The Trypomastigote Small Surface Antigen from *Trypanosoma cruzi* improves treatment evaluation and diagnosis in pediatric Chagas disease

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; TSSA, trypomastigote small surface antigen from *T. cruzi*; TSSA-ELISA, Recombinant TSSA-based ELISA; tELISA, total parasite homogenate-based ELISA; IHA, indirect hemagglutination assay; NX, Nifurtimox; BZ, Benznidazole; GST, Glutathione S-transferase; F2/3, purified fraction enriched in highly antigenic α-galactosyl epitopes from the *T. cruzi* bloodstream trypomastigote coat, SAPA, Shed acute-phase antigen from *T. cruzi*
Chagas disease is caused by the protozoan parasite *Trypanosoma cruzi*. Assessment of parasitological cure upon treatment with available drugs relies on achieving consistent negative results in conventional parasitological and serological tests, which may take years to assess. Here, we evaluated the use of a recombinant *T. cruzi* antigen termed TSSA as an early serological marker of drug efficacy in *T. cruzi*-infected children. A cohort of 78 pediatric patients born to *T. cruzi*-infected mothers was included in this study. Solely 39 of them were infected with *T. cruzi*, and were immediately treated with trypanocidal drugs. Serological responses against TSSA were evaluated in infected and non-infected populations during the follow-up period using an in-house ELISA test, and compared to conventional serological methods. Anti-TSSA antibody titers decreased significantly faster than anti-whole parasite antibodies detected by conventional serology in both *T. cruzi*-infected patients undergoing effective treatment and in those not infected. This differential kinetics allowed a significant reduction in the required follow-up periods to evaluate therapeutic responses or to rule out maternal-fetal transmission, respectively. Finally, we present the case of a congenitally-infected patient with atypical course, in which TSSA provided an early marker for *T. cruzi* infection. In conclusion, we showed that TSSA was efficacious both for rapid assessment of treatment efficiency and for early negative diagnosis in infants at risk of congenital *T. cruzi* infection. Based upon these findings we propose the inclusion of TSSA for refining the post-therapeutic cure criterion and other diagnostic needs in pediatric Chagas disease.
INTRODUCTION

Chagas disease, caused by the protozoan *Trypanosoma cruzi*, is a life-long disease for which no vaccines are yet available. With ~6 million people already infected and up to 70 million individuals at risk of infection, Chagas disease constitutes one of the most important parasitic disease in Latin America; and an emerging threat to global public health (1, 2). *T. cruzi* transmission occurs when humans are exposed to the contaminated feces of blood-sucking triatomine vectors, through the ingestion of tainted food/beverages (3), via blood transfusion or organ transplantation (1) or congenitally (4, 5). According to epidemiological data, maternal-fetal transmission occurs in ~5% of *T. cruzi*-infected mothers, which leads to ~15,000 new congenital cases per year (5).

Only two trypanocidal drugs, benznidazole (BZ) and nifurtimox (NX) are currently available for chemotherapy. Both are oral compounds that may display adverse effects (i.e. allergic dermatitis) and that cannot be used to treat pregnant women due to their uncertain teratogenic risks (5). Most importantly, BZ and NX show high efficacy solely if administered at the onset of infection (6). The only accepted criterion of cure relies on consistent negative results using conventional parasitological and serological tests (2). However, a significant proportion of patients are negative for parasitological techniques prior to treatment, thus making a subsequent negative result uninformative. PCR-based methods were proven useful in certain clinical situations usually associated to patent blood parasitemia such as congenital infections or disease reactivation in immunosuppressed patients (7-10). However, they remain to be clinically validated and are not yet available in regular health care centers. Moreover, some apparent false positive results due to transplacental transfer of maternal parasite DNA have been described (7).

