cardiac	0		NS
HC6	31	BA	Indeterminate	0	2	T3-T4	U11	65	MG	Cardiodigestive	43.4%	2	T5-T10r
HC7	59	SP	Cardiac	38%	3	T1-T9f-T10	U13	31	MG	Cardiac	40%	2	T1-T6
HC10	57	BA	Indeterminate	0		NS	U15	70	MG	Cardiac	0		NS
HC12	42	CE	Indeterminate	8.2%	1	T1	U16	37	GO	Cardiodigestive	0		NS
HC13	42	MG	Cardiodigestive	26%	2	T1-T2	U17	71	SP	Cardiodigestive	3.8%	1	T1
HC14	37	SP	Myocarditis	65.4%	4	T1-T2-bN17-bN19	U18	72	MG	Cardiodigestive	65.5%	2	T12-T16
HC18	31	MG	Indeterminate	14.28%	1	T1	U19	76	MG	Cardiodigestive	0		NS
HC19	55	AL	Indeterminate	20%	2	T1-T2	U20	63	MG	Cardiac	0		NS
HC20	32	AL	Indeterminate	0		NS	U21	51	MG	Cardiodigestive	0		NS
HC21	42	GO	Neurologic	66.6%	3	T1-T7-T9	U22	64	MG	Cardiac	4.8%	1	T1
HC22	27	GS	Neurologic	100%	2	T5-T8	U23	47	GO	Cardiac	0		NS
HC23	28	BA	Indeterminate	NP	3	T6-T11-T13	U24	67	MG	Cardiac	0		NS
HC24	43	BA	Cardiac	NP	2	T1-T4	U25	57	MG	Cardiac	0		NS
HC25	25	BA	Cardiodigestive	NP	2	T1-T2	U26	72	MG	Cardiac	0		NS
HC27	42	BA	Indeterminate	NP	1	T1	U27	52	MG	Cardiac	0		NS
CP1	38	MG	Indeterminate	0		NS	U28	78	MG	Cardiac	23.8%	2	T2-T5
CP2	58	GO	Indeterminate	19.2%	1	T3	U29	69	MG	Cardiac	0		NS
CP3	48	SP	Indeterminate	0		NS	U30	49	MG	Cardiac	0		NS
CP5	46	PA	Cardiac	0	1	bT2	U31	67	BA	Cardiac	41.2%		NS
CP8	40	SP	Indeterminate	NP	2	T5-T10	U32	50	MG	Cardiac	0		NS
CP9	43	SP	Indeterminate	NP	2	T1-T4	U33	40	MG	Cardiac	0		NS
U4	42	MG	Indeterminate	0	4	bT2-cT3-cT6-cT8	U34	67	MG	Cardiac	4%		NS
U5	47	MG	Cardiac	60%	4	N10-T4-bT8-bT9	U35	65	MG	Cardiodigestive	0		NS
U6	36	MG	Neurologic	100%	7	T10-T11-T16-T25-bT22-bT3-bT5	U36	58	MG	Cardiac	0		NS
U7	43	MG	Indeterminate	7.7%	2	T1-T2	HC17	40	MG	Indeterminate	0		NS
U12	48	MG	Neurologic	89.3%	4	T5-T19f-T20f-T23d	HCC25	38	BA	Indeterminate	NP	1	T1
RJ1	28	AL	Indeterminate	47.5%	3	I3-I4-T2	17HCC	30	BA		NP	1	T2
RJ2	44	ES	Indeterminate	40%	1	I1	HCC40	28	MG	Digestive	NP	1	T1
							HCC41	29	MG	Cardiac	NP	1	T3
							HCC42	45	MG		NP	2	T3-T4
							HCC43	42	BA	Indeterminate	NP	1	T1
							HCC44	31	MG	Cardiac	NP	1	T1
							HCC26				NP	1	T1
							HCC31	39	SP	Indeterminate	NP	1	N2

* HIV = human immunodeficiency virus.
† MG = Minas Gerais; BA = Bahia; SP = São Paulo; CE = Ceara; PE = Pernanbuco; GO = Goias; AL = Alagoas; GS = Rio Grande do Sul; PA = Parana; ES = Espiritu Santo.
‡ Number of positive nymphs/total nymphs. NP = parasitemia not determined.
§ NS = no stock isolated.

nymphs of *Triatoma infestans,* a major vector in South America, are fed on the putative infected patient, and the feces of this vector are checked for the presence of *T. cruzi* at the 30th, 60th, and 90th days after feeding.

