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Congenital Chagas disease in Santa Cruz department, Bolivia is dominated by *Trypanosoma cruzi* lineage V

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Abstract

Background: This study identified *Trypanosoma cruzi* discrete typing units (DTUs) in maternal and infant specimens collected from two hospitals in Bolivia, using conventional genotyping and DTU-specific serotyping.

Methods: Specimens from 142 mothers were used, including 24 seronegative and 118 seropositive individuals; 29 women transmitted *T. cruzi* to their infants. Maternal and infant parasite loads were determined by quantitative real-time PCR. Maternal sera were tested with an in-house parasite lysate ELISA and serotyped by a lineage-specific peptide ELISA, targeting the trypomastigote small surface antigen (TSSA). *T. cruzi* genotypes in infected infants were determined by a triple PCR-RFLP assay.

Results: All infant specimens were genotyped as TcV. Maternal parasite loads and absorbance values by the lysate ELISA were significantly higher for transmitters compared to non-transmitters. Among seropositive mothers, 65.3% had positive results by the TSSA II/V/VI peptide ELISA. No significant difference in reactivity to TSSA II/V/VI was observed for transmitters compared to non-transmitters (79.3% *vs.* 60.7%, respectively).

Conclusions: Our findings reinforce the difficulty in obtaining sufficient sample numbers and parasite DNA to investigate the interaction between parasite genetics and risk of congenital transmission and argue for the inclusion of DTU-specific serotyping in prospective studies.

Keywords: Congenital transmission, *Trypanosoma cruzi*, Chagas disease, DTUs, PCR-RFLP, ELISA, Bolivia, TcV, TcVI, TSSA

Introduction

Trypanosoma cruzi, the parasite that causes Chagas disease, is transmitted when the feces of an infected triatomine vector enters through broken skin or intact mucosa (Bern, 2015). Alternate routes, via blood components, organ transplantation and congenital transmission, have taken on more prominence as effective vector control programs have been established in most affected regions of Latin America. Of new *T. cruzi* infections, an estimated 22% is now attributed to mother-to-child transmission (WHO, 2015). Control requires pre- or perinatal identification of infected women, followed by evaluation of their infants on multiple occasions over the first year of life (Messenger and Bern, 2018). Diagnostic testing for infection in early infancy is best performed by molecular techniques, but these are rarely available in the most highly endemic areas. Direct microscopy, even with concentration, has low sensitivity (Messenger and Bern, 2018).

Bolivia has the highest prevalence of *T. cruzi* infection in the world. In Santa Cruz department, infection prevalence among reproductive age women can be as high as 15-20% in the capital city and 45-50% in towns and villages of the hyperendemic Chaco region (Kaplinski et al., 2015). Barriers to effective screening programs range from biological features of the disease to logistical challenges within health systems (Messenger and Bern, 2018).

Recent reviews have identified maternal parasite load as the primary determinant of vertical *T. cruzi* transmission (Bustos et al., 2019; Klein et al., 2021). Although *T. cruzi* genetic lineages (or discrete typing units; DTUs) have been hypothesized to alter congenital transmission risk, direct data are sparse. Familial clustering has been reported, with mothers of one congenitally infected child being significantly more likely to transmit to that child's siblings than mothers without previous transmission (Sanchez Negrette et al., 2005). Selection for parasite strains more apt to cross the placenta was hypothesized to underly this observation. However, maternal factors such as age, parity or immune response could alter parasite load, independent of lineage.

