SUPPLEMENTAL MATERIAL

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Molecular methods. *Measures to limit/avoid carry-over contamination.* The main source of false-positive results is sample contamination by product carryover from previous PCR reactions targeting the same amplicon. To limit/avoid this risk, we took the following measures:

- Guanidine EDTA blood (GEB) maternal and cord blood samples were stored separately from all other reagents used in DNA extraction or PCR and were only manipulated in the room dedicated to DNA extraction.
- The chamber of the Maxwell instrument for DNA extraction was cleaned with alcohol and treated by ultraviolet (UV) lighting for 40 minutes before DNA extraction and for 30 minutes after use.
- The following steps were performed in four physically separated laboratories: 1) DNA extraction, 2) preparation of PCR mixes and controls and addition of template DNA, 3) PCR amplification, and 4) gel electrophoresis. Access to these rooms is highly restricted to technicians performing the PCRs.
- Dedicated pipettes and disposable laboratory coats and materials were used at each step. After amplification, PCR tubes were opened only in laboratory #4. No one was

allowed to return from laboratory #4 to any other laboratory on the same day.

- Commercial reagents (which cannot be contaminated by *T. cruzi* DNA, as it is not an environmental microbe) were aliquoted in a room located on a separate floor from laboratories #1–4 before being stored in laboratory #2 for use. Each aliquot was used only once.
- PCR set-up in laboratory #2 was performed in two separate PCR workstations equipped with UV lighting that were turned on for 40 minutes before working with the samples (one workstation to prepare PCR mixes and the other to handle samples and controls).
- Rigorous handling conditions were implemented, including the use of aerosol-resistant pipette tips. Benches and nondisposable materials were decontaminated with diluted bleach before use, and laboratory coasts and gloves were frequently changed.

In addition to the risk of carry-over contamination existing for all PCRs, there is a risk for cord blood samples to be contaminated by parasite DNA from maternal blood covering the umbilical cord during delivery. To limit this risk, the surface of the umbilical cords was cleaned with a sterile dry gauze before cord blood was collected.

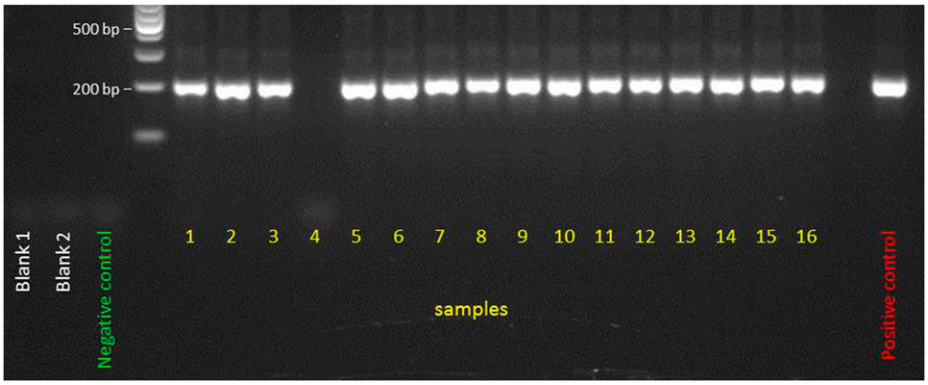
DNA extraction and quality. DNA extraction from maternal and umbilical cord GEB samples was performed with the automated Maxwell® RSC Instrument (Promega Benelux, Leiden, The Netherlands) using standard elution volume (SEV) or low elution volume (LEV) Blood DNA Kit (Promega) following the instructions of the manufacturer. DNA was extracted from 300 µL of maternal GEB samples with LEV kit and eluted in 100 µL of elution buffer, with 85 µL of eluate recovered. For cord blood samples, DNA was extracted with the SEV kit from 200 µL GEB samples and eluted in 300 µL elution buffer, with 200 µL of eluate recovered. DNA concentrations were measured by spectophotometry at 260 nm with the NanoDrop ND-1000 spectrophotometer. The purity of extracted DNA was assessed by measuring absorbance at 230 and 280 nm. The absence of PCR inhibitors in the DNA was assessed by amplifying a fragment of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (see Supplemental Figure 1). In the rare case of a negative result, the sample was discarded and the DNA was re-extracted (e.g., sample 4 in Supplemental Figure 1).

