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Direct molecular profiling of minicircle signatures and lineages of *Trypanosoma cruzi* bloodstream populations causing congenital Chagas disease

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Abstract

Congenital transmission of *Trypanosoma cruzi* may occur in some or all the gestations from a *T. cruzi*-infected mother. Variable rates of congenital transmission have been reported in different geographical areas where different parasitic strains predominate, suggesting that parasitic genotypes might play a role in the risk of congenital transmission. Moreover, in cases of transmission it is unknown if the whole maternal *T. cruzi* population or certain clones are preferentially transmitted by the transplacental route. In this study, bloodstream *T. cruzi* lineages were identified in blood samples from congenitally infected children, transmitting and non-transmitting mothers and unrelated Chagas disease patients, using improved PCR strategies targeted to nuclear genomic markers. *T. cruzi* IId was the prevalent genotype among 36/38 PCR-positive congenitally infected infants, 5/5 mothers who transmitted congenital Chagas disease, 12/13 mothers who delivered non-infected children and 28/34 unrelated Chagas disease patients, all coming from endemic localities of Argentina and Bolivia. These figures indicate no association between a particular genotype and vertical transmission. Furthermore, minicircle signatures from the maternal and infants' bloodstream trypanosomes were profiled by restriction fragment length polymorphism of the 330-bp PCR-amplified variable regions in seven cases of mothers and congenitally infected infants. Minicircle signatures were nearly identical between each mother and her infant/s and unique to each mother-infant/s case, a feature that was also observed in twin deliveries. Moreover, allelic size polymorphism analysis of microsatellite loci from populations transmitted to twins showed that all clones from the maternal polyclonal population were equally infective to both siblings.

Keywords: Transplacental transmission; Phylogenetic lineage; Real time PCR; *Trypanosoma cruzi* clonality; Microsatellite loci

1. Introduction

Due to the increasing control of the transmission of *Trypanosoma cruzi* mediated by vector, blood transfusion and organ transplant, congenital Chagas disease (CCD) has

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emerged as a public health problem (World Health Organization, 2002). Due to migration movements from endemic areas to vector-free suburban and urban centers, CCD is becoming increasingly responsible for the urbanization of Chagas disease. Congenital Chagas disease can be suspected in any child born to a *T. cruzi*-infected pregnant woman at any stage of infection (Freilij and Altcheh, 1995; World Health Organization, 2002; Schijman, 2006). Infected mothers may transmit the parasite in one, some or all their gestations, and may also infect some or all of the siblings in multiple deliveries (Freilij and Altcheh, 1995; Burgos et al,. unpublished data). The transmission rates of CCD vary in different geographical areas, ranging from 0.1% in regions of Brazil and Argentina to 7% or more in some areas of Bolivia, Chile and Paraguay (World Health Organization, 2002; reviewed in Schijman, 2006).

Little is known about the mechanisms of transplacental transmission. Congenital Chagas disease may result from a complex equilibrium between maternal immune response, placental factors and features of the parasitic strains. *T. cruzi* is classified as a single species although there is great genetic and phenotypic diversity among isolates. Natural infections are constituted by multiple clones with different biological properties such as virulence and tissue tropism (Macedo and Pena, 1998). Based on biochemical and molecular markers, *T. cruzi* has been classified into six discrete genetic subdivisions or lineages, designated as *T. cruzi* I, *T. cruzi* IIa, *T. cruzi* IIb, *T. cruzi* IIc, *T. cruzi* IId and *T. cruzi* IIe (Brisse et al., 2000). The existence of a preferential association of certain parasite lineages with vertical transmission can be hypothesised. Moreover, in cases of transmission a subset of the maternal parasite population could be more successful in reaching the growing fetuses and establishing the infection. In this context, we aimed to characterise the bloodstream *T. cruzi* lineages and populations that were transmitted from infected mothers to their CCD children using PCR-based strategies that were improved for direct analysis of peripheral blood samples.

2. Materials and methods

2.1. Patients

All *T. cruzi*-infected patients included in this study were PCR-positive for the 330-bp variable regions of the minicircle genome (Degrave et al., 1988), which was carried out as detailed in Section 2.3.

2.1.1. Congenital Chagas disease-related group

Forty-seven children with congenital Chagas disease (CCD) (25/22 males/females, mean age: 2.3 years; range 1 day to 12 years) and seven mothers (mean age 29.8 years, range 18–40 years) were studied. All CCD children were born in Buenos Aires city, which is non-endemic for Chagas disease. Their mothers acquired *T. cruzi* in endemic localities of Argentina (provinces of Santiago del Estero, Chaco, Córdoba and Santa Fé) and Bolivia (Sucre and Potosí). They were at the indeterminate chronic phase of Chagas disease.

The patients were admitted to the study between the years 2000 and 2006, when the children were referred for diagnosis of *T. cruzi* infection and treatment to hospital Ricardo Gutierrez, a tertiary care pediatric referral center in Buenos Aires city. Peripheral blood samples for molecular analysis of *T. cruzi* populations were withdrawn when the patients received their diagnosis of CCD.

