Risk Factors and Primary Prevention of Congenital Chagas Disease in a Nonendemic Country

Laura Murcia, Bartolomé Carrilero, M. Jose Munoz-Davila, M. Carmen Thomas, Manuel C. López, and Manuel Segovia

Background. In this longitudinal cohort study we evaluated the congenital transmission of Chagas disease (CD) in a nonendemic area. The aim of this work was to analyze the predictive value of a *Trypanosoma cruzi*–positive polymerase chain reaction (PCR) result in pregnant women for the diagnosis of vertical transmission and to evaluate the use of PCR as a tool for early detection of infection.

Methods. The offspring of 59 seropositive pregnant mothers were followed up. The parasitological status of mothers was studied by PCR in a total of 64 pregnancies; 10 of these women had received treatment before pregnancy. Sixty-five infants (including a pair of twins) were monitored at 0, 6, 9, and 12 months of age by PCR and serology. In cases of congenital transmission, hemoculture and parasite lineage typing were performed.

Results. Nine infants had acquired CD congenitally. This represents a transmission rate of 13.8% among seropositive mothers (9 infected newborns of 65 total live births). All infants were infected with *T. cruzi* discrete typing unit V strain. A statistically significant correlation was found between *T. cruzi* vertical transmission and a positive PCR result during pregnancy (31%; 9 infected newborns in 29 live births). No infected infants were detected among 10 mothers who were treated before they became pregnant, compared with 16.4% (9 of 55 live births) among untreated mothers.

Conclusions. PCR is a useful tool for the detection of congenital CD, and the treatment of infected women of childbearing age seems to be useful for preventing vertical transmission.

Keywords. congenital; Chagas; *Trypanosoma cruzi*; treatment; prevention.

Chagas disease (CD) is a parasitic zoonosis caused by *T. cruzi* and is endemic in 21 countries of Latin America. It affects 8 million persons globally [1]. The parasite is transmitted through the feces of an infected triatomine, by blood transfusion or organ transplantation, congenitally [2–4], and by oral ingestion of contaminated food or drink [5]. CD is also a health problem in nonendemic countries where there is an influx of immigrants from endemic areas [6–8]. Thus, legal requirements to rule out *Trypanosoma cruzi* transmission in blood and organs donors have been implemented in Spain and other European countries [6, 9, 10].

The congenital transmission rate reported in Latin American countries varies from 0.7% to 18.2% [11]. Several congenital cases have been reported in nonendemic countries [8, 12], although the lack of mandatory screening and the fact that most infected newborns are asymptomatic means that its incidence is unknown. Diagnosis of congenital infections is based on parasitological and serological techniques [13]. The etiological treatment of congenitally infected newborns is effective [14]; nevertheless, it must begin as early as possible to avoid progression toward the chronic
phase of the disease [14, 15]. Treatment of infected women of reproductive age has also been proposed as a way of interrupting disease transmission [16].

Of all European countries, Spain receives the largest number of immigrants from Latin America [17]. Latin American women of child-bearing age make up 10.2% of the fertile-age population in Murcia. Of these, 20.9% come from Bolivia, a country with high rates of vertical transmission [18].

In this longitudinal cohort study we analyzed bloodstream samples from 59 pregnant women with chronic CD and their offspring to assess the rate of congenital infection in a nonendemic area and to evaluate the risk factors and parasite strains involved in vertical transmission.

METHODS

Subjects and Data Collection
Patients underwent serological diagnostic testing for CD and clinically characterized, treated, and clinically followed up after treatment at the Hospital Virgen de la Arrixaca, Murcia, Spain, from January 2007 to December 2011. A total of 59 T. cruzi–seropositive pregnant women were enrolled in this study. They came from Bolivia (57 women; 96.6%) and Paraguay (2 women; 3.4%) and ranged in age from 15 to 43 years (mean age ± standard deviation, 29.1 ± 6.5 years).

