Towards improving early diagnosis of congenital Chagas disease in an endemic setting

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Short title: Congenital Chagas disease in Bolivia

Summary: Congenital *Trypanosoma cruzi* transmission is now estimated to account for 22% of new infections. Though the proportion of T. cruzi infected infants with clinical signs has fallen from the 1990s, but symptomatic congenital Chagas disease still represents a significant, albeit increasingly challenging to detect, public health problem.

Abstract

Background: Congenital *Trypanosoma cruzi* transmission is now estimated to account for 22% of new infections, representing a significant public health problem across Latin America and internationally. Treatment during infancy is highly efficacious and well tolerated, but current assays for early detection fail to detect >50% of infected neonates and 9 month follow-up is low.

Methods: Women presenting for delivery in two urban hospitals in Santa Cruz department, Bolivia were screened by rapid test. Specimens from infants of infected women were tested by microscopy (micromethod), quantitative PCR (qPCR) and IgM trypomastigote excreted-secreted antigen (TESA)-blots at birth and 1 month, and by IgG serology at 6 and 9 months.

Results: Among 487 infants of 476 seropositive women, congenital *T. cruzi* infection was detected in 38 infants of 35 mothers (7.8%). In cord blood, qPCR, TESA-blot and micromethod sensitivities/specificities were 68.6%/99.1%, 58.3%/99.1% and 16.7%/100%, respectively. When birth and 1 month results were combined, cumulative sensitivities reached 84.2%, 73.7% and 34.2%, respectively. Low birth weight and/or respiratory distress were reported in 11 (29%) infected infants. Infants with clinical signs had higher parasite loads and were significantly more likely to be detected by micromethod.

Conclusions: The proportion of *T. cruzi* infected infants with clinical signs has fallen from the 1990s, but symptomatic congenital Chagas disease still represents a significant, albeit increasingly challenging to detect, public health problem. Molecular methods could facilitate earlier diagnosis and circumvent loss to follow-up but remain logistically and economically prohibitive for routine screening in resource-limited settings.

Keywords congenital, Chagas disease, *Trypanosoma cruzi*, Bolivia, diagnostics

Introduction

Successful regional control initiatives have dramatically decreased the prevalence of Chagas disease from an estimated 18 million in 1990 to <6 million infections in 2015 [1]. With the marked decline in vector-borne transmission, congenital infections are now estimated to account for 22% of new cases [1]. The Bolivian Gran Chaco region has the highest *Trypanosoma cruzi* seroprevalence in the world; the majority of adults are infected, including 20-50% of women of child-bearing age [2]. Women infected as children remain at risk of vertical transmission throughout their child-bearing years, and congenitally-infected women can transmit to their children, thus sustaining the cycle across generations in the absence of the vector [3]. Without treatment, 20-30% of chronic *T. cruzi* infections progress to irreversible, potentially fatal cardiomyopathy and/or gastrointestinal disease; congenital infection is assumed to carry the same long-term risk [4].

Trypanocidal chemotherapy during infancy is highly efficacious and well tolerated [5]. However, many biological and operational issues complicate timely diagnosis and treatment of congenital Chagas disease. Infected infants are asymptomatic or have non-specific signs, so detection requires laboratory screening. In endemic regions, detection relies on a complex, multi-step algorithm, beginning with maternal serological screening and followed by testing of multiple infant specimens over 6 to 12 months. Early specimens are evaluated by microscopy in concentrated cord or peripheral blood collected in microhematocrit tubes (often referred to as "micromethod") [6, 7]. For infants not diagnosed early, one or more specimens must be tested by IgG serology after 6-9 months, once maternal antibodies have cleared [3]. The micromethod, even when optimally executed, fails to detect more than half of infections [8, 9], and in control

programs, fewer than 20% of infants complete the 9 months of follow-up needed for unequivocal diagnosis [10, 11].

Historically, congenital Chagas disease has been associated with high morbidity and mortality rates, ranging from low birth weight, prematurity and low Apgar scores to meningoencephalitis, anaemia, thrombocytopenia and respiratory distress syndrome [12, 13]. More recent cohort data report a decline in severe congenital cases [12]. Although factors driving improvements in clinical outcome remain largely unknown, secular trends in nutrition and prenatal care may play a role [12]. Higher levels of neonatal parasitaemia have been reported to be associated with more severe disease [14].