2.1.2. Patients unrelated to congenital Chagas disease patients

Thirty-two *T. cruzi*-infected pregnant women who did not transmit CCD (mean age 29.1 years, range 18–42 years) were analysed at the time of delivery. They were attended at the service of Obstetrics of Hospital Rivadavia in Buenos Aires, between 2002 and 2005. All them were at the indeterminate phase of Chagas disease and acquired *T. cruzi* in endemic regions of Argentina and Bolivia. We also tested samples that amplified the variable region of the kinetoplastid minicircle DNA (vkDNA-PCR positives) from 44 Chagas disease patients unrelated to the CCD patients, coming from localities of Argentina and Bolivia for follow-up of *T. cruzi* infection between 2000 and 2006.

The study was approved by the Ethical Committees of the participating Institutions with written informed consent.

2.2. Diagnosis criteria

Infants younger than 7 months old were diagnosed by means of microscopic examination of bloodstream trypomastigotes using the microhematocrite test (Freilij and Altcheh, 1995). Diagnosis of *T. cruzi* infection in mothers and in children older than 7 months of age was assessed by means of two positive serological assays, an indirect haemagglutination (Lab Polychaco, Buenos Aires, Argentina) and an ELISA (Wiener, Rosario, Argentina) (Schijman et al., 2003).

The vertical route of transmission of *T. cruzi* was assumed if the infected child: (i) was born to an infected mother, (ii) had never received a blood transfusion and (iii) had never lived in an endemic area.

Infected children were treated with benznidazole (Radanil, Roche, Buenos Aires, Argentina) at 5–8 mg/kg/day in two daily doses for 60 days, without adverse events.

2.3. Blood-based PCR detection of *T. cruzi* minicircle DNA

Two millilitres of peripheral blood from paediatric patients and 10 mL from adult patients were collected and immediately mixed with 1 vol. of 2× lysis buffer containing 6 M guanidine hydrochloride (Sigma, St. Louis, USA) and 200 mM EDTA, pH 8.0 (GE). The resulting GE-blood lysate (GEB) was boiled, allowed to stand at room temperature overnight and stored at 4 °C. Total DNA was purified from 100 or 500 µl aliquots of GEB

Table 1
PCR strategies and conditions for identification of *Trypanosoma cruzi* lineages in human blood samples

PCR	Target	Primer				PCR mix			Termocycler				PCR amplicon size (bp)	*T. cruzi* lineage	Sensitivity
		Name	Sequence	Ref.	Position	Primers	dNTPs	MgCl	D	A	E	C			DNA amount
SL-IRac	Spliced leader	UTCC	CGTACCAATATAGTACAGAAACTG	This article	546–570				94	70	72	3	150	Tc I	1 pg
									94	68	72	3	200	Tc II a-c	1 pg
						1.5	250	3	94	66	72	3			
	Intergenic region	TCac	CTCCCCAGTGTGGCCTGGG	This article	368–386				94	64	72	3	157	Tc II b-d-e	1 pg
									94	62	72	33			
SL-IR II	Spliced leader	UTCC	CGTACCAATATAGTACAGAAACTG	This article	546–570				94	70	72	3			
									94	68	70	3			
						1.5	250	3	94	66	72	3	425	Tc II b-d-e	5 pg
	Intergenic region	TC1	TCCGCCACCTCCTTCGGGCC	[a]	See[a]				94	64	72	3			
									94	62	72	33			
SL-IR I	Spliced leader	UTCC	CGTACCAATATAGTACAGAAACTG	This article	546–570				94	62	72	3			
						1.5	250	3	94	60	72	3	475	Tc I	5 pg
	Intergenic region	TC2	CCTGCAGGCACACGTGTGTG	[a]	See[a]				94	58	72	35			
24Sα rDNA	D7 domain 1st round	D76	GGTTCTCTGTTGCCCCTTTT	[a]	See[a]	4	250	3	94	64	72	2	275	Tc I	NA
									94	62	72	2			
		D75	GCAGATCTTGGTTGGCGTAG	[a]	See[a]				94	60	72	2	275-290	Tc II	
									94	58	72	35			
	Heminested 2nd round	D76	GGTTCTCTGTTGCCCCTTTT	[a]	See[a]	5	250	2	94	60	72	3	125	Tc I	1 pg
									94	57	72	3	140	Tc II b	100 fg
													125-140	Tc II d	100 fg
		D71	AAGGTGCGTCGACAGTGTGG	[a]	See[a]				94	55	72	35	140	Tc II e	100 fg
A10	1st round	pr1	CCGCTAAGCAGTTCTGTCCATA	This article	35–47	0.5	250	3	94	60	72	35	690	Tc II b	NA
		p6	GTGATCGCAGGAAACGTG	[b]	See[b]								630	Tc I Tc II a-c-d-e	
	Heminested 2nd round	pr1	CCGCTAAGCAGTTCTGTCCATA	This article	35–47	0.5	250	3	94	60	72	35	580 tm = 80.2 ± 0	Tc II b	10 pg
		pr3	TGCTTTATTACCCCATGCCACAG	This article	525–548								525 tm = 82 ± 0.2	Tc I Tc II a-c-d-e	1 pg

Primers- positions of SL-IRac, SL-IR I and SL-IR II reactions are based on AF050523 sequence (GenBank); primers-positions of A10 reactions are based on AJ133198 sequence (GenBank); NA, not applicable; D, denaturation step; A, annealing step; E, extension step; C, number of cycles.