Conventional PCR assays. Primers. *Trypanosoma cruzi* DNA was detected using the specific primer sets **Tcz1–Tcz2**, which amplify a 188-base pair (bp) segment of the highly repetitive genomic satellite DNA (satDNA) and **121–122**, which amplify a 220 bp sequence present in kDNA minicircles.[22,51,52] Sequences of primers were Tcz1: 5′-CGA GCT CTT GCC CAC ACG GGT GCT-3′, Tcz2: 5′-CCT CCA AGC AGC GGA TAG TTC AGG-3′, 121: 5′-AAA TAA TGT ACG GGK GAG ATG CAT GA-3′, 122: 5′-GGT TCG ATT GGG GTT GGT GTA ATA TA-3′. PCR amplification of a fragment of the human GAPDH gene was performed using the primers pair ID 3428 available in the public database RTPrimerDB (http://www.rtprimerdb.org). It gives an amplicon of 199 bp. All primers were from Life Technologies Europe (Gent, Belgium).

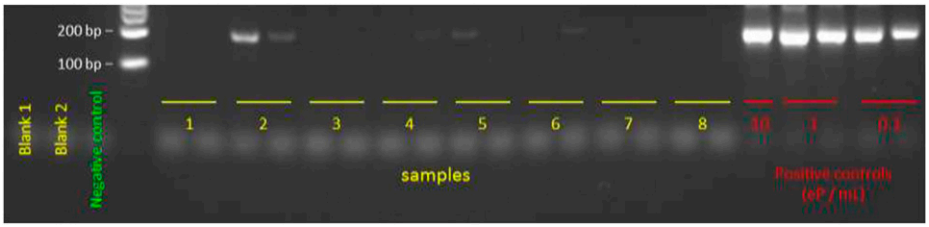
PCR amplification. To detect parasite DNA, each sample was tested in duplicate using the two *T. cruzi*-specific primer sets **Tcz1–Tcz2** and **121–122**. Two microlitres (DNA from maternal samples) or 4 µL (DNA from cord blood samples) of DNA template was added to each reaction in a total volume of 20 µL containing 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotides triphosphate (dNTP), 0.5 µM of each primer (Tcz1–TcZ2 or 121–122), and 0.5 U/assay of Taq polymerase. Taq polymerase and dNTPs concentrations were reduced to 0.4 U/assay and 0.15 mM, respectively, for Tcz1–Tcz2 PCR assays performed on samples extracted with LEV kit. The cycling program with Tcz1–Tcz2 included an initial denaturation at 94°C for 5 minutes, 35 amplification cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, and a terminal extension at 72°C for 10 min. Amplification with 121–122 primers was as follows: denaturation at 95°C for 5 minutes, 35 amplification cycles at 95°C for 30 s, 55.5°C for 30 s, and 72°C for 1 minute, and a terminal extension at 72°C for 1 minute. GAPDH amplification was performed under the same conditions described previously with Tcz1–Tcz2 primers.

The Hot start polymerase GoTaq® G2, dNTP Solution Mix, $MgCl_2$, and buffer were from Promega. Amplifications were performed using the BioRad T100 thermal cycler (Bio-Rad Laboratories, Temse, Belgium) in 0.2-mL thinwalled PCR tubes.

Detection of specific amplicon bands. Five microlitres of PCR amplification product were submitted to gel electrophoresis in 2% agarose gels and tris-acetate-EDTA (TAE) buffer (40 mM Tris, 40 mM acetate, 1 mM EDTA) in the presence of 0.5 g/mL of GelRed nucleic acid stain (VWR



Supplemental Figure 1. Polymerase chain reaction with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers giving an amplicon of 199 bp. Purified parasite DNA was used as negative control and DNA purified from an uninfected individual as positive control. Blanks are nuclease-free water. Sixteen samples were tested per run. In this case, GAPDH was not amplified from sample 4, and extraction was repeated.



Supplemental Figure 2. Polymerase chain reaction with Tcz1–Tcz2 primers giving the specific amplicon of 188 bp. Purified parasite DNA from 10, 1, and 0.1 parasites/mL was used as a positive control. A blood sample from an uninfected individual was used as a negative control. Blanks are nuclease-free water.

