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Vertical transmission of *Trypanosoma cruzi* infection: quantification of parasite burden in mothers and their children by parasite DNA amplification

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ABSTRACT

The relationship between parasite burden and vertical transmission of *Trypanosoma cruzi* was studied in pairs of chronically infected women and their children in a non-endemic area. Parasitemia was quantified by quantitative polymerase chain reaction (qPCR) in the peripheral blood amplifying a nuclear *T. cruzi* DNA and expressed as equivalent amounts of CL Brener parasites DNA per ml (eP/ml). Similar levels of parasitemia were found in non-transmitting pregnant women and in non-pregnant women: 1.8 ± 0.5 and 1.5 ± 0.7 eP/ml, respectively. In women pregnant with infected children parasitemia was 11.0 ± 2.7 eP/ml ($n = 20$). In 12 of their neonates the infection was detected by microscopic observation of the parasites in peripheral blood in the 1st month of age. These children had variable levels of parasitemia ($13\,000 \pm 7000$ eP/ml), that were about 600-fold higher than that found in their mothers. To our knowledge, this is the first quantitative evaluation of parasitemia in these three groups of women and in their congenitally infected children. These parasite quantifications could be a basis to plan the control of mother-to-child transmission of *T. cruzi*.

1. Introduction

Trypanosoma cruzi, the protozoan parasite that causes Chagas disease, is transmitted mainly by blood-sucking vector insects that can be controlled by insecticide spraying of houses. When vectorial and transfusional transmission is controlled, the importance of mother-to-child transmission increases. Congenital infection is the only mode of *T. cruzi* transmission that cannot be prevented, as pregnant women cannot be prescribed trypanocidal treatments because of the latter's possible teratogenic effects.

In Argentina, *T. cruzi* is vertically transmitted in about 10% of pregnancies in seropositive women.[1–3] Early diagnosis of children under 8 months relies on detection of the parasite by microscopy, because specific IgG antibodies can be transferred by their mothers. If parasitologic assays fail to detect the infection, serologic assays allow detection of additional cases of congenital transmission later on, when the maternal antibodies disappear. This laboratory screening is complex for resource-poor endemic areas and as the congenital infections are mostly asymptomatic or cause nonspecific symptoms, most cases go undetected.

Mother-to-child transmission of *T. cruzi* infection is related to several factors, such as maternal responses,[2] placental histopathology, parasite burden[4–6] and intraspecific characteristics of *T. cruzi*. Regarding parasitemia, positive *T. cruzi* hemocultures are more frequent in mothers

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with congenitally infected children than in those who do not transmit the infection.[4] High parasitemia in pregnant women, as determined microscopically, is also associated with a higher prevalence of infected newborns delivered.[7,8]

Quantitative polymerase chain reaction (qPCR) has been reported as a promising tool for the detection and quantification of *T. cruzi* in tissues of experimentally infected mice.[9] The technique has also been successfully used to quantify parasitemia in human infections caused by *T. cruzi*[10] and other pathogenic parasites.[12–14] At delivery, qPCR has demonstrated higher levels of parasitemia in women who delivered infected children than in non-transmitting mothers.[5,6]

In the present study, we carried out qPCR during pregnancy in women chronically infected with *T. cruzi* who delivered congenitally infected children, in infected women who delivered non-infected children and in non-pregnant seropositive women, in order to better design the control of congenital transmission of *T. cruzi* infection.