[a] Souto et al. (1996).

[b] Brisse et al. (2000).

from paediatric and adults patients, respectively, as previously reported (Schijman et al., 2003). A hot-start PCR procedure, targeted to the 330-bp variable regions of the *T. cruzi* kinetoplastid minicircle genome (vkDNA), was carried out as follows: 5 µl of extracted DNA were added to a 25 µl of reaction mix. Final concentrations were: buffer 1×, 3 mM $MgCl_2$, 250 µM of each deoxyribonucleotide triphosphate (dNTP), 2.5 µM of each primer 121 (5′-AAA TAATGTACGGG(T/G)GAGATGCATGA-3′) and 122 (5′-GGTTCGATTGGGGTTGGTGTAATATA-3′) and 0.6 U of platinum Taq DNA polymerase (Invitrogen, USA). Amplification was carried out in a MJR PTC-100 thermocycler (MJ Research, Watertown, MA, USA) as follows: one step of 3 min denaturation at 94 °C; five cycles at 68 °C for 45 s, 72 °C for 45 s, 94 °C for 45 s; 35 cycles at 64 °C for 45 s, 72 °C for 45 s, 94 °C for 45 s; and a final extension step at 72 °C for 10 min. PCR products were analysed by agarose gel electrophoresis followed by ethidium bromide staining and UV visualization.

2.4. Blood-based PCR identification of *T. cruzi* lineages

Parasite lineages were identified from vkDNA-PCR-positive blood samples using a combination of PCR strategies targeted to nuclear genomic markers, which were modified to improve their performance for direct identification of lineages in human blood, as illustrated in Table 1 and Fig. 1.

Amplification of the intergenic region of spliced leader genes (SL-IR): three independent hot-start PCR reactions, named SL-IR I, SL-IRac and SL-IR II, were carried out for a first classification of *T. cruzi* populations in three groups of lineages: Tc I, Tc IIa/c and Tc IIb/d/e, respectively (Table 1). SL-IRac PCR strategy was developed to differentiate between populations belonging to *T. cruzi* IIa/c lineages and the other groups (*T. cruzi* I and IIb/d/e). This PCR amplified a band of 200 bp from reference Tc IIa and Tc IIc stocks, a band of 150 bp from reference Tc I and around 157 bp for Tc IIb, Tc IId and Tc IIe stocks.

SL-IR I allowed amplification of a fragment of 475-bp product from *T. cruzi* I populations using primers TC2 (Souto et al., 1996) as sense primer and UTCC as antisense. SL-IR II allowed amplification of a 425-bp product from *T. cruzi* IIb/d/e lineages using sense primer TC1 (Souto et al., 1996) and UTCC as antisense (Table 1). In samples with negative findings using the three mentioned tests, heminested PCR was carried out using TCC (Souto et al., 1996) and TC1 from SL-IR II reaction tubes or TCC and TC2 from SL-IR I reaction tubes, as described previously (Marcet et al., 2006).

Amplification of the D7 domain of the 24Sα ribosomal RNA genes: a dimorphic region within the D7 domain was amplified by hot-start heminested PCR to distinguish between Tc IId and Tc IIb/Tc IIe groups. The first round PCR was performed using D75 and D76 primers in a 50 µl vol. reaction. The heminested round was carried out using 1 µl of the first round PCR in a 30 µl vol. reaction using primer pair D71-D76 (Table 1).

SL-IR and 24Sα rDNA PCR products were analysed in 3% agarose gels (agarose 1000, GibcoBRL/life technologies, USA) and UV visualization after ethidium bromide staining.

