

CONGENITAL CHAGAS DISEASE IN BOLIVIA IS NOT ASSOCIATED WITH DNA POLYMORPHISM OF *TRYPANOSOMA CRUZI*

MYRNA VIRREIRA, CRISTINA ALONSO-VEGA, MARCO SOLANO, JUAN IJENA, LAURENT BRUTUS, ZULEMA BUSTAMANTE, CARINE TRUYENS, DOMINIQUE SCHNEIDER, FAUSTINO TORRICO, YVES CARLIER, AND MICHAL SVOBODA*

Laboratoire de Chimie Biologique, Faculté de Médecine, Université Libre de Bruxelles (U.L.B.), Brussels, Belgium; Facultad de Medicina, Universidad Mayor de San Simon (U.M.S.S.), Cochabamba, Bolivia; Unidad de Neonatología, Hospital "San Juan de Dios", Tarija, Bolivia; Institut de Recherche pour le Développement (IRD, France), Mother and Child Health Research Unit (UR10), La Paz, Bolivia; Laboratoire de Parasitologie, Faculté de Médecine, Université Libre de Bruxelles (U.L.B.), Brussels, Belgium

Abstract. This study aims to typify the *Trypanosoma cruzi* (sub)lineage(s) in umbilical cord blood of congenitally infected Bolivian newborns, using PCR amplifications of "Region Markers", mini-exon or kDNA fragments followed by hybridization or sequencing. New probes were also designed to distinguish three variants within the TcII sublineage. The I Ib, I Id, or I Ie *T. cruzi* sublineages, as well as different variants of the I Id sublineage, were detected in infected neonates, whereas mixed infections were not found. The frequencies of the I Id sublineage were similar in neonates (95.1%) and adults of the same area (94.1%). The I Id-infected newborns displayed either asymptomatic, or severe and fatal clinical forms of congenital Chagas disease, as well as low or high parasitemia. Altogether these data show that *T. cruzi* DNA polymorphism, based on the presently available markers, is not associated with the occurrence of congenital infection or the development of severe clinical forms of congenital Chagas disease.

INTRODUCTION

The flagellate protozoa *Trypanosoma cruzi* is the agent responsible for Chagas disease, infecting 16–18 million people in Latin America. Parasites are mainly transmitted by blood-sucking bugs releasing excreta containing infectious agents, by transfusion of infected blood, or from mothers to their fetuses.¹ In endemic countries where national programs of vectorial control and selection of blood donors have been developed, congenital infection with *T. cruzi* has evolved as the main route of uncontrolled parasite transmission from one generation to another, remaining a pressing public health problem.^{2,3} Besides large regional variations in the rates of materno-fetal transmission of *T. cruzi*, from 1% in Brazil to 4–12% in Argentina, Bolivia, Chile, and Paraguay, the morbidity and mortality of congenital infection also varies from asymptomatic to severe and mortal clinical forms of disease.^{3,4} Host factors, such as the level of placental defenses, and/or the maternal and fetal capacities to develop a specific immune response able to control the parasitic multiplication, can be involved in such differences.^{5,6} Another possibility relates to the parasitic molecular polymorphism, since *T. cruzi* evolves in the form of independent parasitic lineages, displaying different biologic properties.^{7–9} Likewise, it can also be expected that *T. cruzi* lineage(s) of higher virulence might be transmitted more easily across the placenta and/or be more pathogen for the fetus/neonate, as suggested by previous experimental studies.^{10–12}

There is a consensus to recognize 2 major lineages of *T. cruzi*: TcI and TcII.¹³ The lineage TcII can be subdivided in 5 sublineages: I Ia, I Ib, I Ic, I Id, and I Ie on the basis of genotypical and phenotypical properties estimated by various methods.^{14,15} It has been also reported that the sublineages I Ia and I Ic derive from the fusion of the ancestral (sub)lineages I and I Ib, and that the sublineages I Id and I Ie come from an hybridization between the sublineages I Ib and I Ic.^{16–19}

The lineage TcI predominates at the North of Amazon basin with *Didelphis* species as natural hosts, whereas armadillos host the lineage TcII, which predominates in the south cone region of America.²⁰ Both lineages can infect humans, and, though the sublineage I Id is predominantly circulating in the south cone countries, mixed infections with TcI (clonet 20) and TcII (clonet 39) have been mainly reported in Bolivia and Chile.^{21–24}

The aim of our study was to typify the *T. cruzi* (sub)lineage(s) of congenitally infected Bolivian newborns to determine if the occurrence of infection and/or the severity of congenital Chagas disease in fetus/neonate can be influenced by the parasitic DNA polymorphism. Umbilical cord blood were analyzed using previously described primers and probes, as well as new sensitive probes designed to distinguish variants within the I Id sublineage in small amounts of infected blood.

MATERIALS AND METHODS

Patients and blood samples. *Patients.* The present work considers newborns congenitally infected with *T. cruzi*, and adult patients chronically infected coming from the Bolivian departments of Cochabamba and Tarija, considered as areas endemic for *T. cruzi* infection. Mortality and clinical data of newborns (birth weights, gestational age, Apgar scores, occurrence of hepato-splenomegaly, edema, respiratory distress syndrome, or neurologic alterations) were collected as previously described.⁴ They allowed classifying the congenitally infected newborns into asymptomatic, symptomatic, or fatal cases. All mothers were asymptomatic. This study has been approved by the scientific/ethics committees of the "Universidad Mayor de San Simon" and the "Université Libre de Bruxelles", and written consent of the informed mothers/patients was obtained before blood collection.

