MAJOR ARTICLE

Molecular Identification of *Trypanosoma cruzi* Discrete Typing Units in End-Stage Chronic Chagas Heart Disease and Reactivation after Heart Transplantation

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Background. One hundred years after the discovery of Chagas disease, it remains a major neglected tropical disease. Chronic Chagas heart disease (cChHD) is the most severe manifestation. Heart transplantation is the proper treatment for end-stage heart failure, although reactivation of disease may result after receipt of immunosuppressive therapy. *T. cruzi* strains cluster into 6 discrete typing units (DTUs; I–VI) associated with different geographical distribution, transmission cycles and varying disease symptoms. In the southern cone of South America, *T. cruzi* II, V, and VI populations appear to be associated with Chagas disease and *T. cruzi* I with sylvatic cycles.

Methods. Molecular characterization of DTUs, *T. cruzi* I genotypes (on the basis of spliced-leader gene polymorphisms), and minicircle signatures was conducted using cardiac explant specimens and blood samples obtained from a cohort of 16 Argentinean patients with cChHD who underwent heart transplantation and from lesion samples obtained from 6 of these patients who presented with clinical reactivation of Chagas disease.

Results. Parasite persistence was associated with myocarditis progression, revealing *T. cruzi* I (genotype Id) in 3 explant samples and *T. cruzi* II, V, or VI in 5 explant samples. Post–heart transplantation follow-up examination of bloodstream DTUs identified *T. cruzi* I in 5 patients (genotypes Ia or Id) and *T. cruzi* II, V, or VI in 7 patients. *T. cruzi* I, V, and VI were detected in skin chagoma specimens, and *T. cruzi* V and VI were detected in samples obtained from patients with myocarditis reactivations. Multiple DTUs or genotypes at diverse body sites and polymorphic minicircle signatures at different cardiac regions revealed parasite histotropism. *T. cruzi* I infections clustered in northern Argentina (latitude, 23°S–27°S), whereas *T. cruzi* II, V, or VI DTUs were more ubiquitous.

Conclusions. Multiple DTUs coexist in patients with Chagas disease. The frequent finding of *T. cruzi* I associated with cardiac damage was astounding, revealing its pathogenic role in cChHD at the southern cone.

One hundred years after its discovery, Chagas disease remains a major neglected tropical disease, affecting 8 million *Trypanosoma cruzi*–infected people in countries of endemicity and emerging in regions where it is not endemic as a result of migratory movements [1, 2]. Chagas disease has a variable clinical course, for which the most common manifestation is heart disease (cChHD), which affects ~25% of persons with Chagas disease and represents the leading cause of myocarditis worldwide [1, 3]. Heart transplantation is a valid treatment for end-stage heart failure, although reactivation of Chagas disease may result as the main complication of necessary concomitant immunosuppressive therapy [4, 5]. The pleomorphism of Chagas disease manifestations was attributed to the diversity of *T. cruzi* strains by Carlos Chagas himself [6]. Natural parasite popu-

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lations have a complex multiclonal structure [7], with evidence of genetic exchange among distantly related lineages [8]. The lineages were classified into the 6 major discrete typing units (DTUs) I and IIa–IIe, [9], which were recently renamed *T. cruzi* I–VI [10] and which have a variety of geographical distributions and ecoepidemiological associations [9–19]. *T. cruzi* I is major cause of Chagas disease in northern South America and Central America and prevails in sylvatic cycles throughout the continent [12, 13]. In contrast, in the southern cone, *T. cruzi* II, V, and VI are implicated in Chagas disease [12–19]. Studies of parasite genetic diversity in cChHD are extremely important in understanding its pathogenesis. Most studies that have examined the association of DTUs with manifestations of Chagas disease characterized parasite isolates recovered from patients' blood samples, precluding detection of natural populations with cardiac tropism. In this context, we applied sensitive molecular strategies to characterize *T. cruzi* DTUs at sites of myocarditis in cardiac explant and peripheral blood specimens obtained from a cohort of patients with end-stage cChHD who underwent heart transplantation, as well as from follow-up blood and tissue samples obtained from patients who had reactivation of Chagas disease.

MATERIALS AND METHODS

Patients. Sixteen patients with cChHD (12 men; mean age, 47.07 years; age range, 13–61 years) were admitted consecutively at Hospital Universitario Fundación Favaloro in Buenos Aires, Argentina, during the period 1998–2008 to undergo orthotopic heart transplantation. Diagnosis of *T. cruzi* infection was based on demonstration of anti–*T. cruzi* reactivity in $\geqslant 2$ of 3 standard serological tests (enzyme-linked immunosorbent assay, indirect hemagglutination test, and immunofluorescence test). Determination of cChHD was based on clinical and electrocardiographic findings. Patients 1–11 had end-stage cChHD, whereas patients 12–16 had cChHD and concomitant cardiac diseases of different etiologies—namely, endomyocardial fibrosis, ischemic heart disease, valvular heart disease, noncompaction cardiomyopathy, and familiar cardiomyopathy. All patients were in New York Heart Association classes III and IV. Echocardiographic studies revealed moderate-to-intense dilation of the cavities: the mean left ventricular end-diastolic diameter was 67.0 ± 9.8 mm. The mean left ventricular ejection fraction, determined by radionuclide ventriculography, was 21.0% ± 9.8%. Twelve patients (patients 3, 5–12, and 14–16) underwent heart transplantation categorized as status 1, and the remaining 4 were treated as elective transplantation candidates; the surgical procedure was biatrial in 8 patients (patients 1–4, 6, 7, 15, and 16) and bicaval in the remaining patients.

For comparative analysis of the prevalence of particular DTUs between patients with cChHD and those with indeterminate Chagas disease (idCD), we examined 95 blood and 27 serum samples obtained from 95 Argentinean patients with idCD who had been referred from Rivadavia and Gutierrez hospitals in Buenos Aires and from Universidad Nacional del Nordeste in Chaco.

