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

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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 TcIId sublineage. The Ib, IId, or Iic T. cruzi sublineages, as well as different variants of the IId sublineage, were detected in infected neonates, whereas mixed infections were not found. The frequencies of the IId sublineage were similar in neonates (95.1%) and adults of the same area (94.1%). The IId-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. 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. 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. 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. Another possibility relates to the parasitic molecular polymorphism, since T. cruzi evolves in the form of independent parasitic lineages, displaying different biologic properties. 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.

There is a consensus to recognize 2 major lineages of T. cruzi: TcI and TcII. The lineage TcII can be subdivided in 5 sublineages: Ila, Ib, Iic, IId, and IIe on the basis of genotypical and phenotypical properties estimated by various methods. It has been also reported that the sublineages Ila and Iic derive from the fusion of the ancestral (sub)lineages I and Ib, and that the sublineages IId and IIe come from an hybridization between the sublineages Ib and Iic. 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. Both lineages can infect humans, and, though the sublineage IId 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.

The aim of our study was to typify the T. cruzi (sub)lineage(s) of congenitally infected Bolivian newborns to determine 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 IId 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. 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. Congenital infection with T. cruzi was sought for by direct microscopic examination of blooduffy coat col-
lected in 3–4 microhematocrit heparinized tubes, as previously described. The detection limit of such parasitological detection was estimated to 40 parasites/mL. The number of positive microhematocrit tubes containing parasites, as well as the number of detected parasites per tube allowed a semiquantitative 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 Simón) 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, Ib, II, and Ie (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 Ia and Ic have not been detected in human infection in this country. 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 described 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, Ia, Ib, Ic, and Ie, using the 3 pairs of primers described by Brisse et al. 29 The primers p3 (5′-GGC TGC ATC GCA GGA GAG TGT CC-3′) and p6 (5′-GGC TGC ATC GCA GGA GAG TGT A-3′), after 40 cycles (94°C for 20 seconds, 57°C for 20 seconds, 72°C for 30 minutes) and a final elongation of 7 minutes, give amplification only for TcIa, Ib, and Ic (657 bp), and not for the other lineage/sublineages. The primers p7 (5′-CGC TGC AGA ACC AAC AAG AAG TTG) and p8 (5′-GGC TGC AGA ACC AAC AAG AAG TTG) and p9 (5′-GGC TGC AGA ACC AAC AAG AAG TTG), 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, Ic, and IIa amplicon sizes (455, 474, and 355 bp, respectively) from I, Ib, and Ie (all of 410 bp). Finally, the primers p9 (5′-TGC GCT CAT CCT GCT GGT TTA CCG-3′) and p10 (5′-TCC 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 AGC GGG KGA GAT GCA TGA) and Tc122 (5′-GTT TCG ATT GTT GGT GGT GTT GGA TTA ATC TA) complementary to the constant domain of kDNA minicircles. 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 sequences: (i) TcI: forward (5′-CAG CGC CAC AGA AAG TGT T-3′); reverse (5′-GCT CCT TCA TGG TTG CGT C-3′); (ii) TcII: forward (5′-CCT CCT GTG TTT TCC GGT GT-3′); reverse (5′-ACA CTT CCG AGG 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

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**Table 1**

<table>
<thead>
<tr>
<th>TcI</th>
<th>TcIIa</th>
<th>TcIIb</th>
<th>TcIIc</th>
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acetate, 1 mM EDTA), in the presence of 0.5 μg/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 (Schleimer & Schuell), dried and crosslinked on UV-Appitek transluminator 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 strains34,35: (i) the strains SO34, SP104, and P209 of lineage I; (ii) the strains CBB, Tu18, Esmeraldo, Mas, and 1iv of sublineage IIb; and (iii) the CL Brenner 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 “Wizard 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 μCi of [γ-32P]ATP (3000 Ci/mnmole) and 0.5 μl (5 units) of T4 nucleotide kinase in the final volume of 10 μl. The reaction was stopped by addition of 60 μl of buffer SET (100 mM NaCl, 20 mM Tris-HCl (pH 8), 10 mM EDTA) and the mix-

![Figure 1](image-url)
ture was heated at 95°C for 2 minutes. The purified mixture of hypervariable kDNA fragments (about 25 ng) was labeled using 50 μCi of [α-32P]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× SSC, 0.5% SDS, 5× Denhart’s solution (1% ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin). After 1 hour of prehybridization, 250 μl of denatured 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× 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× SSC, 0.1% SDS solution at 65°C. Detection of the 32P-labeled probes was performed by exposure to Kodak Film X-Omat Blue XB-1 (PerkinElmer Life Sciences).

Cloning and sequencing. Polymerase chain reaction products were cloned using the pCR®II TOPO TA-cloning kit (Invitrogen) according to the manufacturer’s recommendations. One μl of PCR product was incubated for 30 minutes at room temperature with 25 μl of TOPO10 competent bacteria. Transformed bacteria were then selected in LB plate containing 75 μg ampicillin/ml. Plasmids from randomly selected colonies were isolated by alkaline lysis mini-preparations 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 Siences).