Diagnosis of T. cruzi infection in mothers was made using 2 serological tests performed with different antigens [19]—the indirect immunofluorescence assay (Inmunofluor Chagas; Biocientífica) and the enzyme-linked immunosorbent assay (T. cruzi ELISA test system; Ortho Clinical Diagnostics). Mothers with positive results for both serological tests were considered infected by T. cruzi. The presence of parasites in mothers and newborns was assayed by polymerase chain reaction (PCR) amplification of the kinetoplast minicircle DNA of T. cruzi in peripheral blood (see below). For the diagnosis of congenital infections, serology and PCR were performed at 0–2, 6, 9, and 12 months of life. Congenital infection was considered when parasites were detected by PCR and/or hemoculture at any age or when serological findings remained positive at 12 months of life [13]. The “congenital transmission rate” was defined as the number of congenital infections divided by the total number of live births.

Newborns were examined to evaluate the clinical manifestations related to symptomatic congenital CD. Thus, the presence of skin lesions, neurological signs, symptoms of respiratory distress syndrome, cardiovascular disorders, and digestive disturbances such as hepatosplenomegaly were analyzed.

Benznidazole Treatment
Infants and mothers were orally treated with benznidazole, at 10 mg/kg body weight per day in newborns and 100 mg 3 times a day in women for 60 days [20]. Serology and PCR were performed at the time of diagnosis and 60, 150, and 360 days after therapy. Patients were considered cured when conventional serological findings were negative in 2 successive tests [19].

DNA Extraction and Kinetoplast Minicircle PCR Amplification
Peripheral blood samples were collected from T. cruzi–infected women and infants (10 or 2 mL, respectively). DNA extraction and PCR detection of the 330–base pair variable regions of the T. cruzi kinetoplast minicircle genome were carried out as described by Murcia and colleagues [21].

Parasite Culture
Total peripheral blood (200 μL) from newborns was incubated in liver infusion tryptone medium/Novy-MacNeal-Nicolle biphasic medium, supplemented with 10% heat-inactivated fetal calf serum at 28°C and microscopically examined weekly to visualize parasites.

Genomic DNA Purification
The isolated parasites from 6 newborns of the 7 studied were grown, collected by centrifugation at 3500 rpm for 15 minutes at room temperature, and washed twice with phosphate-buffered saline (pH 7.4). Parasite pellets were resuspended in lysis buffer (10 mmol/L Tris–hydrochloric acid [pH 7.6], 10 mmol/L ethylenediaminetetraacetic acid, 100 mmol/L sodium chloride, 0.5% sodium dodecyl sulfate), and digested for 30 minutes at 37°C in the presence of 100 mg/mL RNase A (Roche Diagnostics). Proteinase K (Roche Diagnostics) was then added to a final concentration of 0.3 mg/mL and incubated at 55°C for 30 minutes, with regular inversion. Genomic DNA concentration and integrity were determined by means of agarose-gel electrophoresis and spectrophotometric detection using a NanoDrop 1000 spectrophotometer (Thermo Scientific).

PCR Amplifications for Identification of Discrete Typing Units
The lineage identification of the parasite isolated from congenitally infected newborns was assayed by PCR of the mini-exon, the A10–e fragment, and the 18S and 24S genes. The PCR amplification of the mini-exon and 24Sα ribosomal RNA (rRNA) was performed as described by Brisse and colleagues [22]. PCR for amplification of the size-variable domain of the 18S rRNA sequence was carried out as described by Clark and colleagues [23]. Amplification of the A10–e fragment was performed by PCR, as described by Brisse and colleagues [24].

Statistical Analysis
The χ2 test was used to compare qualitative variables. Relationships were considered significant at P < .05. All statistical tests were performed with SPSS 15.0 software.
Ethical Considerations

The study was reviewed and approved by the Ethical Committee of the Hospital Virgen de la Arrixaca. Written informed consent was obtained from all patients enrolled in the study.

RESULTS

Congenital Transmission in Newborns

A total of 59 seropositive pregnant women were included in the study. We observed 1 twin birth, and 5 mothers became pregnant twice during the 5-year study period. Diagnosis and follow-up involved 65 infants. Nine newborns were found to have acquired the disease congenitally. Thus, among seropositive mothers, the congenital transmission rate was 13.8% (9 infected newborns of 65 live births). All the infected children were born to Bolivian mothers.