This study fulfilled all principles expressed in the Declaration of Helsinki. It was approved by the review boards of the participating institutions, and written informed consent was obtained from patients or a person in charge.

Analysis of heart explants. Fifteen explanted hearts were weighed (mean weight, 456.3 ± 102.6 g) and fixed for 72 h in 10% phosphate-buffered formaldehyde. After fixation, transmural sections of the whole circumference of the left and right ventricle at a plane equidistant from the base to the apex were collected and embedded in paraffin. Sections from 4 regions of each heart were selected for histological and molecular analysis: the apex, the left and right ventricular free walls, and the interventricular septum. A 5-μm-thick section from each region was stained with hematoxylin and eosin and Masson's trichrome stain.

The diagnosis of myocarditis was defined according to the Dallas criteria [20]. For statistical analysis, the amount of inflammatory infiltrate was semiquantified as absent (score 0), mild (score 1), moderate (score 2), and severe (score 3) and its distribution as focal, confluent, or diffuse. The percentage of fibrosis was semiquantified as mild (<10%; score, 1), moderate (10%–20%; score, 2), and severe (>20%; score, 3) [21].

Immunosuppressive treatment. Patients received triple-drug immunosuppressive therapy that included calcineurin inhibitors (cyclosporine or tacrolimus), steroids, and third drug, which could be either azathioprine, mycophenolate mofetil, or rapamycin target inhibitors. Two patients (patients 12 and 13) received rabbit anti–thymocyte globulin induction therapy because of renal failure. Immunosuppressive drug dosages were adjusted for blood levels, in response to toxicity, rejection, and Chagas disease reactivation. Cardiac rejection was characterized in endomyocardial biopsy samples in accordance with the International Society for Heart and Lung Transplantation updated grading system [22]. Acute cellular rejection of grade $\geqslant 2$ or rejection in combination with hemodynamic instability was treated with intravenous methylprednisolone (1000 mg per day for 3 days).

Monitoring of patients. Before or at the time of heart transplantation, blood and serum samples were obtained. After heart transplantation, blood samples and endomyocardial biopsy specimens were collected on a protocol basis [4]. Blood samples were tested by the method of Strout [23], kinetoplastid DNA (kDNA) polymerase chain reaction (PCR), and spliced leader (SL) PCR (see "PCR-based monitoring" below); skin and endomyocardial biopsy samples were processed as explant tissues, and reactivation of Chagas disease was diagnosed by observation of *T. cruzi* nests.

Nucleic acid extraction. Ten mL of peripheral blood mixed with an equal volume of GE buffer (6 M of guanidine hydrochloride [Sigma] and 0.2 M of EDTA [pH, 8.0]) was processed for PCR [17]. Paraffin-embedded tissue sections (10 μm), flanking those observed by histological examination, were processed using the QIAmp tissue kit (Qiagen, Valencia, CA) and DNA integrity was checked by β-globin PCR [24].

PCR-based monitoring. The 330-bp minicircle variable region (kDNA) was amplified from 100-μL guanidine-EDTA-blood (GEB) or 10-μm tissue slices, as reported elsewhere [17]. The intergenic spacer of spliced-leader genes (SL-PCR) was amplified from 100 μL of GEB [4]. Detection limits for kDNA-PCR and SL-PCR were ~0.05 and ~10 parasite equivalents/mL of blood, respectively.

Identification of* T. cruzi *DTUs,* T. cruzi *I genotypes, and minicircle signatures. DTUs I to VI [10] were identified using improved PCR strategies (Figure 1 and Table 1), as follows: (1) SL intergenic region (IR) SL PCR was used to distinguish *T. cruzi* I (150 bp), II, V, and VI (157 bp) from *T. cruzi* III and IV (200 bp); (2) heminested SL-IR-I was used to identify *T. cruzi* I DTUs (350 bp), and hemi-nested SL-IR-II was used to identify *T. cruzi* II, V, and VI DTUs (300 bp); (3) hemi-nested PCR of 24S α-ribosomal DNA was used to distinguish *T. cruzi* V (125 or 125 + 140 bp) from *T. cruzi* II and VI (140 bp); and

Table 1. Polymerase Chain Reaction (PCR) Strategies for Identification of *Trypanosoma cruzi* Discrete Typing Units in Human Blood and Tissue Samples

	Primer		PCR Mix			Termocycler			
PCR, target	Name	Sequence	Primer, μmol/L	dNTPs, μmol/L	MgCl, mmol/L	D	A	E	C
SL-IR									
Spliced leader intergenic region	UTCC	CGTACCAATATAGTACAGAAACTG	1.5	250	3	94	70	72	3
	TCac	CTCCCCAGTGTGGCCTGGG				94	68	72	3
						94	66	72	3
						94	64	72	3
						94	62	72	33
SL-IR II									
Spliced-leader intergenic region, first round	UTCC	CGTACCAATATAGTACAGAAACTG	1.5	205	3	94	70	72	3
	TC1	TCCGCCACCTCCTTCGGGCC				94	68	72	3
						94	66	72	3
						94	64	72	3
						94	62	72	33
Heminested second round (SL-IR II HN)	TC	CCCCCCTCCCAGGCCACACTG	4	250	3	94	67	72	5
	TC1	TCCGCCACCTCCTTCGGGCC				94	65	72	5
						94	63	72	5
						94	61	72	28
SL-IR I									
Spliced-leader intergenic region, first round	UTCC	CGTACCAATATAGTACAGAAACTG	1.5	250	3	94	62	72	3
	TC2	CCTGCAGGCACACGTGTGTG				94	60	72	3
						94	58	72	35
Heminested second round (SL-IR I HN)	TC	CCCCCCTCCCAGGCCACACTG	4	250	3	94	67	72	5
	TC2	CCTGCAGGCACACGTGTGTG				94	65	72	5
						94	63	72	5
						94	61	72	28
24Sα-rDNA									
D7 domain first round	D76	GGTTCTCTGTTGCCCCTTTT	4	250	3	94	64	72	2
	D75	GCAGATCTTGGTTGGCGTAG				94	62	72	2
						94	60	72	2
						94	58	72	35
Heminested second round (24SαrDNA HN)	D76	GGTTCTCTGTTGCCCCTTTT	5	250	2	94	60	72	3
	D71	AAGGTGCGTCGACAGTGTGG				94	57	72	3
						94	55	72	35
A10 real-time PCR									
First round	Pr1	CCGCTAAGCAGTTCTGTCCATA	0.5	250	3	94	60	72	35
	P6	GTGATCGCAGGAAACGTG							
Heminested second round (HN)	Pr1	CCGCTAAGCAGTTCTGTCCATA	0.5	250	3	94	60	72	35
	Pr3	TGCTTTATTACCCCATGCCACAG							