Obtaining population-level genotypic information is limited by sample sizes, collection of sufficient biological material, including paired mother-infant specimens, and suboptimal sensitivity and cross-reactivity of current genotyping techniques (Messenger et al., 2015). To date, most congenital genotyping studies have been performed in Argentina, Bolivia and Chile, and reflect the predominance of TcII/V/VI lineages observed among chronic adult infections (Messenger et al., 2015). However, congenital transmission occurs across the endemic range of all major *T. cruzi* lineages, including TcI (Buekens et al., 2018), and the latter DTU circulates sympatrically in the Southern Cone, albeit less frequently than TcII/V/VI. As an evaluation nested within a cohort study of congenital Chagas disease in Santa Cruz department, Bolivia, the objective was to identify *T. cruzi* DTUs present during congenital transmission, by

characterizing specimens from infants born to transmitters and non-transmitter mothers, using both genotyping (PCR-RFLP) and serotyping techniques (in-house ELISAs to detect *T. cruzi* infection and DTU-specific peptide ELISAs).

Methods

The institutional review boards of Hospital Universitario Japones; Universidad Catolica Boliviana; Universidad Peruana Cayetano Heredia; Asociación Benéfica Proyectos en Informática, Salud, Medicina y Agricultura; Centers for Disease Control and Prevention; and Johns Hopkins Bloomberg School of Public Health approved the protocol. Approval to perform secondary data analyses was granted by the London School of Hygiene and Tropical Medicine. All women in the study provided written informed consent for their own and their infants' participation. The consent form included explicit agreement to the storage and future use of specimens for evaluation of novel diagnostic techniques.

Maternal and infant specimens were collected during a 4-year cohort study of congenital Chagas disease in Hospital Japones in Santa Cruz de la Sierra and the Municipal Hospital of Camiri in Camiri, described in detail elsewhere (Kaplinski et al., 2015; Messenger et al., 2017). In the initial screen, maternal *T. cruzi* infection was confirmed using two rapid tests, *Trypanosoma* Detect or Chagas Detect Plus (InBios, Seattle, Washington) and PolyChaco indirect hemagglutination assay (IHA; Lemos Laboratories, Santiago del Estero, Argentina) at a single dilution of 1:16. Discordant results were resolved by testing sera using IHA with multiple dilutions, Chagatest lysate ELISA or Recombinante 3.0 ELISA (both from Wiener Laboratories, Rosario, Argentina). Confirmed infection required positive results by two or more tests (Bern, 2015). During the original data generation, DNA was extracted from maternal blood and neonatal umbilical cord blood and tissue. Parasite load was determined by quantitative real-time PCR (qPCR), as previously described (Piron et al., 2007; Kaplinski, 2015).

A total of 1851 women were screened during the parent study; of these, 476 had confirmed *T. cruzi* infection. The original cohort thus included 476 seropositive women and their 487 infants (Messenger et al., 2017). The 118 seropositive mother-infant pairs in the current analysis comprised a subset of the full cohort, based on availability of specimens at the time of the laboratory analysis reported here. Specimens from 24 seronegative women were included as a negative control group. qPCR data were available for 10 of the transmitting and 55 of the non-transmitting seropositive mothers included the current analysis.

Maternal sera were tested by an in-house ELISA, to confirm *T. cruzi* infection, using lysate produced by liquid nitrogen lysis of strain Chaco 23 col4 (TcII) epimastigotes (Bhattacharyya et al., 2014). Maternal serotyping, to detect major *T. cruzi* DTUs, was undertaken using five synthesized peptides targeting the trypomastigote small surface antigen (TSSA) (TSSApep-I, TSSApep-II/V/VI, TSSApep-III, TSSApep-IV and TSSApep-V/VI) in an ELISA, according to Bhattacharyya et al., 2014. Briefly, Nunc MaxiSorp® 96-well microplates were incubated overnight at 4°C with: (i) avidin, diluted in carbonate-bicarbonate buffer pH 9.6 (Sigma-Aldrich) (for binding to biotin bound to synthesized peptides; TSSApep-I, TSSApep-II/V/VI, TSSApep-III, TSSApep-IV and TSSApep-V/VI), at a concentration of 1µg/100µl/per well; or (ii) *T. cruzi* lysate (Chaco 23 col4) at a concentration of 0.2µg/100µl/per well, as a positive control (run in parallel as standard for all assays). The next day, avidin and lysate that did not bind to the plate were removed by washing with 1X PBS/0.05% Tween20 three times. Plate wells were blocked by the addition of 200µl 2% skimmed milk and incubated for 2 hours (1 hour at 37°C and 1 hour at room temperature, under constant agitation). At the end of the incubation, plates were washed three times, and peptides were added at 1µg/100µl/per well, diluted in 1X PBS/Tween20 plus 2% skimmed milk and incubated at 37°C for one hour. At the end of the incubation, 100µl/well of 1:200 serum samples, diluted in blocking buffer were added and incubated at 37°C for one hour. At the end of the incubation, plates were washed six times and a secondary anti-human IgG antibody labelled with horseradish peroxidase was added at a dilution of 1/15000 and incubated at 37ºC for 1 hour. At the end of the incubation, plates were washed six times and 100µl of substrate/chromogen (TMB) was added and plates were incubated in a dark room for 5 minutes. Finally, 50µl 2M H_2SO_4 was added and plates were read at 450nm in a VERSA max microplate reader (Molecular Devices, USA).

