Acta Tropica 147 (2015) 31–37

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Diagnosis of congenital *Trypanosoma cruzi* infection: A serologic test using Shed Acute Phase Antigen (SAPA) in mother–child binomial samples

Bibiana J. Volta[a], Graciela Russomando[b,**], Patricia L. Bustos[a], Karenina Scollo[a], Ana M. De Rissio[a], Zunilda Sánchez[b], Rita L. Cardoni[a], Jacqueline Bua[a,c,*]

[a] *Instituto Nacional de Parasitología (INP) Dr. M. Fatala Chaben, Paseo Colón 568 (1063), Administración Nacional de Laboratorios e Institutos de Salud Dr. C.G. Malbrán, Buenos Aires, Argentina*
[b] *Instituto de Investigaciones de Ciencias en Salud, Universidad Nacional de Asunción, Asunción, Paraguay*
[c] *CAECIHS, Universidad Abierta Interamericana, Buenos Aires, Argentina*

ARTICLE INFO

Article history:
Received 6 October 2014
Received in revised form 21 March 2015
Accepted 27 March 2015
Available online 3 April 2015

Keywords:
Trypanosoma cruzi
Congenital infection
Pregnant women
Infants
Shed Acute Phase Antigen
SAPA
Serology

ABSTRACT

Chagas congenital infection is an important health problem in endemic and non-endemic areas in which *Trypanosoma cruzi*-infected women can transmit the parasite to their offspring. In this study, we evaluated the antibody levels against the *T. cruzi* Shed Acute Phase Antigen (SAPA) in 91 binomial samples of seropositive pregnant women and their infected and non-infected children by ELISA. In 70 children without congenital *T. cruzi* transmission, the titers of anti-SAPA antibodies were lower than those of their seropositive mothers. In contrast, 90.5% of 21 congenitally infected children, at around 1 month of age, showed higher anti-SAPA antibody levels than their mothers. Subtracting the SAPA-ELISA mother OD value to the SAPA-ELISA child OD allowed efficient detection of most *T. cruzi* congenitally infected children immediately after birth, when total anti-parasite antibodies transferred during pregnancy are still present in all children born to seropositive women. A positive correlation was observed between parasitemia levels in mothers and infants evaluated by quantitative DNA amplification and anti-SAPA antibody titers by ELISA. As SAPA serology has proved to be very efficient to detect *T. cruzi* infection in mother–child binomial samples, it could be of extreme help for early diagnosis of newborns, in maternities and hospitals where DNA amplification is not available. This prompt diagnosis may prevent drop out of the long-term follow-up for future diagnosis and may ensure early trypanocidal treatment, which has proved to be efficient to cure infants with congenital Chagas disease.

1. Introduction

Chagas disease, caused by the hemoflagellate parasite *Trypanosoma cruzi*, affects around seven to eight million people (WHO, 2014). Control of triatominae vectors with insecticides and screening of blood donors are performed in most endemic countries, but prophylaxis for vertical transmission is limited. It is a consensus that congenital *T. cruzi* infection will be a pressing public health problem for at least the next 20 years, when the pool of infected women of child-bearing age will decrease to insignificant levels. Mother-to-child transmission of *T. cruzi* is considered of great epidemiological importance in endemic and non-endemic countries (Jackson et al., 2009; Buekens et al., 2013), with more than 15,000 new congenital infected cases per year (Pan American Health Organization, 2006). Congenital transmission, which cannot be prevented as etiological treatment of infected women is not recommended during pregnancy, occurs in 4–12% of infected pregnant women (Russomando et al., 2005; Carlier and Torrico, 2003; Bua et al., 2013).

The diagnosis of *T. cruzi* infection during pregnancy is performed by conventional serologic assays (WHO, 2002; Otani et al., 2009; Carlier et al., 2011). Although chronically infected pregnant women usually have low parasitemia, we have previously observed that mothers that delivered congenitally infected newborns had higher parasitic loads than those that delivered non-infected children (Bua

* Corresponding author at: Instituto Nacional de Parasitología (INP) Dr. M. Fatala Chaben, Paseo Colón 568 (1063), Administración Nacional de Laboratorios e Institutos de Salud Dr. C.G. Malbrán, Buenos Aires, Argentina. Tel.: +54 11 4331 4010; fax: +54 11 4331 7142.
** Corresponding author. Tel.: +595 21 424 520; fax: +595 21 480 185.
E-mail addresses: grusso@rieder.net.py (G. Russomando), jacbua@yahoo.com (J. Bua).

http://dx.doi.org/10.1016/j.actatropica.2015.03.026
0001-706X/

et al., 2012). Risk factors for congenital *T. cruzi* infection are poorly understood and no clear association of this mode of transmission with factors such as maternal age, number of previous deliveries, parasite genotype or geographic origin has been found up to date (Carlier et al., 2011; Bua et al., 2013). There is a risk factor for congenital *T. cruzi* infection, when the pregnant mother lives in an endemic area with high burden of vectors.

In newborns congenitally infected with *T. cruzi*, the conventional diagnostic methods are the visualization of bloodstream parasites in the buffy coat after blood centrifugation in capillary tubes, microhematocrit (Freilij and Altcheh, 1995), or in Eppendorf tubes, INP micromethod (De Rissio et al., 2010). When parasites cannot be detected in the first control, children have to be followed-up, with a second control at around 6 months of age. If they continue to be negative for parasite detection, infected children can be diagnosed by two serologic techniques (Indirect Immunofluorescence, hemagglutination or enzyme linked immunosorbent assay – ELISA) after 10 months of age, to avoid detection of passively acquired antibodies (according to the Argentine Chagas National Program, 2013, Ministry of Health, Argentina) or as described for other endemic areas (Carlier et al., 2011). This concept is the gold standard for congenital diagnosis.