NOTE. For primer sequence reference, see Burgos et al [17]. A, annealing; C, number of cycles; D, denaturation; dNTPs, deoxynucleotide triphosphate; E, extension.

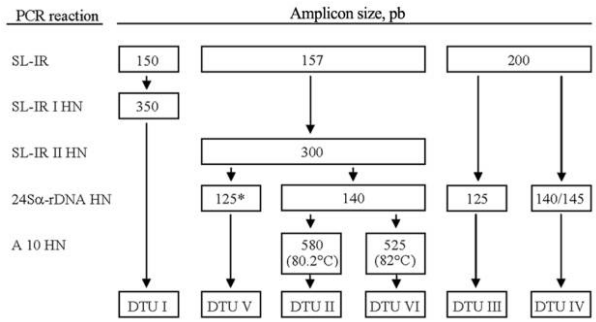

Figure 1. Polymerase chain reaction (PCR) flowchart for identification of *Trypanosoma cruzi* discrete typing units (DTUs) directly from human blood and tissue samples. For PCR specifications, see Table 1. Flow chart indicates amplicon size in pb and melting temperature in °C. A-10 HN, heminested reaction for the A-10 fragment performed by real-time PCR; SL-IR, spliced-leader intergenic region; SL-IR I and II HN, heminested reaction from SL-IR I and II PCRs, respectively; 24Sα rDNA HN, heminested amplification of the D7 domain of the 24Sα ribosomal RNA genes.

(4) heminested real-time PCR targeted to A10 [25] was used to discriminate *T. cruzi* II from *T. cruzi* VI. Samples that yielded positive SL-IR-II PCR results but negative 24S α-ribosomal DNA PCR results were reported as *T. cruzi* II, V, or VI. Those samples that amplified the 140-bp 24Sα rDNA fragment but that had negative results of real-time PCR targeted to A10 were reported as *T. cruzi* II or VI (Figure 1).

The 350-bp hemi-nested SL-IR-I PCR products from *T. cruzi* I were subjected to sequencing and alignment for identification of genotypes *T. cruzi* Ia–Id, as described elsewhere [26]. Purified kDNA-PCR products (1.5 μg) were digested with 5 U of *Msp*I plus *Rsa*I enzymes, and restriction fragment–length polymorphism patterns were visualized after polyacrylamide gel electrophoresis [17].

Western blot with Trypomastigote small surface antigens (TSSAs). Serological reactivities against TSSA I and II recombinant antigens were assayed to identify patients infected with *T. cruzi* I (TSSA I) or *T. cruzi* II, V, or VI (TSSA II) [12, 14]. Glutathione S-transferase (GST)–TSSA- I and GST-TSSA-II, V, or VI recombinant antigens [14] and GST as controls were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; with 3 μg of each antigen) and transferred to polyvinlidene fluoride (PVDF) membranes (Amersham, UK). After blocking the filters with phosphate-buffered saline (PBS) with 5% nonfat dried milk, 1:100 dilutions of serum samples in PBS with 1% nonfat dried milk were incubated for 3 h at room temperature. Bound antibodies were detected with a 1:3000 dilution of a secondary rabbit anti-human immunoglobulin G antibody coupled to horse radish peroxidase (HRPO; Dako) and the subsequent addition of 3, 3′-diaminobenzidine (Sigma) and H_2O_2 both diluted in PBS.

Statistical analysis. Categorical variables were compared using the χ^2 test or the Fisher exact test, and continuous variables were compared using the unpaired *t* test or Mann-Whitney *U* test. *P* values <.05 were considered to denote statistical significance. Pathological and clinical data were analyzed using the SPSS software, version 11.0 (SPSS).

RESULTS

This study applied a PCR-based algorithm targeting 3 polymorphic genomic markers to identify the 6 *T. cruzi* DTUs directly from kDNA-PCR–positive clinical samples (cardiac explant specimens, blood samples, and specimens from reactivation sites) from the patient cohort (Table 1 and Figure 1).

Studies of cardiac explant specimens. Table 2 shows epidemiological and pathological features of the 16 Argentinean patients with cChHD who underwent heart transplantation. Amplification of parasite DNA and DTU identification occurred more frequently in cardiac explant specimens with higher degrees of myocarditis ($P = .003$) (Table 2 and Figure 2*A*). Amastigote nests were observed only in 2 explant samples, in sections with diffuse, severe myocarditis (Table 2 and Figure 3). Of the 15 studied heart explant samples, DTUs were identified in 8 samples (54%). Of these 8 samples, infection with DTUs II, V, or VI was observed in 5 (62.5%), whereas DTU I parasites were observed in 3 (37.5%). All DTU I populations were characterized as genotype Tc Id (Genbank accession numbers GQ398805–10, FJ713358, and FJ713366).