Diagnosis of T. cruzi infection. Infection in adult patients was assessed using standard parasite-specific serological tests (ELISA, hemagglutination, and immunofluorescence) and PCR using the Tcz1/Tcz2 primers, performed as previously described.²⁵ Congenital infection with *T. cruzi* was sought for by direct microscopic examination of blood buffy coat col-

* Address correspondence to Michal Svoboda, Laboratoire de Chimie Biologique, Faculté de Médecine, U.L.B. 808 route de Lennik CP 611, B-1070 Bruxelles, Belgium. E-mail: msvobod@ulb.ac.be

lected in 3–4 microhematocrit heparinized tubes, as previously described.⁴ The detection limit of such parasitological detection was estimated to 40 parasites/mL.²⁶ The number of positive microhematocrit tubes containing parasites, as well as the number of detected parasites per tube allowed a semi-quantitative estimation of parasitemia, as either low (40–400 p/mL) or high parasitemia (> 400 p/mL).

Blood samples. A total of 41 umbilical cord blood samples of newborns congenitally infected with *T. cruzi* were collected in the following Bolivian maternities: German Urquidi (University Hospital Viedma, Universidad Mayor de San Simon) in Cochabamba (*N* = 18), hospital “San Juan de Dios” in Tarija (*N* = 11), district hospital in Yacuiba, department of Tarija (*N* = 12). Seventeen blood samples of Tcz1/Tcz2-positive PCR adults unrelated to the studied newborns. Collected blood (1–5 mL) was immediately mixed with the same volume of guanidine-HCl 6M, EDTA 0.1 M (pH 8).

***T. cruzi* reference strains.** Thirty-one *T. cruzi* stocks (prepared as epimastigotes grown in LIT medium), belonging to the 4 main *T. cruzi* lineages I, IIb, IID, and IIe (4–6 stocks per lineage, as indicated in Table 1), previously cloned in the laboratory of Dr. M. Tibayrenc (CNRS-IRD, Montpellier, France), have been selected for this study. They represent the eco-epidemiologic genetic diversity of *T. cruzi* in Bolivia, since the sublineages IIa and IIc have not been detected in human infection in this country.^{27,28} They were kindly given by Dr. M. Tibayrenc.

DNA extraction. DNA was isolated from 0.2 ml of guanidine samples (previously boiled for 15 minutes), using either the phenol-chloroform method as previously described²⁵ or the “QIAamp DNA Blood Mini Kit” (Qiagen) according to the manufacturer’s protocol. Purified DNA was dissolved in 50 µl of TE (10mM Tris-HCl, 1mM EDTA, pH 8.0).

Polymerase chain reaction amplifications. All PCR were performed in duplicate, using the MyCycler from BIO-RAD.

PCR amplifications of “Sequence Characterized Region Markers” (SCAR). These markers are *T. cruzi* DNA fragments (probably nuclear), whose polymorphism analysis allows unequivocal typing of TcI, IIa, IIb, IIc, IID, and IIe, using the 3 pairs of primers described by Brisse et al.²⁹ The primers p3 (5'-GTG ACT GCA GCA AGT AGT CC-3') and p6 (5'-GTG ATC GCA GGA AAC GTG A-3'), after 40 cycles (94°C for 20 seconds, 57°C for 20 seconds, 72°C for 3:00 minutes) and a final elongation of 7 minutes, give amplification only for TcIIc, IID, and IIe (657 bp), and not for the other lineage/sublineages. The primers p7 (5'-GGC TGC AGA ACC AAT AAG AAA TG) and p8 (5' GGC TGC AGA AAG GGA AAT CCG AAG-3'), after 47 cycles (94°C for 20 seconds, 50°C for 20 seconds, 72°C for 3 minutes) and a final elongation of 7 minutes allow to distinguish the sublineage IIa, IIc, and IID amplicon sizes (455, 474, and 355 bp, respec-

tively) from I, IIb, and IIe (all of 410 bp). Finally, the primers p9 (5'-CCT GCT CAT CCT CGT GCT TTA CGG-3') and p10 (5' TGC TCA TCG ACA ACA TTT TAT TGG-3'), using 50 cycles (94°C for 20 seconds, 50°C for 20 seconds, 72°C for 3 minutes) and a final elongation of 7 minutes, allow to separate easily TcIIb (1170 bp) from the other lineage/sublineages (1080 bp). We used Accuprime PCR kit (Invitrogen) for p7 and p8 amplification. We used GoldStar (Eurogentech) kits for others amplifications. The positive controls for SCAR typing came from the reference strains mentioned in Table 1.

PCR amplifications of minicircle kDNA fragments. These PCR allowed to obtain the amplicons necessary to perform the hybridizations mentioned later in this article. They used the previously described primers Tc121 (5'-AAA TAA TGT ACG GGG KGA GAT GCA TGA) and Tc122 (5'-GGT TCG ATT GGG GTT GGT GTA ATA TA) complementary to the constant domain of kDNA minicircles.³⁰ The PCR were performed in 20 µl (total volume) containing 1.75 mM MgCl₂, 0.2 mM of each dNTP, 0.5 µM of each primer Tc121 and Tc122, 1.25 units of “GoTaq” DNA Polymerase (Promega), and 1 µl of extracted DNA. Forty amplification cycles (94°C for 20 seconds, 57°C for 10 seconds, 72°C for 30 seconds) followed by a final elongation of 7 minutes allowed to obtain 320 bp amplicons. The region nt 40–220 of these fragments corresponds to the highly variable domain of kDNA minicircles. Consequently, the 320 bp amplicon band actually contains hypervariable and constant kDNA sequences.

PCR amplifications of nDNA mini-exon. The sizes of amplicons of mini-exon domains being different for TcI and TcII, their amplification allows the simultaneous detection of both lineages. In our hand, the previously described primers TC1/TC2/TC³¹ gave good results (amplicons of 350 bp and 300 bp for TcI and TcII, respectively) for DNA samples extracted from cultured parasites, but many unspecific amplicons with DNA extracted from human blood. To improve the sensitivity of the detection in blood samples, we designed 2 pairs of new primers based on published mini-exon sequences³²: (i) TcI: forward (5'-CAG CGC CAC AGA AAG TGT T-3'); reverse: (5'-GCT CCT TCA TGT TTG TGT CG-3'); (ii) TcII: forward (5'-CTT CCT GTG TTT TCC GGT GT-3'); reverse: (5'-ACA CTG GGG AGG GGT CAG-3'). Using 40–50 cycles according to the program described previously for Tc121-Tc122, they gave amplicons of 220 bp and 130 bp for the lineages TcI and TcII, respectively, allowing a clear identification of TcI/TcII mixtures in different proportions (Figure 2A). The 4 primers could be present all together in the same amplification reaction (multiplex PCR).