Parasite isolation and culture. Each *T. cruzi* isolate was taken from only one positive nymph. They were cultured and passaged weekly in biphasic medium (NNN/liver infusion tryptose [LIT], 10% fetal calf serum, 50 μg/ml of gentamicin). Seventy isolates were obtained from the 29 HIV+ patients, and 23 isolates were obtained from the 18 HIV- patients. Table 1 shows the origin of the 93 isolates obtained in this study. When the stocks were well adapted to culture conditions, they were bulk-cultured in pure LIT medium in disposable plastic vials. They were harvested by centrifugation and kept at −70°C until isoenzyme analysis.

Isoenzyme analysis. Parasite pellets were mixed with an equal volume of enzyme stabilizer solution (2 mM dithiothreitol, 2 mM amino-n-caproic acid, 2 mM EDTA)[10] and kept on ice for 20 min. This mixture was then centrifuged at 13,000 × *g* for 10 min at 4°C. The pellets of broken cells were discarded, and the supernatants containing the water-soluble enzymes were retained for isoenzyme analysis.

Multilocus enzyme electrophoresis on cellulose acetate plates was performed according to the procedure of Ben Abderrazak and others.[11] The following 20 enzyme systems were used: aconitase (ACON; E.C.4.2.1.3), alanine aminotransferase (ALAT; E.C.2.6.1.2), diaphorase (DIA; E.C.1.6.2.2), glyceraldehyde-3-phosphate dehydrogenase (GAPD; E.C.1.2.1.12), glutamate dehydrogenase NAD+ (GDH-NAD+; E.C.1.4.1.2), glutamate dehydrogenase NADP+ (GDH-NADP; E.C.1.4.1.4), aspartate aminotransferase (GOT; E.C.2.6.2.1), glucose-6-phosphate dehydrogenase (G6PD; E.C.1.1.1.49), glucose-phosphate isomerase (GPI; E.C.5.3.1.9), isocitrate dehydrogenase (IDH; E.C.1.1.1.42), leucine aminopeptidase (cytosol aminopeptidase) (LAP; E.C.3.4.11 or 13), malate dehydrogenase (MDH; E.C.1.1.1.37), malic enzyme (ME; E.C.1.1.1.40), mannose phosphate isomerase (MPI; E.C.5.3.1.8), nucleoside hydrolase (inosine) (NHi; E.C.2.4.2.-), substrate: inosine, peptidase 1 (PEP-1; E.C3.4.22.3 [formerly E.C.3.4.4.12]), substrate: leucyl-leucyl-leucine, peptidase 2 (PEP-2; E.C.3.4.22.4 [formerly E.C.3.4.4.24]), substrate: leucyl-L-alanine, 6-phosphogluconate dehydrogenase (6PGD; E.C.1.1.1.44.), phosphoglucomutase (PGM; E.C.2.7.5.1), and superoxide dismutase (SOD; E.C.1.15.1.1).

Random amplified polymorphic DNA (RAPD) analysis. Random amplified polymorphic DNA procedures were performed according to those of Williams and others.[12] We used seven 10-mer primers that were selected from prior studies according to their reproducibility and discriminative power[13] (Brisse S. University of Montpellier, Montpellier, France, unpublished data). Their sequences (GGCTGCAGAA, CAGCCCAGAG, ACGGCAAGGA, ACCAGGGGCA, GGTGCTCCGT, CCATTCCCCA, GTGACGTAGG) are identical to the F13, N7, R20, N2, N20, R10, and A8 primers produced by Operon Technologies, Inc. (Alameda, CA). The polymerase chain reaction (PCR)–amplified DNA fragments were separated by electrophoresis in 1.6% agarose gels and stained with ethidium bromide.

Genetic and phylogenetic analysis. The analysis of 20 enzyme systems led to the identification of 21 genetic loci since ME corresponds to two different loci.[6] For a given *T. cruzi* stock and for a given locus, each isoenzyme band was given a value of 1 (presence) or 0 (absence). For a given stock, the 21 loci led to the identification of a given zymodeme, or distinct enzyme profile. These zymodemes can be considered as multilocus genotypes in which the allelic composition remains hypothetical. However, population genetic analysis is still possible since in linkage disequilibrium statistics, the only condition required is the independence of genotypes observed at different loci, and the identification of alleles is not indispensable.[14] Each RAPD gel band was coded with a number, starting with 1 for the slowest band (largest DNA fragment). Each stock was thus represented by a set of numbers for each primer.