This necessary long-term follow-up to determine if an infant born to a *T. cruzi*-infected mother is healthy is very difficult to achieve, as control adherence is 20–30% in rural endemic areas (Russomando et al., 2005; Sosa-Estani, 2005) and 44% in urban areas (De Rissio et al., 2010). Treatment with 5–10 mg/kg per day benznidazole (Russomando et al., 1998) or 10–15 mg/kg per day Nifurtimox (Freilij and Altcheh, 1995), for 2 months is successful in most treated infants, as indicated by the progressive decline of antibodies. Early detection and treatment become a relevant issue of public health, considering that early drug treatment is curative, and the fact that up to 30% of infected and non-treated children irreversibly progress toward the chronic phase of Chagas disease (WHO, 2002; Carlier et al., 2011).

As a prompt diagnosis of infected infants assures early trypanocidal treatment, effective in 100% of treated children when administered during the first year of life and with no side effects observed (Altcheh et al., 2011) the diagnosis of Chagas congenital infection should be improved.

Although molecular approaches amplifying the *T. cruzi* DNA in infants born to *T. cruzi* infected mothers have demonstrated very high sensitivity (Russomando et al., 1998; Virreira et al., 2003; Bern et al., 2009; Bua et al., 2013), these methods are still difficult to be established in the public primary healthcare in rural endemic areas, being the parasitological molecular diagnosis only performed in specialized laboratories.

A simple serologic test for early detection of *T. cruzi* congenital infection is the detection of anti-SAPA (Shed Acute-Phase Antigen) IgG antibodies (Affranchino et al., 1989; Russomando et al., 2010; Mallimaci et al., 2010). An ELISA system with SAPA has been developed at the University of Asunción, Paraguay, and is currently used in the Public Health system in Paraguay for the diagnosis of congenital Chagas infection. The assessment of congenital transmission is at 3 months of age with the detection of IgG antibodies against SAPA (Russomando et al., 2005, 2010).

In this work, we analyzed the levels of IgG antibodies against SAPA in mother–child binomial serum samples and whether there was any correlation with the parasite loads of the patient. Our final goal was to predict congenital parasite transmission in children at birth or around 1 month after delivery, using serologic methods in laboratories or maternities where routine facilities for molecular biology techniques such as *T. cruzi* DNA amplification are not possible.

Table 1
Age and time of pregnancy (expressed as mean values ± standard deviation) of the women studied, at the moment of blood collection.

N	M+ B−	M+ B+
	70	21
Age (in years)	29.5 ± 6.3	28.9 ± 6.1
Gestational time (in months)	5.9 ± 1.6	5.4 ± 1.7
Born in:		
Argentina	47 (67%)	16 (76%)
Bolivia	13 (19%)	4 (19%)
Paraguay	10 (14%)	1 (5%)

Seropositive women included in this study.

2. Materials and methods

2.1. Participants

This retrospective study was performed with patients of the Instituto Nacional de Parasitología (INP), Dr. "M. Fatala Chaben" (Buenos Aires, Argentina), the reference center for diagnosis of Chagas disease in Argentina. The study populations were pregnant women infected with *T. cruzi* and their newborns followed-up until diagnosed as *T. cruzi*-infected or non-infected. The population of pregnant women included 91 pregnant women recruited from January 2008 to December 2011. All of them were in the asymptomatic phase of the chronic *T. cruzi* infection, resided in the city of Buenos Aires or surrounding areas, had not been treated with trypanocidal drugs, had not received any blood transfusion in the year before the study, and had not traveled to any Chagas disease endemic area. Out of these, 70 women (47 born in Argentina, 13 in Bolivia and 10 in Paraguay) delivered non-infected children and 21 (16 born in Argentina, 4 in Bolivia and 1 in Paraguay) transmitted the *T. cruzi* infection to their offspring, as confirmed by follow-up (Table 1).

Samples were analyzed in groups, as follows: Group 0: samples from 70 pregnant women and their non-infected children followed-up for 1 year; Group 1: 10 binomial samples from mothers and infected newborns diagnosed as infected in the first control, around 1 month after delivery, by the INP micromethod (De Rissio et al., 2010); Group 2: six binomial samples from mothers and their infected children diagnosed as infected in the second parasitological control by the INP micromethod at 6 months of age; Group 3: five binomial samples from mothers and their infected babies diagnosed as infected at the third control by specific anti-*T. cruzi* serology at around 1 year old (Fig. 1).

Informed written consent was obtained from all women recruited for this study to obtain both their blood samples and blood samples from their children. The study was approved by the Ethics Committee of the Instituto Nacional de Enfermedades Infecciosas ANLIS Carlos G. Malbrán (Buenos Aires) and another external independent Committee (Hospital Público Nacional de Pediatría Dr. Fernando Barreiro, Misiones, Argentina). All procedures were carried out according to the declaration of Helsinki.

2.2. Diagnosis of T. cruzi infection

2.2.1. Serologic tests

Sera from the participants were tested for the presence of *T. cruzi* specific antibodies by indirect hemagglutination (IHA), indirect immunofluorescence (IFA) and enzyme linked immunosorbent assay (ELISA), as previously described (De Rissio et al., 2010).