Analysis of PCR products. They were analyzed in gel electrophoresis (1.2% agarose in TAE buffer: 40 mM Tris, 40 mM

TABLE 1
Used reference strains for the different (sub)lineages of *T. cruzi*

TcI	TcIIa	TcIIb	TcIIc	TcIID	TcIIe
Cutia cl1	92122102R	Tu18 cl93	CM17	MN cl2	Cl Brener
SP 104 cl1	CanIII cl1	CBB cl3	M5631 cl3	Bug2148 cl1	P63 cl1
P209 cl93	Stc 26R	Mas cl1	M6241	SO35 cl5	Tula cl2
SO34 cl4	Stc 35R	Ivv cl14	85847	TPK 1	X154/7
OPS21	Stc 50R	Esmeraldo cl3		SC43	VMV 4
X10 cl21				RN PCR0	

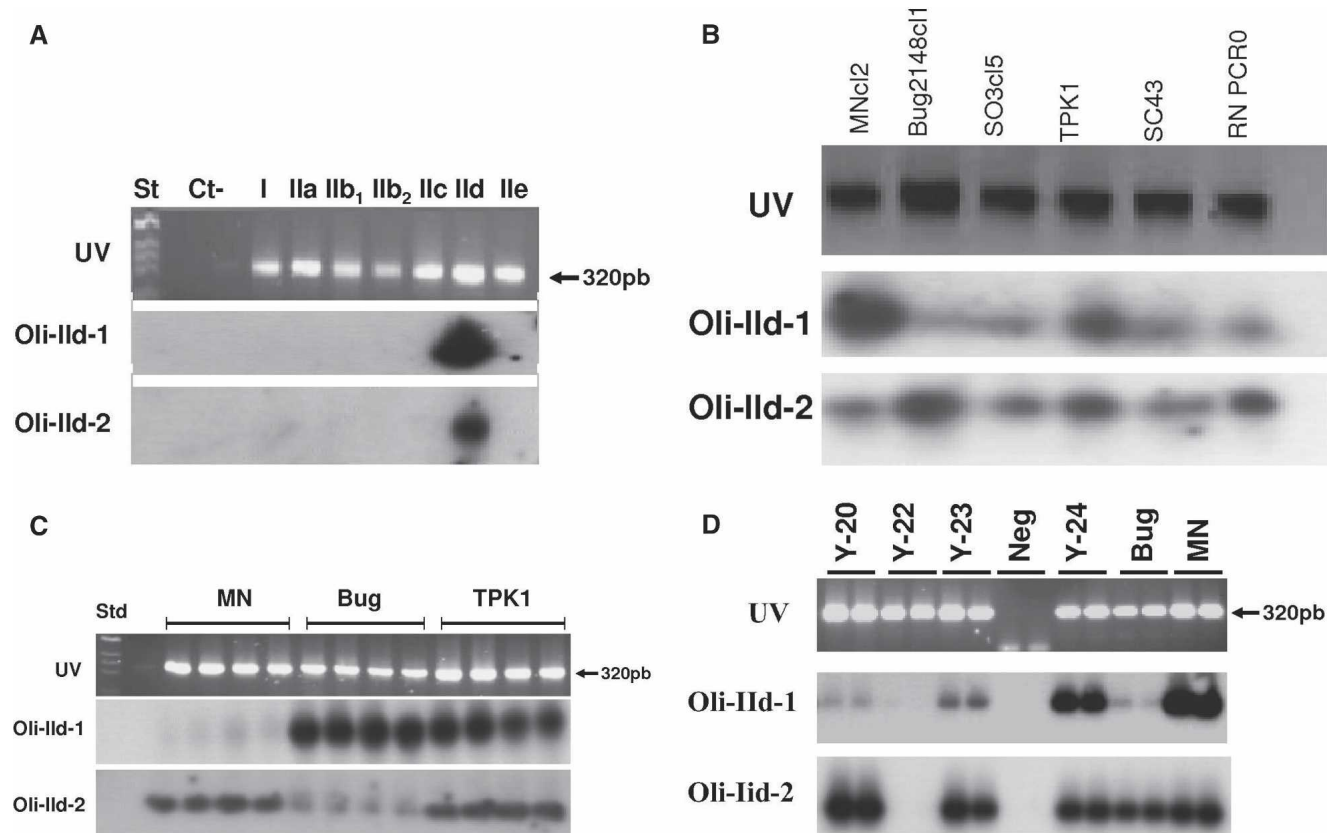


FIGURE 1. Relative hybridization of kDNA amplicons with synthetic oligonucleotide probes. The hypervariable regions of *T. cruzi* kDNA were amplified and the intensity of amplicon fluorescence (first line) is compared with autoradiography after hybridization with indicated oligodeoxynucleotides probe Oli-IId-1 and Oli-IId-2 (second and third lines). **A**, Analysis of DNA extracted from 6 reference strains of different (sub)lineages: X10, CanIII, Esmeraldo, Tu18, M5631, MNcl2, and CL Brener. **B**, Analysis of 5 different strains of the same lineage IId. **C**, Analysis of amplicons made in quadruplicate from DNA extractions performed from the same reference strains 1 year later of the same 3 reference IId strains MNcl2, Bug2148, and TPK1. **D**, Analysis of DNA extracted from umbilical blood (Y-20, Y-22, Y-23, Y-24) compared with the hybridization of 2 reference strains MNcl2 and Bug2148.

acetate, 1 mM EDTA), in the presence of 0.5 μ g/mL of ethidium bromide. Gels were examined using the Appitek transilluminator (UVP), and the amplicons were visualized using Bio1D software (Wilber-Lourmat).