Conventional serological techniques such as enzyme-linked immunosorbent assay (ELISA) that use crude parasite homogenates are routinely used to assess post-therapeutic responses. However, and even in successful treatments, seronegativization
may take months to years to assess (11). Conventional serology methods display low predictive value for diagnosis and/or treatment evaluation of congenital infections until 8-9 months after birth due to the passive transfer of maternal antibodies (4, 5). Aiming to develop reliable post-therapeutic markers, different biochemical and serological approaches have been explored (12-27). The latter included the evaluation of T. cruzi antigenic fractions or defined antigens that elicit serological responses with different qualitative, quantitative and/or kinetic properties. Overall, the best results were obtained with the F2/3 fraction (28, 29), which consists of highly antigenic α-galactosyl epitopes from the surface coat of bloodstream trypomastigotes (30). Several methodological drawbacks (i.e. costly and difficult purification procedures) however preclude its routine implementation in clinical settings.

In previous works, we characterized a surface adhesion molecule from T. cruzi bloodstream trypomastigotes termed TSSA (Trypomastigote Small Surface Antigen) (31-33). TSSA elicits a strong humoral response during human infections (31, 34-36), and has been validated for Chagas disease serodiagnosis (37). At variance with F2/3, most of anti-TSSA antibodies are directed to peptide epitopes (33, 36, 38), thus enabling the straightforward production of a highly pure diagnostic reagent in engineered bacteria. Here, we evaluated the potential use of recombinant TSSA as a novel serological marker of drug efficacy in T. cruzi-infected children.
MATERIALS AND METHODS

Study Population and screening for T. cruzi infection

A cohort of 78 children from both sexes and born to T. cruzi-infected mothers was recruited for this study. All of them were screened for T. cruzi infection and followed up at the Servicio de Parasitología-Chagas, Hospital de Niños ‘Dr Ricardo Gutierrez’ following current normatives. Briefly, T. cruzi infection in children over 8 months of age was diagnosed using two conventional serological tests: an ELISA that use crude parasite homogenates (Wiener Chagatest-ELISA or tELISA) and an indirect hemagglutination assay, IHA (Wiener Chagatest-HAI). Both are validated, commercial tests widely used in clinical settings. Infection in children under 8 months of age was assessed by the microhematocrit method (4). In case of positive results, they were immediately treated. In case of negative parasitological results, children were called for a medical appointment at 3, 6 and 9 months of age. Serum samples were taken at each time-point and analyzed by conventional serological tests. Those patients displaying negative results for conventional serology at 9 months were considered non-infected whereas those displaying positive results were immediately treated. Most of the participating children were born and raised within the urban limits of Buenos Aires, Argentina, an area free of vector-borne parasite transmission, and hence most likely acquired T. cruzi infection congenitally.

Treatment and follow-up

T. cruzi-infected children were treated with BZ (5-8 mg/kg/day, b.i.d) or NX (10-15 mg/kg/day t.i.d) (39). Infants’ doses were provided as fractioned tablets (100 mg BZ tablets, Abarax; Elea, Argentina or 120 mg NX tablets, Lampit; Bayer) and treatment was open label for 60 days. Medication was provided in monthly batches, and compliance was assessed by tablet counting at each visit. Caregivers were also provided with a treatment
diary to record doses administered, times of doses, symptoms, and problems associated
to the treatment. Serum samples were taken at diagnosis (pre-treatment), at 7, 30 and 60
days (during treatment) and every 3-6 months after treatment (follow-up). A detailed
clinical history, physical examination, and laboratory routine tests were conducted during
treatment (39), and *T. cruzi* conventional serology was carried out in every medical visit
along the follow-up. DNA was purified from whole blood samples and used as template for
a Multiplex Real-Time PCR targeting a 166-bp segment from *T. cruzi* satellite DNA as
described (40).