Genotypes in infected infants were determined by a triple PCR-RFLP assay, targeting *COXII* + *Alu*I, 24Sα rRNA and the SL-IR, according to Zingales et al., 2012.

Categorical variables were compared using Mantel-Haenszel Chi square or Fisher's Exact test, as appropriate. Distributions of continuous variables were compared using the Wilcoxon rank sum test. The relationship between lysate optical density (OD) values and maternal parasite load was tested in a linear regression model. All analyses were conducted in SAS 9.4.

Results

Specimens from 24 seronegative and 118 seropositive women were included in the current analysis; 29 women transmitted *T. cruzi* to their infants. Results by the in-house lysate ELISA showed 100% concordance with the serological results generated at the time of the original data collection. Among infected women, both OD values by the lysate ELISA (median 2.64 *vs.* 2.23,

respectively; $p<0.0001$) (Figure 1) and parasite loads by qPCR were significantly higher among transmitters compared with non-transmitters (median 115.24 *vs.* 27.043 par-eq/ml, respectively; $p=0.0057$), as reported in our previous publications (Kaplinski et al., 2015; Messenger et al., 2017). The relationship between lysate OD values and parasite load did not reach statistical significance (p=0.128 by linear regression).

Among seropositive mothers, 65.3% (77/118) had positive results by TSSA II/V/VI peptide ELISA. For transmitters compared to non-transmitters, 79.3% (23/29) *vs.* 60.7% (54/89) reacted to the TSSA II/V/VI peptide ($p=0.12$), and OD distribution did not differ significantly for transmitters vs non-transmitters (median 0.5401 *vs.* 0.5086, respectively; $p=0.1925$).

Among 25 infected newborns with available specimens, 17 umbilical cord tissue and 13 cord blood specimens had parasitic loads greater than 10^4 par-eq/ml, the limit of detection for DNA-based genotyping in our lab. All infant specimens were genotyped as TcV (Figure 2) based on bands visualized at 81bp and 294bp for COII + *Alu*I, 150bp for the SL-IR and 110bp for 24α rRNA.

Discussion

TcV was the only DTU detected in infected infants, consistent with previous reports from Bolivia (Messenger et al., 2015; Virreira et al., 2006). Further work is needed to characterize intra-DTU diversity associated with congenital transmission. The majority of seropositive mothers reacted to TSSA II/V/VI, confirming the predominance of these lineages in Bolivia (Messenger et al., 2015; del Puerto et al., 2010); however, as we lack a TSSA V-specific peptide, due to shared motifs among TcII/V/VI from common ancestry, we are unable to unequivocally confirm that these mothers are only infected with this particular DTU. Given the challenges of obtaining specimens adequate for conventional genotyping, serotyping represents a promising technique to screen exposed populations on a larger scale (Bhattacharyya et al., 2019; Murphy et al., 2019), especially when used as a low-cost rapid test (Bhattacharyya et al., 2018) and combined with a recently improved epitope specific to TcI (Murphy et al., 2020). Screening with the latter assay could help resolve the role of TcI in congenital infection but was unfortunately unavailable at the time of our study. The relative likelihood of congenital transmission in areas with TcI predominance compared to those with TcII/V/VI predominance remains a matter of debate (Buekens et al., 2018).