2. Materials and methods

2.1. Participants

Around 700 women, pregnant and non-pregnant, non-infected or infected with *T. cruzi*, were studied during the period 2008–2011. Diagnosis of *T. cruzi* infection was carried out at the Instituto Nacional de Parasitología (INP) Dr M Fatala Chaben-ANLIS, the reference center for diagnosis in Argentina. At the INP, venous blood was withdrawn from pregnant and non-pregnant women, either non-infected or infected with *T. cruzi*. Pregnant women who presented for diagnosis at INP were interviewed and, when they were seropositive, their children diagnosed. We included in our study all the mothers of infected children; whereas women were randomly selected from among the seropositive mothers of non-infected children and non-pregnant infected or non-infected women. Sixty percent of seropositive women (pregnant with infected and non-infected children, and non-pregnant) were from Argentina, half of them born in the northern endemic areas; 35% came from Bolivia and 5% from Paraguay. No significant differences were found in the nationality distribution of the three groups of *T. cruzi* seropositive women. All the infected women and children included in the study resided in the non-endemic area, did not travel to the endemic area, and did not receive any blood transfusions in the year before the study. They received medical assistance in public hospitals located in non-endemic urban areas of Buenos Aires and surrounding cities. The infected women were in the asymptomatic phase of the chronic *T. cruzi* infection and had not been treated with trypanocidal drugs.

2.2. Diagnosis of T. cruzi infection

The presence of specific antibodies in sera was determined by indirect hemagglutination (IHA), indirect immunofluorescence (IFA) and enzyme-linked immunosorbent assay (ELISA). IHA and IFA were carried out with two-fold serial dilutions of sera and considered reactive when a dilution $\geq$1/32 was positive. The ELISA was carried out with a 1/200 dilution of the samples incubated in microplates precoated with *T. cruzi* antigens. The optical density (OD) of the ELISA was quantified and the sample considered positive when OD$\geq$0.200. Women were considered infected when at least two of the serological tests were positive, according to the criteria of the WHO and the Argentinean guidelines.

Peripheral blood from infants of the infected mothers was obtained during their first, sixth and 12th months of age, or until *T. cruzi* infection was detected. The presence of *T. cruzi* in the children's blood was determined by the INP micromethod.[3] Briefly, 0.5–1.0 ml of heparinized blood was centrifuged in Eppendorf tubes. The buffy coat was distributed between two slides and examined carefully by light microscopy at $\times$400 magnification for at least 30 min. Serological assays were also carried out. The infected children were referred for trypanocidal treatment. No blood samples from drug-treated children were further considered in this study.

2.3. DNA extraction from human blood samples

The blood samples collected from the infected women and infants (5 ml and 1 ml respectively) were mixed with one volume of buffer containing equal volumes of guanidine hydrochloride 6 M (Sigma Chemical Co., St Louis, MO, USA) and EDTA 0.1 M, pH8 (GEB), kept at room temperature for 1 week and then at 4 °C until use. DNA was isolated from 0.2 ml of GEB mixture using illustra blood Mini columns (GE Healthcare Life Sciences, Uppsala, Sweden) and eluted in 0.2 ml, according to the manufacturer's protocol. A bacterial commercial plasmid, pQE (Qiagen, Valencia, CA, USA), obtained from a midi-preparation and purified by the Qiagen Plasmid Midi kit (Qiagen), was used as an internal standard of DNA extraction. Purified plasmid DNA was linearized with *Pvu* II restriction enzyme and 2 ng added to each GEB sample before extraction.

2.4. Quantitative DNA amplification

The ABI 7500 thermocycler (Applied Biosystems, Carlsbad, CA, USA) was used to amplify a *T. cruzi* satellite DNA of 140 bp flanked by the Sat Fw and Sat Rv oligonucleotides, highly conserved in the parasite genome.[11] Duplicated samples were run with these primers and a commercial kit, SYBR GreenER qPCR SuperMix Universal, with integrated uracil DNA glycosylase (UDG) (Invitrogen, Life Technologies, Grand Island, NY, USA). DNA amplifications were performed using 8 μl of human extracted DNA as template in a 20 μl final volume.