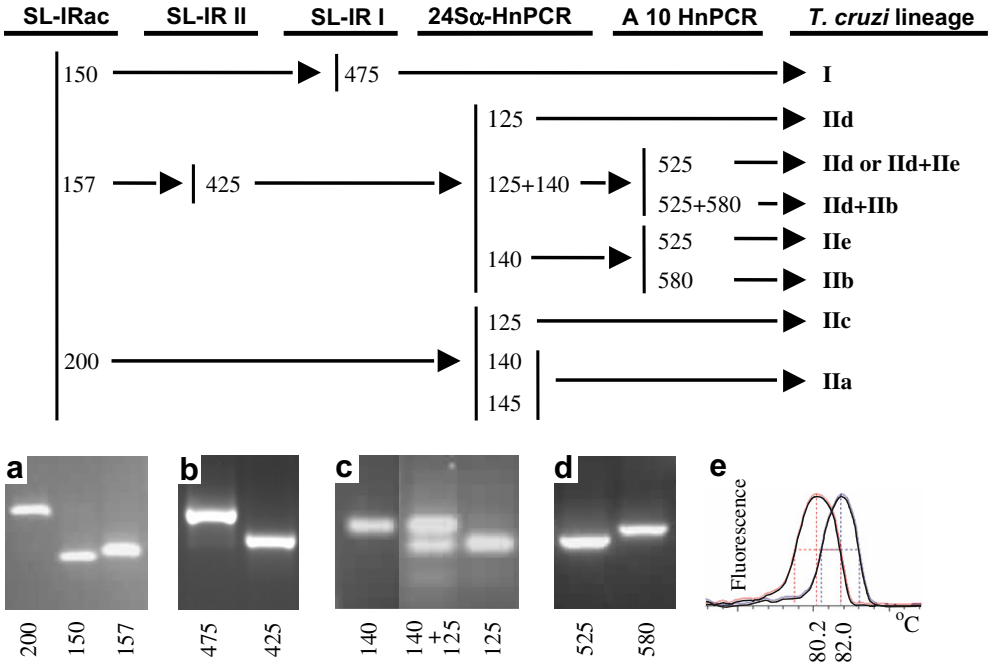

Fig. 1. PCR flowchart for identification of *Trypanosoma cruzi* lineages in human blood samples. SL-IR, spliced leader intergenic region; Hn, heminested. Numbers are expressed in bp. (a–d) Agarose gel electrophoresis patterns of the SL-IR, 24Sα rDNA and Hn-A10 amplification products. (e) Denaturation peaks obtained after real time heminested PCR of the A10 fragments.

Nested Amplification of A-10 fragment was carried out once SL-IR and 24Sα rDNA PCR strategies gave products indicative of Tc IIb/Tc IIe lineages (SL-IRac: 157 bp, SL IR-II: 425 bp and 24Sα rDNA: 140 bp, Table 1). In order to enhance the sensitivity of the original A10-PCR method (Brisse et al., 2000) used to distinguish between Tc IIb (A10 negative) from Tc IIe (A10 positive, 657-bp product) in culture isolates, we designed internal primers that amplified a 580-bp heminested product for lineage Tc IIb and 525-bp heminested products for the remaining lineages.

The first round of amplification was carried out using a novel primer Pr1 (Table 1) and P6 (Brisse et al., 2000). The heminested round was performed using new primers Pr1 and Pr3 by real time PCR in a MJR-Opticon II device (MJ Research, USA). The heminested amplicon of 580 bp (Fig. 1d) resolved as a denaturing peak with a melting temperature of 80.2° ± 0.2 (Fig. 1e) and amplicons of 525 bp resolved as denaturing peaks of temperature 82 °C ± 0.2.

Reference *T. cruzi* stocks used as controls were: Tc I (X-10, TCC and CA-1 K98); Tc IIa (Can III); Tc IIb: (Tu 18); Tc IIc: (M5631), Tc IId (MnCl2) and Tc IIe (Cl- Brenner, RA). They were kindly provided by Patricio Diosque (Instituto de Patología Experimental, Universidad Nacional de Salta, Argentina), Stella M. Gonzalez Cappa (Facultad de Medicina, Universidad de Buenos Aires, Argentina) and Michel Tibayrenc (UR62 ''Genetics of Infectious Diseases'', IRD Centre, Montpellier, France).

2.5. Analysis of minicircle signatures

Restriction fragment length polymorphism (RFLP)-PCR profiling was performed with 2 μg of purified vkDNA amplicons (Wizard SV Gel and PCR Clean-Up System, Promega, WI, USA) that were digested with 5 U of MspI + RsaI restriction enzymes for 3 h at 37 °C. The digestion products were visualised after 10% PAGE and silver staining.

Genetic distances among RFLP-PCR profiles were estimated using the Jaccard's coefficient (JD) (Jaccard, 1901). The Jaccard's distance was estimated under the formula: $D = 1 - (a/(a + b + c))$, where a is the number of bands that are common to the two compared profiles, b is the number of bands in the first profile and absent in the second, and c is the number of bands absent in the first profile and present in the second. Intra-family JDs were estimated by comparing the profiles obtained from samples of pairs of mother-infants. Inter-family JDs were estimated comparing the minicircle profiles obtained from the mothers' samples.

2.6. Sequence analysis of the constant region of the minicircle genome

The 120-bp constant region of the minicircle (ckDNA) was amplified as reported (Marcet et al., 2006). Purified amplicons were cloned into the pGEM-T easy vector (Promega, MA, USA) for sequence analysis. Homologous sequences from *T. cruzi* strains (Y, CL and CA-1) available at the GenBank were included. Sequence alignment was conducted using MEGA version 3.1 (Kumar et al., 2004) to construct a Neighbor-Joining tree (Saitou and Nei, 1987).

2.7. Microsatellite PCR assay

Full nested-PCR targeted to sequences flanking microsatellite repeats (Macedo et al., 2001) for the loci TcTAT20, TcTAC15, TcATT14 and TcAAAT6 were carried out from DNA obtained from the blood samples, as recently described (Valadares et al., unpublished data). To determinate the allele sizes, 1–3 μl of PCR fluorescent products were analysed in 6% denaturing polyacrylamide gels of an ALF sequencer (GE Healthcare, Milwaukee, Wisconsin, USA). The interpretation of the patterns was according to Oliveira et al. (1998).