To depict the genetic relationships among the stocks, Jaccard's genetic distances were computed.[15] Jaccard's distance was estimated by the formula $D = 1 - [a/(a + b + c)]$, where a = the number of bands that are common to the two compared genotypes, b = the number of bands present in the first genotype and absent in the second, and c = the number of bands absent in the first genotype and present in the second. A dendrogram was generated by the unweighted pair group method with arithmetic averages (UPGMA).[16]

To reliably estimate the phylogenetic position of the stocks isolated, five fully characterized reference stocks that were cloned in the laboratory were added to the phylogenetic analysis: MN cl2, Esmeraldo cl3, Tu 18 cl2, Tehuentepec cl2, and the CL Brenner clone F11SS included in the *T. cruzi* genome project.[17] They represent the formerly described clonal genotypes 39, 30, 32, 12, and 43, respectively.[6]

Population structure was analyzed by linkage disequilibrium statistics (nonrandom association of genotypes at different loci) according to the tests proposed by Tibayrenc and others.[14] These tests are based on the null hypothesis that genetic recombination occurs randomly in the population under survey (panmixia). Statistically significant departures from panmictic expectations are taken as circumstantial evidence that the population has a clonal structure. Means to detect the biases due to geographic distance have been proposed by Tibayrenc and others.[18] The UPGMA analysis and linkage disequilibrium tests have been computed with the genetics software designed in our laboratory (S. Noël).

RESULTS

Clinical patterns of Chagas' disease observed in HIV+ patients. Uncommon clinical forms of chronic Chagas' disease (meningoencephalitis and myocarditis) that showed reactivation of Chagas' disease were present only in the HIV+ patients. Four patients developed a meningoencephalitis form and one patient developed a myocarditis form.[19] These clinical forms were characterized by the detection of trypomastigote forms by microscopic examination of peripheral blood.

Xenodiagnosis. A complete xenodiagnosis protocol was performed on a subset of 57 patients; a positive xenodiagnosis result was recorded in 31 (47.4%). Twenty (74%) of 27 HIV+ patients were positive for Chagas' disease, whereas only 11 (37%) of 30 HIV- patients were positive for Chagas' disease. This difference was statistically significant ($P < 10^{-2}$ by chi-square test). Moreover, in the population of

TABLE 2
Levels of parasitemia in the different groups of patients*

Clinical forms	No parasitemia		Low parasitemia (0 < NP ≤ 10%)		Medium parasitemia (10% < NP ≤ 40%)		High parasitemia (40% < NP ≤ 60%)		Very high parasitemia (60% < NP)	
	HIV+	HIV−	HIV+	HIV−	HIV+	HIV−	HIV+	HIV−	HIV+	HIV−
Indeterminate†	6	1	1	1	5		1		1	
Cardiac†	1	13		3	1	3	2	1		
Cardiodigestive†		4	1	1	3			1		1
Digestive†		1								
Myocarditis‡									1	
Neurologic‡									4	
Subtotal	7	19	2	5	9	3	3	2	6	1
	26%	63%	7.4%	17%	33.3%	10%	11.1%	7%	22.2%	3%
	No parasitemia + low parasitemia				Medium + high + very high parasitemias					
	HIV+		HIV−		HIV+		HIV−			
Total	33%		80%		67%		20%			

* NP = number of positive nymphs; HIV = human immunodeficiency virus.
† Chronic clinical forms.
‡ Clinical reactivation forms.

patients with a positive xenodiagnosis, the proportion of *T. cruzi*-positive *T. infestans* nymphs was higher in the HIV+ patients than in the HIV- patients (44% [204 of 459] versus 25.5% [61 of 239]), respectively. Again, the difference was statistically significant ($P < 10^{-3}$ by chi-square test).

Level of parasitemia. Parasitemia estimated on the subset of 57 patients was ranked into five groups (Table 2): 1) not detected (all nymphs are negative); 2) low (number of positive < 10%); 3) medium (number of positive nymphs 10%–< 40%); 4) high (number of positive nymphs 40%–< 60%); 5) very high (number of positive nymphs ≥ 60%).

In the HIV+ group, 66.6% of the patients had medium or higher parasitemias, whereas this percentage was 20% only in the HIV- group. This difference was statistically significant ($P < 2 \times 10^{-2}$ by chi-square test). The five patients that had clinical reactivation of the disease showed very high levels of parasitemia. The percentage of positive nymphs was 100%, 100%, 66.6%, and 89.3% (average = 89%) for the four cases of meningoencephalitis and 65.4% for the case of myocarditis. There was a statistically significant association ($P = 1.3 \times 10^{-5}$ by chi-square test) between a very high level of parasitemia (> 60%) and cases of Chagas' disease reactivation. Of the seven HIV+ patients with a negative xenodiagnosis result, six (86%) had no chagasic symptoms (indeterminate phase).