An initial step of 50 °C for 2 min was used for UDG activation, followed by a step of 95 °C, 10 min for UDG inactivation and hot-start DNA polymerase activation. The qPCR DNA amplification was achieved after 40 cycles (95 °C, 15 s; 60 °C, 60 s). The melting temperature of the satellite sequence amplified DNA was 80–82 °C. Another qPCR was performed to amplify the internal standard plasmid with primers flanking a 175-bp fragment, pQE rev. GTTCTGAGGTCATTACTGG and pQE fw. CGGATAACAATTTCACACAG. Recovery of pQE DNA was 70–95% and the parasite load was normalized according

to these amounts of recovery.[11] Epimastigotes of *T. cruzi*, clone CL Brener, TcVI, were used as standard. A volume of 10 ml of a GEB mix of a seronegative pregnant woman was spiked with 10^7 parasites/ml. The extracted DNA was 10-fold diluted from 10^6 to 0.1 parasite/ml and the qPCR amplification was performed in 2 μl of each dilution as above. The parasite curve, negative samples and non-template DNA were included in every determination.

2.5. Statistical analysis

Receiver-operating characteristic (ROC)[15] curve analysis was carried out to obtain a cut-off value for our quantitative parasitological method, using the MedCalc statistical software, version 11.5.0.0 (available at www.medcalc.org). Differences between groups were examined by the Kruskal–Wallis test and the Dunn post-test for multiple comparisons, using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). A p value of <0.05 was considered statistically significant.

3. Results

Parasitemia was evaluated by qPCR in three groups of seropositive women: women pregnant with infected children, women pregnant with non-infected children and non-pregnant women. The women's ages and their levels of specific antibodies did not differ significantly between the groups; neither did the gestational time at sampling in the first two groups (Table 1). Patient quantitative parasitemias were referred to a standard parasite curve using *T. cruzi* CL Brener clone, TcVI. The cycle thresholds (Ct, defined as the number of cycles in which the fluorescent signal crosses the threshold or exceeds the background level) of the samples were interpolated in the plot versus the log of parasite concentrations. The slope of several independent experiments was −3.5857 (Figure 1).

The levels of parasite equivalents in the seropositive women pregnant with non-infected children (1.8 ± 0.5 eP/ml, mean ± SE) were not statistically different from those of non-pregnant infected women (1.5 ± 0.7 eP/ml). However, parasitemia was significantly higher in the mothers of congenitally infected children (11.0 ± 2.7 eP/ml; $p < 0.05$, Kruskal–Wallis test and Dunn post-test for multiple comparisons) (Figure 2). In seronegative pregnant women, parasitemia was 0.04 ± 0.01 eP/ml (n = 20), which generated a detection limit of 0.14 eP/ml (ROC curve analysis).

In order to determine the best discriminant cut-off value to predict parasite infection, we analyzed the quantitative parasitemia data of the entire female population under study. The ROC curve analysis[15] (see 'Materials and methods' section) identified a parasite load equal to 0.14 eP/ml (area under curve = 0.812; sensitivity = 100%; specificity = 75.4%), as the best cut-off value to predict and identify a *T. cruzi* infected patient. In our study, five women pregnant with non-infected children, nine seropositive non-pregnant women and one woman pregnant with an infected child were under the qPCR cut-off.

Seropositive pregnant women were asked to come back for the diagnosis of their children during the first, sixth and 12th month after delivery (Table 2). In non-infected children, parasites were not detected by the INP micromethod and the neonates were seronegative at 1 year of age. Twelve of the infected children were diagnosed by the INP micromethod in the first control. In six children, microscopic diagnosis was negative in the first month after delivery, but parasites were found during the sixth month. In two cases, child samples were not available until the child was 1 year old, when the positive serology confirmed the *T. cruzi* infection. Finally, in one child, parasites were not detected by the INP micromethod performed in the