We conducted a cohort study of pregnant women and their infants in two hospitals in Bolivia from 2010 to 2014 [2]. The objectives of the current analysis were to evaluate the performance of diagnostic tests applicable early in infancy and to describe the clinical manifestations of congenital Chagas disease in an endemic setting.

Materials and methods

Ethics statement

The protocol was approved by the Institutional Review Boards of Hospital Universitario Japones (HUJ) (protocol #006), Universidad Catolica Boliviana, Universidad Peruana Cayetano Heredia (UPCH) (protocol #56907), Asociación Benéfica Proyectos en Informática, Salud, Medicina y Agricultura (AB PRISMA), Centers for Disease Control and Prevention (protocol #5829) and Johns Hopkins Bloomberg School of Public Health (IRB #2644). Approval to perform secondary data analyses was granted by the London School of Hygiene and Tropical Medicine (LSHTM)

(#5483). All study women provided written informed consent for their own and their infants' participation.

Study population

The study was conducted in Hospital Japones in Santa Cruz de la Sierra (population ~1.7 million), and the Municipal Hospital of Camiri, (population ~30,000), 300 km south of Santa Cruz. Vector-borne *T. cruzi* transmission is absent in the most urbanized zones, but many rural migrants have moved to the cities in recent decades. In addition, many women living in villages with continued vector-borne transmission give birth in the hospital in Camiri. Trained study nurses enrolled women presenting for delivery, obtained informed consent and collected demographic, clinical and epidemiological data.

Diagnosis of maternal T. cruzi infection

Maternal venous blood was collected in serum separator tubes, centrifuged and screened by two rapid tests, *Trypanosoma*Detect or Chagas Detect Plus (InBios, Seattle, WA) and PolyChaco indirect hemagglutination assay (IHA; Lemos Laboratories, Santiago del Estero, Argentina) at a single dilution of 1:16. Sera were subsequently tested by IHA with multiple dilutions and Chagatest lysate ELISA, with Recombinante 3.0 ELISA to resolve discordant results (both ELISAs from Wiener Laboratories, Rosario, Argentina). Confirmed infection required positive results by two or more tests [31].

Diagnosis of congenital T. cruzi infection

A study nurse attended the delivery of each rapid test-positive woman. Cord blood and umbilical tissue were collected at birth; infant venous blood was collected at 30, 180 and 270 days after birth. Infant specimens were evaluated using three techniques: micromethod, IgM trypomastigote excreted-secreted antigens (TESA)-blots and qPCR [6-8]. For micromethod,

blood was aliquoted in 4-6 heparinized microhematocrit tubes, sealed, and processed by centrifugation (12,000 rpm for 7 minutes) followed by microscopic examination. Six and 9 month specimens were tested by the same IgG serological assays as maternal specimens.

Parasite load was measured by quantitative real time polymerase chain reaction (qPCR) in 500 μl blood specimens and 25 mg specimens of umbilical tissue. DNA was extracted using standard phenol-chloroform for specimens processed prior to January 2012 [15] and afterward using an automated Qiacube system and Qiagen DNA extraction kits (Qiagen, Hilden, Germany). qPCR was conducted according to published methods, using primers Cruzi 1 (5'–ASTCGGCTGATCGTTTTCGA–3') and Cruzi 2 (5'–AATTCCTCCAAGCAGCGGATA–3') to amplify a 166 bp fragment of nuclear satellite DNA [8, 16]. The probe Cruzi 3 (5'–CACACACTGGACACCAA–3') was labeled with 5´FAM (6 – carboxyfluorescein) and 3´MGB (minor groove binder). TaqMan Human RNase-P detection reagent (Applied Biosystems) was included as an internal control; results were considered valid only if the internal control was efficiently amplified. A non-template negative control was included in each PCR run. PCR standard curves were generated by inoculating a blood clot specimen with 1 x 10^6 *T. cruzi* Y strain trypomastigotes, followed by DNA extraction and serial dilutions. The detection limit was determined to be 1 parasite/ml. A positive result was defined by a Ct value below the Ct value of the detection limit standard, which fell consistently between 37 and 38 cycles. Parasite loads in individual specimens were calculated based on the standard curve included in each batch run.

