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Predictive role of polymerase chain reaction in the early diagnosis of congenital *Trypanosoma cruzi* infection

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ABSTRACT

The efficacy of specific chemotherapy in congenital Chagas disease before the first year of life ranges between 90 and 100%. Between this age and 15 years of age, the efficacy decreases to around 60%. Therefore, early infection detection is a priority in vertical transmission. The aim of this work was to assess whether polymerase chain reaction (PCR) plays a predictive role in the diagnosis of congenital Chagas disease as compared to conventional parasitological and serological methods. To this end, we studied a total of 468 children born to *Trypanosoma cruzi* seroreactive mothers came from Argentina, Bolivia and Paraguay, who lived in the city of Buenos Aires and suburban areas (Argentina), a non-endemic area of this country. These children were assessed by PCR from 2004 to 2009 with the specific primers Tcz1 and Tcz2, and 121 and 122. PCR allowed detecting 49 *T. cruzi*-positive children. Eight of these 49 children were excluded from the analysis: six because they did not complete follow-up and two because the first control was performed after 12 months of age. Parasitological methods allowed detecting 25 positive children, 7 of whom had been earlier diagnosed by PCR (1.53 ± 2.00 vs. 6.71 ± 1.46 months; $p = 0.0002$). Serological methods allowed detecting 16 positive children, 12 of whom had been earlier diagnosed by PCR (1.46 ± 1.48 vs. 11.77 ± 4.40 months; $p < 0.0001$). None of the children negative by PCR was positive by serological or parasitological methods. This study shows that PCR allows early diagnosis in congenital Chagas disease. At present, an early positive PCR is not indicative for treatment. However, a positive PCR would alert the health system to search only those infected infants diagnosed by early PCR and thus generate greater efficiency in the diagnosis and treatment of congenital *T. cruzi* infection.

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1. Introduction

Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi*, is one of the main sanitary problems in Latin America (Salas et al., 2007). This parasite is transmitted mainly by blood-sucking insect vectors, from infected mothers to their children, blood transfusions and organ transplants. Congenital infection is the second main transmission route of *T. cruzi* (Carlier and Truysens, 2010). In Argentina, since 1993 until the present time, the seroprevalence of pregnant women has declined steadily from 11.8% in 1994 to 1995, 9.1% in 1996 to 1997, 6.8% in 2000 (Chagas Program, Vector Coordination, unpublished data) to 4% in 2012 (Chuit and Segura, 2012).

In 2010, regarding Chagas disease, the Sixty-third World Health Assembly recommended promoting "the development of public health measures in disease-endemic and non-endemic countries with special focus on endemic areas, for the prevention of transmission through blood transfusions and organ transplantation, early diagnosis of congenital transmission and management of cases" (World Health Assembly, 2010). In addition, diagnosis of blood parasites by different methods and serological anti-*T. cruzi* tests as from 8 months of age was defined as the gold standard for diagnosis of congenital Chagas disease in the absence of transmission by vectorial and blood transfusion routes (Carlier et al., 2011).

Newborns are diagnosed by parasite microscopic detection in the first 6 months post-birth. Serological methods are not applicable because specific anti-*T. cruzi* antibodies can be transferred from their mothers. Serological methods are clearly useful after this period, when maternal antibodies disappear. At least 12 months of follow-up is recommended to complete the diagnosis of *T. cruzi* transmission (Brutus et al., 2010; De Rissio et al., 2010).

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Early diagnosis of congenital *T. cruzi* infection in newborns is essential (Moya et al., 1985; Moya, 2005) because children have a good tolerance in a long-term treatment and present high indices of cure (Britto, 2009).

polymerase chain reaction (PCR) has been reported as a sensitive tool to detect *T. cruzi* DNA in experimentally infected mice and patients (Virreira et al., 2003; Cummings and Tarleton, 2003; Diez et al., 2008; Burgos et al., 2009; Svoboda et al., 2011). PCR procedures to detect *T. cruzi* in human blood samples have been evaluated and standardized in an international multicenter study (Schijman et al., 2011), but have not yet been validated for the diagnosis of congenital infection (Carlier et al., 2011).