Hybridizations. Southern blot. Gels were denatured by incubation in 0.5M of NaOH, 1.5 M NaCl for 1 hour, and then neutralized twice for 15 minutes each with 0.5 M Tris-HCl, 1.5 M, NaCl (pH 7.4). DNA amplicons resulting from the Tc121/Tc122 PCR amplification were transferred by capillarity (type "sandwich") to nylon membranes Nytran N (Schleier & Schuell), dried and crosslinked on UV-Appitek transilluminator screen for 1 minute.

Synthetic oligodeoxynucleotide probes for variants of lineage IId. To study the molecular polymorphism of the sublineage IId in amplicons derived from the Tc121/Tc122 PCR, we have designed new synthetic probes based on the sequences of various IId strains previously analyzed by Telleria et al.³³ (GenBank accession number: AJ747914–AJ748069). Their results indicated that 90% of kDNA sequences of the variable domain of sublineage IId could be grouped into 3 major sequence classes. The sequences inside each class were highly homologous, but markedly different from the sequences of both others classes. The proportion of these sequence classes is however different in various strains of the sublineage IId. We selected the following oligodeoxynucleotide probes,

named Oli-IId-1 (5'-TGG TTG TGT AGT AGA ATG TTG TG-3') and Oli-IId-2 (5'-GTA TGT TGA TTG TTG ATT TGA GTT G-3').

kDNA hypervariable region probes for the (sub)lineages I, IIb, and Ile. To identify the (sub)lineages I, IIb, and Ile, we used the previously described probes obtained from the kDNA hypervariable region of reference strains^{34,35}: (i) the strains SO34, SP104, and P209 of lineage I; (ii) the strains CBB, Tu18, Esmeraldo, Mas, and Ivv of sublineage IIb, and (iii) the CL Brener strain of the sublineage Ile. All probes were prepared similarly as follows: each strain was amplified separately with the CV1/CV2 primers, and amplicons were digested with the restriction enzymes Sau96I and ScaI. Mixtures of digested hypervariable fragments for each considered (sub)lineage were purified by electrophoresis on 2% agarose gel and extracted from the gel using the Promega "Wizar SV Gel and PCR clean Up system" kit.

Probe labeling. The oligodeoxynucleotides were phosphorylated by [γ -³²P]ATP (ICN) using a T4 nucleotide kinase. The oligodeoxynucleotides (10 pM) were incubated for 15 minutes at 37°C, in the manufacturer provided buffer, with 25 μ Ci of [γ -³²P]ATP (3000 Ci/mmol) and 0.5 μ l (5 units) of T4 nucleotide kinase in the final volume of 10 μ l. The reaction was stopped by addition of 60 μ l of buffer SET (100 mM NaCl, 20 mM Tris-HCl (pH 8), 10 mM EDTA) and the mix-

ture was heated at 95°C for 2 minutes. The purified mixture of hypervariable kDNA fragments (about 25 ng) was labeled using 50 μ Ci of [α - 32 P]dATP (6000 Ci/mM), using the "Prime-a-Gene Labelling System" kit according to the manufacturer's recommendations (Promega).

Hybridization. The hybridization of amplicons transferred onto membranes was performed at 55°C in 6 $\times$ SSC, 0.5% SDS, 5 $\times$ Denhart's solution (1% ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin). After 1 hour of prehybridization, 250 μ l of denaturated sonicated haring sperm DNA (0.1 mg/ml) was added. After an additional incubation of 20 minutes, the hybridization was started by the addition of labeled oligonucleotides and the mixture was incubated overnight at 55°C. Hybridized membranes were washed sequentially 3 times at room temperature in 1 $\times$ SSC, 0.1% SDS solution, and, for synthetic probes, twice each 15 minutes at 55°C in the same wash solution, whereas random primed hypervariable probes were washed in 2 $\times$ SSC, 0.1% SDS solution at 65°C. Detection of the 32 P-labeled probes was performed by exposure to Kodak Film X-Omat Blue XB-1 (PerkinElmer Life Sciences) at -80°C.

Cloning and sequencing. Polymerase chain reaction products were cloned using the pCRII TOPO TA-cloning kit (Invitrogen) according to the manufacturer's recommendations. One μ l of PCR product was incubated for 30 minutes at room temperature with 25 μ l of TOPO10 competent bacteria. Transformed bacteria were then selected in LB plate containing 75 μ g ampicillin/mL. Plasmids from randomly selected colonies were isolated by alkaline lysis minipreparations and tested for the presence of a 320 bp insert by EcoRI digestion. Sequencing was performed using M13 primer with the Dye Terminator Cycle Sequencing Ready Reaction kit on ABI-PRISM Automated DNA Sequencer (PerkinElmer Life Sciences).

RESULTS

***T. cruzi* typing by polymerase chain reaction amplification of "Sequence Characterized Region Markers."** We first determined the *T. cruzi* (sub)lineages present in umbilical cord blood by SCAR PCR amplification.²⁹ These markers allowed unambiguous *T. cruzi* typing for only 8 of 15 randomly selected DNA samples extracted from umbilical blood, showing all of them as sublineage TcIIId. DNA amplification performed from the other 7 cord blood extracts (6 of them having parasitemia $\leq$ 150 parasites/mL) gave us only nonspecific bands, indicating the SCAR typing as not sensitive enough for typing blood samples displaying low parasitemia.

***T. cruzi* typing by hybridization of synthetic oligodeoxynucleotide probes identifying variants of sublineage IIId.** The sublineage IIId, which appeared to be largely predominant in our samples, has recently been shown as heterogeneous.³³ To identify the IIId sublineage and to specify the variants of this sublineage present in the umbilical cord samples, we have developed new synthetic 5'- 32 P-labeled oligodeoxynucleotide probes allowing a sensitive hybridization of kDNA minicircle-derived amplicons (obtained from Tc121/Tc122 PCR).