Studies of blood samples. Before or at time of heart transplantation, kDNA-PCR results were positive in blood samples obtained from 5 (38.46%) of 13 patients (patients 5–9) (Table 3). In contrast, SL-PCR results were negative, probably as a result of low parasitic loads. Thus, DTU identification was achieved by means of the TSSA immunological marker. Patients 1 and 3 demonstrated anti-TSSA I reactivity, patient 6 had anti-TSSA I and II reactivity, and the remaining patients had anti-TSSA II reactivity (Table 3). Comparison of TSSA-based DTU identification in a group of 27 patients with indeterminate Chagas disease revealed a higher frequency of anti-TSSA I reactivity in patients with cChHD ($P = .034$) (Figure 2*B*).

The 5 patients with kDNA-PCR–positive blood samples findings before or at the time of heart transplantation had persistently positive kDNA-PCR results during post–heart transplantation follow-up. Besides, in 7 of 8 patients whose samples were kDNA-PCR negative, positive PCR results occurred 7–21 days after heart transplantation, indicating an increase in the parasite load. The single patient with a negative kDNA-PCR result was patient 15, who was treated with benznidazole at the age of 6 years (Table 3).

Spliced leader-PCR–based monitoring revealed elevations in the parasite load 7–70 days after heart transplantation in 8 patients, 6 of whom experienced reactivation of Chagas disease 38–98 days after heart transplantation (patients 1–6; mean in-

Table 2. Epidemiological Features and Histological and Molecular Analysis of Cardiac Explant Specimens Obtained from Patients with Chronic Chagas Disease

	Heart explant analysis							
Patient, heart explant section	Myocarditis	Fibrosis	Nest	Proportion of positive kDNA-PCR results	*Trypanosoma cruzi* DTU	Patient sex/age, years	Procedence	Route
1								
Apex	Moderate, diffuse	Moderate	No	1/2	–	F/48	Sgo Estero (27°S, 64°W)	Vectorial
Right ventricle	Severe, diffuse	Severe	No	0/2	…	…	…	…
Interventricular septum	Severe, diffuse	Severe	No	1/2	–	…	…	…
Left ventricle	Severe, diffuse	Severe	No	0/2	…	…	…	…
2								
Apex	Mild, focal	Severe	No	0/2	…	F/59	Buenos Aires (34°S, 57°W)	Transfusional
Right ventricle	Moderate, diffuse	Severe	No	0/2	…	…	…	…
Interventricular septum	Mild, focal	Severe	No	1/2	II/VI	…	…	…
Left ventricle	Severe, diffuse	Severe	No	1/2	II/V/VI	…	…	…
3								
Apex	Severe, diffuse	Severe	No	0/2	…	M/46	Chaco (27°S, 58°W)	Vectorial
Right ventricle	Moderate, diffuse	Moderate	No	1/2	I	…	…	…
Interventricular septum	Severe, diffuse	Severe	No	1/2	Id	…	…	…
Left ventricle	Moderate, diffuse	Moderate	No	0/2	…	…	…	…
4								
Apex	No	Mild	No	0/2	…	F/61	La Pampa (37°S, 64°W)	Vectorial
Right ventricle	No	Moderate	No	0/2	…	…	…	…
Interventricular septum	Mild, focal	Severe	No	2/2	II/V/VI	…	…	…
Left ventricle	No	Moderate	No	0/2	…	…	…	…
5								
Apex	Severe, diffuse	Severe	No	1/2	–	M/42	La Pampa (36°S, 64°W)	Vectorial
Right ventricle	Severe, diffuse	Mild	No	2/2	II/V/VI	…	…	…
Interventricular septum	Severe, diffuse	Severe	Yes	2/2	II/VI	…	…	…
Left ventricle	Severe, diffuse	Severe	No	2/2	II/VI	…	…	…
6								
Apex	Severe, diffuse	Moderate	Yes	1/1	Id	M/45	Jujuy (23°S, 64°W)	Vectorial
Right ventricle	Mild, diffuse	Moderate	No	0/1	…	…	…	…
Interventricular septum	Severe, diffuse	Moderate	No	1/1	–	…	…	…
Left ventricle	Severe, diffuse	Moderate	No	0/1	…	…	…	…
7								
Apex	Mild, diffuse	Moderate	No	0/2	…	M/59	La Pampa (36°S, 64°W)	Vectorial
Right ventricle	Moderate, diffuse	Moderate	No	1/2	–	…	…	…
Interventricular septum	Moderate, diffuse	Moderate	No	1/2	II/V/VI	…	…	…
Left ventricle	Severe, diffuse	Severe	No	1/2	II/VI	…	…	…
8								
Apex	Moderate, diffuse	Severe	No	1/1	–	M/45	Chaco (27°S, 57°W)	Vectorial
Right ventricle	Moderate, diffuse	Severe	No	0/1	…	…	…	…
Interventricular septum	Severe, diffuse	Severe	No	1/1	–	…	…	…
Left ventricle	Moderate, diffuse	Moderate	No	0/1	…	…	…	…
9								
Apex	Mild, diffuse	Severe	No	1/2	–	M/54	Sta Fe (32°S, 60°W)	Vectorial
Right ventricle	Mild, diffuse	Mild	No	0/2	…	…	…	…
Interventricular septum	Moderate, diffuse	Moderate	No	0/2	…	…	…	…