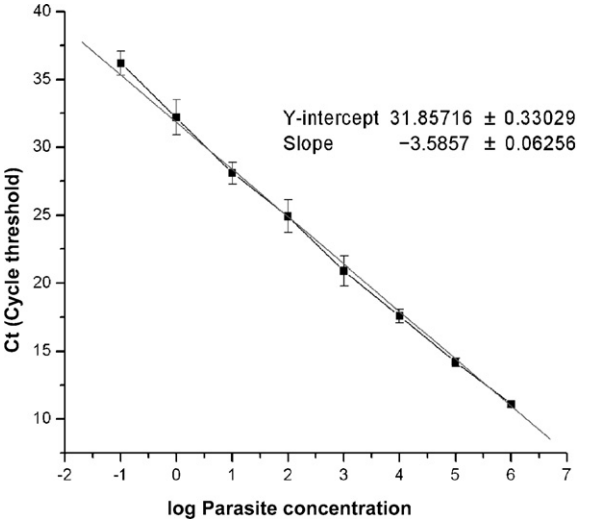

Figure 1. Standard curve reflecting the dynamic range of the *Trypanosoma cruzi* satellite amplified DNA fragment (0.1–10^6 p/mL). Each point represents the average number of six independent experiments performed. DNA of 10-fold serial dilutions of a seronegative pregnant woman spiked with CL Brener clone epimastigotes and expressed as the logarithm of the parasite concentration per milliliter of blood. Slope = −3.5857, $r^2 = 0.9983$.

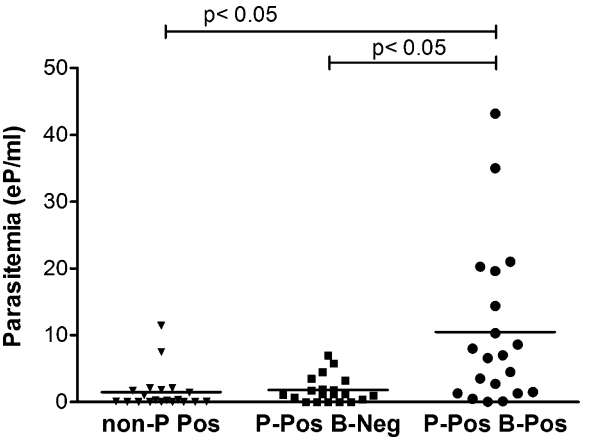

Figure 2. *T. cruzi* parasite burden in 60 seropositive women, who were either not pregnant (non-P-Pos; n = 20), pregnant with a non-infected child (P-Pos B-Neg; n = 20) or pregnant with an infected child (P-Pos B-Pos; n = 20). Parasitemia, expressed as the number of CL Brener parasites per ml of blood that contain equivalent amounts of DNA to the sample (eP/ml), was significantly higher in seropositive women pregnant with infected children than in infected women pregnant with non-infected children or in non-pregnant infected women.

Table 1
Vertical transmission of *Trypanosoma cruzi*: the three groups of seropositive women included in the study

	Non-pregnant infected women (n = 20)	Women pregnant with non-infected children (n = 20)	Women pregnant with infected children (n = 20)
Age (years)[a]	34 ± 12	26 ± 8	29 ± 7
Gestation (months)[a]	–	6.7 ± 1.5	5.1 ± 1.5
IHA[b]	6.4 ± 1.3	7.0 ± 0.9	6.8 ± 1.0
IFA[b]	6.4 ± 1.3	7.1 ± 0.9	7.0 ± 0.9
ELISA[b]	349 ± 71	358 ± 77	337 ± 72

[a] No significant differences between the groups were detected in maternal age or gestation (Kruskal–Wallis test and Dunn post-test); values are mean ± SD.
[b] No significant differences between the groups were detected in the results of the serologic tests (Kruskal–Wallis test and Dunn post-test).
Seropositive women were diagnosed by three serological tests: indirect hemagglutination (IHA), indirect immunofluorescence (IFA) and enzyme linked immunosorbent assay (ELISA). IHA and IFA results are expressed as the $\log_2$ of the inverse of their titer and considered reactive when ≥5. Results of ELISA are expressed as the optical density at 490 nm ×1000 and considered reactive when ≥200.