IgM Western blots were performed on sera from cord and 30 day blood, using trypomastigote excreted-secreted antigens (TESA-blots) [17]. Ladder-like bands at 130-200 kDa on IgM TESA-blots demonstrate antibodies to Shed Acute Phase Antigens (SAPA), indicating acute or congenital infection. Bands below 95 kDa are considered non-specific. Infant sera from

the 180 and 270 day specimens were analyzed by IHA and ELISA, as previously described for confirmation of *T. cruzi* infection.

We considered an infant to have unequivocal congenital infection if he or she had positive results by micromethod, positive PCR in two specimens collected at different time points, or positive PCR plus positive IgM TESA-blot. Infants were also considered infected based on positive serology at 6 months with IHA titer >=1:128 and ELISA absorbance value > 0.7 or positive serology at 9 months by IHA and ELISA with cut-offs based on the manufacturer's specifications. In our earlier study, we found that 78% of uninfected infants had positive results by Chagatest ELISA at 6 months but all absorbance values were <0.7 [8]. Neonatologists managed infected infants in compliance with the Bolivian National Control Program guidelines, which recommends antitrypanosomal treatment based on positive results by microhematocrit or serology at 6 or 9 months [7].

Data analysis

Data analyses were conducted in STATA/SE 13.1 and SAS 9.0. Performance characteristics of diagnostic tests were calculated with binomial 95% confidence intervals following standard statistical methods. The significance of differences in categorical variables was tested using Chi square or Fishers exact test, depending on expected cell counts. Significance testing for continuous variables used the Wilcoxon two sample test with normal approximation. Multivariable models were constructed using forward stepwise logistical regression, testing variables with P<0.10 in univariate analyses.

Results

T. cruzi infection was confirmed in 476 (25.7%) of 1851 women screened; 465 infected women delivered singletons and 11 had twin births, yielding 487 infants at risk of infection. Congenital

T. cruzi infection was diagnosed in 38 infants of 35 mothers, including three sets of concordantly infected twins (7.8% of at risk infants) (Table 1). Of the 38 infected infants, 32 (84.2%) were detected by qPCR in the first month of life; of these, 28 also had positive results by TESA-blot and 13 by micromethod in one of the early specimens.

Thirteen babies presented borderline or low PCR loads in a single specimen (Table S1). Four of these infants had infection ruled out based on negative serology at 6 or 9 months, while the other 9 were lost to follow-up, despite multiple contact attempts. Of the 13 specimens with definite or possible false positive PCR results, 4 were processed on a single day in 2012 and 6 on a single day in 2014, suggesting potential laboratory contamination events. Four babies were treated by a hospital neonatologist based on positive IHA at 6 months; their ELISA results were negative in 3 cases and showed low positive absorbance in the fourth. The National Control Program recommends treatment based on IHA titers of 1:128 or higher and positive ELISA at 6 months [9], but ELISA is not routinely conducted in this hospital.

Test performance characteristics were calculated for the 491 infants of seropositive mothers (Table 2). The 17 infants in Table S1 were counted as uninfected for these calculations. Quantitative PCR, TESA-blot and micromethod displayed sensitivities of 68.6%, 58.3% and 16.7% in cord blood, and 73.1%, 54.8% and 25.9%, in 1 month specimens, respectively. The sensitivity of qPCR in umbilical tissue was 69.7%. When birth and 1 month results were combined, cumulative sensitivities reached 84.2%, 73.7% and 34.2% for qPCR, TESA-blot and micromethod, respectively. All assays had high specificity (Table 2).