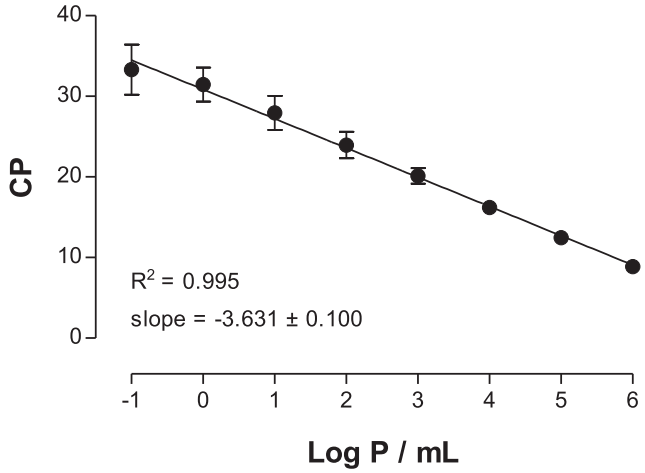
International, Leuven, Belgium), which is markedly more sensitive (and safer) than the classically used ethidium bromide for DNA detection, particularly for low size DNA amplicons.[53] The 100 bp BenchTop DNA ladder (Promega) ranging from 100 to 1,000 bp was used as a DNA size marker. The gels were examined using the GenoSmart imaging system with a GenoView transilluminator (VWR International). Gel photographs were taken with overexposure to detect any trace of *T. cruzi* DNA amplicon. The result of the PCR was noted positive whatever the intensity of the amplicon band, even if it was faint (i.e., a negative result means that no specific amplicon band could be seen even by strongly increasing the brightness of the gel image).

Control of PCR performances and absence of contamination. Blanks (nuclease-free water), negative control (DNA extracted from GEB samples from uninfected individuals), and positive control (DNA purified from *T. cruzi* trypomastigotes obtained by culture,[54] Tulahuen strain–genotype VI) were included in each PCR run of eight duplicate samples. Two blanks were performed, one before (blank 1) and one after treatment of samples and positive controls. Positive controls were used at *T. cruzi* concentrations of 10, 1, and 0·1 equivalent parasite (eP)/mL. All PCR runs gave an intense amplicon band with the positive controls 10 and 1 eP/mL. *Trypanosoma cruzi* DNA used at 0·1 eP/mL gave a detectable band in 80% and 93% of PCR runs with Tcz1–Tcz2 and 121–122 primers, respectively (Supplemental Figure 2). Any PCR run showing a faint specific amplicon band in blanks or negative control, or showing no specific band in the positive control "Tc 1 eP/mL," was discarded and repeated. Altogether, our protocols for DNA extraction and PCR, the sensitivity of DNA staining on gels, and the exposure settings for gel analysis led to an ultrasensitive conventional PCR.

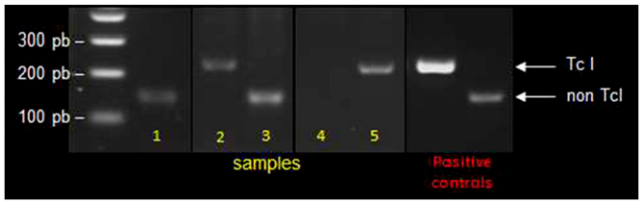
Real-time PCR. Real-time PCR (qPCR for quantitative PCR) was performed using a LightCycler® 480 system (Roche Diagnostics) according to the manufacturer's instructions. Reactions were performed in 25 µL final volume with 160 nM *T. cruzi* primers Tcz1–Tcz2 (same as described previously) and Perfecta SYBRGreen SuperMix (Quanta Biosciences, VWR). Each PCR reaction contained 5 µL of purified blood DNA (diluted 5-fold just before use) or 5 µL *T. cruzi* DNA standard (see hereunder). The amplification protocol consisted of a denaturation phase at 95°C for 5′ (RampRate 4.40 µC/second) followed by 50 cycles of amplification (95°C 3′ [RampRate 4.40 µC/second], 65°C 1′ [RampRate 2.20 µC/second]).[55]

Fluorescence emission was measured at the end of each elongation step. A melting curve phase program was applied with a continuous fluorescence measurement between 50°C and 95°C (RampRate 2.20 µC/second). The identity of the amplified products was checked by analysis of the melting curve carried out at the end of amplification. Each LightCycler run contained a negative control (no DNA added to the reaction). Each DNA sample was quantified in duplicate. Duplicate values for each DNA sample were averaged and parasite equivalent load was calculated by plotting the crossing point values against each standard of known concentration and calculation of the linear regression line of this curve. Supplemental Figure 3 shows the mean of standard curves of all qPCR performed. Results are expressed as eP/mL and were multiplied by the dilution factor of samples (×5).

Standards were generated from blood obtained from an uninfected individual initially spiked with 10^8/mL *T. cruzi* trypomastigotes (previously obtained in supernatant of infected fibroblasts and cryopreserved) from Tulahuen strain (TcVI



Supplemental Figure 3. *Trypanosoma cruzi* DNA standard curve obtained by real-time polymerase chain reaction (PCR) with Tcz1–Tcz2 primers. Results are expressed as the Log of the number of parasites per millilitre of blood and represent the average of the 46 PCR runs performed to test all samples.