The aim of this work was to assess the predictive ability of PCR to detect congenital *T. cruzi* infection as compared to conventional parasitological and serological methods to obtain greater efficiency in strategies to detect infections by this main route of transmission.

2. Materials and methods

2.1. Patients and diagnosis

The study was carried out in 468 children born to mothers with reactive serology for Chagas disease diagnosed from 2004 to 2009, at the Instituto Nacional de Parasitología “Dr Mario Fatała Chaben” (National Parasitology Institute of Argentina), a reference health care center of Chagas disease, which is located in Buenos Aires, a non-endemic urban area of Argentina. All patients enrolled in this study were characterized as having urban Chagas disease based on their current residence in Buenos Aires, a non-endemic area of our country without vectorial transmission and with control of blood banks.

Maternal infection was assessed using conventional serological methods as indirect hemagglutination assay (IHA), enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence assay (IFA). These techniques were performed and standardized with “in-house” antigens according to international and domestic rules.

The diagnosis protocol of congenital Chagas disease included three controls: between birth and 30 days of age, and at 6 and 12 months of age. The parasitological diagnosis was performed through a micro-method (Mm) developed and validated at our institution. Briefly, 0.5 ml of blood was collected by venipuncture in an Eppendorf tube with a drop of heparin and centrifuged at 3000 rpm for 1 min. The buffy coat between the sera and the blood cells was used in at least four smears with 22 mm × 22 mm coverslips, and a reading was obtained at 400× (De Rissio et al., 2010).

2.2. Case definition

A child was considered to have congenital *T. cruzi* infection either when parasitemia was positive at any time of the follow-up or when serology was clearly reactive above the cut-off value in two serological tests in children between 6 and 12 months of age. A child was considered to be free of congenital infection when serology was non-reactive as from the 10th month on.

The follow-up was considered incomplete when the second control before 10 months was negative by parasitological and serological methods, or when the absence of a third control after 10 months did not allow children's medical discharge from the protocol. Children were excluded either when they had received blood transfusions or when they had been born or had been in transit in endemic areas.

2.3. Blood collection

Peripheral blood (2 ml) was collected from each patient: 1 ml was used for serological diagnosis, 0.5 ml for parasitological diagnosis and 0.5 ml for PCR, and immediately mixed with equal volume of 6 M guanidine HCl/0.2 M EDTA solution pH 8.

2.4. DNA extraction

The guanidine-EDTA blood (GEB) mixture was stored at room temperature for at least 3 days and then stored at 4 °C until processing. The mixture was heated for 15 min in boiling water to break the minicircles (Britto et al., 1993), then maintained at room temperature overnight, and finally stored at 4 °C until DNA extraction. Briefly, 200 µl of GEB was mixed with 200 µl of water and 200 µl of phenol-chloroform-isoamyl alcohol (25:24:1, v/v) and precipitated with glycogen (Centurion-Lara et al., 1994) and cool ethanol at –20 °C (Gomes et al., 1999). The dried pellets were suspended in 50 µl of ultra-pure water. Each sample was extracted in duplicate. Each time, blood samples from healthy individuals were intercalated in order to secure the quality of the preparation.

2.5. PCR detection

The PCR was performed with previously described primers: 121 and 122, which amplify a 330-bp sequence from kinetoplast DNA (k), and Tcz1 and Tcz2, which amplify a 188-bp sequence from nuclear sequence (n) (Moser et al., 1989; Sturm et al., 1989; Virreira et al., 2003). A fragment of the human β-actin housekeeping gene, βActF/βActR, was used to ensure the quality of DNA extraction. Nucleotide sequence and temperature profiles are shown in Table 1.