Six of the 9 congenitally infected infants (66.6%) were born at term and did not show any clinical manifestations of symptomatic congenital CD. The other 3 were symptomatic. Twin brother 1 presented with low birth weight, abdominal distension with hepatosplenomegaly, signs of jaundice, and myocarditis with signs of cardiac failure and respiratory distress. Twin brother 2 did not suffer the same severe clinical course but had a low birth weight and splenomegaly. The other symptomatic newborn had low Apgar scores (3 and 6, respectively) at 1 and 5 minutes, low birth weight, abdominal distension with hepatosplenomegaly, purpuric lesion, cardiac failure, severe pulmonary hypertension, and respiratory distress [8].

Diagnostic Follow-up

*T. cruzi* DNA was detected by PCR in the bloodstream of 9 infected infants, confirming vertical transmission. Of these infants, 5 had a positive PCR result in their first months of life; in the other 4, because blood samples were not taken at birth, the diagnosis by PCR was performed at age 6 months (3 infants) or 12 months (1 infant) (Table 1). The parasite was isolated by hemoculture in 3 of the 5 infants with infection diagnosed by PCR at age 0–2 months and 2 of the 3 with diagnosis at 6 months. Culture was also positive in the infant who had both positive serological and PCR results at 12 months of age (Table 1).

Among uninfected infants, all had negative PCR results throughout the diagnostic follow-up, and the negative serological results at 8 and/or 12 months of age ruled out *T. cruzi* infection.

Typing of Parasites Isolated From Congenitally Infected Newborns

Analysis of the PCR amplification products of the nontranscribed spacer of the mini-exon genes showed a specific band of *T. cruzi* discrete typing units (DTUs) II, III, IV, V, and VI, and the analysis of the size-variable domain of the 18S rRNA sequence identified the parasites as belonging to *T. cruzi* DTU II, III, or V. The PCR amplification products of the D7 divergent domain of the 24Sα rRNA showed that they belonged to *T. cruzi* DTU V. In addition, the PCR amplification of the A10-e fragment confirmed that all the newborns were infected by a *T. cruzi* DTU V strain.

Treatment Follow-up of Congenitally Infected Infants

All congenitally infected newborns were treated with benznidazole. Serology and PCR during the posttreatment follow-up period were performed in 8 of the 9 infected infants. All newborns showed good tolerance to benznidazole (no side effects

### Table 1. Diagnostic Follow-up by Parasite Growth, Serology, and Polymerase Chain Reaction in 9 Infants With Congenital Chagas Disease

<table>
<thead>
<tr>
<th>Infant</th>
<th>Age at Diagnosis, Months</th>
<th>Parasite Growth</th>
<th>Age 0–2 Months</th>
<th>Age 6 Months</th>
<th>Age 9 Months</th>
<th>Age 12 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>IFI</td>
<td>ELISA</td>
<td>PCR</td>
<td>IFI</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>Yes</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>Yes</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>Yes</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>Yes</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>No</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>No</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>12</td>
<td>Yes</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
</tr>
</tbody>
</table>

Plus signs indicate positive results.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IFI, indirect immunofluorescence assay; ND, not done; PCR, polymerase chain reaction.
were observed) and all cases were cured. PCR results became negative in 7 infants who had initially positive results and remained negative until the end of follow-up. One newborn in whom treatment was not administered correctly had a negative PCR result at 60 days and a positive result at 150 days. Consequently, the treatment had to be carefully supervised to ensure administration of the complete course. Subsequent PCR results were negative at all times throughout the posttreatment follow-up, which was considered an indication of cure (Table 2).

**Treatment of Infected Women to Prevent Vertical Transmission**

Ten of the 59 pregnant women had their infection diagnosed before they became pregnant. Consequently, they had been treated for CD before the study. None of their 10 offspring was born infected. The PCR follow-up and the timelines from treatment to pregnancy are shown in Table 3.

Interestingly, 1 untreated mother gave birth to an infected infant and, after her infection was diagnosed and treated, gave birth to a second, healthy infant (Figure 1). In contrast with the congenital transmission rate among treated mothers, which was 0%, the rate among untreated mothers was 16.4%, because 9 infants were infected, from a total of 55 live births (Figure 1). Consequently, untreated mothers were more likely to transmit the infection to their offspring. However, probably because of the small number of treated mothers in our study, there was not a statistically significant correlation between the treatment of infected women and the prevention of vertical transmission ($P = .1989$; 1-sided Fisher test).