Table 2
Diagnosis of *T. cruzi* infection in the congenitally infected children of 20 seropositive pregnant women

Mothers	Children	Diagnosis of congenital infection	Child's blood sample available for qPCR[a,b]
1	A	In 1st month by qPCR	Sample 1
2	B	In 1st month by INP micromethod	Sample 1
3	C	In 1st month by INP micromethod	Sample 1
4	D	In 1st month by INP micromethod	Sample 1
5	E	In 1st month by INP micromethod	Sample 1
6	F	In 1st month by INP micromethod	Sample 1
7	G	In 1st month by INP micromethod	Sample 1
8	H	In 1st month by INP micromethod	Sample 1
9	I	In 1st month by INP micromethod	Sample 1
10a,10b	J, K[c]	In 1st month by INP micromethod	Sample 1 (from one child only)
11	L, M[d]	In 1st month by INP micromethod	Sample 1 (from each twin)
12	N	In 6th month by qPCR (performed because antibody levels were higher in Sample 2 than in Sample 1)	Samples 1 and 2
13	O	In 6th month by INP micromethod	Samples 1 and 2
14	P	In 6th month by INP micromethod	Samples 1 and 2
15	Q	In 6th month by INP micromethod	Samples 1 and 2
16	R	In 6th month by serological tests. Antibody levels were higher in Sample 2 than in Sample 1	Sample 1
17	S	In 6th month by qPCR	Sample 2
18	T	Checked for first time at 10 months. Diagnosed by serological tests	No qPCR determination
19	U	Checked for first time at 9 month. Diagnosed by serological tests	9th month sample
20	W	In 12th month by serological assays	Samples 1, 2 and 3

qPCR: quantitative PCR; INP: Instituto Nacional de Parasitología (INP) Dr M Fatala Chaben-ANLIS.
[a] For results, see Figure 3.
[b] Sample 1: taken in 1st month after delivery; Sample 2: taken in 6th month after delivery; Sample 3: taken in 12th month after delivery.
[c] From two pregancies.
[d] Twins.

first, sixth and 12th month after delivery, and the *T. cruzi* infection was confirmed only by positive serology.

The 12 infants diagnosed in the first month after delivery by the INP micromethod had highly variable levels of parasites: 13 000 ± 7000 eP/ml (mean ± SEM), which reflected an average 600-fold increase over the parasitemia of their mothers ($p<0.05$, Mann–Whitney test) (Figure 3). However, there was no correlation between the mean of parasitemias of mothers and children (correlation coefficient $r = 0.55$, $p>0.05$). Parasites were detected in 24 of the 26 determinations by qPCR as shown in Figure 3. In contrast, the INP micromethod yielded positive results in only 15 out of 28 determinations of paralleled samples ($p<0.05$, χ^2 test). In children with very high parasite loads (Figure 3, K–M), 1/10 and 1/100 dilutions of the extracted DNA were also quantified. The dilutions yielded similar parasite loads, ruling out dimers of satellite amplifications.[11]

4. Discussion

We quantified parasitemia by qPCR during pregnancy in women chronically infected with *T. cruzi* who delivered congenitally infected children, and in infected women who delivered non-infected children. In women pregnant with non-infected children parasitemia did not increase as a result of pregnancy, as parasitemia was similar to that in non-pregnant seropositive women. However, in mothers pregnant with infected children, the parasite burden increased about sixfold compared to that in the other two groups studied. These data suggest that parasite burden is a major factor related to transmission.

A higher level of parasitemia in mothers of infected children than in non-transmitting mothers has been previously reported in two studies carried out in Bolivia,[5,6] in which qPCR was used to evaluate parasite levels in peripheral