Parasitemia, immunodeficiency, and HIV symptomatology. Among the 22 HIV+ patients, there was no association between the level of parasitemia and the number of CD4 cells. Conversely, a highly significant association ($P < 10^{-3}$ by chi-square test) between high levels of parasitemia and HIV clinical forms (A, B, and C) according to the Centers for Disease Control (Atlanta, GA) classification[20] (Figure 1).

***Trypanosoma cruzi* genotype identification and phylogenetic relationships.** The *T. cruzi* isolate from one HIV+ patient (HC12) showed an apparent mixture of *T. cruzi* and *T. rangeli,* which is a nonpathogenic species. This was shown by several enzyme loci exhibiting juxtaposed patterns from both species (Figure 2). This patient had an indeterminate clinical form of Chagas' disease and his parasitemia was low (8.2%). Due to the difficulty in interpreting patterns of mixtures of genotypes, this isolate was excluded from the phylogenetic and population genetic analysis.

All *T. cruzi* isolates identified in the present study were closely related to one of the formerly described clonal genotypes (30, 32, 39, or 43).[6] Since the methods of identifi-

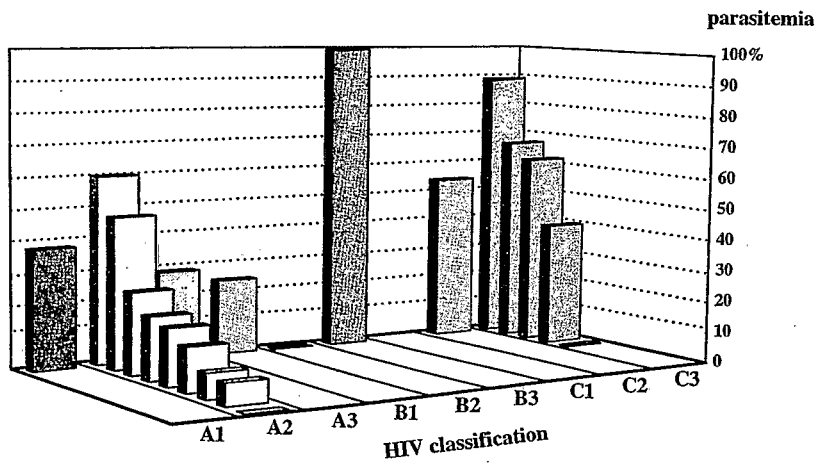

FIGURE 1. Parasitemia levels and classification of human immunodeficiency virus (HIV) (1992)[20] infection for 22 patients.

FIGURE 2. Electrophoretic patterns of the malate dehydrogenase enzyme system for some parasite stocks included in the present study. Lane 1, *Trypanosoma cruzi* (CP8); 2, *T. cruzi* (CL); 3, *T. cruzi* (CP5T2); 4, *T. cruzi* (MN cl2); 5, *T. cruzi* (HC24T1); 6, *T. rangeli* (ref. RGB); 7, mixture of *T. cruzi* and *T. rangeli* (HC12); 8, *T. cruzi* (Tu18 cl2); 9, *T. cruzi* (HC23T6); 10, *T. cruzi* (HC22T8).

cation have been improved (21 isoenzyme loci instead of 15), it is impossible to determine if the *T. cruzi* genotypes identified in the present study are identical to formerly recognized ones. Nevertheless, with the help of reference stocks, it is possible to conclude that they are at least genetically similar. The stocks isolated from 89% (25 of 28) of the HIV+ patients were closely related to either clonal genotypes 30 or 32, with a predominance of the genotype 32 (22 of 25). This predominance was also observed in the HIV- patients, in which 94% (17 of 18) of the stocks were closely related to clonal genotype 32. The remaining stocks isolated from HIV+ patients were also closely related to formerly described genotypes.[6] Two of these stocks were related to clonal genotype 43, and one was related to clonal genotype 39. In the HIV- group of patients, only one stock from one patient was related to clonal genotype 39. Genotypes 39 and 43 are easily identifiable by their specific heterozygous, three-banded pattern for the GPI locus. Figure 3 shows two UPGMA dendrograms computed from the genetic distances recorded between all stocks, including reference stocks, based on multilocus enzyme electrophoresis and RAPD analysis.