The amplification reactions were achieved in a volume of 25 µl, consisting of 2.5 µl of the 10× Taq Platinum buffer (100 mM Tris–HCl, pH 8.3; 500 mM KCl), 2.5 µl of a dNTP mixture (200 µM of each), 50 pmol of each primer and 1, 0.3, and 0.5 units of Taq Platinum (Invitrogen Life Technologies) and 3.5 mM, 2 mM and 1.5 mM of MgCl₂ solution for each set of primers 121/122, Tcz1/Tcz2 and βActF/βActR respectively and 2.5 µl (approximately 100 ng) of the human genomic DNA per sample.

Sensitivity was determined by analyzing DNA of each dilution obtained as follows: blood trypomastigotes from the Tulahuen *T. cruzi* strain were added to non-infected human blood in a concentration of 10⁵ parasites/ml of reconstituted blood (GEB), and the resulting spiked blood was serially diluted 10-fold with non-infected GEB to cover a range between 10⁵ and 0.4 equivalent parasites/ml.

Gel electrophoresis was performed using 2% agarose and TAE buffer in the presence of 0.5 µg/ml of ethidium bromide.

2.6. Statistical analysis

All results were analyzed using the GraphPad Prism software 6.0. Confidence intervals were calculated and the *p*-value was considered significant at least 0.05. Data are expressed as mean ± SD with a 95% confidence interval. To evaluate consistency between diagnosis methods, we calculated sensitivity and specificity, as well as positive and negative predictive values.

2.7. Bioethical criteria

Infected mothers were invited to be included in the protocol of congenital *T. cruzi* infection and allow the follow-up of their children. Informed consent forms were signed by all mothers after detailed interviews. Infected mothers were given descriptive and explanatory material on Chagas disease, and the value of monitoring and the recovery of their potentially infected children by

Table 1**Q4** Primer sets, sequences and cycling conditions for PCR assays.

Primers	Sequences	Temperature profile	Origin of DNA	Size
121	AAATAATGTACGGG(T/G)GAGATGCATGA	95 °C 4 min; 68 °C 2 min; 40 cycles 95 °C 30 s; 68 °C 30 s	K	330 bp
122	GGTTCGATTGGGGTTGGGTAATATA	72 °C 10 min	N	188 bp
Tcz 1	CGAGCTCTTGTCCACACGGTGCT	95 °C 4 min; 60 °C 2 min; 33 cycles 95 °C 30 s; 60 °C 30 s, 72 °C 30 s		
Tcz 2	CCTCAAGCAGCGGATAGTTCAGG	72 °C 10 min	HousekeepingGenes	289 bp
β Act F	CGGAACCGCTCATTGCC	95 °C 4 min; 68 °C 2 min; 33 cycles 95 °C 30 s; 60 °C 30 s, 72 °C 30 s		
β Act R	ACCCACACTGTGCCCATTA	72 °C 10 min		

means of specific treatment were explained in detail. All children with congenital *T. cruzi* infection were treated with benznidazole. The Bioethics Committee of the National Institute Fatale Chaben approved the study and the informed consent form.

3. Results

Of the 468 children born to mothers with reactive serology for Chagas disease diagnosed from 2004 to 2009, 297 were born by natural delivery, 169 by cesarean section and two for which this information was not available. Twenty of the 468 births were premature. The kind of birth, the nulliparity/multiparity of women and the weight of children at birth led to no significant differences in vertical transmission (data not shown).

The age distribution of the enrolled mothers showed that most of them were between 21 and 35 years old (Fig. 1A). Regarding their country of origin, 294 out of the 463 mothers (63.5%) had been born in Argentina, 131/463 (28.3%) in Bolivia, and 38/463 (8.20%) in Paraguay. In Argentina, 124/463 (26.8%) of the infected pregnant women studied were from Buenos Aires, 55/463 (11.9%) from Chaco and 32/463 (6.9%) from Santiago del Estero (Table 2). Regarding the children studied, 82.05% (384/468) were under six months in the first control (Fig. 1B).

Table 2

Origin of the studied mothers and congenital transmission detected by PCR.