2.2.2. Parasitological tests

Blood samples were taken from infants for parasitological tests in three different controls in 1 year follow-up: before 1 month

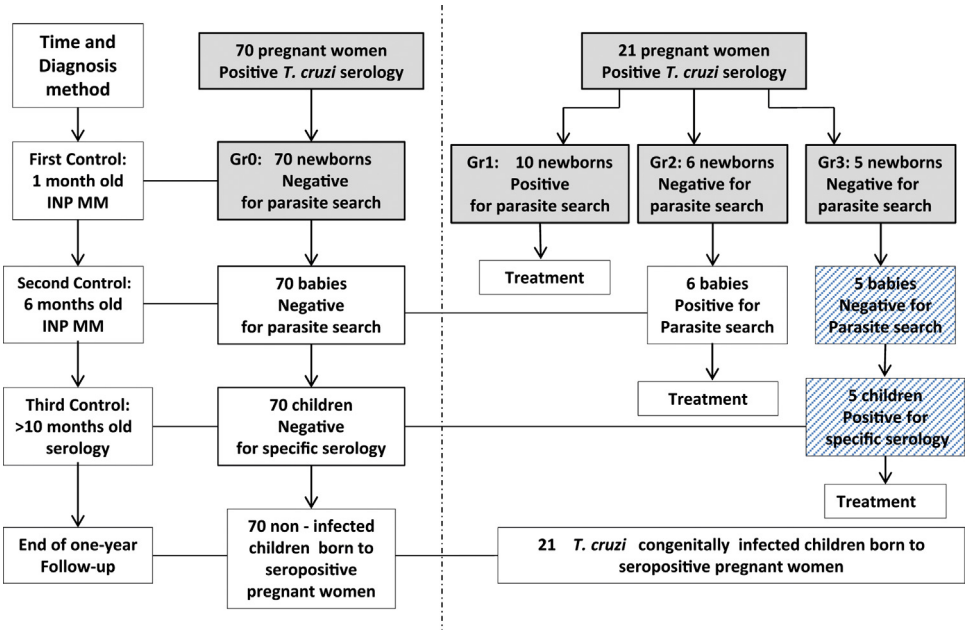

Fig. 1. Groups of samples obtained from 91 *T. cruzi* infected pregnant women and their offsprings during one-year follow-up for the diagnosis of the congenital infection. Binomial mother and child samples at 1 month of age tested for anti-SAPA antibodies are those in shaded boxes. Samples used for comparison of levels of anti-SAPA antibodies along one year follow up are those in stripped boxes. Gr = Group 0 = non-infected children 1, 2 and 3 = infected children diagnosed in the first, second and third control, respectively. INP MM = parasitological micromethod test developed at the Instituto Nacional de Parasitología, Buenos Aires, Argentina.

of age, at 6 months of age and around one year old. If they were diagnosed as *T. cruzi*-infected, they were immediately referred to trypanocidal treatment.

2.2.2.1. Microscopic observation. The presence of *T. cruzi* in children's blood was determined by the INP micromethod. Briefly, 0.5–1.0 mL of heparinized blood was centrifuged in Eppendorf tubes (3000 rpm for 1 min). The buffy coat was distributed in two slides and examined carefully by light microscopy at 400× for at least 20 min (De Rissio et al., 2010).

2.2.2.2. Quantitative DNA amplification. Peripheral blood collected from infected women and their infants (5 and 1 mL respectively) was mixed with the same volume of Guanidine-HCl 6 M, EDTA 0.1 M, pH 8 (GEB) and DNA was extracted with a commercial kit, as previously described (Bua et al., 2012). DNA was amplified by real time PCR (qPCR) in an ABI 7500 thermocycler (Applied Biosystems, Carlsbad, CA, USA), using a commercial kit SYBR® GreenER® qPCR SuperMix Universal, with primers Sat Fw and Sat Rv flanking the satellite nuclear DNA (Duffy et al., 2009). Another qPCR was performed to amplify an internal standard plasmid to calculate recovery of the DNA extracted. Epimastigotes of *T. cruzi*, clone CL Brener, DTU VI, were used for spiking blood from seronegative pregnant women with 10-fold parasite concentrations, as previously detailed (Bua et al., 2012, 2013). Parasite curve, negative samples and non-template DNA were included in every qPCR determination. Samples were run in duplicate.

2.3. SAPA-ELISA

The Shed Acute Phase Antigen (SAPA) was recombinantly constructed in the pGEX vector and 70 ng/well of the purified recombinant protein was used in an ELISA system as previously described (Russomando et al., 2005, 2010). Serum samples were diluted 1:50 and 50 μL were incubated in each well in duplicate. Sera and second antibody solution were incubated for 1 h at room temperature, and three washes were subsequently performed. After 30 min incubation with developing solution, the reaction was stopped with 1 N H_2SO_4 (50 μL/well). Optical density (OD) was measured at 405 nm using an automated ELISA reader Mindray MR96A Biomedical Electronics (Shangai Co. Ltd. China). Three independent positive and negative control sera were included in each plate. Results are expressed as the mean of duplicate samples. The cut-off value 0.3 for SAPA-ELISA positivity was previously determined in a study with 222 sera from non-infected women (Russomando et al., 2005).

2.4. Statistical analysis

Anti-SAPA antibody levels were not normally distributed, so data were presented as the medians with interquartile ranges (IQR). The non-parametric Kruskal–Wallis test and the Dunn post-test for multiple comparisons were used for differences between groups. Other data were presented as mean and standard deviation and differences between groups were examined using *T* test. Correlations were examined using the Spearman's rank test. All data analyses were performed using GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). A p value of <0.05 was considered statistically significant.

3. Results and discussion

3.1. Anti-SAPA antibodies levels in T. cruzi-infected women and their non-infected children

Antibody levels against the *T. cruzi* trypomastigote Shed Acute Phase Antigen (SAPA) were evaluated by ELISA in 91 pregnant *T. cruzi*-seropositive women, 70 of whom gave birth to non-infected children and 21 of whom gave birth to *T. cruzi* congenitally infected children. These two groups of women were not significantly different regarding their age and months of pregnancy at the moment of blood collection (Table 1). The mean age of the pregnant women under study was similar to that of a previous report with SAPA-ELISA in Tierra del Fuego, Argentina (Mallimaci et al., 2010).

