Diagnosis of Congenital Chagas Disease Using an Iron Superoxide Dismutase Excreted as Antigen, in Mothers and Their Children During the First Year of Life

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Background: Chagas disease caused by Trypanosoma cruzi is endemic in Latin America. Human infection is mainly spread by Triatominae insects. Other forms of transmission are congenital, blood transfusion and organ transplantation.

Methods: Anti-T. cruzi antibodies were determined by enzyme-linked immunosorbent assay (ELISA) and Western blot (WB) in 155 serum samples from mothers and their babies. Indirect immunofluorescence (IFA) and a commercial test were used to validate efficacy of a specific ELISA–iron-excreted superoxide dismutase assay. Sera from babies were collected at 6 and 12 months, whereas maternal samples were obtained after delivery. Calostrum and umbilical cord samples were simultaneously obtained.

Results: Anti-T. cruzi antibodies were detected in 8 (5.16%) mothers by ELISA-WB, in 7 (4.51%) using IFA and in 1 (0.64%) by a commercial kit. Nine (5.80%) 6-month-old children were positive by ELISA-WB and 7 (4.51%) by IFA; negative results were obtained when the commercial kit was used. At 12 month of age, 15 (9.67%) children were positive by ELISA-WB, 13 (8.38%) by IFA and 1 (0.64%) by the commercial test. Antibodies were detected in 4 mothers whose children were serologically negative. Four other mothers and their children were positive, but only one of them had detected antibodies in umbilical cord up to 12 months, thus assuming vertical transmission.

Conclusions: The use of iron-excreted superoxide dismutase as antigen in serologic tests for detection of T. cruzi yielded promising results as diagnostic procedure.

Key Words: congenital transmission, iron-excreted superoxide dismutase, serological test, Trypanosoma cruzi

Chagas disease, a major endemic parasitic disease in Latin America, is caused by the protozoa Trypanosoma cruzi. The transmission from invertebrate vectors to mammals is done by bedbugs of the subfamily Triatominae (Hemiptera: Reduviidae), distributed from the southern of the United States and northern Mexico to Argentina and Chile.1,2 Other means of infection include congenital transmission, blood transfusion, organ transplant, oral, and accidental infections of laboratory staff.3–5

The control for the transmission of Chagas disease is based mainly on controlling the vector spread and also on screening blood samples from blood banks. Early diagnosis of congenital transmission is considered fundamental.5–10 Congenital infection may go undetected because pregnant women infected by T. cruzi are not aware of the infection because of lack of information in endemic areas, and it can be at the chronic, asymptomatic stage.

Surveys have been conducted searching for the keys to the prevalence of congenitally transmitted Chagas disease in both endemic11–13 and nonendemic areas.14–16 Some studies have focused on decreasing the spread of the congenital disease transmission and have reported that proper treatment heals congenitally infected infants, demonstrating the effectiveness of the treatment in newborns.17

The first reported case of congenital transmission in Mexico was in 1998.18 Subsequently, other reports have appeared showing the prevalence of anti-T. cruzi antibodies in mothers, but the presence of congenital infection was ruled out.13,19,20 A study determined that the maternal–fetal overall transmission rate was 4.08%, demonstrating the high prevalence of congenital transmission in Mexico and confirming that the potential risk of congenital Chagas disease transmission constitutes a major way in which the disease spreads, posing a serious health problem.21

Currently, in Mexico, pregnant women are routinely screened, but a Chagas disease test is not included in that screening. Our research group has long-term experience using serologic tests to diagnose Chagas disease, based on the immunogenic properties of the T. cruzi partially purified iron-excreted superoxide dismutase protein (FeSODe).22–24 Therefore, the main goal of the present study was to evaluate the use of the FeSODe in-house enzyme-linked immunosorbent assay (ELISA) and Western blot (WB) to diagnose congenital Chagas disease transmission.

MATERIALS AND METHODS

Study Population

All gestating women over 18 years old attended from February to July 2012 by the gynecologist and obstetrician service at the General Hospital of Merida, state of Yucatan, in southeastern Mexico, were included in the survey.

All pregnant mothers agreed to participate in the study by signing a letter of consent, which was approved as ethical protocol by the Ethics Committee for Research of the University of Granada (Spain).