**Recombinant TSSA-based ELISA (TSSA-ELISA)**

The glutathione S-transferase (GST)-fusion protein bearing the antigenic region (residues
24 to 62) of *T. cruzi* (CL Brener) TSSA has been described (36). GST-TSSA was
expressed in *Escherichia coli* and purified from the soluble fraction to almost homogeneity
by a single glutathione affinity chromatography step (36). Flat-bottomed 96-well Nunc-
Immuno plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 80 µL of
GST-TSSA dissolved in carbonate buffer (pH 9.6) at 0.25 µg/mL and processed for a
previously validated, colorimetric TSSA-ELISA as described (36). Serum samples were
assayed in duplicate at 1:500 dilution, and those displaying [mean - 3 SD] value greater
than the corresponding cutoff value (calculated as the [mean + 3 SD] of 4 samples from
healthy children born to non-Chagasic mothers) were considered reactive. Reactivity of
samples used to determine the cutoff ranged from 0.06 to 0.12 absorbance units (36). For
comparison purposes, cutoff and sample values were expressed as a percentage of a
positive control (a chronic Chagasic patient yielding 0.8-1.4 absorbance units) included in
each assay (36). The overall performance of our TSSA-ELISA has been extensively
validated (37). When indicated, anti-SAPA (shed acute-phase antigen from *T. cruzi* (41,
42)) IgG responses were evaluated by an in-house ELISA (36).
Statistical treatment of results

A linear regression model was used to examine the course of antibody levels over time. Because tELISA and TSSA-ELISA OD values were highly variable among different patients, even among those from the same group, they were expressed as a percentage of the OD value of the first specimen: pre-treatment sample (for patients from Groups 1 and 2) or the corresponding mother sample (for patients from Group 3). Reactivity of negative samples was expressed as zero. For each group of patients and each method, a slope parameter (with 95% confidence interval, CI) was calculated based upon time-point data for which at least one patient per group was positive. In cases when two or more consecutive samples were non-reactive by either tELISA or TSSA-ELISA, the date of the first negative sample was considered as the time of seronegativization for this method.

Kaplan-Meier curves and linear regression analysis were plotted and compared using Log-rank (Mantel Cox) test to obtain median time of seronegativization and ANCOVA, respectively; both available in GraphPad Prism 5 software (version 5.01 for Windows; San Diego, CA, USA). CI were calculated using SPSS® Statistics (IBM® Versión20).

Ethics statement

The study protocol was approved by the research and teaching committee, and the bioethics committee from the Hospital de Niños ‘Dr Ricardo Gutierrez’. Written informed consent was required from each patient’s legal representatives as well as assent from the patient, if applicable. All samples were decoded and de-identified before they were provided for research purposes.
RESULTS

A total of 78 children (4 days to 10 years-old) born to T. cruzi-infected mothers were included in this study. Thirty eight of them were initially diagnosed as infected with T. cruzi, and were coursing either the acute or the early chronic phase of Chagas disease, with no evidence of cardiac abnormalities or any other Chagas disease-associated pathology. These 38 T. cruzi-infected patients were split into 2 groups based on their age range at diagnosis. Group 1 comprised 26 T. cruzi-infected children (0.59 to 9 years-old, median: 4.5 years-old) that were diagnosed by conventional serology whereas Group 2 comprised 12 T. cruzi-infected babies (8 to 143 days-old, median: 36 days-old) that were diagnosed by parasitological tests. A total of 430 serum samples were obtained from these patients during treatment/follow-up (median: 12 samples per patient). Samples were analyzed by conventional serology and, whenever possible, by PCR. The average follow-up time (and range) for these patients was 36 months (14.57-111.53 months).

Chemotherapy was considered successful in every T. cruzi-infected patient, based on a steady decrease in conventional serology values along the follow-up period and, in most cases (24/26 from Group 1 and 5/12 from Group 2), also based on PCR negativization.

Group 3 included 40 infants (4 to 118 days-old, median: 31 days-old) born to T. cruzi-infected mothers. At variance with Group 2 patients, these patients rendered negative results for parasitological tests. A total of 148 samples were analyzed by conventional serology during the follow-up (median: 4 samples per follow-up).