We found that 24 out of the 70 mothers that delivered non-infected children (35%) showed negative levels of anti-SAPA IgG

Table 2
T. cruzi seropositive women, mothers of non-infected children (Gr0) were grouped according to the titers of anti-SAPA antibodies, OD higher and lower than 0.4. Pregnant women of *T. cruzi* infected offspring were grouped according to the age that their children were diagnosed as infected: Gr1 = at the first control, around 1 month of age; Gr2 = at the second control, about 6 months old, and Gr3 = mothers of children diagnosed only by serology, at around one year old. Samples from children were withdrawn in every control visit but the results presented are those of the first control at 1 month of age (see Fig. 1). Levels of antibodies against the SAPA recombinant antigen are shown as the median OD at 405 nm value × 1000. The parasitic load is shown as parasite equivalents/mL. Medians and IQR values were calculated with Graph Pad Prism 5.0 program.

	M+ B–	B– 1st C	M+ B–	B– 1st C	M+ B+	B+ 1st C	M+ B+	B+ 1stC	M+ B+	B+ 1st C
	OD > 0.4	Gr0	OD < 0.4	Gr0	Gr1	Gr1	Gr2	Gr2	Gr3	Gr3
Number of values	34	34	36	35	10	10	6	6	5	5
SAPA antibodies										
25% percentile	470	243	172	105	205	875	333	569	283	330
Median	548	331	257	152	283	1569	431	953	379	386
75% percentile	837	467	328	204	398	2135	802	2114	537	1089
Parasitic load										
Median (Pe/mL)	1.71	Neg	Neg	Neg	6.8	2828	2.6	69.8	3	0.3

Anti-SAPA antibodies and parasitemia in *T. cruzi* seropositive women and in their non-infected children, and seropositive women who delivered congenitally infected children.

(OD values below the cut-off of the assay, 0.3), in accordance with the results of a previous study in Paraguay (Russomando et al., 2005).

With the aim to evaluate the clearance of maternal anti-SAPA IgG in non-infected children, 70 mother–child binomial serum samples were analyzed in two groups: samples from pregnant women with SAPA-ELISA OD values higher and lower than 0.4. Pregnant women (34/70, 49%) were positive for ELISA-SAPA with OD values higher than 0.4, with a median OD = 0.548 (IQR = 0.470–0.837) and a maximum OD = 1525. Their non-infected children showed a median OD value of 0.331 (IQR = 0.243–0.467) for anti-SAPA antibodies in the first month of age (Table 2, Fig. 2). Women with anti-SAPA antibodies with OD values equal to or lower than 0.4 (36/70, 51%) showed a median OD = 0.257 (IQR = 0.172–0.328) and their non-infected babies were all negative for SAPA antibodies at birth or around 1 month after delivery (median = 0.152; IQR = 0.105–0.204) (Table 2, Fig. 2A).

3.2. *Parasitic load in* T. cruzi*-infected pregnant women who did not congenitally transmit the parasite*

Women who had anti-SAPA IgG titers with an OD > 0.4 had detectable parasitic load (1.71 Pe/mL), by quantitative amplification of *T. cruzi* satellite nuclear DNA, whereas those with low or negative anti-SAPA antibody titers (OD < 0.4) had either undetectable parasitic load or loads below our quantitative PCR sensitivity (Bua et al., 2012) (Table 2).

It is worth noting that all non-infected children in our study were negative for anti-SAPA antibodies at 6 months of age, independently of the OD level observed in their mothers (data not shown). This is in accordance with a previous study in which non-infected children were negative by ELISA-SAPA at 3 months of age (Russomando et al., 2010).

3.3. *Conventional serology and SAPA-ELISA reactivity in* T. cruzi *– seropositive pregnant women*

In this work, we found no differences in the levels of antibodies measured by IHA or IFA comparing seropositive pregnant women who gave birth non-infected children, with high and low SAPA antibody titers. Although higher levels of anti-*T. cruzi* total antibodies by ELISA were observed in women with anti-SAPA OD > 0.4, no significant correlation was found between the conventional *T. cruzi* ELISA and SAPA-ELISA results (low SAPA mothers, $r = 0.22$; high SAPA mothers, $r = 0.41$) (Table 3).

In previous studies, most of the mothers of *T. cruzi*-infected children had undetectable levels of anti-SAPA antibodies (Reyes et al., 1990; Mallimaci et al., 2010). In this work, we studied a larger sample size of mothers of infected and non-infected children, some of which showed titers with OD > 0.4 at 405 nm, in agreement with another report (Russomando et al., 2005).

3.4. *Anti-SAPA antibody levels in* T. cruzi*-infected women with congenitally infected children*

The titers of antibodies against SAPA were also evaluated by ELISA in the 21 seropositive women that transmitted the *T. cruzi* infection to their children. These pregnant women had a median OD value of 0.358 (IQR = 0.265–0.474). Higher anti-SAPA antibodies, detected by ELISA, were found in their 21 infected babies which had a median OD value of 1.026 (IQR = 0.555–2.054) in their first control. In our study, all the mothers who congenitally transmitted the parasite were in the chronic phase of the infection and showed a parasite load of 4.9 Pe/mL, whereas their offspring had a median parasite load of 167 Pe/mL. Results of anti-SAPA titers and parasitemia from the total of pregnant women and their infected babies are not shown in Table 2, to simplify the amount of data shown.

The 21 babies with congenital Chagas infection were grouped according to the moment of conventional *T. cruzi* diagnosis: Groups 1, 2 and 3, previously detailed in Section 2 and pictured in Fig. 1. Regarding their anti-SAPA antibody levels, infants diagnosed as *T. cruzi*-infected in the first control by INP micromethod (10 out of 21) showed a median OD titer of 1.569 (IQR = 0.875–2.135), and their parasite load was quantified with a median of 2828 Pe/mL (Table 2).