Origin of the mothers (Argentinean provinces or neighboring countries)	Number of mothers	Number of children	Positive children	% of transmission
Jujuy ^a	13	14	4	30.8
Formosa	11	11	3	27.2
Caba	38	38	8	21.1
Misiones	11	11	2	18.2
Salta	14	14	2	14.3
Chaco ^a	55	56	6	10.9
Paraguay	38	38	4	10.5
Buenos Aires ^b	86	88	9	10.5
Bolivia	131	131	11	8.4
SGO del Estero	32	32	0	0
Tucuman	9	9	0	0
Corrientes	7	7	0	0
Catamarca	1	1	0	0
Cordoba	3	3	0	0
Entre Rios	6	6	0	0
La Rioja	1	1	0	0
Mendoza	3	3	0	0
San Juan ^a	2	3	0	0
WD	2	2	0	0
Total	463	468	49	10.47

^a One mother with twins.^b Two mothers with twins.

Forty-nine of the 468 (10.65%) children displayed a positive PCR. The provinces with highest percentages of vertical transmission of *T. cruzi* infection were Jujuy (30.8%), Formosa (27.2%), Misiones (18.2%), Buenos Aires (10.5%) and Chaco (10.9%). No positive children were found in the remaining provinces. Infection rates in Paraguay and Bolivia were 10.5% and 8.4%, respectively (Table 2).

The limit of detection using the primers 121/122 was 0.002 parasites per assay equivalent to 2 parasites/5 ml of infected human blood similar results were obtained using the primers Tcz1/Tcz2 (data not shown).

Eight out of the 49 PCR-positive children were excluded from the analysis: six because they did not complete the monitoring and two because the first controls were done after 17 and 18 months of age. Then, the predictive ability of PCR for diagnosis compared with conventional methods was evaluated in 41 children. Twenty-six out of these 41 children were found positive by Mm: 18 of them were simultaneously positive by Mm and PCR in the first control, at an average of 2.79 ± 3.26 months of age, while 7 were detected earlier by PCR than Mm, at 1.63 ± 2.0 months vs. 6.71 ± 1.49 months of age ($p = 0.0002$), respectively (Fig. 2A).

Sixteen out of the 41 children were diagnosed by serology after the sixth month. Three of them were simultaneously positive by serology and PCR, while thirteen were detected earlier by PCR than by serology (1.44 ± 1.48 vs. 11.77 ± 4.40 months; $p < 0.0001$) (Fig. 2B).

When comparing the overall diagnosis of congenital infection in the 41 children between PCR and conventional methods, the diagnosis by PCR was at 2.57 ± 2.99 months whereas that by conventional methods was at 6.71 ± 5.11 months ($p < 0.0001$) (Fig. 2C).

Twenty six out of the 41 (63.41%) children were diagnosed before the first month of age by PCR, seven before 12 days and two in the first 7 days of life. At the end of the follow-up, diagnosis was confirmed in all of them (Fig. 3).

Five sets of twins were born. One of these sets was PCR-positive at two months of age: one of the twins had parasitemia evidenced by Mm at that time while the second showed parasitemia 2 months later.

Of the 468 children enrolled, 419 were PCR-negative. Of these negative children, 123 did not complete the follow-up. The analysis of these data allowed us to estimate that the prevalence was 12.17% [95% confidence interval (CI): 8.97–16.25%]. Sensitivity was 100% (95% CI: 89.33–99.78%) and specificity was 100% (95% CI: 98.4–99.97%) (Table 3).

4. Discussion

In the present study, the blood samples were preserved with guanidine, a chaotropic agent that improves DNA availability and integrity. These parameters were validated even 9 years after the samples were obtained (data not published). The phenol–chloroform method was used for DNA extraction. This