***Trypanosoma cruzi* clonal genotypes and clinical forms of Chagas' disease.** We have tested by chi-square analysis all possible associations between clinical forms of Chagas' disease and *T. cruzi* clonal genotypes in both HIV+ and HIV- patients. The clinical categories were cardiac, cardiodigestive, indeterminate, chronic Chagas' disease, and disease reactivation (neurologic or myocarditis). Only three *T. cruzi* genotypes considered: 30, 32, and 39 + 43. All test results were not statistically significant.

***Trypanosoma cruzi* population structure in HIV+ and HIV- patients.** We made various subdivisions in the total set of *T. cruzi* stocks and performed linkage disequilibrium tests[14,18] not only on the whole population of parasite stocks, but also within each subpopulation so defined (Tables 3 and 4). This design had three objectives. The first was to avoid possible bias due to clonal propagation of the same genotype within the same patient. Thus, in certain subpopulations, we have counted only once identical genotypes isolated from the same patient (B in Tables 3 and 4). The second was to separate true clonal evolution from epidemic clonality (short-term propagation of ephemeral clones in a sexual species).[21] Therefore, as recommended by Maynard Smith and others,[21] we performed the statistical analysis by taking as the unit of analysis each distinct genotype (C in Tables 3 and 4) rather than all stocks (A in Tables 3 and 4). The third was to test the hypothesis[21] that a possible linkage disequilibrium seen in *T. cruzi* stocks isolated from HIV+ patients is due to cryptic speciation between clonal genotypes 30 and 32 on the one hand, and 39 and 43 on the other. For this, as recommended by Maynard Smith and others,[21] we performed part of the statistical analysis on the stocks related only to clonal genotypes 30 and 32 (A' in Tables 3 and 4). There were only five stocks (corresponding to three patients) with either genotypes 39 or 43, which would have made any statistical analysis for them meaningless. Lastly, since the goal of this study was not to analyze the population structure of the whole species, but rather, the population structure of the fresh isolates obtained in the present study, the reference stocks have been eliminated from the population genetic analysis.

Tables 3 and 4 show the results for different subpopulations of parasite stocks based on multilocus enzyme electrophoresis and RAPD analysis, respectively. In most cases, including those stocks that were isolated from HIV+ patients, the results are highly significant, which is an indication of strong linkage disequilibrium.

DISCUSSION

Our results show a significant association between HIV infection and positivity for Chagas' disease by xenodiagnosis. Moreover, the results of xenodiagnosis showed that HIV+ patients tend to have much higher parasitemia levels than HIV- patients. This result suggests that immunodepression leads to a release of the parasite in circulating blood. This is consistent with experimental studies that showed in *T. cruzi* murine infection a high parasitemia accompanying depletion of CD4 and CD8 cells.[22,23] Depression of cellular immunity due to aging could explain the fact that xenodiagnosis positivity tended to increase with the age in the population of HIV- patients analyzed. This is in agreement with the results of Coura and others.[24]

Parasitemia levels in immunocompromised patients showed no correlation with the number of CD4 cells. These observations suggest a modulation of T helper lymphocytes and the effector cell function. According to Sher and others,[25] a down-regulation of the cell immunity could be attributed to the action of a set of inhibitory cytokines produced by T lymphocytes and other cell types. The main inhibitory lymphokines are interleukin-4 (IL-4), IL-10, and transforming growth factor-β; IL-4 and IL-10 are dominant in many situations of chronic or exacerbated parasitic infection. Regulation of the parasitic infection seems to depend on the equilibrium between the T helper 1 (Th1) cells producing interferon-γ and IL-2 and Th2 cells producing mainly IL-4 and IL-10.[25] Patients with high levels of parasitemia probably had greater production of IL-4 and IL-10, and consequently an uncontrolled parasitic infection due to the antagonist effect of interferon-γ. Silva and others[22] have observed in the immunocompromised murine model an increase in IL-10 in *T. cruzi* coinfection with murine leukemia virus.[22] A quantification of cytokine production in coinfected patients could verify this hypothesis. Despite the fact that parasitemia was not correlated with the number of CD4 cells, our study showed a clear association between parasitemia levels and