Table 2. ***(Continued.)***

Patient, heart explant section	Heart explant analysis					Patient sex/age, years	Procedence	Route
	Myocarditis	Fibrosis	Nest	Proportion of positive kDNA-PCR results	*Trypanosoma cruzi* DTU			
Left ventricle	Severe, diffuse	Severe	No	1/2	−	…	…	…
10								
Apex	NA	NA	NA	NA	NA	M/39	Salta (25°S, 65°W)	Vectorial
Right ventricle	NA	NA	NA	NA	NA	…	…	…
Interventricular septum	NA	NA	NA	NA	NA	…	…	…
Left ventricle	NA	NA	NA	NA	NA	…	…	…
11								
Apex	Mild, diffuse	Severe	No	0/1	…	M/43	Chaco (27°S, 61°W)	Vectorial
Right ventricle	Moderate, diffuse	Moderate	No	0/1	…	…	…	…
Interventricular septum	Moderate, diffuse	Moderate	No	1/1	I	…	…	…
Left ventricle	Severe, diffuse	Severe	No	1/1	Id	…	…	…
12								
Apex	No	Moderate	No	1/1	II/V/VI	M/39	Chaco (27°S, 58°W)	Vectorial
Right ventricle	No	Moderate	No	1/1	II/V/VI	…	…	…
Interventricular septum	No	Moderate	No	0/2	…	…	…	…
Left ventricle	No	Moderate	No	0/1	…	…	…	…
13								
Apex	No	Moderate	No	0/2	…	M/56	Entre Rios (31°S, 58°W)	Vectorial
Right ventricle	No	Mild	No	1/2	−	…	…	…
Interventricular septum	No	Severe	No	0/2	…	…	…	…
Left ventricle	No	Severe	No	0/1	…	…	…	…
14								
Apex	Mild, focal	Mild	No	0/2	…	F/51	San Juan (31°S, 68°W)	Vectorial
Right ventricle	Mild, focal	Mild	No	0/2	…	…	…	…
Interventricular septum	No	Mild	No	0/2	…	…	…	…
Left ventricle	No	Mild	No	0/2	…	…	…	…
15								
Apex	Mild, diffuse	Severe	No	0/2	…	M/13	Córdoba (32°S, 62°W)	Congenital
Right ventricle	Moderate, focal	Severe	No	0/1	…	…	…	…
Interventricular septum	Mild, focal	Severe	No	0/1	…	…	…	…
Left ventricle	Mild, focal	Severe	No	0/1	…	…	…	…
16								
Apex	No	Moderate	No	0/2	…	M/45	Sgo Estero (27°S, 64°W)	Vectorial
Right ventricle	No	Mild	No	0/2	…	…	…	…
Interventricular septum	No	Moderate	No	0/2	…	…	…	…
Left ventricle	No	Moderate	No	1/2	−	…	…	…

NOTE. DTU, discrete typing unit; NA, sample not available; PCR, polymerase chain reaction; −, negative.

terval, 72 days) (Table 3). In patients who had reactivation of Chagas disease, positive SL-PCR results occurred subsequent to positive kDNA-PCR results in consecutive blood samples, 28–78 days earlier than reactivation of Chagas disease. Accordingly, sustained positive SL-PCR results served as an early marker of reactivation of Chagas disease ($P = .007$) (Table 3).

After heart transplantation, *T. cruzi* I was identified in blood samples obtained from 5 (31%) of 16 patients (patients 1–3,

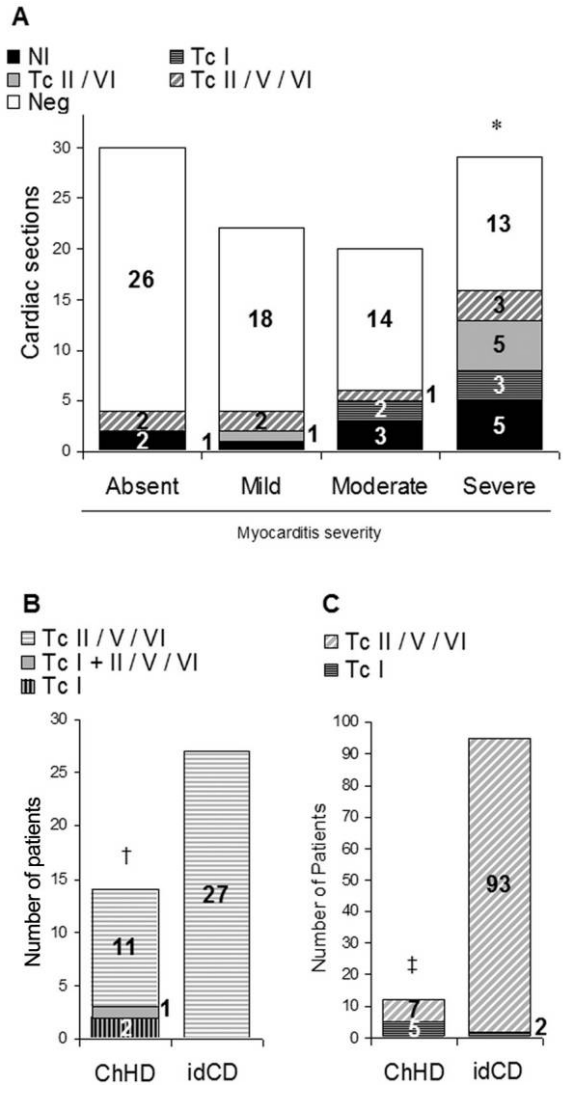

Figure 2. *Trypanosoma cruzi* discrete typing unit (DTU) findings in cardiac samples obtained from patients with Chronic Chagas heart disease (cChHD) and comparison of blood DTU prevalence in patients with cChHD versus those with indeterminate Chagas disease (idCD). *A,* DNA persistence and DTU identification in cardiac explant specimens with different degree of myocarditis. *White areas,* kDNA polymerase chain reaction (PCR)–negative sections (Neg); *black areas,* cardiac sections with kDNA-PCR–positive findings where DTU could not be identified (NI); *shadowed areas,* kDNA-PCR–positive sections where *T. cruzi* DTU was characterized. DNA persistence was higher in cardiac explant specimens with severe myocarditis (*$P = .003$). *B,* Anti–Trypomastigote small surface antigen reactivity of cChHD and idCD serum samples (†$P = .034$). *C,* Blood DTUs in patients with cChHD versus idCD after heart transplantation identified by means of the PCR algorithm in Figure 1 (‡$P < .001$).