As previously, we observed higher parasite load associated with congenital transmission (Kaplinski et al., 2015; Carlier and Truyens, 2015; Bustos et al., 2019), and for the first time,

significantly higher levels of anti-*T. cruzi* antibodies in women who transmitted compared to those who did not. Anti-*T. cruzi* antibodies mediate extracellular parasite elimination by complement-dependent and independent lysis and phagocytosis of opsonized parasites (Kemmerling et al., 2019). Higher levels in transmitters may reflect an enhanced serological response to higher circulating parasitemia (Bustos et al., 2019; Buss et al., 2020). With congenital transmission affecting a variable, but small, proportion of infected mothers (1-10%) among endemic regions (Howard et al. 2014), PCR assays must be conducted on specimens from a large number of women to have sufficient statistical power to investigate the interaction between parasite genetics and congenital transmission risk. DTU-specific serotyping, which requires fewer costs and specialized infrastructure and is less constrained by low maternal parasitaemia, may represent a more feasible method of *T. cruzi* lineage detection among prospective maternal cohorts as well as in other population-based surveys.

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Conflict of interest

No conflict of interest to declare.

Author contributions

LS, LAM, TB, RHG, MAM and CB designed the study. LS, HM and MV performed the experiments, with materials supplied by LAM, TB, RHG, RC, RB, MAM and CB. LS LAM, TB, RHG, HM, MV, MAM and CB were responsible for data analysis and interpretation. LAM and CB drafted the manuscript, which was revised by all co-authors. All authors read and approved the final manuscript.

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Figure 1. Recognition of maternal seropositive sera to *T. cruzi* in-house lysate (A) or TSSA II/V/VI lineage-specific peptide (B) between congenitally transmitting and non-transmitting mothers. Each data point represents the A_{450} of the mean reaction of duplicates of each serum sample per assay. Medians for each group are represented by solid lines. M+B- = mother seropositive, baby seronegative; M+B+ = mother seropositive, baby seropositive.

Figure 2. Amplification of PCR-RFLP assays with *T. cruzi* reference strains (A-D): Chaco 23 col4; TcII, JR c14; TcI, A18; TcIII, ERA; TcIV, Bug2148; TcV, CL Brener; TcVI; CN: negative control) and congenital specimens (E-H), alongside a 100 bp molecular ladder (M). (A) *COXII* reference DTUs; all bands are 375 bp. (B) *COXII* + *Alu*I reference DTUs; TcII: 81 bp + 212 bp; TcI: 30 bp + 81 bp + 264 bp; TcIII: 81 bp + 294 bp; TcIV: 81 bp + 294 bp; TcV: 81 bp + 294 bp; and TcVI: 81 bp + 294 bp. (C) 24Sα rRNA reference DTUs; TcI: 110 bp; TcII: 125 bp; TcIII: 110 bp; TcIV: 120 bp; TcV: 110 bp or 110 bp + 125 bp; TcVI: 125 bp. (D) SL-IR reference DTUs; TcI: 150 bp; TcII: 150 bp; TcIII: 200 bp; TcIV: 200 bp; TcV: 150 bp; TcVI: 150 bp. (E) *COXII* congenital specimens #1-9 + negative control (#10). (F) *COXII* + *Alu*I congenital specimens #1-9 + negative control (#10). (G) 24Sα rRNA congenital specimens #1- + negative control (#9). (H) SL-IR congenital specimens #1- + negative control (#9).