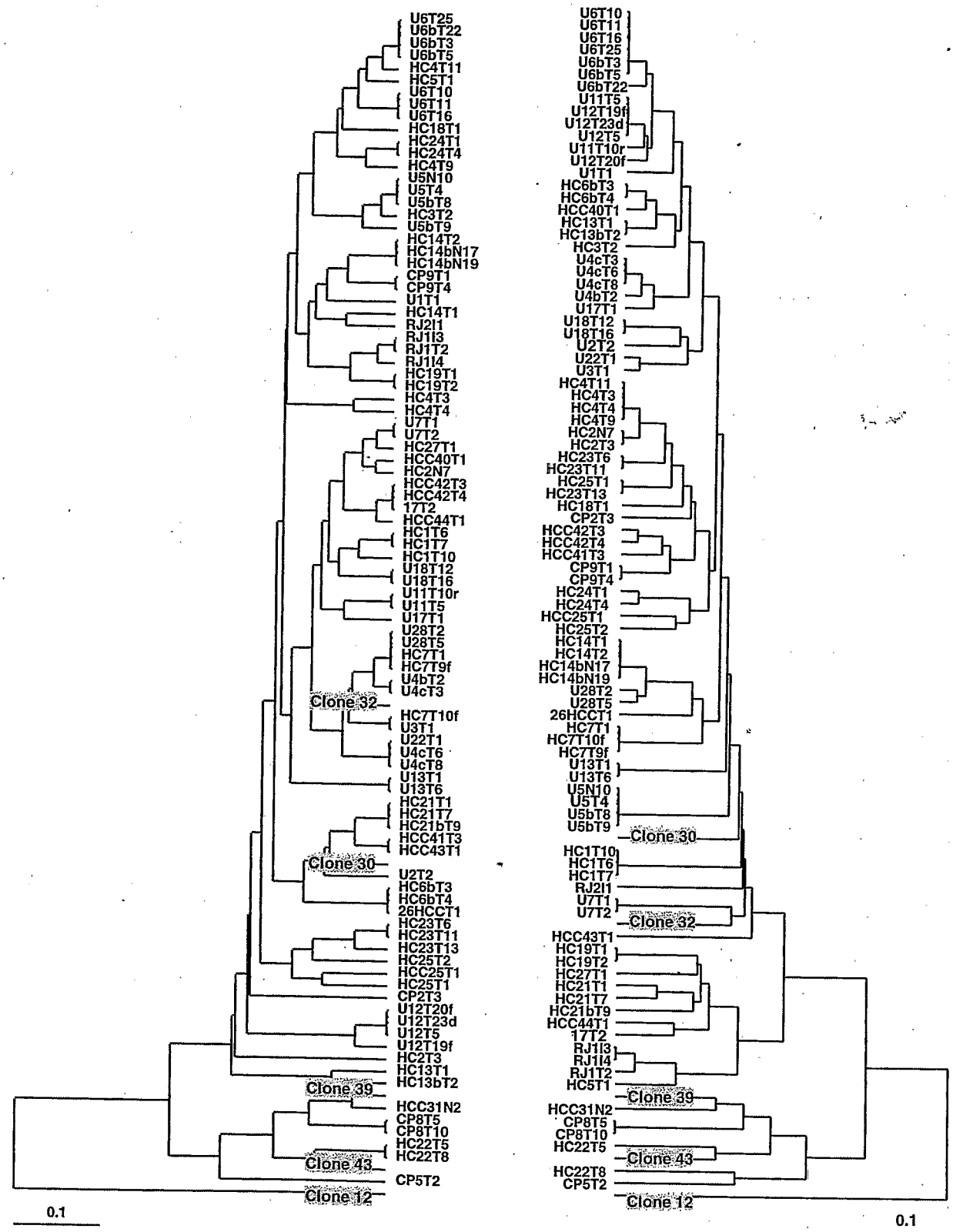

FIGURE 3. Unweighted pair group method with arithmetic averages dendrograms based on multilocus enzyme electrophoresis (**right**) and random amplified polymorphic DNA analysis (**left**) and derived from Jaccard's genetic distances (indicated by the scale) of 97 *Trypanosoma cruzi* stocks, including the five reference stocks.

TABLE 3
Results of the linkage disequilibrium tests based on multilocus enzyme electrophoresis in the different groups of patients*

	All patients				HIV+ patients				HIV− patients			
Size†	92 A	86 A′	69 B	64 C	69 +A	64 +A′	50 +B	50 +C	23 −A	22 −A′	19 −B	17 −C
Test d1	1.5×10^{-5}	6.7×10^{-5}	3.5×10^{-2}	(−)	7.09×10^{-5}	4.59×10^{-4}	(−)	(−)	0.01	0.023	0.027	(−)
Test d2	0.14	0.47	0.96	(−)	0.037	0.20	(−)	(−)	0.05	0.09	0.37	(−)
Test e	$< 10^{-4}$	$< 10^{-4}$	0.15	(−)	$< 10^{-4}$	$< 10^{-4}$	(−)	(−)	$< 10^{-4}$	3×10^{-4}	0.061	(−)
Test f	$< 10^{-4}$	$< 10^{-4}$	$< 10^{-4}$	$< 10^{-4}$	$< 10^{-4}$	$< 10^{-4}$	$< 10^{-4}$	$< 10^{-4}$	0.007	0.21	0.058	0.084