3. Results

3.1. PCR-based identification of bloodstream parasite lineages

PCR procedures targeted to polymorphic nuclear genomic sequences (Table 1) were carried out on vkDNA-PCR positive DNA preparations from blood samples of CCD-related and -unrelated groups of patients coming from endemic regions of Argentina and Bolivia. Lineages of *T. cruzi* were identified following the PCR-based flow-chart described in Fig. 1.

We have identified *T. cruzi* lineages in samples of 38/47 (80.8%) CCD infants, 5/7 (71.4%) CCD-transmitting mothers, 13/32 (40.6%) women who delivered uninfected newborns and 34/44 (77.3%) Chagas disease patients unrelated to CCD patients (Table 2).

Trypanosoma cruzi I (SL-IRac: 150 bp; SL-IR I: 475 bp, 24Sα rRNA group 1: 125 bp) was detected in one CCD non-transmitting mother and in three CCD-unrelated patients. *T. cruzi* IIa genotypes were not detected. *T. cruzi* IIb (SL-IRac: 157 bp, SL-IR II: 425 bp, 24Sα rRNA group 2: 140 bp and A-10 580 bp) was detected in one CCD infant. *T. cruzi* IIc genotypes were not detected. *T. cruzi* IId populations (SL-IRac: 157 bp, SL-IR II: 425 bp, 24Sα rRNA group 1/2: 125 bp + 140 bp or 24Sα rRNA group 1: 125 bp, Fig. 1) were identified in blood samples from 36/38 (94.7%) CCD infants, in all five CCD-transmitting mothers, in 12/13 (92.3%) CCD non-transmitting mothers and in 28/34 (82.35%) CCD-unrelated patients. *T. cruzi* IIe (SL-IRac: 157 bp, SL-IR II: 425 bp, 24Sα rRNA group 2: 140 bp and A-10 525 bp) was only detected in two CCD-unrelated patients. Overlapping infections of *T. cruzi* I and *T. cruzi* IId and/or IIe (SL-IRac: 150–157 bp; SL-IR I: 475 bp, SL-IR II: 425 bp, 24Sα rRNA group 1/2: 125 bp + 140 bp and A10: 525 bp) were detected in one CCD infant and in one CCD-unrelated patient (Table 2).

Table 2
Distribution of *Trypanosoma cruzi* lineages in PCR-positive blood samples of congenital Chagas disease (CCD) infants, CCD-transmitting mothers, CCD-non-transmitting mothers and CCD-unrelated Chagas disease patients

Study groups	Number of samples	Tc I (%)	Tc IIa	Tc IIb (%)	Tc IIc	Tc IId (%)	Tc IIe (%)	Tc I + Tc IId/e (%)
CCD children	38	0	0	1 (2.6)	0	36 (94.8)	0	1 (2.6)
CCD mothers	5	0	0	0	0	5 (100)	0	0
CCD non-transmitting mothers	13	1 (7.7)	0	0	0	12 (92.3)	0	0
CCD unrelated patients	34	3 (8.8)	0	0	0	28 (82.4)	2 (5.9)	1 (2.9)

Tc I, *T. cruzi* lineage I; Tc II a/b/c/d/e: *T. cruzi* lineage II, sublineages a, b, c, d and e, respectively.

The remaining tested patients' samples were PCR-negative by means of the SL-IR procedures and therefore were not analysed further. *T. cruzi* IId was the predominant lineage in both CCD-related and unrelated individuals (two-tail Fisher exact test, $P > 0.05$).

3.2. Profiling of minicircle signatures from maternal and infants' bloodstream trypanosomes

Minicircle signatures from bloodstream parasite populations of seven cases of *T. cruzi* IId-infected mothers and CCD infants were profiled by RFLP-PCR of the 330-bp vkDNA amplicons amplified from the DNA preparations used for identification of lineages. To increase the possibility of detecting vkDNA sequence variability, we performed double digestions with MspI + RsaI (Fig. 2). The comparison between minicircle signatures transmitted from mothers to siblings revealed nearly identical intra-family profiles, showing the vertical transmission of the maternal bloodstream parasite population (Fig. 2a, M and CCD). This was also observed in two cases of CCD twins (Fig. 2a, cases 6 and 7). The intra-family JD ranged from 0 to 0.3 (median JD = 0.06, Fig. 2b). The major intra-family JD was detected between populations from the mother and daughter of case 3, whose samples were collected 1 year after delivery.

On the other hand, the minicircle signatures were unique to each family, revealing the genetic diversity of *T. cruzi* populations belonging to the same lineages, even among mothers born in the same endemic regions (cases 2, 4, 6 and 7 from Chaco Province, Argentina). Accordingly, the inter-family JDs were higher than the intra-family JD, ranging from 0.2 to 0.83 (median JD: 0.583, Fig. 2b).