Eleven (28.9%) infected infants showed one or more signs of illness; 7 (63.6%) had 2 or more signs (Table S2). Infected infants were significantly more likely to have birth weight <

2500 grams, respiratory distress, appear premature on physical examination, and/or be hospitalized at birth (Table 3). All congenital infection and clinically manifest infection were both associated with younger maternal age. In multivariable regression models, the odds of low birth weight were significantly higher for twins and *T. cruzi* infected infants (odds ratio (OR) and 95% confidence intervals (CI) 11.4 [4.5, 29.1 and 2.7 [1.1, 6.8] respectively). Similar results were found for all clinical illness (OR 7.1 [2.8, 17.6] and 2.5 [1.1, 5.8] for twins and congenital *T. cruzi* infection, respectively). Many of the key variables (low birthweight, twin birth, respiratory distress, hospitalization) were significantly correlated with each other.

Infected infants with clinical signs had significantly higher parasite loads in cord blood and umbilical cord tissue. Among ill infants, the cumulative sensitivity of micromethod in cord and 1 month blood was 63.4%, compared to 22.2% among their asymptomatic counterparts (Table 4). Positive results by qPCR or TESA-blot were not associated with clinical presentations.

Discussion

Regional Chagas disease control initiatives have achieved remarkable success over the past 25 years, but several challenges remain [18]. Improved tools, or novel combinations of existing interventions, will be needed to achieve further reductions in incidence [19, 20]. Accurate early detection and treatment of congenital *T. cruzi* infection are crucial to this effort. Treatment in infancy results in high cure rates and few side effects, but based on the low sensitivity of the micromethod and poor follow-up rates, more than half of infected infants go unrecognized in current programs [8, 10, 11]. Even in our study, with dedicated study nurses conducting active searches, 6 infected infants whose conventional testing was negative were lost to follow-up and never treated.

In our study, nearly one-third of infected neonates presented with signs attributable to congenital Chagas disease, but none were severely ill. Our data place this cohort of infants on a continuum with previous reports: the proportion of symptomatic congenital infections has continued to fall, from 55% of infants born in 1992-1994 and 45% in 1999-2001 [12], to 29% in our study in 2010-2014. Some of this decline is likely attributable to improvements in prenatal care and nutrition in Bolivia; the rate of low birth weight also declined in infants of uninfected mothers [12]. Other factors responsible for these changes are unknown. Consistent with other studies, symptomatic infants had significantly higher parasite loads than asymptomatic infants [14, 21]. The higher sensitivity of the micromethod in sick infants compared to asymptomatic ones is the direct result of these higher parasite burdens. Most recent studies have not included clinical data [21, 22], or have described all infected infants as asymptomatic [8, 23]; however, the lack of findings may reflect the small numbers of infected infants in most studies (usually <10). A recent large study included 125 infected infants, but did not report clinical status data [24]. Our data demonstrate that symptomatic congenital Chagas disease has not disappeared and represents a significant unrecognized public health problem.

We evaluated the operational performance and feasibility of three diagnostic techniques for detection of congenital Chagas disease in the first month of life. The micromethod, the only widely available test for this purpose, demonstrated low sensitivity. Specimen quality differed depending on the time between collection and processing; for example, if the birth occurred during the evening or night, microscopic examination was delayed until the following day, when trypomastigotes were no longer motile and were less likely to be detected. However, the micromethod has the important advantage of affording unequivocal diagnosis, particularly of symptomatic infants, enabling the initiation of immediate treatment before mother and child left

the maternity ward. For this reason, the micromethod remains a valuable tool, and it would be premature to abandon it before routine access to more sophisticated, sensitive molecular assays can be assured.

The most sensitive technique was qPCR, which has emerged as a promising diagnostic test, particularly for infections with parasite burdens below the detection limit of microscopy [25]. Nevertheless, multiple infant specimens were still needed to achieve optimal detection. In addition to the considerable equipment and expertise requirements, rigorous quality control is essential for molecular results to be reliable. Some of our apparent false positive results may have been due to transplacental transfer and transient persistence of maternal parasite DNA, as described previously [8, 26]. However, the temporal clustering of 10 of the 13 positive results suggests specimen contamination as a more parsimonious explanation, and emphasizes the difficulties of sustaining quality control even in a research laboratory, let alone a large-scale surveillance program.