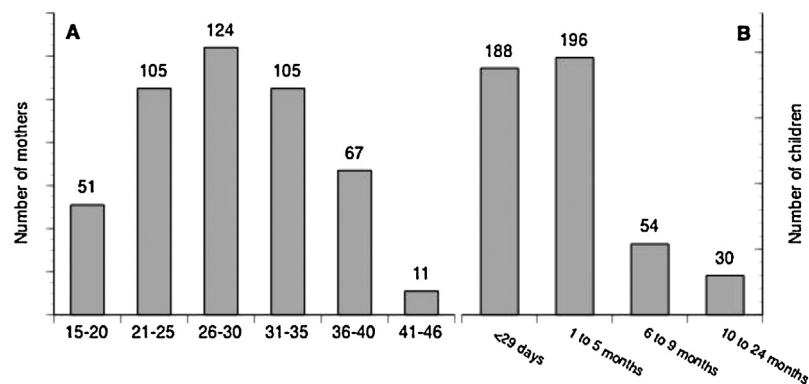


Fig. 1. Comparison of age of mother in intervals. (A) Age intervals of mothers (years, at the moment of the birth), (B) age intervals of children at the moment of the first control.

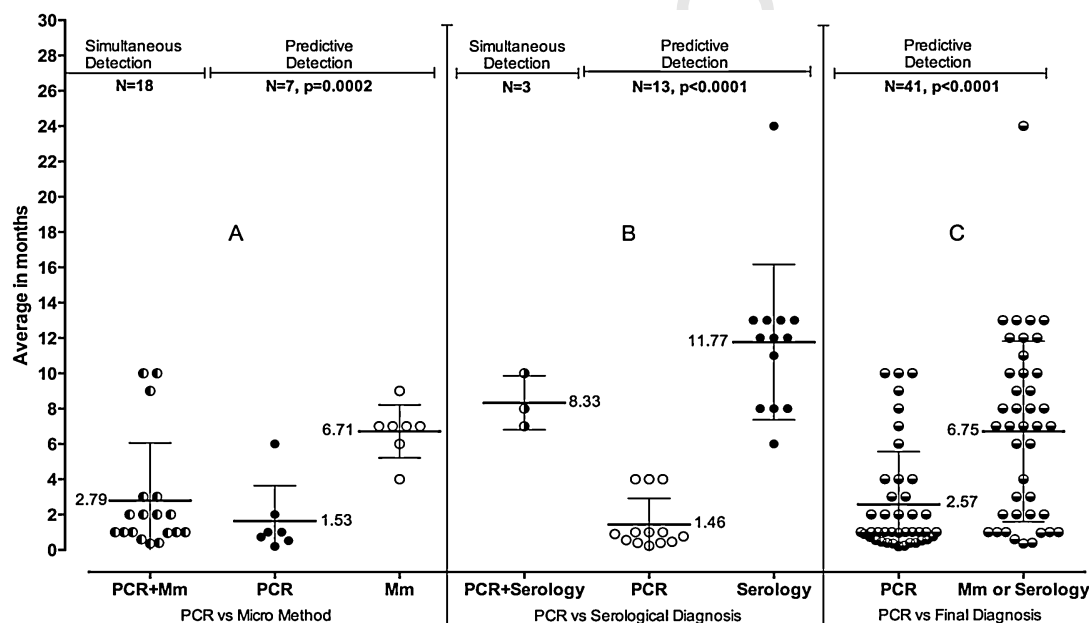


Fig. 2. Predictive detection of *T. cruzi* infection. Comparison of PCR with different diagnostic methods. (A) PCR with Mm. (B) PCR with serological techniques. (C) PCR with the final diagnosis (Mm or serology).