Conventional serology became negative in Group 3 infants at the end of follow-up except for patient REC52, which was accordingly excluded from this group. A flow chart summarizing this information is depicted in Fig. 1; and all relevant demographic, clinical and diagnostic features of every patient included in this study, and of their mothers (when available) are summarized in Tables S1-S4.
TSSA-ELISA for assessing therapy efficacy in *T. cruzi*-infected children

Serological reactivity towards TSSA in *T. cruzi*-infected children was firstly assessed in samples taken at diagnosis (pre-treatment). Nineteen out of 26 (73%) and 10 out of 12 (83%) children belonging to Groups 1 and 2, respectively, yielded positive results (Table S5), and were hence evaluated for anti-TSSA antibody titers along the serologic follow-up. TSSA-ELISA and tELISA results are shown in Tables S1-S2 and Fig. S1; and linear regression analyses upon these data are shown in Fig. 2. Overall, patients from Group 1 showed a steady decrease in anti-*T. cruzi* antibody titers after treatment, which in certain cases led to seronegativization. This decreasing trend was not significantly different when assessed by tELISA or TSSA-ELISA (*p*=.28; Fig. 2A). However, upon stratification of Group 1 by age and hence, by duration of infection, significant differences in the serological regression slopes for either method were detected for younger patients (1-4 years-old; *p*=.01) but not the older ones (4-10 years-old; *p*=0.67) (Figs. 2B and C).

Patients from Group 2 displayed significant differences (*p*=.01) in the serological regression slopes assessed by TSSA-ELISA or tELISA (Fig. 2D).

A total of 22 *T. cruzi*-infected patients (10/26 from Group 1 and 12/12 from Group 2) seronegativized by tELISA following treatment. From these, solely 18 (8 from Group 1 and 10 from Group 2) were TSSA-reactive (Table S5). Interestingly, these 18 patients seronegativized either before (*n*=16) or at the same time (*n*=2) for TSSA-ELISA than for tELISA. Moreover, 3 patients from Group 1 achieved seronegativization in TSSA-ELISA but not in tELISA (denoted as censored cases in Fig. 3A). Kaplan-Meier curves comparing the performance of both methods among seronegativized patients are plotted in Fig. 3. As shown, the median time values of negativization for TSSA-ELISA and tELISA were 8.67 and 32 months, respectively, for Group 1 (*p*<.0001); and 2.21 and 5.4 months, respectively, for Group 2 (*p*=.002). Again, significant differences in the median time values
of negativization for either method were detected for younger patients ($p<0.0001$) but not for the older ones ($p=2$) upon stratification of Group 1 (Fig. 3B).

Comparative analysis of tELISA data indicated that serological regression followed distinct kinetics, being significantly faster ($p<0.0001$) in Group 2 (slope: -10.53) than in Group 1 (slope: -1.527) (Fig. 2). This in turn translated into significantly shorter ($p<0.0001$) time-periods to reach tELISA negativity threshold (median values: 5.4 and 32 months for Groups 2 and 1, respectively) (Fig. 3). In the same line, TSSA-ELISA revealed differences in serological regression slopes (-20.62 and -2.058, respectively, $p=0.07$, Fig. 2) as well as shorter time-periods to achieve seronegativization (2.21 and 8.67 months, respectively, $p=0.001$, Fig. 3) for Group 2 ($n=10$) as compared to Group 1 ($n=11$). Overall, these latter results support previous findings indicating that the decline in anti-$T. cruzi$ antibody titers after chemotherapy is faster in younger children (6, 43).