Biological Samples

Samples of venous umbilical cord blood from the newborns and calostrum and blood from the mothers taken at the moment of delivery were collected and placed in tubes (5 mL). At the sixth and twelfth months after the babies were born, blood samples were taken. The blood was centrifuged at 2000 rpm for 10 min to separate

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serum from globular fraction. Serum was stored and frozen at −20°C until used. Also, the study included 10 and 3 serum samples from individuals diagnosed for Leishmaniasis and malaria, respectively, provided by the Cancun State Centre of Blood (CETS, Mexico), to prove the possibility of cross-reactivity with other trypanosomatids and other parasites. In addition, sera from 10 healthy subjects living in a nonendemic area (Spain) were used as control sera to determine the cut off for individual runs of the ELISA assays.

Parasite Culture

Epimastigote forms of *T. cruzi* H4 (MHOM/MX/2001/H4), isolated from a human patient with severe pathology in the state of Yucatan, were used and cultured on axenic liquid (MTL) medium (Gibco, Alcobendas, Madrid, Spain), supplemented with 10% heat-inactivated fetal bovine serum at 28°C in plastic Falcon flasks.

Extraction and Purification of the Fe-SODe

The Fe-SODe was obtained following to Marin, and then the protein content was determined using Bio-Rad test, based on the Bradford method (Sigma Immunochimical, St Louis, MO), with bovine serum albumin as standard. The Fe-SODe obtained was used as the antigen fraction on both ELISA and WB immune assays.

Serological Tests

ELISA Assay

The semipurified FeSODe fractions, at a concentration of 1.5 μg, were coated onto a 96-well polystyrene plate (Nunc, Penfield, NY) in carbonate–bicarbonate buffer (0.1 M, pH 9.6) for 2 h at 37°C.

ELISA-FeSODe was carried out following the protocol described by López et al. Absorbance was read at 492 nm in a Sunrise microplate reader (Tecan, Las Rozas, Madrid, Spain). All samples were analyzed 3 times. The mean and standard deviation of the optical densities of the negative control sera were used to calculate the cutoff value (mean + 3 × standard deviation).

Western Blot

The antigen fraction of FeSODe (at a concentration of 1.5 μg of protein) was run on isoelectric focusing 3–9 gels and afterwards transferred to nitrocellulose membrane (Hybond C Extra, GE Healthcare, Barcelona, Spain) using the Phast-Transfer kit following the manufacturer’s instructions (PhastSystem handbook). The WB was carried out as described elsewhere.

Indirect Immunofluorescence

All the samples were proved using an indirect immunofluorescence (IFA). Parasites were harvested by centrifugation at 1500 rpm for 10 min, washed 3 times, and suspended in phosphate-buffered saline (PBS) at a ratio of 5 × 10^6 parasites/mL. Samples of the suspension (10 μL) were placed in a CoverWell imaging chamber (Grace Bio-Labs, Bend, OR), allowing the buffer to evaporate, and the cells were fixed with acetone at room temperature. Next, the immunofluorescence staining was performed. First, the parasites were incubated with the sera from patients and the control (positive) sera for 30 min at room temperature (using a final dilution 1:32). The CoverWell was then washed 3 times with PBS and incubated for 30 min at room temperature with the secondary antibody (anti-human IgG conjugated with fluorescein 5-isothiocyanate; Sigma-Aldrich, St Louis, MO) at a dilution of 1:100 in PBS and with Evans blue (0.003%). Finally, the CoverWell was washed and the coverslips put in place with PBS-buffered glycerine (9 volumes of glycerine, per 1 volume of PBS) for quantitative examination under a Motic BA410 fluorescence microscope (Motic, Barcelona, Spain).

Commercial Test

Stick Chagas (Operon SA, Zaragoza, Spain) is an immunochromatographic test capable of detecting antibodies specific for various immunologically relevant *T. cruzi* antigens. The test is based on the immunologic capture of microparticles that are colored and coated with a multi-antigen protein as they pass through a membrane on which the same multi-antigen protein has been immobilized. If the sample contains antibodies against *T. cruzi*, they react with red colloidal particles that are conjugated to the multi-antigen protein. The colloidal particle–antibody complexes then migrate through the chromatography area to the reaction area, producing a red band in the test window.

Statistical Analysis

Data were registered on an Excel sheet (Microsoft, Redmond, WA). Statistical analysis was performed by SPSS Statistics 17.0 software (IBM, NY). Those analyses were based on contingency tables for the study of prevalence, using the statistical χ^2 to demonstrate the relationship between the variations.

The sensitivities and specificities of the serological tests were calculated as in Thrusfield.