Of the three diagnostic tests under evaluation, IgM-TESA blots represented a promising tool with intermediate characteristics [17]. Levels of sensitivity were higher than micromethod and this technique relies on infrastructure readily available for other serological diagnosis. However, blot strips fade quickly over time and must be interpreted immediately, bands can be ambiguous with imperfect reproducibility for a given specimen, and strips need to be produced in-house, raising issues of standardization between batches and laboratories. Maintaining reliable, routine TESA-blot testing in Bolivia proved challenging; the results reported here were generated in our Lima research laboratory. Adaptation to a more field-friendly format such as an ELISA would greatly facilitate wider use of IgM TESA-blot as an early diagnostic test for congenital Chagas disease.

Conventional serological diagnosis has the major disadvantage of delay: early treatment is preferable and programmatic loss to follow-up at 9 months reaches 80% [10, 11]. Negative results at 6 months allow shortened follow-up for some infants, but most are likely to need a specimen at 9 months to effectively rule out infection [8]. As our data show, decisions based on the 6 month results run the risk of exposing uninfected infants to unnecessary treatment. Our follow-up rate was close to 70% while we had full-time research staff regularly tracking study participants, but this dropped to 30% once research staff time was cut back, a rate similar to that achieved by regional screening programs [10, 11]. An unexpected difficulty resulted from the fact that Bolivian cell phone numbers are terminated if not recharged at least every 60 days. Thus, many women were unreachable because their number at the time of delivery was no longer in effect when 6 and 9 month follow-up visits were due. Mobile health initiatives have reported encouraging results in Argentina [27], but are unlikely to achieve high success rates in Bolivia when phone numbers change so frequently.

Maintaining high screening program effectiveness is challenging in the face of the current multi-step algorithm. The Chagas Detect Plus, used in this study for maternal screening, showed excellent performance in both serum and whole blood in evaluations in Bolivia [28], and was recently cleared for diagnostic use by the US Food and Drug Administration. Such point-of-care tests facilitate the identification of mothers at risk of transmission to their infants. The critical unmet need is for a field-friendly test for early detection of congenital infection. The 'ideal' assay would have high sensitivity in a single specimen, preferably at birth, and yield definitive results within a few hours to begin timely treatment and prevent loss to follow-up. A novel experimental technique based on concentration and detection of *T. cruzi* antigens in neonatal urine using nanoparticles may represent a viable, non-invasive alternative, if it can be adapted for

use in the field [29]. Much progress has been achieved in the control of Chagas disease; however, to sustain and build upon these successes, congenital Chagas disease screening strategies will require both diagnostic and programmatic improvements [10].

NOTES:

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Table 1. Diagnostic test results for 38 infants with congenital *Trypanosoma cruzi* infection.

	Conventional diagnosis		qPCR in blood or tissue			IgM TESA-blot		Age (months) at	
Infant	Test	Age	0m	Umb tissue	1m	0m	1m	First positive test	Treatment
1	Serology	9	-	-	-	-	-	6	9
2	Micromethod	1	+	+	+	+	-	0	1
3	Serology	9	+	+	+	-	+	0	9
4	Serology	9	NS	NS	+	NS	+	1	9
5	Serology	9	-	NS	-	-	-	9	Lost to follow-up
6	Micromethod	3	-	+	+	-	-	0	3
7	Micromethod	1	+	+	+	+	-	0	1
8	Serology	11	+	+	NS	+	NS	0	11
9	Serology	9	+	+	NS	-	NS	0	Lost to follow-up
10	Micromethod	1.5	NS	NS	+	NS	-	1	1.5
11	Missed		+	+	NS	+	NS	0	Lost to follow-up
12	Serology	6	-	-	-	-	-	6	6
13A	Micromethod	0	+	+	PT	+	+ (PT)	0	<1
13B	Micromethod	0	+	+	PT	+	+ (PT)	0	<1
14	Micromethod	1	+	+	PT	+	+ (PT)	0	1
15	Serology	6	+	+	+	+	+	0	11
16	Missed		+	BL	NS	+	NS	0	Lost to follow-up
17	Serology	6	-	-	-	-	-	6	6
18	Missed		+	+	NS	+	NS	0	Lost to follow-up
19	Serology	6	+	+	+	+	+	0	6
20	Serology	9	+	+	+	+	+	0	9
21	Serology	11	-	-	-	-	-	11	11
22	Serology	9	-	-	+	-	+	1	9
23	Missed		-	-	+	+	+	0	Lost to follow-up
24A	Serology	6	+	-	+	+	-	0	6
24B	Micromethod	0	+	+	PT	+	- (PT)	0	<1
25A	Serology	6	-	+	+	-	+	0	6
25B	Serology	6	BL	+	+	+	NS	0	6
26	Serology	6	-	-	-	-	-	6	6