8, and 10) (Table 3). The proportion of *T. cruzi* I was higher than that in 95 patients with idCD characterized by the PCR algorithm ($P < .001$) (Figure 2*C*). Moreover, genotype Tc Ia was identified in blood samples obtained from patients 2, 3, and 8 (Genbank accession numbers FJ713371, FJ713382, and FJ713400), and Tc Id was identified in blood samples from patient 10 (FJ713404). *T. cruzi* V was identified in blood samples obtained after heart transplantation from 5 (31%) of 16 patients (patients 4, 6, 9, 12, and 13), *T. cruzi* VI was identified in blood samples obtained from 1 patient (patient 5 before 2 Chagas disease reactivation–related events, which occurred 38 and 395 days after heart transplantation), and *T. cruzi* II/VI was identified in blood samples from 1 patient (patient 7) (Table 3). Analysis of consecutive samples from all patients who underwent heart transplantation and who had sustained positive SL-PCR results revealed the persistence of the same bloodstream DTU, even in patients with other DTUs at different body sites (patients 1–6, 8, and 13).

Studies at sites of reactivation of Chagas disease. Five patients who experienced reactivation of Chagas disease (patients 1–3, 5, and 6) developed skin lesions on the lower limbs, and 1 (patient 4) developed chagasic myocarditis (Table 3). Patients 1–5 were also Strout positive. All 6 were treated with benznidazole, with Strout results becoming negative and with remission of symptoms. However, patient 5 underwent a second episode of reactivation of Chagas disease, which was diagnosed as myocarditis, 395 days after heart transplantation. A second treatment with benznidazole was successful, with negative Strout and PCR results after 3 years of follow-up.

Reactivation of Chagas disease was associated with higher degrees of myocarditis in the explanted heart samples ($P = .036$) and with surgical technique; 5 of 6 patients who had reactivation of Chagas disease underwent biatrial heart transplantation, whereas 7 of 9 patients who did not have reactivation of Chagas disease, who were observed for at least 98 days, underwent bicaval heart transplantation ($P = .041$) (Table 3). Reactivation of Chagas disease was not found to be associated with age, sex, rejection episodes, immunosuppressive treatment regimens, or "pulsed" corticosteroid therapy (Table 3).

T. cruzi I was detected in skin chagoma samples for patients 1 and 3 (genotype Ia; Genbank accession number GU179064), *T. cruzi* V was detected in patients 4 (in endomyocardial biopsy samples) and 6 (skin samples), *T. cruzi* VI was detected in patient 5 (in skin and endomyocardial biopsy samples for the first and second episodes of Chagas disease reactivation, respectively), and *T. cruzi* II, V, or VI was detected in patient 2 (skin samples) (Table 3).

Overall, *T. cruzi* I infections were identified in 7 patients with cChHD (43.75%); in 4 of them (patients 1, 3, 8, and 11), *T. cruzi* I infection was the single infection, and in 3, it overlapped with infection with *T. cruzi* V (patient 6) or II, V, or VI (patients 2 and 10). *T. cruzi* V was identified in 4 patients (patients 4, 9, 12, and 13), *T. cruzi* VI was identified in 1 (patient 5), *T. cruzi* II/VI was identified in 1 (patient 7), and anti-TSSA II reactivity was detected in the remaining 3 PCR-negative pa-