Hybridization with both probes was IIId-specific, since only IIId reference strains hybridized to Oli-IIId-1 and Oli-IIId-2 probes whereas strains belonging to the other 6 different (sub)lineages did not (see Fig. 1A). Within the IIId sublineage

(using 6 different strains), the relative intensity of hybridization of both probes varied markedly from one strain to another, easily allowing typing of IIId variants: the strains MN and Bug hybridized more strongly with Oli-IIId-1 and Oli-IIId-2, respectively, whereas the amplicons from the strain TPK hybridized equally with both probes (Figure 1B). These hybridization patterns were stable and reproducible, since amplicons made in quadruplicate from DNA extracts prepared from the same strains 1 year later, gave similar results (Figure 1C). They allowed classifying of samples as IIId variants MN-like, Bug-like or TPK-like, according to the similitude of their hybridization pattern with the hybridization obtained with both probes and the corresponding reference strains.

This protocol, applied to the 320-bp amplicons obtained from umbilical cord blood samples, allowed to obtain several patterns of hybridization. We reassembled these patterns in 3 groups according to their similitude with the hybridization obtained with Oli-IIId-1 and Oli-IIId-2 probes and the reference strains: MN, Bug, and TPK. Hybridization patterns of some samples are shown in Figure 1D. This method allowed the IIId typing of 39 of the 41 cord blood samples with the following variant distribution: 15 MN-like, 16 Bug-like, and 8 TPK-like. The negative hybridization of both other cord blood samples with Oli-IIId-1 and Oli-IIId-2 probes indicates that they belong to other sublineage(s).

***T. cruzi* typing by multiplex amplification of mini-exon.** To complete the TcI and TcII typification of cord blood samples, and to investigate on possible mixed infections with both major lineages, we used a multiplex amplification of mini-exons with new primers. These primers allowed simultaneous detection of both major lineages in the same sample allowing detection of potential mixed infections, at least in the relative proportion of each to other from 20–80% (Figure 2A). By this method, all cord blood samples were typified as lineage TcII. Figure 2B illustrates this result for one sample, which did not hybridize with any oligonucleotide TcIIId probe. The presence of 130 bp amplicon clearly indicated that this sample belongs to lineage TcII. Infection with TcI and simultaneous infection with both TcI and TcII lineages were not detected in the analyzed cord blood samples.

***T. cruzi* typing by hybridization with hypervariable kDNA probes identifying the (sub)lineages I, IIb, and IIc.** To typify both remaining TcII umbilical cord blood samples that were IIId-negative, and to complete investigation on possible mixed infections, since the multiplex amplification of mini-exon displays a low sensitivity to detect unbalanced mixed infections (data not shown), we hybridized the sample kDNA minicircle-derived amplicons (obtained from Tc121/Tc122 PCR), with hypervariable kDNA probes identifying the (sub)lineages I, IIb, and IIc. As shown in Figure 3A, the probe for lineage I hybridized with the 6 TcI reference strains, and particularly with the reference strains SP104 and SO34, strains frequently used as TcI probes. The probe for the sublineage IIb hybridized only with the 5 IIb reference strains, and neither with the IIa, nor with the IIc reference strains (Figure 3B), nor with reference strains TcIIId and TcIIc (see example in the Figure 3D). The probe for the sublineage IIc hybridized strongly with the corresponding CL Brener strain, but also to a slighter extent with the reference cloned IIb strain Tu18 (Figure 3C) indicating a lower specificity of this probe. The 3 probes for the (sub)lineages I, IIb, and IIc were used with all the cord blood samples.

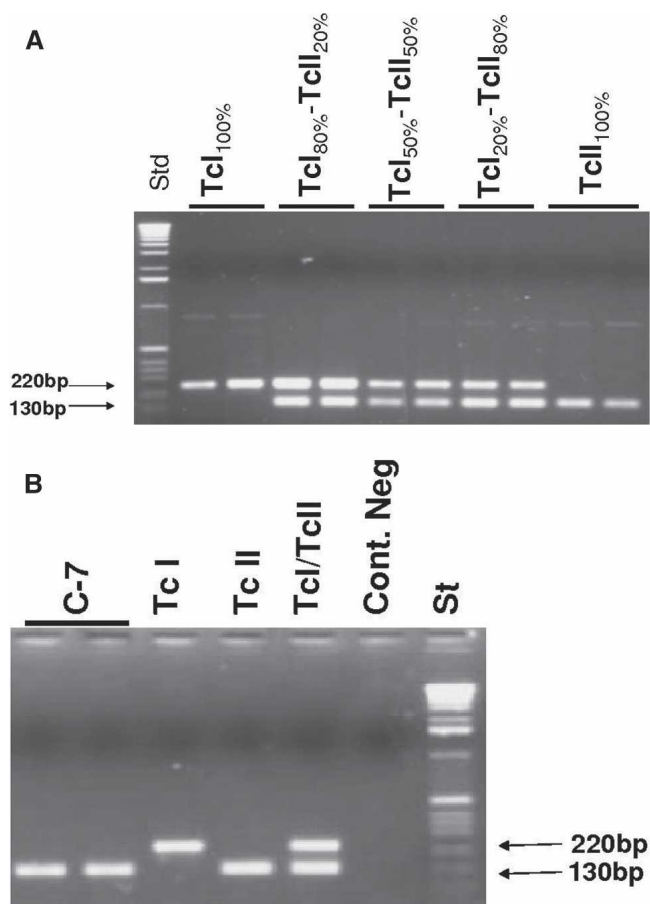


FIGURE 2. Multiplex PCR mini-exon typing. Mini-exon domain was amplified using simultaneous mixture of 4 primers described in Materials and Methods. **A**, Simultaneous amplification of 1 strain of lineage TcI (Cutia cl1) and 1 strain of lineage TcII (MNcl2) mixed in relative proportions TcI-TcII: 100-0%; 80-20%; 50-50%; 20-80%; 0-100%. **B**, Amplification of a umbilical blood sample (C-7) no hybridizing with any synthetic oligodeoxynucleotides probe of sublineage IId, compared with the reference strains of lineage TcI (Cutia cl1) and lineage TcII (MNcl2) alone or together.