27	Serology	9	+	+	+	+	-	0	9
28	Micromethod	1	+	+	+	-	+	0	1
29	Micromethod	0	+	+	PT	+	+ (PT)	0	<1
30	Serology	6	+	+	NS	+	+	0	6
31	Serology	6	+	-	+	-	+	0	6
32	Micromethod	1	+	+	NS	+	-	0	1
33	Serology	9	-	NS	+	-	-	1	9
34	Micromethod	2	+	-	+	-	+	0	2
35	Micromethod	0	+	+	PT	+	+ (PT)	0	<1

NS = no specimen; PT = specimen collected after initiation of treatment; BL = borderline

Table 2. Performance of diagnostic tests for congenital Chagas disease in specimens from infants of infected mothers.

	Micromethod						IgM TESA-blot					
	Cord blood		1 month blood[1]		Cumulative		Cord blood		1 month blood[1]		Cumulative	
Infant status	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos
Uninfected	431	0	301	0	445	0	377	2	275	2	399	4
Infected	30	6	20	7	25	13	15	21	14	12	10	28
Performance % [95% CI]												
Sensitivity	16.7	[6, 33]	25.9	[11, 46]	34.2	[20, 51]	58.3	[41, 75]	46.2	[27, 67]	73.7	[57, 87]
Specificity	100	[99, 100]	100	[99, 100]	100	[99, 100]	99.5	[98, 100]	99.3	[97, 100]	99.0	[97, 100]
PPV	100	[54, 100]	100	[59, 100]	100	[75,100]	91.3	[72, 99]	85.7	[57, 98]	87.5	[71, 96]
NPV	93.5	[91, 96]	93.8	[91, 96]	94.7	[92, 97]	96.2	[94, 98]	95.2	[92, 97]	97.6	[96, 99]

	Quantitative PCR							
	Cord blood		Umbilical tissue		1 month blood[1]		Cumulative	
Infant status	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos
Uninfected	390	10	81	1	257	0	411	11
Infected	12	24	11	23	6	19	6	32
Performance % [95% CI]								
Sensitivity	66.7	[49, 81]	67.6	[49, 83]	76.0	[55, 91]	84.2	[69, 94]
Specificity	97.5	[95, 99]	98.8	[93, 100]	100	[99, 100]	97.4	[95, 99]
PPV	70.6	[53, 85]	95.8	[79, 100]	100	[82, 100]	74.4	[59, 86]
NPV	97.0	[95, 98]	88.0	[80, 94]	97.7	[95, 99]	98.6	[97, 99]

	Conventional serology at 6 months or older									
	IHA >=16 at 6m		IHA >=128 at 6m		EIA at 6m		IHA >=16 at 9m		EIA at 9m	
Infant status	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos
Uninfected	203	50	245	8	159	49	166	7	163	3
Infected	1	14	6	9	0	14	0	12	0	12
Performance % [95% CI]										
Sensitivity	93.3	[68, 100]	60.0	[32, 84]	100	[77, 100]	100	[74, 100]	100	[74, 100]
Specificity	80.2	[75, 85]	96.8	[94, 99]	76.4	[70, 82]	96.0	[92, 98]	98.2	[95, 100]
PPV	21.9	[13, 34]	52.9	[28, 77]	22.2	[13, 34]	63.2	[38, 84]	80.0	[52, 96]
NPV	99.5	[97, 100]	97.6	[95, 99]	100	[98, 100]	100	[98, 100]	100	[98, 100]

PPV, Positive predictive value; NPV, negative predictive value

[1]Six infants treated before 1 month of age excluded from 1 month specimen analyses.