Interestingly, infants of Group 2 had lower levels of anti-SAPA IgG antibodies than those of Group 1, median OD = 0.953, and children of Group 3 showed even lower titers than those of Group 2, with a median OD = 0.386 in their first month after delivery. The parasitic load of babies of Groups 2 and 3 was lower than that of children that were diagnosed by direct parasite visualization

Table 3
Antibody titers measured by indirect hemagglutination assay (IHA), indirect immunofluorescence assay (IFA), and enzyme linked immunosorbent assay (ELISA) in *T. cruzi* seropositive women, mothers of non-infected children. IHA and IFA titers are expressed as the Log_2 of the inverse of the titer and considered reactive when ≥5, and ELISA titers are expressed as the OD at 490 nm × 1000 and considered reactive when ≥200. Mean values ± SD are shown.

Serology of the *T. cruzi* infection	M + B– (OD < 0.4) ($n = 34$)	M + B– (OD > 0.4) ($n = 36$)
IHA	6.8 ± 0.1	6.9 ± 0.1
IFA	6.5 ± 0.2	6.9 ± 0.2
ELISA	330 ± 15	371 ± 14

Conventional and anti-SAPA serology in *T. cruzi*-infected women, that gave birth to non-infected children.

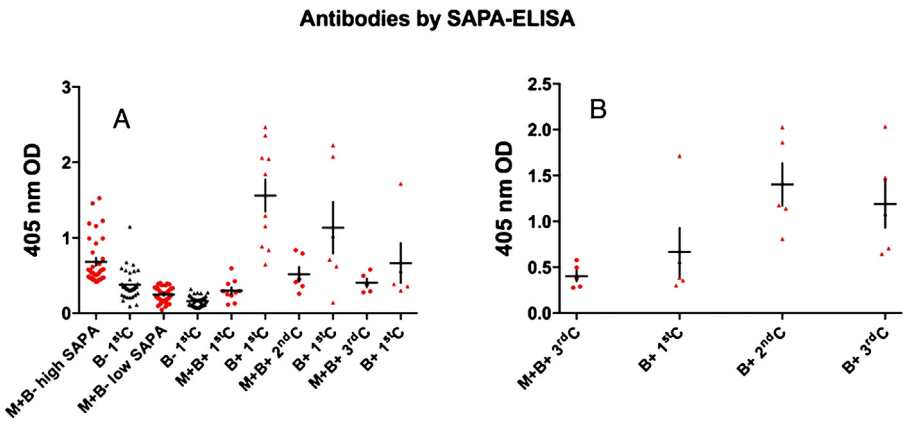

Fig. 2. Serum levels of IgG antibodies against SAPA recombinant antigens, measured as OD at 405 nm, in (A) pregnant seropositive women at the very day of birth: (M+ B− high SAPA), mothers of non-infected children with SAPA OD > 0.4 and their offspring (B− 1st C) at the first control; (M+ B− low SAPA), mothers of non-infected children with SAPA OD < 0.4 and their children at the first control (B− 1st C); pregnant women who gave birth to *T. cruzi* infected children grouped according the age that their offspring could be diagnosed: (M+ B+ 1st C) at the first control, (M+ B+ 2nd C) at the second control (around 6 months old) and the third control (M+ B+ 3rd C). In this last group (B), the results include three controls during one-year follow-up (B+ 1st C), (B+ 2nd C), (B+ 3rd C). In each group the median OD is shown (value × 1000).

in their first control (Table 2). Children of Group 3 ($n = 5$), diagnosed as *T. cruzi* infected by serology after 10 months of age, had a very low parasitemia (0.3 Pe/mL) and a low titer of anti-SAPA antibodies (OD = 0.360) in their first sample at 1 month of age (B+ 1st C) (Table 2, Fig. 2A). In the last group, the levels of anti-SAPA antibodies were studied in the three samples obtained during the one-year follow-up (see Fig. 1 striped boxes). It is worth to note that in the second control at 6 month of age these five children showed very high levels of antibodies anti-SAPA, with a median OD = 1.180 and a high parasitemia 818.30 Pe/mL, but in their third control after 10 months of age, anti-SAPA titers and parasitic load decreased (OD = 1.080 and 147 Pe/mL, respectively). These results are in accordance with our previous observations that concentrations of maternal antibodies transferred to the children begin to decrease around 3–6 months of age (De Rissio et al., 2010) and the number of circulating parasites rapidly increases (Bua et al., 2013). Once *T. cruzi* infected children are able to mount an specific immune response (Volta et al. unpublished results) the parasitic load and anti-SAPA antibodies decrease at around one year old (Fig. 2B). Our results show that infants born to *T. cruzi*-infected mothers exhibit high levels of anti-SAPA antibodies during their first year of life and that the titers of these antibodies are related to the moment at which children are diagnosed by conventional parasitological tests.

The experience in Asunción, Paraguay, proved that every *T. cruzi*-infected child can be diagnosed at 3 months of age (Russomando et al., 2010). It is very interesting that, in another study, anti-SAPA antibodies were found in 81.4% infected children at school-age, meaning that these antibodies could still be detected some years after the initial infection (Brenière et al., 1997).

3.5. Levels of anti-SAPA antibodies correlated with parasitemia

The median values of OD of anti-SAPA antibodies in mothers and their infants seemed to be related to the parasitemia level. Spearman's rank non-parametric correlation was used to analyze SAPA antibody titers and parasitic load variables. After analyzing 87 pairs of values, a Spearman r^2 coefficient = 0.6921 was obtained, with a p value (two-tailed) < 0.0001 meaning a positive correlation of SAPA antibody titers and parasitemia (Fig. 3).