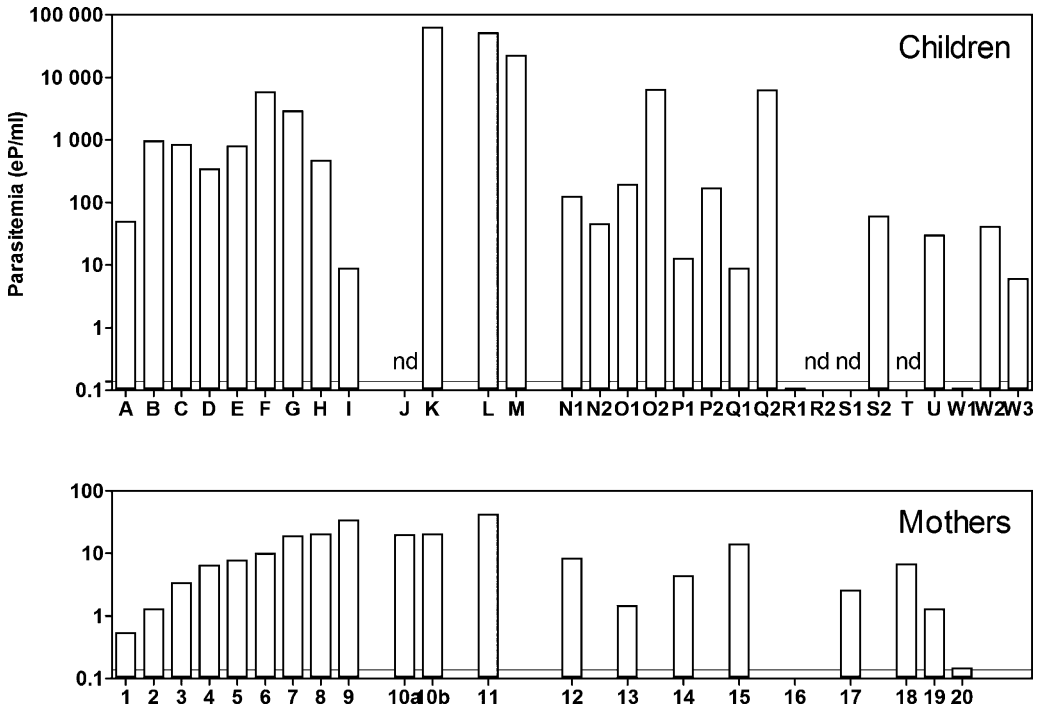

Figure 3. *T. cruzi* parasite burden in pregnant women and their congenitally infected children. Parasitemia in pregnant mothers (cases 1–20) and in their 22 children (cases A–W) is shown (mean values of three independent determinations). Line behind the bars: detection limit (see legend to Figure 1). Cases 10a and 10b are blood samples from the same woman in two different pregnancies; quantitative PCR (qPCR) was carried out in only one of the children (K). Mother 11 delivered twins (L and M).
Parasitemia in the 12 children (A–M) of mothers 1–11, diagnosed in the 1st month after delivery, is shown.
In cases 12–17, children were diagnosed by positive INP micromethod in the 6th month after delivery and the results of the qPCR in the 1st and 6th month after delivery (Samples 1 and 2, respectively) are shown. Parasitemia of mother 16 and her 1-month-old child is below the detection limit and the 6-month sample was not available. In cases 18 and 19, children were diagnosed for the first time at 10 and 9 months of age, respectively, by serological assays, but the sample for qPCR was only available in case 19. In case 20 (child W), no parasitemia was detected by the three parasitologic controls of the INP micromethod; however, qPCR detected parasites in the 6th and 12th month after delivery (Samples 2 and 3), although not in the 1st-month sample.

blood of the mothers at delivery. Our results include the study of a third group of non-pregnant infected women, and the quantitative amplification of a *T. cruzi* satellite DNA fragment compared with a standard quantitative curve of CL Brener clone parasites.

Even though our data highlight increased parasitemia as an important factor related to vertical transmission, the parasite burden was highly variable in mothers of infected children. For example, the parasite load was below the detection limit of qPCR in one of the infected mothers and her infected offspring, but microscopically detected in the child. This may indicate that other factors, such as the type of parasite and the maternal responses,[2] and the physiopathology of the placenta, could play a part in congenital transmission. Our results suggest that qPCR of the pregnant women is not indicated, as some mothers of infected children had low levels of parasitemia similar to those of the mothers of non-infected children.