**TSSA-ELISA for early assessment of congenital $T. cruzi$ transmission**

According to current guidelines, ruling out maternal-to-fetal $T. cruzi$ transmission requires negative results in parasitological tests performed early after birth and in conventional serology carried out at 8-9 months of life, upon clarification of antibodies of maternal origin (5). To explore if TSSA might also improve diagnosis in this area, data from the serologic follow-up of patients from Group 3 were analyzed as before. TSSA-ELISA ($n=36$, since 3 patients were born to TSSA-non-reactive mothers (Table S5)) and tELISA ($n=39$) results are shown in Table S3 and Fig. S1; and linear regression analyses upon these data are plotted in Fig. 4. Both kinds of maternally-transferred antibodies showed a steady declining early after birth, although with significant differences in their regression slopes (Fig. 4A). Accordingly, patients from Group 3 seronegativized either before ($n=31$) or at the same time ($n=5$) for TSSA-ELISA than for tELISA; and displayed significantly different median values of seronegativization (Fig. 4B). Interestingly, patients
from Groups 2 and 3 displayed almost indistinguishable median values of seronegativization assessed either by tELISA ($p=0.33$) or TSSA-ELISA ($p=0.45$) (Fig. 4C), suggesting that, if treated immediately after birth, *T. cruzi*-infected children do not elicit robust, parasite-specific serological responses. In such scenario, the kinetics of seronegativization seems to be majorly driven by the persistence of passively transferred maternal IgG antibodies.

One of the patients originally assigned to Group 3 yielded particular results which deserve to be analyzed separately. This patient, labeled as REC52 (Fig. 1) was born and raised within the urban limits of Buenos Aires, an area free of vector-borne parasite transmission, and did not undergo blood transfusion. Despite being positive for PCR-based methods, REC52 displayed consistent negative results for conventional parasitological tests carried out early after birth (Fig. 5 and Table S4). At 10.5 months of age, and having achieved seronegativization for conventional serological methods, REC52 was declared non-infected and the follow-up was ended. Seronegativization was achieved at 4.2 and 7.2 months of life as measured by TSSA-ELISA and tELISA, respectively, values that are well within the range of non-infected children included in Group 3 (Fig. 4). Unexpectedly, REC52 yielded positive results for TSSA-ELISA at 10.5 months of age (Fig. 5). At this time-point, we also detected reactivity towards SAPA (42), the canonical acute-phase *T. cruzi* antigen (Fig. 5). Positive results for TSSA-ELISA were confirmed at 19 months of age (Fig. 5). At this time-point, REC52 also yielded positive results for tELISA and treatment with BZ was thereby initiated. Following treatment, REC52 showed typical anti-*T. cruzi* antibody decay, indicating therapeutic efficacy (Fig. 5).
DISCUSSION

Identification of novel and reliable post-therapeutic markers is an urgent need in the field of Chagas disease (5, 28, 29). As shown here, TSSA-ELISA provides a significantly better indicator of trypanocidal drug efficacy than currently used serological methods, particularly for newborns and infants (Figs. 2 and 3). Unfortunately, TSSA results are difficult to compare to those reported for other T. cruzi recombinant antigens and/or antigenic fractions, since most of these studies did not involve newborns and infants but older T. cruzi-infected populations (12-21). Nevertheless, when compared to the only similarly designed study that we are aware of, the median times of seronegativization for TSSA (2.21 and 8.67 months for children under and over 8 months of age, respectively) were significantly lower than those recorded for F2/3 (4 and 21.9 months, respectively) (44). As mentioned, F2/3 is so far considered the best alternative serological marker for treatment evaluation in Chagas disease (16, 28, 29).

In addition of providing a novel tool able to shorten follow-up periods following chemotherapy, we also show that TSSA improves diagnosis in infants at risk of congenital T. cruzi infection. Moreover, our findings with REC52, although preliminary, suggest the applicability of TSSA as an alternative early marker for T. cruzi infection in certain clinical situations. Based on PCR results and clinical history, we postulate that REC52 became congenitally infected with T. cruzi, although this infection coursed sub-clinically and below the detection limits of different parasitological and serological tests until ‘reemergence’ probably due to the disappearance of maternal antibodies. As shown in Fig. 5, TSSA-ELISA also displayed better performance than conventional serology for the detection of this infection ‘reemergence’.