In other studies for the diagnosis of *T. cruzi* infection performed in dogs from a Chagas disease endemic area, the highest sensitivity was observed with SAPA recombinant antigen (86.8%) (Floridia-Yapur et al., 2014). This suggests that the antibody levels observed in infected dogs might correlate with the high and persistent parasitemia of these parasite reservoirs (Cimino et al., 2012), which results in a high risk in the transmission of *T. cruzi* to humans. This high level of parasite load in dogs from endemic areas has been recently confirmed by quantitative DNA amplification (Enriquez et al., 2014).

3.6. The differential level of anti-SAPA IgG detected by ELISA in the mother–child binomial samples could be used as prognosis of congenital infection

An interesting previous approach, in which the differential SAPA OD values were calculated in each binomial sample (ELISA-SAPA OD baby – ELISA-SAPA OD mother), could be predictive of a *T. cruzi* congenital infection at 1 month of age. In that report, three infected children showed an index of 1.7 with the median differential subtraction (Mallimaci et al., 2010). In our study, we analyzed 70 binomial samples without *T. cruzi* congenital transmission, in newborns, in which the differential median index was negative: −160 (range, −237 to −81). In contrast, in 21 binomial samples of mothers and their *T. cruzi*-infected children at around 1 month of age, this index was positive: 615 (range, 94–1633) (Table 4). This index, in which the titers of the maternal anti-SAPA antibodies are subtracted from the titer of their offspring, highlights the specific anti-parasite reactivity of the *T. cruzi*-infected infant.

When congenitally infected children were analyzed, all babies from Group 1, diagnosed by microscopic observation of the parasite

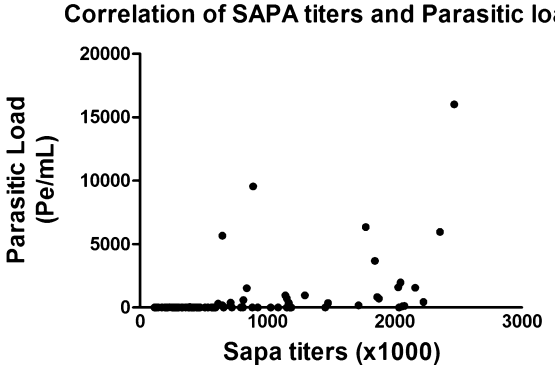

Fig. 3. A Spearman correlation $r^2 = 0.6921$ was obtained analyzing 87 pairs of binomial sample values. $p < 0.0001$ indicated that both variables vary together or significantly correlated. This type of analysis does not create a regression line as non-parametric data were used.

Table 4
The differential OD values were obtained by subtracting of anti-SAPA titer of the mother to the anti-SAPA titer of the child. The indexes are shown with their interquartile range. Samples of all infected babies studied and calculated independently of the time in which they were diagnosed ($n = 21$), and also calculated depending the time of diagnosis (Group 1: at first control, $n = 10$; Group 2: at second control, $n = 6$; Group 3: at third control, $n = 5$) (see Fig. 1).

Differential SAPA OD	(B−) − (M+ B−) All values	(B+) − (M+ B+) All values	(B+) − (M+) Group 1	(B+) − (M+) Group 2	(B+) − (M+) Group 3
No. of binomial samples	70	21	10	6	5
25% Percentile	−237	94	875	13	−97
Median	−160	615	1569	535	67
75% Percentile	−81	1633	2135	1373	717

Median differential values of baby anti-SAPA OD, subtracting the anti-SAPA OD of its mother.

in less than 1 month age, had a positive differential SAPA OD and a very high parasitic load (a median of 2828 Pe/mL) (Table 2). In Group 2, in which children were diagnosed at around 6 months after delivery, 5 out of 6 children (83%) had a positive differential OD index. One child of this Group (baby 1, Table 5) had a negative differential OD = −217 in the retrospective sample at 1 month of age. In Group 3, children diagnosed by serology at around one year old, 4 out of 5 children (80%) displayed a positive differential OD subtracting the mother antibody titers; but one of these children (baby 2, Table 5) also had a negative differential OD = −220. It is important to note that the samples of these 2 out of 21 infected babies (9.5%) with a negative differential OD before 1 month of age, shared the common fact that their parasitic load was below the sensitivity of our quantitative PCR technique (cut off = 0.14 Pe/mL) so they would have been considered negative around birth even for a molecular parasitological diagnosis. But these two infants had a positive differential OD (208 for baby 1 and 1284 for baby 2) after subtracting their mother antibody titers, in their second control sample, at 6 months of age (Table 5). In the second control at 6 months baby 1 was positive by conventional INP micromethod and treatment was started, but baby 2 needed a third control at the age of 10 months to be diagnosed for *T. cruzi* infection by serology. They both could have been diagnosed with SAPA serology in their second control, at 6 months after delivery; or even earlier, if the single infant sample would had been assayed by SAPA serology at 3 months after delivery, as has been previously reported for 100% of *T. cruzi* infected children in Paraguay (Russomando et al., 2010). Moreover, both mothers of these negative babies at the first control, tested during their pregnancy, also had very low parasitic load: 0.5 and 0.15 Pe/mL (Table 5)

We demonstrated that the population under study shows a strong association between the high levels of parasitemia in *T. cruzi* congenitally infected infants, detected by microscopic observation and qPCR, and the levels of anti-SAPA IgG. We also showed that SAPA serology tested in mother–child binomial samples was efficient to detect *T. cruzi* infection in 90.5% of the children analyzed before 1 month of age.

Although 2 out of the 21 infected babies (9.5%) were negative by parasite microscopic observation and exhibited a negative differential binomial SAPA OD after birth, they could have been detected with SAPA serology in their second control, at 6 months after delivery (Table 5), or even earlier, if the sample had been assayed by SAPA serology at 3 months of age, as previously reported in Paraguay, where 100% of *T. cruzi*-infected children can be diagnosed at 3 months of age (Russomando et al., 2010).