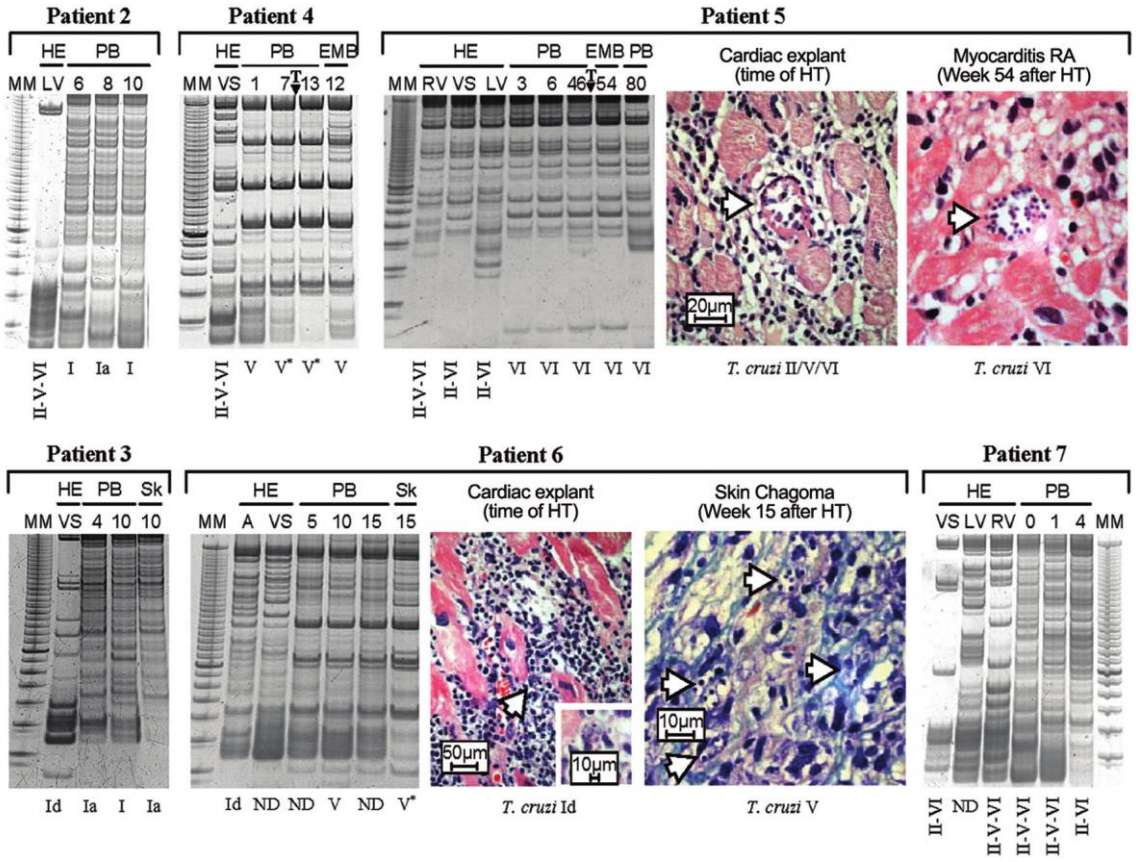

Figure 3. Ten percent polyacrylamide gels stained with Sybr green showing *Trypanosoma cruzi* minicircle signatures at different body sites during chronic Chagas disease (cChHD) follow-up. Sample type and post–heart transplantation (HT) period in weeks are indicated on top, and *T. cruzi* discrete typing unit (DTU) or genotype on the bottom of each line. Histologic images of amastigote nests in cardiac explants and reactivation of Chagas disease lesions of patients 5 (week 54) and 6 (week 15). Inset illustrates free amastigotes from broken nest. A, apex; EMB, endomyocardial biopsy; HE, heart explant; LV, left ventricle; MM, 10-bp DNA ladder; ND, not determined; PB, peripheral blood; RV, right ventricle; Sk, skin chagoma; VS, interventricular septum.

tients (patients 14–16) (Table 3). In the patients infected with multiple *T. cruzi* I plus V or II, V, or VI populations, different tissue distribution of each DTU was found, as follows: (1) in patient 2, anti-TSSA-II reactivity was noted, *T. cruzi* II/VI was noted in heart explant samples, *T. cruzi* Ia was detected in post–heart transplantation blood samples, and *T. cruzi* II, V, or VI was detected in skin chagoma samples; (2) in patient 6, anti-TSSA I and II reactivities occurred, *T. cruzi* Id was detected in heart explant samples, and *T. cruzi* V was detected in post–heart transplantation blood and skin chagoma samples; and (3) in patient 10, anti-TSSA II reactivity occurred, and *T. cruzi* Id was detected in post–heart transplantation blood samples.

Parasite minicircle signatures. Fingerprinting of minicircle signatures (Figure 3) revealed polymorphic populations of a same DTU infecting different patients (patients 4 and 6; *T. cruzi* V in peripheral blood samples) showing intra-DTU diversity. *T. cruzi* I populations depicted a larger number of restriction fragments than did *T. cruzi* V or VI populations, suggesting higher heterogeneity of minicircle classes and/or degrees of clonality (patients 2 and 3 versus patients 4–6) (Figure 3). Moreover, kDNA polymorphism of different DTUs and *T. cruzi* I genotypes detected at different body sites from a same patient (patients 2, 3, and 6; heart explant versus peripheral blood samples) or even in different sections from a same explant (patient 7; interventricular septum and left and right ventricles) denoted the varied histotropism of DTU coinfections. In contrast, in a patient infected with *T. cruzi* VI (patient 5), almost identical signatures were profiled at different body sites (interventricular septum, peripheral blood sample, and endomyocardial biopsy samples).

DISCUSSION

Previous studies of serum samples [14], culture isolates recovered from peripheral blood samples [15, 18], and clinical samples [17, 19] at the southern cone of America revealed prevailing

Table 3. Identification of *Trypanosoma cruzi* Discrete Typing Units (DTUs) in Different Clinical Samples during Follow-Up of Patients with Chronic Chagas Heart Disease Who Underwent Heart Transplantation

	Before transplantation				Posttransplantation follow-up								
					Blood analysis			Reactivation					
Patient	Serum TSSA	Blood[a] kDNA-PCR result	Heart transplantation technique	Heart explant *T. cruzi* DTU	Interval between heart transplantation and positive result, days		*T. cruzi* DTU	Site (no. of days after transplantation)	*T. cruzi* DTU	No. of episodes of acute rejection	No. of corticosteroid pulses	Follow-up period, years	Outcome
					kDNA-PCR	SL-PCR							
1	I	–	Biatrial	NI	21	42	I	Skin (75)	I	5	4	0.25	Death (acute rejection)
2	II	–	Biatrial	II/VI	21	44	Ia	Skin (75)	II/VI	8	8	7.04	Alive
3	I	–	Biatrial	Id	14	33	Ia	Skin (78)	Ia	0	0	6.24	Alive
4	II	–	Biatrial	II/V/VI	7	14	V	Heart (92)	V	1	1	5.01	Alive
5	II	+	Bicaval	VI[b]	…	7	VI	Skin (38); heart (395)	VI; VI	4	5	4.02	Alive
6	I + II	+	Biatrial	Id	…	70	V	Skin (98)	V	0	0	2.14	Alive
7	II	+	Biatrial	II/V/VI	…	–	II/VI[c]	No	No	0	0	0.1	Death (sepsis)
8	NA	+	Bicaval	NI	…	7	Ia	No	No	0	0	1.36	Alive
9	II	+	Bicaval	NI	…	–	V[d]	No	No	1	1	3.18	Alive
10	II	NA	Bicaval	NA	21	50[e]	Id	No	No	0	0	4.21	Alive
11	NA	–	Bicaval	Id	21	–	NI	No	No	4	4	0.71	Death (sudden death)
12	II	–	Bicaval	II/V/VI	21	–	V[d]	No	No	1	1	3.3	Alive
13	II	–	Bicaval	NI	7	42	V	No	No	1	1	3.13	Alive
14	II	NA	Bicaval	-	14	–	NI	No	No	1	1	5.57	Alive
15	II	–	Biatrial	-	–	–	NI	No	No	3	3	4.51	Death (sepsis)
16	II	–	Biatrial	NI	ND	ND	NI	No	No	0	0	7.09	Death (sepsis)