Table 3. Clinical outcomes among singleton infants in the congenital Chagas disease cohort study, Santa Cruz and Camiri, Bolivia.

Characteristic	M+B+ Infected mother - infected infant (N=32)[1]	M+B- Infected mother - uninfected infant (N=252)[2]	M-B- Uninfected mother – uninfected infant (N=1360)[3]	P value M+B+ vs M+B-	P value M+B+ vs M-B-	P value M+B- vs M-B-
Maternal characteristics						
Primiparous	7 (21.9)	48 (19.7)	494 (36.3)	NS	NS	<0.001
Maternal age (median [IQR])	23.5 [19.6, 28.1]	26.9 [22.0, 34.2]	22.7 [19.0, 28.9]	<0.01	NS	<0.001
Cesarean section	19 (59.4)	121 (49.6)	613 (45.1)	NS	NS	NS
PROM	4 (12.5)	16 (6.7)	196 (14.8)	NS	NS	<0.001
Infant characteristics						
Female sex	26 (68.4)	114 (46.7)	658 (48.5)	<0.05	NS	NS
Birth weight (median [IQR])	2980 [2500, 3525]	3300 [3070, 3650]	3300 [2960, 3600]	<0.05	<0.05	NS
Birth weight <2500 grams	7 (21.9)	12 (5.0)	107 (7.9)	<0.01	<0.05	NS
1-minute Apgar score <7	1 (3.1)	11 (4.5)	41 (3.0)	NS	NS	NS
5-minute Apgar score <7	0 (0)	1 (0.4)	4 (0.3)	NS	NS	NS
Premature by exam	6 (18.8)	11 (4.6)	90 (6.6)	<0.01	<0.05	NS
Hospitalized at birth	5 (15.6)	16 (6.7)	131 (9.7)	NS	NS	NS
Respiratory distress	4 (12.5)	10 (4.2)	119 (8.8)	0.07	NS	<0.05

NS, non-significant; IQR, interquartile range; PROM, premature rupture of membranes

[1]Excludes 3 sets of twins, all concordantly infected
[2]Excludes 4 sets of twins, 174 infants with no specimen at 6 or 9 months and 23 infants whose final serology was inconclusive
[3]Excludes 15 sets of twins

Table 4. Characteristics of *T. cruzi*-infected infants with and without clinical signs consistent with congenital Chagas disease.

Characteristic	Infants with clinical signs (N=11)	Infants without clinical signs (N=27)	P value
Median [IQR] maternal age	20.8 [16.3,23.0]	25.5 [22.0, 30.9]	0.04
Median [IQR] parity	2 [1,3]	3 [2,4]	0.04
Cord blood specimen			
Micromethod positive	4/11 (36.4)	2/25 (8.0)	0.06
qPCR positive	9/11 (81.8)	15/24 (62.5)	0.44
Median [IQR] parasites/mL	89,263 [5.4, 571552]	37.9 [0, 10,175]	0.04
IgM TESA-blot positive	8/11 (72.7)	13/25 (52.0)	0.30
Umbilical cord tissue			
qPCR positive	9/11 (81.8)	14/22 (63.4)	0.43
Median [IQR] parasites/mL	859,119 [2133,>10^6]	5.9 [0, 948,591]	0.06
1 month blood specimen[1]			
Micromethod positive	3/7 (42.9)	4/20 (20)	0.33
qPCR positive	4/6 (66.7)	15/19 (79.0)	0.61
Median [IQR] parasites/mL	37,517 [0, 90,855]	342 [1.1, 8720]	0.39
IgM TESA-blot positive	2/7 (28.6)	10/19 (52.6)	0.39
Cumulative 0 and 1 month results			
Micromethod positive	7/11 (63.6)	6/27 (22.2)	0.03
qPCR positive	9/11 (81.8)	23/27 (85.2)	1.00
IgM TESA-blot positive	9/11 (81.8)	19/26 (73.1)	0.69

[1]Six infants (4 with and 2 without clinical signs) treated before 1 month of age excluded from these analyses.