We suggest that for a prompt diagnosis of children born to seropositive mothers, a simple and easy ELISA-SAPA test can be of extreme help in maternities in rural areas. The anti-SAPA IgG reactivity in binomial samples of *T. cruzi* congenitally infected and non-infected children is very accurate and around 90% of the infected children could be diagnosed as a congenital case of *T. cruzi* infection. A laboratory with more complex facilities, in which DNA amplification could be performed, should be proposed to confirm *T. cruzi* infection. A negative differential baby–mother ELISA-SAPA titer should warn and invite mothers to bring their children to a second control at 3 months of age, in which anti-SAPA IgG levels would be positive only for infected children, overcoming a long 9–10-month follow-up serology for the diagnosis of *T. cruzi* congenitally infected children. A timely diagnosis of the Chagas vertical transmission assures a prompt and effective trypanocidal treatment for infected children.

Table 5
Anti-SAPA values and parasitic loads in two infants in which the differential OD index is negative after delivery. Group 2: at second control; Group 3: at third control. ELISA titers are expressed as the OD at 490 nm × 1000. The parasitic load is shown as parasite equivalents/mL.

Samples	SAPA OD		Pe/mL
Pregnant + mother	358		0.5
Baby 1 Group 2		Diff SAPA OD	
1 month old	141	−217	0
6 months old	566	208	6
Pregnant + mother	578		0.15
Baby 2 Group 3		Diff SAPA OD	Pe/mL
1 month old	358	−220	0
6 months old	1862	1284	818

Anti-SAPA antibody titers and parasitic load in two special cases.

Funding

This work was supported by ANLIS Carlos G. Malbran, the Agencia Nacional de Promoción Científica y Tecnológica (FONCyT, PICT 956/07) and PICTO-ANLIS 00136-2011. BV was a research fellow from FONCyT. JB, and RLC are members of CONICET Research Career in Argentina.

Ethical approval

Ethical clearance was obtained from the Ethics Committee of the ANLIS and carried out according to the declaration of Helsinki. Informed written consent was obtained from all women included in the study before blood collection.

Conflict of interest

None declared.

Acknowledgements

We thank the staff of the Department of Diagnosis of the INP Dr. M. Fatala Chaben for their kind collaboration.

References

Affranchino, J.L., Ibañez, C.F., Luquetti, A.O., Rassi, A., Reyes, M.B., Macina, R.A., Aslund, L., Pettersson, U., Frasch, A.C., 1989. Identification of a *Trypanosoma cruzi* antigen that is shed during the acute phase of Chagas' disease. Mol. Biochem. Parasitol. 15, 221–228.

Altcheh, J., Moscatelli, G., Moroni, S., Garcia-Bournissen, F., Freilij, H., 2011. Adverse events after the use of benznidazole in infants and children with Chagas disease. Pediatrics 127, e212–e218, http://dx.doi.org/10.1542/peds.2010-1172

Bern, C., Verastegui, M., Gilman, R.H., Lafuente, C., Galdos-Cardenas, G., Calderon, M., Pacori, J., Del Carmen Abastoflor, M., Aparicio, H., Brady, M.F., Ferrufino, L., Angulo, N., Marcus, S., Sterling, C., Maguire, J.H., 2009. Congenital *Trypanosoma cruzi* transmission in Santa Cruz, Bolivia. Clin. Inf. Dis. 49, 1667–1674, http://dx.doi.org/10.1086/648070

Brenière, S.F., Yaksic, N., Telleria, J., Bosseno, M.F., Noireau, F., Wincker, P., Sanchez, D., 1997. Immune response to *Trypanosoma cruzi* shed acute phase antigen in children from an endemic area for Chagas' disease in Bolivia. Mem. Inst. Oswaldo Cruz 92, 503–507.

Bua, J., Volta, B.J., Velázquez, E.B., Ruiz, A.M., De Rissio, A.M., Cardoni, R.L., 2012. Vertical transmission of *Trypanosoma cruzi* infection: quantification of parasite burden in mothers and their children by parasite DNA amplification. Trans. R. Soc. Trop. Med. Hyg. 106, 623–628, http://dx.doi.org/10.1016/j.trstmh.2012.03.015

Bua, J., Volta, B.J., Perrone, A.E., Scollo, K., Velázquez, E.B., Ruiz, A.M., De Rissio, A.M., Cardoni, R.L., 2013. How to improve the early diagnosis of *Trypanosoma cruzi* infection: relationship between validated conventional diagnosis and quantitative DNA amplification in congenitally infected children. PLoS Negl. Trop. Dis. 17, e2476, http://dx.doi.org/10.1371/journal.pntd.0002476

Buekens, P., Cafferata, M.L., Alger, J., Althabe, F., Belizán, J.M., Carlier, Y., Ciganda, A., Dumonteil, E., Gamboa-Leon, R., Howard, E., Matute, M.L., Sosa-Estani, S., Truyens, C., Wesson, D., Zuniga, C., 2013. Congenital transmission of *Trypanosoma cruzi* in Argentina, Honduras, and Mexico: study protocol. Reprod. Health. 10, 55, http://dx.doi.org/10.1186/1742-4755-10-55

Carlier, Y., Torrico, F., 2003. Congenital infection with *Trypanosoma cruzi*: from mechanisms of transmission to strategies for diagnosis and control. Rev. Soc. Bras. Med. Trop. 6, 767–771.

Carlier, Y., Torrico, F., Sosa-Estani, S., Russomando, G., Luquetti, A., Freilij, H., Albajar Vinas, P., 2011. Congenital Chagas disease: recommendations for diagnosis, treatment and control of newborns, siblings and pregnant women. PLoS Negl. Trop. Dis. 10, e1250, http://dx.doi.org/10.1371/journal.pntd.0001250

Cimino, R.O., Diosque, P., López Quiroga, I.R., Gil, J.F., Nasser, J.R., 2012. Immunoenzymatic evaluation of the recombinant SAPA protein of *Trypanosoma cruzi* in naturally infected dogs. Rev. Argent. Microbiol. 44, 177–181.