RESULTS

A total of 155 mother–child pairs were tested. All mothers were from both the urban and peri-urban areas of the city of Merida, state of Yucatan, Mexico. The average age was 23 ± 4.8 years, the number of pregnancies was 2.2 ± 1.5, and the average weight of newborns was 3.09 ± 0.420 kg.

Samples for analysis were taken after giving birth as follows: colostrum and umbilical cord simultaneously, and after 24 h, sample from each mother was taken. Also, after 6 and 12 months sera from babies were obtained to complete the scheduled protocol, testing a total of 775 samples (155 × 5 samples).

All samples were tested using all 4 serological tests (ELISA-FeSODe, WB, IFA and Stick Chagas commercial test), and samples that were positive to ELISA-FeSODe also proved positive to WB. Samples that were positive to 2 different techniques were considered a true positive.

The seroprevalence of *T. cruzi* infection in mother samples with ELISA-FeSODe/WB was 5.16%, whereas 4.51% was determined using IFA and 0.64% when using the commercial test. From the sample collected at the sixth month of infant life, 5.80% were positive using ELISA-FeSODe/WB, 4.51% using IFA and none when the commercial test was used. The percentages of positive sera collected at the twelfth month were as follows: 9.67% with ELISA-FeSODe/WB, 8.38% using the IFA test and 0.64% using commercial test. (Table 1, Figure 1)

After overall serological analysis, 8 mothers tested positive: 1 sample was positive to ELISA FeSODe/WB (case No 15), 6 proved *T. cruzi*-positive using 3 techniques (ELISA-FeSODe/WB and IFA), and only 1 sample was positive to all 4 techniques (case No 99). Four mothers were found to be positive with these techniques but their children were found negative (cases 33, 74, 99 and 118) at 6 and 12 months of age. With respect to the other 4 positive women, their children tested positive to the same anti-*T. cruzi* antibody tests. Furthermore, in 2 of these positive mothers, samples from umbilical cord (case No 7) and calostrums (case No 14) also tested positive. The study also included 10 and 3 serum samples from mothers diagnosed for Leishmaniasis and malaria, respectively, as control sera to determine the possibility of cross-reactivity with other trypanosomatids and other parasites. In addition, sera from 10 healthy subjects living in a nonendemic area (Spain) were used as control sera to determine the cut off for individual runs of the ELISA assays.

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transmitted congenitally. Therefore, the congenital transmission rate from mother to child was deduced to be 12.5%.

Five of the children (3.22%) registered positive results at the sixth and twelfth month of life, whereas the sera from their mothers in all cases were negative. In 6 of the children (3.87%), the positive results were detected only until the 12th month of age. Thus, a total of 11 (7.09%) of the children showed anti-
*T. cruzi* antibody responses from a group of negative mothers.

The possibility of false positives by the ELISA test exists because of cross-reactivity with other trypanosomatids and other species belonging to the genus *Leishmania*. However, in previous studies, we have shown that FeSODE is species-specific and, therefore, shows no cross reactions with other trypanosomatids or with other pathogenic organisms.30,31 In this study, no cross reactions were detected with *Leishmania* and malaria.

**DISCUSSION**

Based on epidemiological data, it is estimated that ≈243,000 women in Mexico are infected with Chagas disease and that some 1100 newborns are at risk of infection each year.18 Despite that the congenital transmission of *T. cruzi* has been reported in Mexico since 1998, further studies are needed. 18 Maternal seroprevalence found in this study (5.16%) is consistent with other studies conducted in 2 different areas of Mexico.19,21 Nevertheless, if we compare our

### TABLE 1. Results in Maternal Sera at the Time of Delivery and in Their Children at 6 and 12 Months of Age

<table>
<thead>
<tr>
<th>Case</th>
<th>Diagnostic Test</th>
<th>Mother</th>
<th>Child 6 Months</th>
<th>Child 12 Months</th>
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<td></td>
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<td>E</td>
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<td>7*</td>
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<td>118</td>
<td></td>
<td>+</td>
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</table>

*Case shows the presence of anti-*Trypanosoma cruzi* antibodies in colostrum.
†Case shows the presence of anti-*T. cruzi* antibodies in umbilical cord.
CK indicates commercial kit.

**FIGURE 1.** Western blot of representative positive and negative sera against *T. cruzi* Fe-SODE antigen. a and b are children’s sera at 6 and 12 months of age, respectively; C is Fe-SODE activity in isoelectrofocus and staining after the technique of Beyer and Fridovich29; and M is mothers’ sera.