The probe for lineage I showed hybridization with none of the cord blood samples (data not shown). As shown in the Figure 3D, the probe for the sublineage I Ib gave a hybridization signal in only 1 cord blood sample (Y-22). The probe for the sublineage I Ie allowed strong hybridization with 1 cord blood sample (C-7) previously negative with the other typing methods. It also slightly hybridizes with the previously mentioned I Ib-typed sample (Y-22). However, the weak intensity of Y-22 hybridization, with the sublineage I Ie probe, associated the previously mentioned lower specificity of this probe, highly suggest this sample being infected only with the I Ib sublineage.

So, altogether our results obtained with I, I Ib, and I Ie probes did not reveal patent mixed infections with these (sub)lineages in the cord blood samples of congenitally infected neonates.

Complementary typing of *T. cruzi* II sublineages by sequencing. In order to proceed to a complementary analysis of TcI Ib/TcI Ie hybridizing and TcI Id no-hybridizing cord blood samples (C-7 and Y-22), the hypervariable kDNA minicircle domain of this sample was amplified again using Tc121/Tc122

primers. Amplicons were subcloned in pCR2.1 using TA-TOPO-cloning. We sequenced the insert of a dozen of random picked colonies. Sequence comparison showed a clear homology with kDNA sequences of sublineage I Ib for the Y-22 sample and sublineage I Ie for C-7 sample. Reference sequences are available on GenBank (GenBank accession numbers: AJ747914-AJ748069; Telleria et al., in preparation).

Comparison of typified *T. cruzi* (sub)lineages in infected neonates and adults. The sublineage I Id was identified in 39/41 (95.1%) of umbilical cord blood samples. Both other congenital cases were infected with I Ib and I Ie sublineages (1/41 [i.e., 2.4% for each]). Blood samples of chronically infected adults were typified using the multiplex amplification of mini-exon, and hybridizations with probes identifying (sub)lineages I, I Ib, I Id, and I Ie, as for cord blood. The sublineages I Id and I Ie were identified in 16/17 (94.1%) and 1/17 (5.9%) of such adult samples, respectively. The proportions of I Id sublineage were similar in neonate and adult groups (χ^2 test $P > 0.05$). Infection with TcI and simultaneous infection with both TcI and TcII lineages were detected neither in the cord blood, nor in the adult samples.

Typified *T. cruzi* sublineages, morbidity-mortality, and parasitemia of congenital Chagas disease. A possible association between the typified *T. cruzi* (sub)lineages and morbidity-mortality of congenital Chagas disease was also investigated. The distribution of asymptomatic, symptomatic, and fatal forms of the disease among the congenital cases infected with the I Id variants was 21/39 (53.8%), 17/39 (43.6%), and 1/39 (2.6%), respectively, whereas both cases infected with I Ib and I Ie sublineages were asymptomatic. As shown in Table 2, the comparison of the distribution of these clinical forms of congenital Chagas disease among the I Id variants-infected cases did not show significant differences ($P > 0.05$).

A potential association between the typified *T. cruzi* (sub)lineages and the umbilical cord blood parasitemia, estimated from microscopic examination of microhematocrit tubes, was similarly studied. Parasitemia was estimated as low (40-400 parasites/mL) or high (> 400 parasites/mL) in 28/39 (71.8%) and 11/39 (28.2%) of the I Id congenital cases, respectively, whereas parasitemia was low in both I Ib-infected and I Ie-infected neonates. As indicated in Table 2, the frequencies of low and high parasitemias were similar whatever the involved I Id variants (χ^2 test $P > 0.05$).

DISCUSSION

Altogether, our results show that: (i) the I Ib, I Id, and I Ie *T. cruzi* sublineages, as well as different variants of the I Id sublineage, can induce congenital infection; (ii) the frequencies of I Id sublineage are similar in neonates and in adults of the area; (iii) the I Id-infected newborns displayed either asymptomatic, or severe and fatal clinical forms of congenital Chagas disease, as well as low or high parasitemia.

A conjunction of different methods using amplification of both n and kDNA of *T. cruzi*, and hybridization of amplicons present in the studied samples. Our results show that SCAR amplification²⁹ failed to typify parasite in blood samples with parasitemia < 400 parasites/mL. The previously described standard mini-exon PCR³¹ also failed for all the tested cord