De Rissio, A.M., Riarte, A.R., García, M.M., Esteva, M.I., Quaglino, M., Ruiz, A.M., 2010. Congenital *Trypanosoma cruzi* infection. Efficacy of its monitoring in an urban reference health center in a non-endemic area of Argentina. Am. J. Trop. Med. Hyg. 82, 838–845, http://dx.doi.org/10.4269/ajtmh.2010.08-0383

Duffy, T., Bisio, M., Altcheh, J., Burgos, J.M., Diez, M., Levin, M.J., Favaloro, R.R., Freilij, H., Schijman, A.G., 2009. Accurate real-time PCR strategy for monitoring bloodstream parasitic loads in Chagas disease patients. PLoS Negl. Trop. Dis. 3, e419, http://dx.doi.org/10.1371/journal.pntd.0000419

Enriquez, G.F., Bua, J., Orozco, M.M., Wirth, S., Schijman, A.G., Gürtler, R.E., Cardinal, M.V., 2014. High levels of *Trypanosoma cruzi* DNA determined by qPCR and infectiousness to *Triatoma infestans* support dogs and cats are major sources of parasites for domestic transmission. Infect. Genet. Evol. 25, 36–43, http://dx.doi.org/10.1016/j.meegid.2014.04.002

Freilij, H., Altcheh, J., 1995. Congenital Chagas' disease: diagnostic and clinical aspects. Clin. Infect. Dis. 21, 551–555.

Floridia-Yapur, N., Vega-Benedetti, A., Monje Rumi, M., Ragone, P., Lauthier, J., Tomasini, N., d'Amato, A.A., Lopez-Quiroga, I., Diosque, P., Marcipar, I., Nasser, J., Cimino, R., 2014. Evaluation of recombinant antigens of Trypanosoma cruzi to diagnose infection in naturally infected dogs from Chaco region. Argentina. Parasite Immunol 36 (12), http://dx.doi.org/10.1111/pim.12144

Jackson, Y., Myers, C., Diana, A., Marti, H.P., Wolff, H., Chappuis, F., Loutan, L., Gervaix, A., 2009. Congenital transmission of Chagas disease in Latin American immigrants in Switzerland. Emerg. Infect. Dis. 15, 601–603, http://dx.doi.org/10.3201/eid1504.080438

Mallimaci, M.C., Sosa-Estani, S., Russomando, G., Sanchez, Z., Sijvarger, C., Alvarez, I.M., Barrionuevo, L., Lopez, C., Segura, E.L., 2010. Early diagnosis of congenital *Trypanosoma cruzi* infection, using shed acute phase antigen, in Ushuaia, Tierra del Fuego, Argentina. Am. J. Trop. Med. Hyg. 82, 55–59, http://dx.doi.org/10.4269/ajtmh.2010.09-0219

Otani, M.M., Vinelli, E., Kirchhoff, L.V., del Pozo, A., Sands, A., Vercauteren, G., Sabino, E.C., 2009. WHO comparative evaluation of serologic assays for Chagas disease. Transfusion (Paris) 49, 1076–1082, http://dx.doi.org/10.1111/j.1537-2995.2009.02107.x

Pan American Health Organization, OPS/HDM/CD/425-06, Washington 2006. Estimación cuantitativa de la enfermedad de Chagas en las Americas (Quantitative estimation of Chagas disease in the Americas).

Reyes, M.B., Lorca, M., Munoz, P., Frasch, A.C., 1990. Fetal IgG specificities against *Trypanosoma cruzi* antigens in infected newborns. Proc. Natl. Acad. Sci. U. S. A. 87, 2846–2850.

Russomando, G., Tomassone de, M.M.C., Guillen, I., Acosta, N., Vera, N., Almiron, M., Candia, N., Calcena, M.F., Figueredo, A., 1998. Treatment of congenital Chagas disease diagnosed and followed-up by the polymerase chain reaction. Am. J. Trop. Med. Hyg. 59, 487–491.

Russomando, G., Almiron, M., Candia, N., Franco, L., Sanchez, Z., de Guillen, I., 2005. Implementación y evaluación de un sistema localmente sustentable de diagnóstico prenatal que permite detectar casos de transmisión congénita de la enfermedad de Chagas en zonas endémicas del Paraguay. Rev. Soc. Bras. Med. Trop. 38, 49–54.

Russomando, G., Sanchez, Z., Mesa, G., Guillen, Y., 2010. Shed acute-phase antigen protein in an ELISA system for unequivocal diagnosis of congenital Chagas disease. Expert Rev. Mol. Diagn. 10, 705–707, http://dx.doi.org/10.1586/erm.10.70

Sosa-Estani, S., 2005. Congenital transmission of *Trypanosoma cruzi* infection in Argentina. Rev. Soc. Bras. Med. Trop. 38, 29–32.

Virreira, M., Torrico, F., Truyens, C., Alonso-Vega, C., Solano, M., Carlier, Y., Svoboda, M., 2003. Comparison of polymerase chain reaction methods for reliable and easy detection of congenital *Trypanosoma cruzi* infection. Am. J. Trop. Med. Hyg. 68, 574–582.

World Health Organization Control of Chagas Disease, 2002. Report of the WHO Expert Committee. World Health Organ. Tech. Rep Series., pp. 905.

2014. World Health Organization Chagas Disease (*American trypanosomiasis*). WHO Media Center, 2014. Fact sheet N°340, Available at: http://www.who.int/mediacentre/factsheets/fs340/en/ (Updated March 2014).