One issue that needs to be addressed in order to improve the clinical value of TSSA is that of its sub-optimal sensitivity, which may be attributed to variations in the
clinical, immunological and/or immunogenetic features of patients, and/or to differences in the antigenic constitution of the infecting *T. cruzi* strain(s) (45). Structural differences among protein variants encoded by distinct parasite strains have a major impact on TSSA antigenicity (31, 37, 46). In our study, for instance, TSSA prevalence was 86%, which is consistent with previous data (~86-91%, (36, 37)). In the case of the two TSSA non-reactive children from Group 2, however, it is most notably that they were born to TSSA reactive mothers (Table S2). It may be therefore hypothesized that both of them underwent clarification of anti-TSSA antibodies of maternal origin at some point between birth and initial *T. cruzi* infection diagnosis. In such a case, the sensitivity and overall performance of TSSA-ELISA would have been underestimated. Despite these considerations, different alternatives including the use of a mixture of TSSA variants are currently being explored to improve the clinical value of TSSA-ELISA.

Progress towards development of novel and better treatments for Chagas disease has been slow and usually disappointing (47, 48). This scenario fortunately seems destined to change in the coming years with the recent development of robust tools to screen, prioritize and evaluate novel anti-trypanosomal drugs (49-51). Identification of biomarkers able to refine the post-therapeutic criterion is instrumental to fasten the assessment of current trypanocidal chemotherapies and, most importantly, for the development of much needed novel and improved treatments.
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FIGURE 1: Study population, inclusion criteria and group conformation.

FIGURE 2: Serological regression analysis of patients from Group 1 (panel A), younger patients (0.59-4 years-old) from Group 1 (panel B), older patients (4-10 years-old) from Group 1 (panel C) and Group 2 (panel D). tELISA and TSSA-ELISA results (expressed as % of the first sample) are indicated in solid and dotted lines, respectively. Slope (95%CI) and $r^2$ values are indicated for each data set. P: Pre-treatment. ANCOVA analyses were performed to compare slopes.

FIGURE 3: Kaplan-Meier curves of seronegativized patients from Group 1 (panel A) and 2 (panel C). Upon stratification of Group 1 by age, similar analysis was performed for younger (0.59-4 years-old, violet) and older (4-10 years-old, orange) patients (panel B). tELISA and TSSA-ELISA results are indicated in solid and dotted lines, respectively. Median (95%CI) values are indicated for each data set. Censored cases are indicated with square symbols. Log-rank (Mantel Cox) analyses were performed to compare median time of seronegativization. N/A: Confidence Interval was not calculated due to the small number of samples.

FIGURE 4: Serological regression analysis (panel A) and Kaplan-Meier curves comparing seronegativization of patients from Group 3 (panel B) or Group 2 vs Group 3 (panel C, grey and blue lines, respectively), determined either by tELISA (solid lines) or TSSA-ELISA (dotted lines). Slope (95%CI) and $r^2$ values (panel A) or median (95%CI) values (panel B) are indicated for each data set. ANCOVA and Log-rank (Mantel Cox) analyses were performed to compare slopes and median time of seronegativization, respectively.
FIGURE 5: TSSA-ELISA (black circles), tELISA (grey triangles), SAPA-ELISA (light grey squares), IHA and PCR results for patient REC52 are indicated. The black arrow and black dotted line, respectively, indicate treatment initiation and the cutoff determined for both TSSA-ELISA and SAPA-ELISA. N/D, not done.
78 children born to *T. cruzi*-infected mothers

Children over 8 months of age → Children under 8 months of age

Positive serological tests (n=26)

Positive parasitological tests (n=12)

Negative parasitological tests (n=40)

60-day treatment
Serum samples taken at 0, 7, 30 and 60 days

Follow-up
Serum samples taken every 3-6 months

Follow-up
Serum samples taken at 3 and 6 months

Confirmatory conventional serology
at ~ 9 months of age

CASE REC52

Group 1
26 *T. cruzi*-infected children

Group 2
12 *T. cruzi*-infected children

Group 3
39 non-infected children
A

Group 1

Group 1

Group 2

Months after treatment

Seronegativization (%)