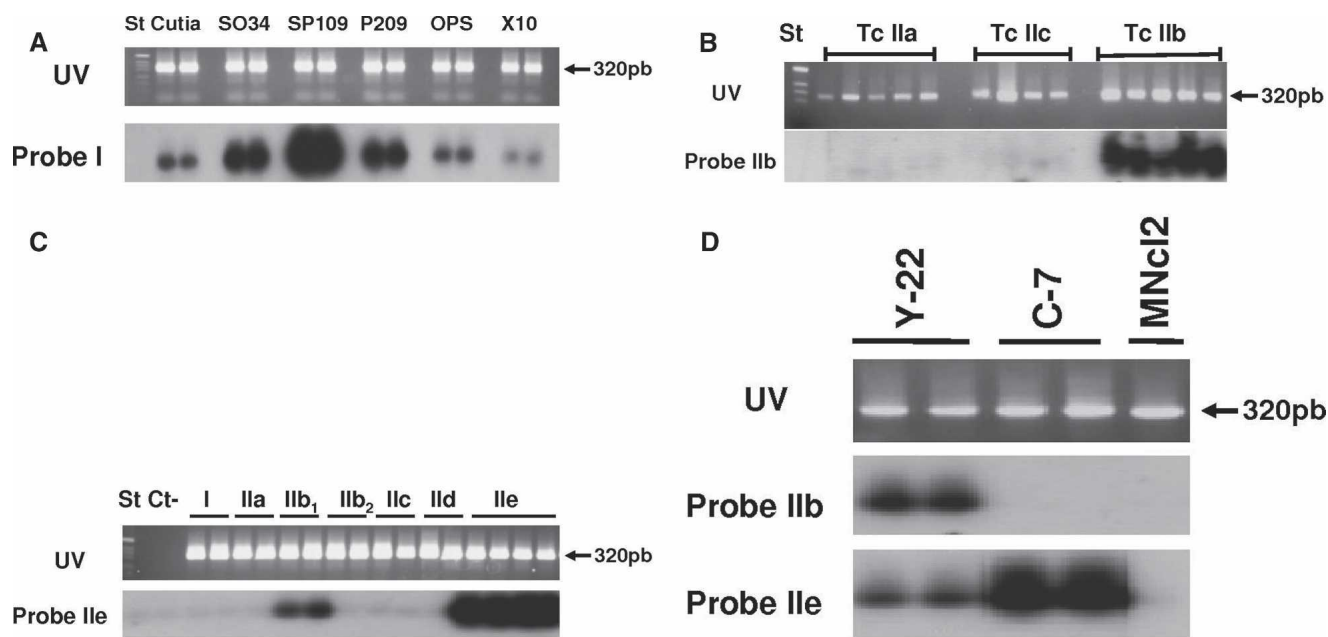


FIGURE 3. Hybridization of kDNA amplicons with kDNA probes. **A**, Amplicon generated from indicated reference strains of lineage TcI were hybridized with random labeled probe prepared from mixture of hypervariable domain of strains: SO34, P209, and SP104. Intensity of amplicon fluorescence (first line) is compared with autoradiography after hybridization with indicated probe (second line). **B**, Amplicon generated from reference strains of lineage IIa, IIc, and IIb (see Table 1) were hybridized with random labeled probe prepared from mixture of hypervariable domain of 5, sublineages IIb, strains (CBB, Tu18, Esmeraldo, Mas, and Iv). Intensity of amplicon fluorescence (first line) is compared with autoradiography after hybridization with indicated probe (second line). **C**, Hybridization of DNA extracted from 6 reference strains of different (sub)lineages: X10, CanIII, Esmeraldo, Tu18, M5631, MNc12, and CL Brener with sublineage IIe probe. Intensity of amplicon fluorescence (first line) is compared with autoradiography after hybridization with indicated probe (second line). **D**, Hybridization of 2 umbilical blood samples (Y-22 and C-7) and MNc12 (sublineage IId) with sublineage IIe probe. Intensity of amplicon fluorescence (first line) is compared with autoradiography after hybridization with indicated probe (second and third lines).

blood samples coming from acute infection and containing from 40 to > 1000 parasites/mL. To improve the sensitivity/specificity of typification, we designed new primers for the mini-exon multiplex amplification, as well as new hybridization probes. In this way, we were able to typify all available cord blood samples in which parasites were previously detected by direct microscopic examination (i.e., displaying ≥ 40 parasites/mL). This indicates the difficulty to easily perform such studies directly in blood samples coming from endemic areas of human *T. cruzi* infection, and the need for more simple and sensitive methodology to typify parasites.

We detected mixed infections neither in the adult-, nor in the cord blood analyzed samples, whereas such co-infections

with different (sub)lineages of *T. cruzi* have been reported in patients from Bolivia^{21,23} and Chile,^{22,24} as well as in tissue samples belonging to Argentinian or Brazilian patients.^{36,37} A low sensitivity/specificity of typification methods we have used for cord blood samples is likely not related to such negative results, since, as mentioned previously, this has been improved. Moreover, detection of mixed infections in Chile and Bolivia, from where our samples also originate, has been performed using standard methodology.^{21–24} Moreover, no artificial bias selecting a dominant (sub)lineage has been introduced in the present study, since we have directly used the blood samples instead of hemoculture in which such selection might occur during parasite multiplication.³⁸ Finally, particular attention was focused in adding immediately HCl-guanidine to cord blood samples to avoid possible degradation of low amount of DNA from minor parasite subpopulations. Since we cannot strictly exclude the possibility of mixed unbalanced infections with minor and hardly detectable parasite subpopulations, our results obtained in cord blood can be considered as representative of the dominant, if not the lone, parasite (sub)lineage infecting newborns.

All the 41 congenital cases that we have analyzed displayed infection with the lineage II, and 39/41 (95.1%) of them with the IId sublineage. Such distribution of infecting sublineages in newborns is similar to that observed in adult patients (16/17, 94.1%) coming from the same Bolivian area. This is in agreement with previous data indicating TcII, and particularly TcIId, as the main (sub)lineage infecting humans in Bolivia, TcI being less frequently encountered in patients living

TABLE 2

Distribution of clinical forms (AS = asymptomatic; S = symptomatic; M = mortal) and estimated parasitemia in congenital cases according to the detected variants of IId *T. cruzi* sublineage (low: 50–400 p/mL; high: > 400 p/mL; results are expressed as: number of detected cases/total number of patients per group; data in parentheses are %)

TcIId	Clinical forms			Parasitemia	
	AS	S	M	Low	High
MN-like	6/15 (40.0)	8/15 (53.3)	1/15 (6.7)	12/15 (80.0)	3/15 (20.0)
Bug-like	11/16 (68.7)	5/16 (31.2)	0/16 (0.0)	12/16 (75.0)	4/16 (25.0)
TPK-like	4/8 (50.0)	4/8 (50.0)	0/8 (0.0)	4/8 (50.0)	4/8 (50.0)

in endemic areas.^{8,21,23} So, the high frequency of the IId sublineage observed in the studied congenitally infected newborns cannot be attributed to a preferential association of this sublineage with congenital infection, but rather as indicative of the general distribution of *T. cruzi* populations in Bolivian patients. However, such distribution might be different in congenital cases observed in other South American endemic regions.