We found no statistically significant difference between the ELISA-FeSODE and WB serological tests, and therefore, we compared these tests as one for statistical analysis. As a result, we determined 100% sensitivity for ELISA-FeSODE/WB and, considering the IFA to be a standard test, we found specificity to be between 98.59% and 99.32%. The Kappa index during the comparison was between 0.86 and 0.93 (see Table 2).

Only 2 of the samples proved positive (1 from a mother and the other from a child) with the commercial test, cases 99 and 21, respectively.

**TABLE 2.** Evaluation of the In-house Serological Tests

<table>
<thead>
<tr>
<th>Reliability</th>
<th>IFA/E-WB*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mother</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>100%</td>
</tr>
<tr>
<td>Specificity</td>
<td>99.32%</td>
</tr>
<tr>
<td>PPV</td>
<td>87.5%</td>
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<tr>
<td>NPV</td>
<td>100%</td>
</tr>
<tr>
<td>Kappa index</td>
<td>0.93</td>
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</tbody>
</table>

*Calculation made take IFA as reference test.
E-WB indicates ELISA SODe-Western blot; NPV, predicted negative value; and PPV, predicted positive value.

Based on epidemiological data, it is estimated that ≈243,000 women in Mexico are infected with Chagas disease and that some 1100 newborns are at risk of infection each year. Despite that the congenital transmission of *T. cruzi* has been reported in Mexico since 1998, further studies are needed.18 Maternal seroprevalence found in this study (5.16%) is consistent with other studies conducted in 2 different areas of Mexico.19,21 Nevertheless, if we compare our
results with the maternal prevalence previously found in Merida, our prevalence is higher (5.16% vs. 0.80%) than values previously published. These results may reflect the advantage of using semipurified protein originating from a local strain of the parasite in ELISA-FeSODc and WB assays. These suggest that the lower sensitivity of the rapid test in Mexico is because of the antigen used in the test to the specific immune response induced for the autochthonous strain of parasites. When they, seeking to increase the sensitivity, used a whole parasite lysate from a local strain H1 of T. cruzi in a noncommercial test, they found no statistically significant difference between the commercial test vs. noncommercial test. The total percentage of the maternal infection that we found in this study (5.16%, 8 mothers) was similar to that published from the states of Jalisco, Oaxaca, and Mexico City. The reason why some mothers transmit the infection to their babies and others do not remains unknown. The same was found in the present study, where only 4 children were positive from 8 positive mothers. On the other hand, only in 1 child (case No 7) was it possible to detect antibodies in the sample of umbilical cord, at the sixth month and at the twelfth month after birth. Given that after 8 months the mother’s antibodies should have disappeared in all infants, this indeed suggests congenital transmission. In order for a case to be considered to be congenital Chagas disease, 2 criteria must be fulfilled: (1) the infant has to be born from a mother with positive serology for T. cruzi determined by 2 different methods; and (2) anti-T. cruzi antibodies must be detected in the newborns (umbilical cord) and remain positive after 9 months of life.

The data compiled in the present work show that vertical transmission is occurring at this region with an overall rate at 12.5% (1/8). Mothers and children who tested positive were referred to the Ministry of Health for trypanocidal treatment. The accurate diagnosis of Chagas disease is a complex process. There is no consensus about which is the best test, although several studies evaluating different techniques have been performed. The sensitivity and specificity observed in ELISA and WB tests demonstrate that the protein FeSODc used as antigen has immunogenic properties and that it becomes an appropriate molecule to be used in Chagas disease diagnosis.

Early infection in children suggests that contact with the vector is possible because of various factors, such as living in a peri-urban area characterized by poverty and low quality of dwellings and also cultural aspects such as cohabitating with domestic animals such as cats and dogs (which act as major reservoirs). This is likely because percentages of infestation by Triatoma dimidiata are above 70%, whose infection range from 21.9% to 45.9% in the Yucatan peninsula.

Health authorities should develop screening for congenital Chagas disease by promoting the implementation of techniques for serological diagnosis of infection by T. cruzi and detection of Chagas infection in pregnant women and their children. The Ministry of Health in Mexico should study and evaluate the incorporation of the Chagas disease test in pregnant women in the routine screening.

The present study confirms the potential risk of nonvector Chagas disease transmission, demonstrating that congenital transmission is an important way in which Chagas disease spreads. The data presented here suggest that it is important to closely supervise the child born from infected mothers at least during the first year of life as a preventive initiative to avoid the propagation of the infection. Furthermore, we suggest the use of the FeSODc from T. cruzi as antigen in serological test for antibody detection because of its promising sensitivity and specificity.

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