3.3. Characterization of parasite populations transmitted to twins

Further characterization of congenitally transmitted trypanosomes was carried out in cases of CCD twins (cases 6 and 7, Fig. 2). Nested-PCR strategies targeted to four nuclear microsatellite loci were carried out to address the clonal complexity of the corresponding parasite populations (Fig. 3a). The samples from case 7 amplified alleles of identical sizes, confirming the high intra-family homogeneity among these populations at the nuclear genomic level (Fig. 3a). Interestingly, three allelic peaks for the loci TcTAC15 and TcAAAT6 were detected (Fig. 3a, 99, 132 and 135 bp, and 259, 271 and 279 bp, respectively). These data are interpreted as a polyclonal population and thus reveal that the clones were transmitted equally to both infected twins. The mother's sample of

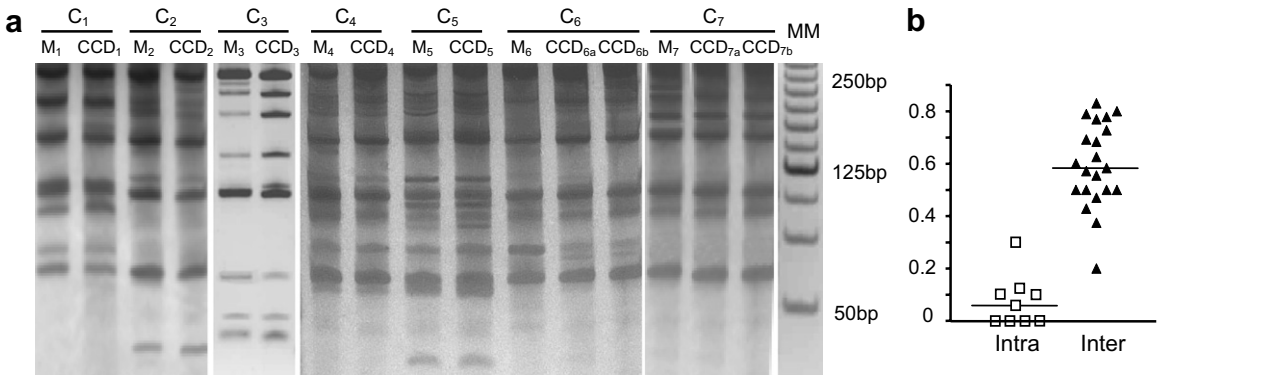

Fig. 2. Minicircle genome-based characterization of maternal and pediatric bloodstream parasite populations. (a) 10% polyacrilamide silver-stained gel showing MspI + RsaI-based restriction fragment length polymorphism-PCR profiles of seven cases (C) comprising a mother (M) and her congenitally infected sibling/s (CCD). Case 1: 42 years old M, 2 months old son; case 2: 30 years old M, 2 months old daughter; case 3: 37 years old M, 12 months old daughter; case 4: 27 years old M, 2 months old daughter; case 5: 26 years old M, 2 days old daughter; case 6: 18 years old M and 16 days old twin sisters; case 7: 24 years old M, 2 months old brother and sister fraternal twins. MM, 25-bp DNA molecular ladder. (b) Intra-family and inter-family Jaccard genetic distances (JD).