Our results show that, independently of their distribution frequencies, 3 different sublineages of *T. cruzi*, IId, IIb, and IIe, can be transmitted from mothers and involved in congenital infection. Moreover, using a new comparative hybridization approach, 3 variants of the IId sublineage could be also detected in congenital cases, confirming the previously expected heterogeneity of this sublineage.^{39,40} Infections with these 3 IId variants induce similar proportions of asymptomatic, severe, and fatal clinical forms of congenital Chagas disease, the latter displaying higher parasitemia. These results, in agreement with previous data obtained with isoenzymatic and/or endonuclease restriction analyses of limited numbers of human congenital infection cases,^{41,42} currently argue against the possibility that a particular *T. cruzi* sublineage/variants, defined using the presently available markers, might preferentially induce congenital infection and be more pathogenic for the fetus/neonate. They are in line with other studies performed in Bolivian children or adults, also unable to associate parasite molecular diversity with the different clinical forms of Chagas disease.^{23,43} If geographical variations in prevalence of clinical forms and morbidity of Chagas disease have been reported, as for instance, the higher frequency of digestive forms in Chile and central Brazil whereas they are uncommon in Venezuela and central America, or the asymptomatic undetermined form frequently observed in Amazon basin, there is at present no demonstration they are related to the genetic diversity of parasites.^{44,45} Altogether, such information suggests that either the presently DNA markers used to identify the (sub)lineages of *T. cruzi* are not sufficiently relevant in relation to human pathology^{9,46,47} or the pathology induced by *T. cruzi*, in human fetus/neonates or in adults should depend more on host resistance/susceptibility governed by its genetic background, as well as environmental and social factors.^{45,48–50} Indeed, recent data from our laboratory show that the capacity of infected fetuses/neonates to mount a parasite-specific IFN- γ response⁵ is associated with their capacity to control parasitemia and clinical forms of Chagas disease.⁵¹

In conclusion, our data suggest that *T. cruzi* DNA polymorphism, based on the presently available markers, is not associated with the occurrence of congenital infection or the development of severe clinical forms of congenital Chagas disease, and that investigation on the eventual role of parasite genetic diversity in human pathology must focus on more relevant parasitic markers.

Received March 30, 2006. Accepted for publication May 26, 2006.

Acknowledgments: The authors thank Marisol Cordova and the staff of the hospital materno-infantil German Urquidi (Cochabamba, Bolivia), Bertha Mendoza, Claudia Bernal, and the staff of the maternity Odon Ortega Flores de Yacuiba (Bolivia) and of the "Unidad de Neonatología" del Hospital Universitario San Juan de Dios (Tarija, Bolivia) for the management of patients; Mary Cruz Torrico, Patricia Rodríguez, Miguel Guzmán, Rudy Parrado, Amílcar Flores, and Tatiana Tellez (CUMETROP/LABIMED, U.M.S.S., Cochabamba, Bo-

livia), Jorge Postigo (UR010-IRD, La Paz, Bolivia) and Dolores Rangel y Nelly Aguado (Hospital Universitario San Juan de Dios and Laboratorio de Epidemiología-SEDES, Tarija, Bolivia) for the serological and parasitological diagnosis of patients. C. Alonso-Vega is fellow of the "Association pour la promotion de l'éducation et la formation à l'étranger" (APEFE, "Communauté Française de Belgique"). This study was supported by the Conseil Interuniversitaire de la Communauté Française de Belgique (CIUF), the « Fonds National de la Recherche Scientifique Médicale » (Belgium, conventions 3.4.595.99/3.4.615.05), and the "Institut de Recherche pour le Développement" (IRD, France). Part of the present data have been presented to the international colloquium: "Infección congénita por *Trypanosoma cruzi*: desde los mecanismos de transmisión hasta una estrategia de diagnóstico y control", held on November 6–8, 2002, at Cochabamba, Bolivia.

Authors' addresses: Myrna Virreira and Zulema Bustamante, Faculté de Médecine, Université Libre de Bruxelles, 808 route de Lennik CP 611, B-1070 Brussels, Belgium, E-mails: mvirreir@ulb.ac.be and ciuf2@bio.umss.edu.bo. Cristina Alonso-Vega, Marco Solano, and Faustino Torrico, Facultad de Medicina, Universidad Mayor de San Simon (U.M.S.S.), Cochabamba, Bolivia, E-mails: cristina@med.umss.edu.bo, msolano@med.umss.edu.bo, and ftorrico@med.umss.edu.bo. Juan Jijena, Unidad de Neonatología, Hospital "San Juan de Dios", Tarija, Bolivia, E-mail: jijena@cosett.com.bo. Laurent Brutus and Dominique Schneider, Mother and Child Health Research Unit (UR10), La Paz, Bolivia, E-mails: laurentbrutus@club-internet.fr and dschneider@club-internet.fr. Carine Truyens and Yves Carlier, Laboratoire de Parasitologie, Faculté de Médecine, Université Libre de Bruxelles (U.L.B.), Brussels, Belgium, E-mails: ctruyens@ulb.ac.be and ycarlier@ulb.ac.be. Michal Svoboda, Laboratoire de Chimie Biologique, Faculté de Médecine, U.L.B. 808 route de Lennik CP 611, B-1070 Brussels, Belgium, Telephone: 32-2-555-62-25, Fax: 32-2-555-62-30, E-mail: msvobod@ulb.ac.be.

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Virreira M., Alonso-Vega C., Solano M., Jijena J., Brutus
Laurent, Bustamante Z., Truyens C., Schneider D., Torrico F.,
Carlier Yves, Svoboda M. (2006)

Congenital Chagas disease in Bolivia is not associated with
DNA polymorphism of *Trypanosoma cruzi*. American Journal
of Tropical Medicine and Hygiene, 75 (5), 871-879

ISSN 0002-9637