The parasite burden in the neonates as diagnosed by microscopic observation of the blood in the first month of age was highly variable, and was 600-fold higher than that found in their mothers. The differences in parasitemia among neonates could be related to the timing of the first parasite contact as well as to the response of the fetus or neonate. It was also found that most of the children diagnosed by microscopic observation had >1000 parasites/ml in the cord blood.[5] The parasite loads of some of the children were $\leq$10 eP/ml, in the group we have studied; therefore, the detection limit has to be specially considered in the choice of the parasitological assay for diagnosis.

The different results in our study compared to those carried out in Bolivia[5,6] may be related to experimental design, for example, amplification of different DNA fragments with kDNA S35/36 or Cruzi 1, 2 and 3 primers, the time of sample collection, etc. We followed *T. cruzi* serologically positive women pregnant with infected and non-infected children, and all baby samples were submitted to parasite quantification. Many researchers in the field think that parasitemia in the congenitally infected child is so high that parasitological methods can diagnose the *T. cruzi* infection. In our study, we found that some infants not diagnosed by microscopy could be diagnosed by qPCR. Furthermore, we showed that even a negative diagnosis in the first control could be a positive parasitological diagnosis at 6 months old by qPCR. To our knowledge, our findings have not been previously demonstrated. We strongly recommend following up neonates with parasitological diagnosis, because if *T. cruzi* diagnosis is performed only by serology at

10 months of age or later, a high percentage of children will not receive early treatment.

In our experimental design a range of parasite detection was performed with *T. cruzi* CL Brener clone, TcVI, and also with *T. cruzi* parasites of other discrete typing units (DTU) (data not shown). With our qPCR protocol the detection limit is around 0.1 eP/ml, in agreement with the best discriminant cut-off value by ROC analysis (0.14 eP/ml). TcV parasites can be reliably quantified by a CL Brener clone, TcVI as the two parasites are similar with regard to the number of satellite sequences and the melting temperature of their corresponding satellite sequence amplicons.[11]

Regarding the types of parasites transmitted in the group of patients studied, we performed PCR lineage genotyping in many mother and child *T. cruzi* isolates, when parasite hemoculture was possible. Most of the isolated parasites were genotyped as TcV. Most women in this study were born in the northern areas of Argentina, Bolivia and Paraguay, where most of the infecting parasites belong to TcV, according to the results obtained by Virreira et al., in which 95% of isolated parasites were TcIId, now called TcV.[5] Thus, lineage variability did not seem to be strongly related to mother-to-child transmission, and other DNA markers should be explored.

The socioeconomic situation in endemic areas would make it difficult to quantitate parasitemia by qPCR in every laboratory, because the cost of each qPCR determination, including internal standard amplifications, assay controls, parasite curves and costs of equipment calibration, is currently around 25US$, However, costs could soon decrease when the development of new reagents makes the technique more affordable, as has occurred with many PCR determinations widely used in clinical medicine.

The very low or undetectable parasitemia found in some cases emphasizes the importance of carrying out parasitological assays in addition to serological tests, for the diagnosis of congenitally infected children. The key role of parasitological assay could be intuitively predicted, but the data gathered in our study confirm it as a necessary element in any design for effective detection of mother-to-child transmission of *T. cruzi*.

Authors' contributions: RLC conceived and designed the work. AMDR, AMR and RLC were responsible for human sample collection; AMDR, AMR and the group of people acknowledged performed the *T. cruzi* serology. JB, BJV and EBV were involved in the quantitative diagnosis of mothers and children under the supervision of RLC and AMDR. JB and RLC interpreted and analyzed quantitative PCR results and carried out the final data analysis. JB, BJV and RLC prepared the first draft of the manuscript; all authors contributed to the revision of the manuscript, and read and approved the final version. JB and RLC are guarantors of the paper.

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Competing interests: None declared.

Ethical approval: The study was approved by the Ethics Committee of the Administración Nacional de Laboratorios e Institutos de Salud (ANLIS) Dr C.G. Malbrán, Buenos Aires, Argentina, and carried out according to the declaration of Helsinki. Informed written consent was obtained from all women included in the study before blood collection.

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