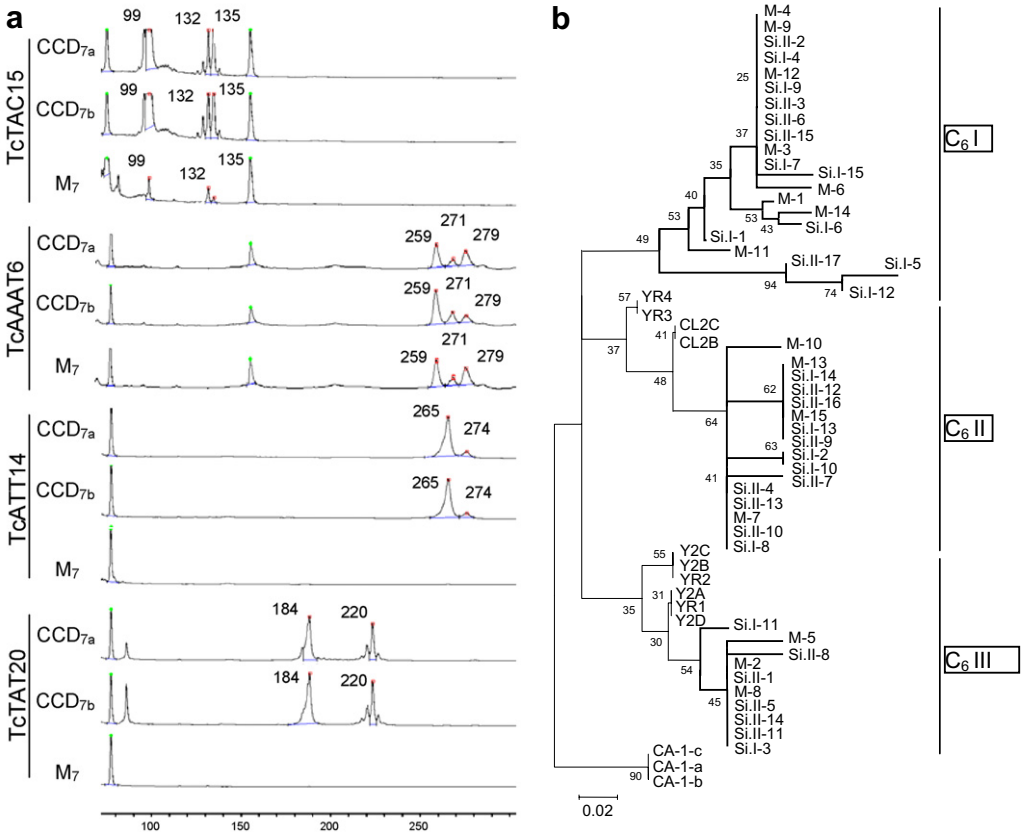

Fig. 3. Analysis of bloodstream parasite populations transmitted to twins. (a) Analysis of allelic polymorphism in microsatellite loci. ALF (Pharmacia-GE) DNA sequencer electrofluorograms showing the amplified fragments obtained by TcAAAT6, TcTAC15, TcTAT20 and TcATT14 microsatellite loci analyses on blood samples from patients of case 7. M_7, mother; CCD, twins. The numbers above the peaks refer to the size of the amplicons in bp. Peaks without sizes correspond to the molecular DNA ladder. (b) Neighbor-joining tree constructed (*P*-distance model, MEGA v. 3.1) from the alignment of the 120-bp constant region of the minicircle sequences (ckDNA) obtained from the patients' samples of case 6 and reference parasite stocks. Wide lines denote branches conforming the clusters of patients' sequences C_6 I, C_6 II and C_6 III. Accession Nos. are DQ873338-52 (mother), DQ873353-69 (sister 2), DQ873370-84 (sister 1), M18814 (Y strain), M19176 (CL strain) and M15512 (CA-1 strain). *Trypanosoma cruzi* lineage I strain CA-1 sequences were used as an outgroup to root the tree. Numbers at the branches show bootstrap values after 100 replications. Si-I and Si-II, sisters 1 and 2; M, mother.

case 7 was negative by TcTAT20- and TcATT14- PCR, and the samples from all patients in case 6 gave no PCR products from the tested loci, probably because their parasitemias were lower than the detection limits of the mentioned PCR procedures. Thus, to analyse the *T. cruzi* population in case 6, sequences of the 120-bp constant regions of the ckDNA from the patient's blood samples were aligned. The Neighbor-Joining tree placed maternal and neonatal parasite sequences within three major clusters (Fig. 3b). Cluster C_6I grouped maternal and neonatal sequences from both twins. Cluster C_6II grouped sequences of C_6 as well as sequences from *T. cruzi* II strains CL and Y, being the sequences from the patients' populations more related among them than with those from the reference strains. Cluster C_6III showed grouping of maternal and neonatal sequences separated from branches gathering sequences of the Y strain. This topology suggested that the minicircle classes detected in maternal bloodstream populations were also represented in both CCD sisters' samples.

4. Discussion

Trypanosoma cruzi lineages have mostly been identified from cultured stocks using biochemical and molecular markers (Souto et al., 1996; Fernandes et al., 1998; Brisse et al., 2000, 2001; Diosque et al., 2003). These analyses may underestimate the parasite diversity present in natural infections, because of competence among strains during culture expansion. These PCR-based strategies were later modified for direct identification of lineages from tissue specimens (Freitas et al., 2005; Burgos et al., 2005; Virreira et al., 2006a), triatomine feces (Marcet et al., 2006) and umbilical cord samples (Virreira et al., 2006b). To enable direct identification of lineages in peripheral blood samples from patients at the indeterminate chronic phase of Chagas disease it was necessary to increase the PCR sensitivity of these strategies. For that, hot-start heminested or nested procedures using Taq platinum DNA polymerase were applied from 500 µl of Guanidine-EDTA blood lysates. In addition, the original multiplex SL-PCR assay that uses TC1 and TC2 as sense and TCC as antisense primers

(Souto et al., 1996) was modified by the replacement of the antisense primer (Table 1). The new UTCC primer allowed higher sensitivity and identification of parasites belonging to lineages Tc IIa and Tc IIc, which amplify a differential fragment of 200 bp (SL-IR ac, Fig. 1a). Furthermore, to increase the possibility of detecting potential overlapping infections by different lineages, three independent reactions were performed; SL-IR I amplified only Tc I populations, SL-IR II amplified Tc IIb/d/e (Fig. 1b) and SL-IRac allowed distinction between Tc IIa/c and the other 4 four lineages.