NOTE. IR, intergenic region; NA, not available; ND, not done; NI, not identified; PCR, polymerase chain reaction; SL, spliced leader; TSSA, trypomastigote small surface antigen (TSSA II does not discriminate antigens from DTUs II, V or VI); +, positive; –, negative.

[a] PCR-based DTUs in pre–heart transplantation blood samples were not determined because SL-PCR results were negative in all tested samples.

[b] DTU VI was identified by kDNA signatures.

[c] Three hundred–bp heminested SL-IR-PCR and 140-bp HN 24Sα-rDNA PCR.

[d] Three hundred–bp heminested SL-IR-PCR and 125-bp heminested 24sα-rDNA PCR.

[d] A single SL-PCR–positive blood sample.

T. cruzi II, V, and VI human infections, reinforcing the concept that *T. cruzi* I does not cause Chagas disease in the region. This longitudinal study integrated findings from cardiac tissues, blood samples, and Chagas disease reactivation–associated lesions of a cohort of cChHD patients undergoing heart transplantation and revealed a more complex scenario: 7 patients were infected with *T. cruzi* I, 4 harbored single *T. cruzi* I infections, and 3 were coinfected with other DTUs, which were detectable at different body sites and periods of follow-up. Such a significant proportion of *T. cruzi* I cardiac populations among Argentinean patients with cChHD was astonishing. Sequencing of SL-IR amplicons allowed confirmation of DTU I findings, further revealing genotypes Tc Ia and Tc Id [26]. To our knowledge, this is the first report of Tc I genotypes identified in clinical samples obtained from patients with Chagas disease. Tc Id was identified in all 3 genotyped cardiac explant samples, even in samples from a patient with Tc Ia in blood and skin chagoma specimens, suggesting genotype histotropism.

Before or at the time of heart transplantation, blood samples from most *T. cruzi* I–infected patients had negative kDNA-PCR and SL-PCR results, indicative of lower parasite loads than II, V, or VI populations (Table 3). This may explain the low prevalence of *T. cruzi* I in blood samples obtained from Argentinean patients with idCD and the fact that it was mostly detectable in cardiac tissue or blood samples in persons receiving immunosuppression therapy, in agreement with recent findings in one case with cerebral Chagas disease due to AIDS [27].

T. cruzi I was identified in patients with cChHD from the northern provinces of Chaco and Santiago del Estero (latitude, 27°S toward the north; longitude, 57°W–64°W), mixed *T. cruzi* I and II, V, or VI was identified in the northern provinces of Salta and Jujuy (latitude, 23°S–25°S; longitude, 63°W–65°W), *T. cruzi* II/VI or VI in La Pampa (latitude, 36°S; longitude, 64°W), and *T. cruzi* V was more ubiquitous (latitude, 27°S–37°S; longitude, 58°W–64°W) (Table 1).

Our analysis of cardiac explant samples provides additional evidence of parasite persistence involvement in myocarditis and cChHD pathogenesis, confirming previous immunochemical and molecular studies [28–30].

Reactivation was associated with myocarditis severity and parasite persistence in explanted heart samples. In fact, cardiac explant samples with detectable nests (*T. cruzi* Id and *T. cruzi* VI) and with higher proportion of PCR-positive sections belonged to patients who experienced reactivation of Chagas disease. In addition, reactivation of Chagas disease appeared linked to the heart transplantation technique, being more common among patients subjected to biatrial transplantation than among those undergoing bicaval heart transplantation [31]. The biatrial technique determines that portions of the atrium of the receptor are not explanted. Palomino et al [32] observed high intensities of inflammation in the posterior wall of the right atrium in 12 hearts mapped in detail. A similar picture could be occurring in the atrium of our patients who underwent heart transplantation, favoring parasite proliferation and invasion of the implanted heart causing myocarditis.

In the present study, skin lesions were observed in 5 of 16 patients and myocarditis was observed in 2 of 16 patients—proportions similar to those in other cohorts [5, 33]. Reactivation was associated with earlier and sustained positive blood SL-PCR results accompanying increases in the parasite load, as demonstrated by quantitative real-time PCR in samples from patients 3–5 [34], showing the potential of quantitative real-time PCR to assist physicians in treatment decisions before onset of reactivation of Chagas disease. Reactivation can be treated with benznidazole [5, 35], allopurinol [36] or, very recently, with posaconazole [37]. In our cohort, patients who had reactivation of Chagas disease and who were infected with any *T. cruzi* DTU responded successfully to current benznidazole treatment. Only patient 5 experienced a second Chagas disease reactivation episode; this patient was infected with a monoclonal *T. cruzi* VI population, as demonstrated by studies of microsatellite loci polymorphism [38], indicating that the population that reemerged at the second reactivation of Chagas disease was not eradicated after the first round of treatment. Increasing data support the view that *T. cruzi* I and II are ancient parental lineages [39], whereas *T. cruzi* V and VI are polyphyletic products of $\geqslant$1 hybridization event [8, 39–41]. Our findings support the concept that cChHD can be caused by multiple parasite clones belonging to both parental and hybrid DTUs, conforming a natural scenario for interspecific recombination events and DTU evolution.

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