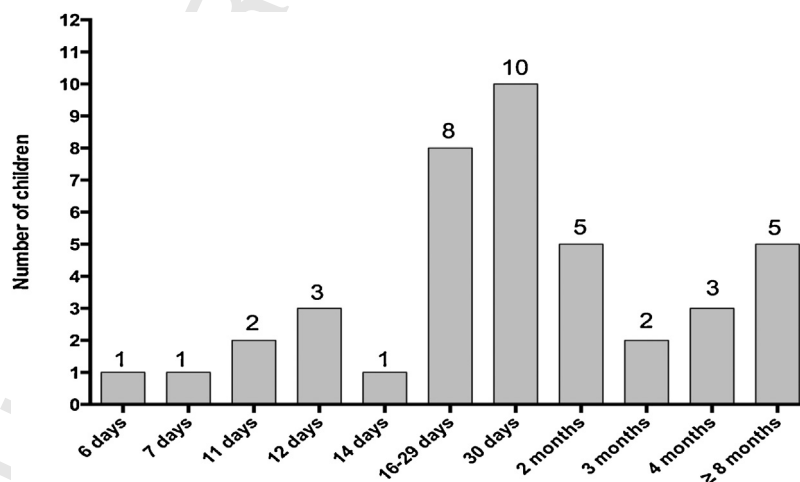


Fig. 3. Age of positive PCR newborns at the moment of controls.

Table 3

Statistical analysis of results. (A) Comparison of PCR with the final diagnosis (Mm or serology), (B) statistical parameters.

(A) Predictive capability of the PCR				
Diagnosis by PCR		Referential diagnosis		
		Positive	Negative	Total
	Positive	41	0	41
	Negative	0	296	296 ^a
(B)		95% C.I.		
		Lower limit		Upper limit
	Prevalence	12.17%	8.97%	16.25%
	Patients correctly identified	100.00%	98.60%	99.87%
	Sensitivity	100.00%	89.33%	99.78%
	Specificity	100.00%	98.40%	99.97%
	Predictive positive value	100.00%	89.33%	99.78%
	Predictive negative value	100.00%	98.40%	99.97%

^a Patients correctly identified with conventional diagnosis (419 negatives – 123 do not completed follow-up).

method showed good sensitivity and specificity although its performance relies on hazardous organic solvents and skilled operators (Sambrook and Russell, 2001; Moreira et al., 2013). At present, commercial kits based on silica-membrane technology are recommended for better performance in DNA purification (Schijman et al., 2011).

Congenital infection is the second main way of *T. cruzi* transmission. In this context, international consensus recommends neonatal screening because early diagnosis allows early treatment ensuring therapeutic efficacy (WHO, 2002). The control strategy of congenital *T. cruzi* infection relies on serological detection of infected pregnant women, monitoring and diagnosis of congenital transmission along the first year of life, and treatment and follow-up of infected children to demonstrate drug efficacy (De Rissio et al., 2010; Carlier et al., 2012).

In Argentina, it has been estimated that between 1100 and 1300 children infected with *T. cruzi* are born every year. Hence, congenital transmission is considered, at present, the most important route in the production of new cases. According to the National Health Surveillance system, 158–255 annual cases are diagnosed and notified, which means that 80% to 85% of children are not timely diagnosed (Chuit and Segura, 2012). This sub-diagnosis, a failure in the effective implementation of the overall strategy of congenital infection may be improved by enforcing PCR, as demonstrated in this study, because its predictive value would reduce the loss of diagnosis and follow-up of children born to infected mothers.

At present, direct parasitological methods are the only ones available to diagnose newborns up to 6 months of age, but the efficacy of these methods depends on the skill of the operator. In addition, the parasitic load in some children is below the limits of conventional parasitological methods (40 parasites/ml) (Torrico et al., 2005). In the present study, PCR allowed predicting a child in the first control at 23 days of life. PCR was also positive in the second control at 6 months of age, whereas parasitological and serological assays were negative. Diagnosis was confirmed by serological assays at 24 months of age when the children came back to the last monitoring. In our hands, PCR was 100 times more sensitive than conventional parasitological methods.

Previous recommendations established the diagnosis of congenital *T. cruzi* infection supported by the visualization of parasites in blood or the serological diagnosis by at least two techniques as from 8 months of age as the gold standard (Carlier et al., 2011). In

our hands, 25.3% of children with negative or discordant serology (reactive by only one test) during the first 6–9 months of age were subsequently confirmed to be infected by the congenital route. Ten-month-old infants born to *T. cruzi*-infected mothers were the clear boundary between the presence and absence of congenital *T. cruzi* infection (De Rissio et al., 2010).