* HIV = human immunodeficiency virus.
† Number of stocks analyzed; A = all stocks are included in the analysis; A′ = all stocks but genotypes 39 and 43; B = repeated genotypes are counted only one time if they are found in the same patient; C = each different clonal genotype is counted only one time; (−) = not feasible (no repeated genotypes).

HIV clinical evolution. These results suggest an interaction between *T. cruzi* and HIV virus. To our knowledge, this result dealing with human clinical cases has never been previously published. Silva and others have described such an interaction in the murine model of acquired immunodeficiency syndrome.[22] Their study describes the activation of *T. cruzi* infection by murine leukemia virus (MuLV) as well as the aggravation of MuLV infection by *T. cruzi*. A study using the HIV-1 p24 ELISA to test the culture medium and on the viral load could confirm this hypothesis.

The reactivation of Chagas' disease in HIV+ patients has been attributed to the immunodepression caused by the virus.[1–5,19] The results of our study are consistent with this hypothesis since high levels of parasitemia (> 60%), as measured by the quantification of the xenodiagnosis, have been observed in the five cases of diseases reactivation that we could follow for this parameter.

High parasitemias seem to play an important role in the reactivation of Chagas' disease, as well as in the clinical form of this reactivation. It is worth nothing that the meningoencephalitis clinical form had a parasitemia level higher than the myocarditis clinical form. Moreover, there was an association in HIV+ patients between either weak or average parasitemia and the absence of chagasic symptoms, but this association was not statistically significant.

Our results suggest that high levels of parasitemia play an important role in the reactivation of Chagas' disease in HIV+ patients. To our knowledge, this hypothesis has never been tested. These results again fuel the debate on Chagas' disease pathogenesis. Studies of Chagas' disease, especially those dealing with cardiomyopathy[26] in immunocompetent patients, suggest that the chagasic lesions may have an autoimmune origin. Nevertheless, other studies based on PCR detection of the parasite show the presence of *T. cruzi* in heart tissue sections from immunocompetent patients with cardiac inflammation.[27,28] Our results are in agreement with those of Levin underlining the important role of the parasite in the pathogenesis of Chagas' disease.[29]

The apparent direct role of the parasite in the clinical forms of reactivation leads to a re-evaluation of treatment for chronic chagasic patients. During the chronic phase of the disease, the efficiency of antichagasic drugs is controversial, whereas during the acute phase of the infection, treatment with benznidazole or nifurtimox is indicated for immunocompetent patients since treatment reduces the clinical course of the disease and prevents death by myocarditis and meningoencephalitis.[30] Treatment in immunocompromised patients may be effective in controlling clinical manifestations, in reducing parasitemia, and in controlling tissue damage related to reactivation of Chagas' disease.[31,32]

We suggest as a practical implication of this result that it is necessary to quantify the xenodiagnosis results to determine which patients should be treated with specific antichagasic drugs to prevent such reactivations due to the actual presence of the parasite. The limit we propose when all triatomine bugs are checked separately is that 50% or more of these bugs should be positive for *T. cruzi*.

An important initial result of this study is that the overall phylogenetic diversity of the stocks isolated from both HIV+ and HIV- patients is relatively limited. First, as shown in Figure 3, all stocks isolated in the present study are ranked into only one of the main phylogenetic subdivisions identified by Tibayrenc.[33] The other main subdivision is represented only by the reference stock Tehuantepec and not by any fresh isolate from the present study (Figure 3). Second, within this main phylogenetic subdivision, clonal genotypes 30, 32, 39 and 43, the only ones to be represented in the stocks isolated in the present study, are closely related to each other.[6,32] Lastly, 89% of the stocks isolated from HIV+ patients and 94% of the stocks isolated from HIV- patients are closely related to either clonal genotype 30 or 32, which are very closely related to each other[6,33] (Figure 3).