Supplemental Figure 4. Multiplex polymerase chain reaction for *Trypanosoma cruzi* genotyping in TcI vs. non-TcI discrete typing units with MV1 to MV4 primers, giving amplicons of 220 and 130 bp, respectively. Positive controls were DNA purified from *T. cruzi* epimastigotes of genotype I (strain X10/I) or VI (strain CL Brener). Results for five samples are shown.

SUPPLEMENTAL TABLE 1

Parasitological examination and cord blood polymerase chain reaction (PCR) and *Trypanosoma cruzi* serology at 10 months in infants with congenital transmission, Argentina, Honduras, and Mexico, 2011–2013.

Country	Subject ID	Stat-Pak cord blood	T-Detect cord blood	ELISA Wiener maternal blood	Direct parasitological examination cord blood	Direct parasitological examination at 4–8 weeks	Cord blood PCR	Stat-Pak at 10 months	T-Detect at 10 months	ELISA Wiener at 10 months	ELISA Hemagen at 10 months	Maternal conventional PCR	Maternal quantitative PCR (eP/mL)
Argentina	1	+	+	+	–	–	+	+	+	+	+	+	2.534
Argentina	2	+	+	+	–	–	+	+	+	+	+	–	0.579
Argentina	3	+	ND	+	–	–	+	+	+	+	+	+	10.738
Argentina	4	+	+	+	–	+	+	–	–	–	–	+	13.785
Argentina	5	+	+	+	–	–	+	+	+	+	+	+	8.109
Argentina	6	+	+	+	+	ND	+	–	–	–	–	+	1.855
Argentina	7	+	+	+	+	ND	+	–	–	–	–	+	6.948
Argentina	8	+	+	+	–	–	–	+	+	+	+	+	3.996
Argentina	9	+	+	+	–	–	+	+	+	+	+	+	41.712
Mexico	10	+	+	+	+	ND	+	–	–	–	ND	+	2.689
Mexico	11	+	+	+	–	–	+	+	–	–	+	+	1.470

ELISA = enzyme-linked immunosorbent assay; ND = not done.

DTU). Spiked blood was lysed by the addition of one volume of guanidine hydrochloride 6 M—EDTA 0.2 M and DNA was extracted as described previously. The standard curve was prepared with serial 10-fold dilutions of purified DNA in water. The standard curve (range 10^6 to 10^{-1} parasites/mL—Supplemental Figure 3) was repeated on each microplate.

Trypanosoma cruzi *genotyping by multiplex PCR.* The mini-exon gene's intergenic region was amplified using a multiplex PCR, with the primers described in Virreira et al.,[11] which we have named MV1 to MV4. It allowed us to discriminate the *T. cruzi* genotypes (or discrete typing unit [DTU]) present in blood samples as TcI and non-TcI (comprising TcII to TcVI DTUs) by giving amplicons of 220 and 130 bp, respectively (Supplemental Figure 4). Two microlitres (DNA from maternal samples—LEV samples) or 4 µL (DNA from cord blood samples—SEV samples) of DNA template were added to each reaction in a total volume of 25 µL containing 0.5 µM of each primer, 1.75 mM $MgCl_2$, 0.1 (LEV samples) or 0.15 mM (SEV samples) of each dNTP and 0.25 (LEV samples) or 0.38 U/assay (SEV samples) of Taq polymerase. Each sample was tested in duplicates. Each PCR run comprised blanks (water), a negative control (DNA extracted from GEB samples from uninfected individuals), and positive controls. Positive controls were DNA extracted from *T. cruzi* epimastigotes of genotype I (strain X10/I) or VI (strain CL Brener) available thanks to a gift from M. Lewis and M. Miles (London School of Hygiene and Tropical Medicine, United Kingdom).

Sequence analysis. To obtain enough material for sequencing, purified DNA from GEB samples was amplified by two successive runs of the multiplex PCR described previously for genotyping. Amplicons were sequenced at Beckman Coulter Genomics by the Sanger method,[56] using each of the PCR primers in separate reactions (MV1 and MV2 when TcI genotype was identified in the sample, MV3 and MV4 when non-TcI was identified). Sequences were analyzed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences were edited manually and aligned with *T. cruzi* reference sequences for DTU assignment (TcI or non-TcI DTUs) using MEGA version 5.2 software.[25]

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