In some Tc IId-infected samples, the 24Sα rDNA-PCR amplified both 125- and 140-bp ribosomal DNA bands (Fig. 1c). Because Tc IIb and Tc IIe strains also amplify the 140 bp 24Sα rDNA fragment (Brisse et al., 2001), we cannot discard the possibility of Tc IId infections mixed with Tc IIb and/or Tc IIe. Accordingly, DNA preparations from blood samples of six Tc IId-infected CCD infants that amplified both 24Sα rDNA sequences were tested by a novel AluI-based RFLP procedure from an amplified fragment of the mitochondrial cytochrome oxidase subunit II gene. Five of six samples were CO II haplotype B, which corresponds to hybrid lineages Tc IId or Tc IIe, whereas one sample revealed haplotypes B and C, which indicates Tc IId/e and Tc IIb overlapping infections (data not shown). These findings suggest that a proportion of populations amplifying both 24Sα rDNA sequences are conformed by at least Tc IId and Tc IIb lineages.

The majority of *T. cruzi* populations detected in this study belonged to *T. cruzi* lineage IId, in both CCD-related and unrelated patients from the same regions of endemicity. Thus, the predominance of Tc IId in the studied CCD-related groups cannot be ascribed to a preferential link of this lineage with vertical transmission, but rather is indicative of the distribution of bloodstream *T. cruzi* genotypes in these geographical areas. Moreover, minicircle signatures from mother-infants cases infected with *T. cruzi* IId were unique to each case, also suggesting no association of vertical transmission with a particular bloodstream strain pattern.

The prevalence of Tc IId in CCD patients of our study is in agreement with recent work performed in Bolivian CCD newborns, using sequence characterised region markers and miniexon gene amplification tests from umbilical cord samples (Virreira et al., 2006b). Also, in the mentioned report the distribution of lineages detected in CCD cases was similar to that of the general population in the region. Indeed, *T. cruzi* IIb/d/e genotypes are prevalent in humans and the domestic vector *Triatoma infestans* from southern cone countries of South America (de Luca D'oro et al., 1993; Barnabe et al., 2001; Di Noia et al., 2002; Burgos et al., 2005; Freitas et al., 2005; Marcet et al., 2006; Virreira et al., 2006b).

Another aspect involved in vertical transmission is enhanced maternal parasitemia (Menezes et al., 1992; Hermann et al., 2004). It is noteworthy that five of seven (71.4%) CCD-transmitting mothers (women with indeterminate Chagas disease) were PCR-positive when tested using the lineage-specific nuclear genomic markers, whereas 13/32 (40.6%) tested CCD non-transmitting mothers were PCR-positive by the same tests. Moreover, two of the five PCR-positive CCD-transmitting mothers were co-infected with human immunodeficiency virus (HIV), whereas all 32 non-transmitting women were HIV negative. This suggests that the CCD-transmitting women in our study exhibited relatively higher parasitic loads than the CCD non-transmitting mothers and that enhanced parasitemia may be linked to vertical transmission. In this context, it is worth noting that the only CCD neonate infected with *T. cruzi* I and *T. cruzi* II d/e populations had perinatal acquired immunodeficiency syndrome (AIDS); her mother's samples were not available for lineage identification. Interestingly, *T. cruzi* I was detected in bloodstream of five out of nine immunosupressed heart transplanted patients from Argentina, and in two of seven patients with AIDS, in these cases as mixed infections with *T. cruzi* II (Burgos et al., unpublished data). These recent data show that Tc I populations exist more frequently in human populations coming from the Southern cone of South America but these strains would display lower parasitemias than *T. cruzi* IId/e populations, at least during the indeterminate and chronic phases of the infection. Accordingly, in the *T. cruzi*-infected mother, HIV-driven immunosupression could have favored enhancement of parasitemia leading to the transplacental passage of both *T. cruzi* I and *T. cruzi* IId/e genotypes.

The high intra-family similarity between maternal and children's *T. cruzi* signatures was observed between maternal and infants' samples collected from 2 days to 1 year after delivery. This suggests that the genetic composition of bloodstream populations is stable in the infected infants from the event of in-uterus transmission up to the period when the infant's infection has evolved to the indeterminate chronic phase. The minicircle patterns of the maternal bloodstream populations also persisted during the same periods.

The analysis of microsatellite polymorphism allows us to determine the number and the composition of parasite clones present in a given biological sample (Macedo et al., 2001; Valadares et al., unpublished data). This strategy enabled us to assess the polyclonal composition of the parasite populations involved in case 7. This characterization could not be achieved in samples from case 6, probably because their parasitic loads were below the detection limits of these assays, which are based on single copy nuclear markers. However, sequence analysis of the constant regions of the minicircles in samples from this family suggested that the maternal populations were equally infective to both CCD sisters, in agreement with RFLP-PCR profiling (Fig. 2a).

Altogether, the data reported in this study do not support a direct association between the *T. cruzi* lineage or minicircle signature with the occurrence of congenital infection. In cases of transmission, our findings reveal that

the whole maternal bloodstream population, even if composed of multiple clones, were transmitted, a fact that was also observed in twin deliveries. Finally, the high degree of intra-family minicircle homogeneity detected between populations of mothers and CCD siblings, as well as between CCD twins, opens new possibilities for epidemiological surveys of emergent cases of Chagas disease, in which the sources of transmission need to be elucidated.

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