TABLE 4
Results of the linkage disequilibrium tests based on random amplified polymorphic DNA in the different groups of patients*

	All patients				HIV+ patients				HIV− patients			
Size†	92 A	86 A′	59 B	57 C	69 +A	64 +A′	38 +B	37 +C	23 −A	22 −A′	21 −B	21 −C
Test d1	2.6×10^{-10}	5.6×10^{-10}	2.1×10^{-3}	(−)	2.1×10^{-10}	5.3×10^{-10}	1.0×10^{-3}	(−)	2.63×10^{-4}	4.1×10^{-4}	(−)	(−)
Test d2	$< 10^{-4}$	$< 10^{-4}$	0.36	(−)	$< 10^{-4}$	$< 10^{-4}$	0.22	(−)	0.07	0.11	(−)	(−)
Test e	$< 10^{-4}$	$< 10^{-4}$	0.07	(−)	$< 10^{-4}$	$< 10^{-4}$	0.22	(−)	2.0×10^{-3}	6.1×10^{-3}	(−)	(−)
Test f	$< 10^{-4}$	10^{-4}	0.031	0.04	$< 10^{-4}$	$< 10^{-4}$	0.07	0.05	0.7	0.7	0.6	0.6

* HIV = human immunodeficiency virus.
† Number of stocks analyzed; A = all stocks are included in the analysis; A′ = all stocks but genotypes 39 and 43; B = repeated genotypes are counted only one time if they are found in the same patient; C = each different clonal genotype is counted only one time; (−) = not feasible (no repeated genotypes).

Clone 39 was present in one HIV+ patient and one HIV- patient. Both patients were born in Parana State in southern Brazil. Clone 43 was present in two HIV+ patients who were born in the southern Brazil (Sao Paulo, Rio Grande do Sul). These observations suggest that *T. cruzi* genotype distribution is dependent on the area where the patient was born rather than the HIV status.

Since the *T. cruzi* stocks isolated from four patients were related only to either clonal genotypes 39 or 43 and there were only three HIV- patients native from southern Brazil, it is impossible to know, with the present set of stocks, whether clonal genotypes 39 and 43 are more frequent in HIV+ patients than in HIV- patients. Moreover, there was no statistically significant difference in genotype distribution between HIV+ and HIV- patients. All stocks isolated in the present study, including those from HIV+ patients, are closely related to clonal genotypes previously identified.[6] This shows that immunodepression due to HIV infection has not led to the establishment of new *T. cruzi* genotypes.

The lack of new genotype in our stocks could be explained by the chronology of the Brazilian coinfections. All patients recruited in cities were probably infected first with *T. cruzi* in rural areas during their childhood since vector transmission of Chagas' disease is nearly nonexistent in urban areas whereas HIV contamination is essentially urban. Our patients were in contact with the HIV virus when they emigrated to cities. According to this chronology, *T. cruzi* genotypes were not modified by subsequent HIV infection, the immune system having yet played its filter role. However, the fact remains that repartition of *T. cruzi* genotypes is not drastically modified in HIV+ patients, a result that to our knowledge has not been previously observed.

There was a slightly higher *T. cruzi* genotype (zymodeme) diversity in HIV+ patients than in HIV- patients, although this difference was not statistically significant. There was also no significant association between given *T. cruzi* genotypes and different clinical forms of Chagas' disease.

A strong linkage disequilibrium is persistent, not only in the whole population of *T. cruzi* stocks analyzed, but also in those stocks isolated from HIV+ patients, and in most of the various subpopulations designed to avoid the bias due to either clonal propagation in the same patient or epidemic clonality.[21] Moreover, most test results remained significant in the population of stocks from HIV+ patients corresponding only to clonal genotypes 30 or 32, which shows that linkage disequilibrium of *T. cruzi* stocks from HIV+ patients is not due to cryptic speciation. The more parsimonious hypothesis to account for these results is that a clonal population structure is conserved in the *T. cruzi* stocks isolated from this set of HIV+ patients. This is consistent with the results obtained with another parasite, *Leishmania infantum*.[34] Again, to our knowledge, this is the first time that *T. cruzi* population structure in HIV+ patients has been extensively by convenient population genetic tests. This result was obtained through the analysis of culture-adapted parasites; however, the bias due to culture selection must be considered as a factor. Nevertheless, the same criticism can be made for all studies dealing with population genetics of pathogens, and with current methods, no multilocus analysis can be done without culturing the pathogen.[9]

Our results have shown that high parasitemia seems to play a major role in the reactivation of Chagas' disease in HIV+ patients, that clinical forms of this disease have no association with given genotypes of the parasite, and that *T. cruzi* genetic diversity and population structure show no drastic differences in isolates from HIV+ compared with those from HIV- patients, despite the immunodepression.

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