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 TcIId. DNA amplification performed from the other 7 cord blood extracts (6 of them having parasitemia ≤ 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 oligodeoxyribonucleotide probes identifying variants of sublineage IId. The sublineage IId, which appeared to be largely predominant in our samples, has recently been shown as heterogeneous.33 To identify the IId sublineage and to specify the variants of this sublineage present in the umbilical cord samples, we have developed new synthetic 5′-32P-labeled oligodeoxyribonucleotide probes allowing a sensitive hybridization of kDNA minicircle-derived amplicons (obtained from Tc121/Tc122 PCR).

Hybridization with both probes was IId-specific, since only IId reference strains hybridized to Oli-IId-1 and Oli-IId-2 probes whereas strains belonging to the other 6 different (sub)lineages did not (see Fig. 1A). Within the IId sublineage (using 6 different strains), the relative intensity of hybridization of both probes varied markedly from one strain to another, easily allowing typing of IId variants: the strains MN and Bug hybridized more strongly with Oli-IId-1 and Oli-IId-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 latter, gave similar results (Figure 1C). They allowed classifying of samples as IId variants MN-like, Bug-like or TPK-like, according to the similarity 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-IId-1 and Oli-IId-2 probes and the reference strains: MN, Bug, and TPK. Hybridization patterns of some samples are shown in Figure 1D. This method allowed the IId 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-IId-1 and Oli-IId-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 typed as lineage TcII. Figure 2B illustrates this result for one sample, which did not hybridize with any oligonucleotide TcII 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 IId. To typify both remaining TcII umbilical cord blood samples that were IId-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 made in quadruplicate from DNA extracts prepared with both probes and the corresponding reference strains. We reassembled these patterns in 3 groups according to their similitude with the hybridization obtained with Oli-IId-1 and Oli-IId-2 probes and the reference strains: MN, Bug, and TPK. Hybridization patterns of some samples are shown in Figure 1D. This method allowed the IId 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-IId-1 and Oli-IId-2 probes indicates that they belong to other sublineage(s).

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In order to proceed to a complementary analysis of the IIb and IIe 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 IIb variants was 21/39 (53.8%), 17/39 (43.6%), and 1/39 (2.6%), respectively, whereas both cases infected with IIb and IIe sublineages were asymptomatic. As shown in Table 2, the comparison of the distribution of these clinical forms of congenital Chagas disease among the IId 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 IId congenital cases, respectively, whereas parasitemia was low in both IIb-infected and IIe-infected neonates. As indicated in Table 2, the frequencies of low and high parasitemias were similar whatever the involved IId variants (χ² test P > 0.05).

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 IIb gave a hybridization signal in only 1 cord blood sample (Y-22). The probe for the sublineage IIe 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 IIb-typed sample (Y-22). However, the weak intensity of Y-22 hybridization, with the sublineage IIe probe, associated the previously mentioned lower specificity of this probe, highly suggest this sample being infected only with the IIb sublineage.

So, altogether our results obtained with I, IIb, and IIe 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 TcIIb/TcIIe hybridizing and TcIIId 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 IIb for the Y-22 sample and sublineage IIe 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 IId was identified in 39/41 (95.1%) of umbilical cord blood samples. Both other congenital cases were infected with IIb and IId 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, IIb, IId, and Ile, as for cord blood. The sublineages IId and Ile were identified in 16/17 (94.1%) and 1/17 (5.9%) of such adult samples, respectively. The proportions of IId sublineage were similar in neonate and adult groups (χ² 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. Altogether, our results show that: (i) the IIb, IId, and Ile T. cruzi sublineages, as well as different variants of the IId sublineage, can induce congenital infection; (ii) the frequencies of IId sublineage are similar in neonates and in adults of the area; (iii) the IId-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 was necessary to complete the typing of parasite (sub)lineages present in the studied samples. Our results show that SCAR amplification failed to typify parasite in blood samples with parasitemia < 400 parasites/mL. The previously described standard mini-exon PCR also failed for all the tested cord
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 \textit{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 \textit{T. cruzi} have been reported in patients from Bolivia\cite{21,23} and Chile,\cite{22,24} as well as in tissue samples belonging to Argentinian or Brazilian patients.\cite{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.\cite{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.\cite{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

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<th>TcIId</th>
<th>Clinical forms</th>
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<td>AS (asymptomatic)</td>
<td>S (symptomatic)</td>
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<tr>
<td>MN-like</td>
<td>6/15 (40.0)</td>
<td>8/15 (53.3)</td>
</tr>
<tr>
<td>Bug-like</td>
<td>11/16 (68.7)</td>
<td>5/16 (31.2)</td>
</tr>
<tr>
<td>TPK-like</td>
<td>4/8 (50.0)</td>
<td>4/8 (50.0)</td>
</tr>
</tbody>
</table>

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 Ila, Iic, and IId (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 Irv). 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 Iic 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 Iic probe. Intensity of amplicon fluorescence (first line) is compared with autoradiography after hybridization with indicated probe (second and third lines).
in endemic areas. 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*, Ib, IId, and IJe, 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. 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, 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. 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. 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, or the pathology induced by *T. cruzi*, in human fetuses/neonates or in adults should depend more on host resistance/susceptibility governed by its genetic background, as well as environmental and social factors. Indeed, recent data from our laboratory show that the capacity of infected fetuses/neonates to mount a parasite-specific IFN-\( \gamma \) response is associated with their capacity to control parasitemia and clinical forms of Chagas disease.

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.

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