Our data show the predictive ability of PCR to diagnose congenital *T. cruzi* infection; 25 children were diagnosed by parasitological methods, 72% (18/25) of whom were simultaneously diagnosed by PCR and Mm, and 28% (7/25) of whom had been previously diagnosed (4 months before) by PCR and later confirmed by Mm from 4 to 9 months. The standards assays for diagnosis of congenital Chagas disease in infants aged >10 months are *T. cruzi* specific serological methods (De Rissio et al., 2010). In this work, 12/16 children were diagnosed by PCR before 6 months of age. This early detection makes possible to start treatment, considering that, if done before the first year of life, etiological treatment is highly effective (around 90–100%) and drug tolerance is better. It has been reported that adverse drug reaction and patient age are strongly correlated (Altcheh et al., 2011).

We have previously reported that 68.9% of children are diagnosed before 6 months (De Rissio et al., 2010). When applying predictive PCR strategy, we were able to diagnose 63.41% of positive children in the first month of life and subsequently confirm them by conventional methods. Other authors have also shown that PCR assays are able to diagnose the infection earlier than microscopic examination in peripheral blood buffy coat (Russomando et al., 1998; Schijman et al., 2003).

Early diagnosis is necessary not only for effective treatment but also because the generally low economic status of the infected population makes it difficult to adhere to follow-up and children's medical care after delivery is generally made in health centers usually located in peripheral zones. Most studies emphasize that 80% of children are lost to follow-up after the age of 6 months and that less than one of two congenital cases is correctly diagnosed and treated (Brutus et al., 2010). Most of the studies indicate the difficulty in convincing mothers to bring their infants back for follow-up, with a poor competency of primary care systems in performing routine follow-up activities and keeping track of infected children (Blanco et al., 2000). In our previous report, 55.8% of children were lost to follow-up (De Rissio et al., 2010). However, in this study, only 27% of children were lost. This decrease in lost percentage was possible because an exhaustive search of children was conducted when PCR results were predictively positive.

Although PCR is a more expensive technique than conventional methods, it is necessary to consider its cost-effectiveness. As already mentioned, the effectiveness of the treatment depends on the age of the child. Thus, early detection of infection allows early treatment because it shortens the follow-up and reduces the resources required. A child without infection ensures an adult without disease and thus, the Health System will not have to bear the costs of an adult with Chagas disease. Despite the long time elapsed since the first researches on the detection of infection with *T. cruzi* by PCR were published (Moser et al., 1989; Sturm et al., 1989), no commercial kits have yet been developed for this purpose. We hope this study encourages commercial suppliers to develop molecular diagnostic assay kits for Chagas disease testing.

The demonstration of the predictive ability of PCR was the base to define a rational strategy of transference to the national sanitary system. A multicenter collaborative study for the transfer and validation of PCR has been recently performed in eight Argentine provinces with different endemicity. A total of 16 out of the 434 (3.7%) children born to mothers infected with *T. cruzi* showed a positive result by PCR. The quality control of the transferred PCR showed 100% concordance (Velázquez et al., 2011).

It has been recently suggested that a positive PCR at birth cannot be interpreted as indicative of an active infection, especially when only DNA traces are detected (Howard et al., 2013). In the present study, all positive PCR were later confirmed by parasitology or serology. Further, other authors have shown that naked DNA in mice could not survive more than minutes by the action of blood nuclease (Kawabata et al., 1995). These data support that positive PCR results are related to an active congenital infection.

Our results show that PCR provides an early and safe diagnosis. This agrees with that found by other authors (Gomes et al., 1999; Riera et al., 2006) even with low amount of parasites in blood (Carlier et al., 2012). On the other hand, we found no false negative or false positive children. At present, this early diagnosis of the infection would allow alerting on a possible congenital transmission of *T. cruzi* and indicating to the Health System the need to perform exhaustive actions to search the infection in PCR-positive children. In the near future, if the results obtained in this work are reproduced in appropriate number of laboratories and health centers, a positive PCR could indicate, by itself, the etiological treatment of children.

Uncited reference

Virreira et al. (2007).

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