### Table 2. Serological and Polymerase Chain Reaction Posttreatment Follow-up in Infants With Congenital Chagas Disease

<table>
<thead>
<tr>
<th>Infant</th>
<th>60 Days After Treatment</th>
<th>150 Days After Treatment</th>
<th>360 Days After Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IFI</td>
<td>ELISA</td>
<td>PCR</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>4</td>
<td>−</td>
<td>ND</td>
<td>−</td>
</tr>
<tr>
<td>5</td>
<td>ND</td>
<td>ND</td>
<td>−</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>7</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>8</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>9</td>
<td>ND</td>
<td>ND</td>
<td>−</td>
</tr>
</tbody>
</table>

Plus signs indicate positive results; minus signs indicate negative results.

Abbreviations: CM, continued monitoring; ELISA, enzyme-linked immunosorbent assay; IFI, indirect immunofluorescence assay; ND, not done; PCR, polymerase chain reaction.

* Patient 7 was followed up twice after her first treatment. Treatment failure was diagnosed on the basis of a positive PCR result at 150 days, and a second treatment and posttreatment follow-up were performed.

### Table 3. Polymerase Chain Reaction Follow-up of Previously Treated Mothers

<table>
<thead>
<tr>
<th>Mother Before Treatment</th>
<th>End of Treatment</th>
<th>Duration After Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>150 Days</td>
<td>1 Year</td>
</tr>
<tr>
<td>1</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>3</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>7</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

Plus signs indicate positive results; minus signs indicate negative results.

Abbreviation: PCR, polymerase chain reaction.

* Test during follow-up by which time the mother had become pregnant.

### PCR as a Prognostic Tool for Congenital Transmission of CD

The parasitological status was studied by PCR in a total of 64 pregnancies, 10 of which involved previously treated mothers. Among untreated women, 28 cases had a positive PCR result (51.8%). Eight mothers with a positive PCR result transmitted the parasite (9 congenitally infected infants, including a pair of twins). However, there was no transmission to infants from mothers with a negative PCR result. This represents a 31% congenital transmission rate among mothers with a positive PCR result (9 infected newborns in 29 live births) and no congenital transmission among mothers with a negative PCR result (no infected newborns in 26 live births) (Figure 1).

PCR results were negative for all mothers who had been treated before pregnancy and none of them transmitted the infection to their offspring. Moreover, in 1 of the transmitting mothers with a positive PCR result, PCR results became negative after treatment and remained negative during a second gestation in which infection was not transmitted. There was a statistically significant correlation between vertical *T. cruzi* transmission and a positive PCR result during pregnancy ($P = .0046$; $\chi^2$ test).

### DISCUSSION

Diagnosis and prevention of congenital transmission of CD is becoming an important challenge in endemic and nonendemic areas. In this study the rate of placental transmission was 13.8%, this finding is similar to those in other studies on Bolivian infected mothers, in which a high prevalence of *T. cruzi* infection and a high rate of congenital transmission have...
been reported [25]. The number of congenital cases diagnosed in this study was higher than that found in other Spanish areas, where vertical transmission rates of 7.3% and 0% have been reported [26, 27].

This finding may be attributed to the meticulous screening of the infected mothers and their newborns carried out in this study, as well as the sensitivity of the parasitological technique used, which allowed the diagnosis of both symptomatic and asymptomatic cases [21, 25]. Thus, all confirmed congenital cases had a positive PCR result at 0 and 2 months of age. Therefore, PCR should be considered a useful tool for the early diagnosis of T. cruzi infection in infants born to seropositive women. Because the clearance of maternal antibodies occurs at 8–12 months of age, conventional serology does not have sufficient prognostic value to early diagnosis of congenital CD [28]. Nevertheless, even though a positive PCR result during the first 12 months of life is a direct indication of infection, follow-up serology is essential when results are negative, because a negative PCR result does not necessarily mean that the parasite is absent.

It has been proposed that PCR performed soon after birth could amplify the DNA of dead parasites from infected mothers [11, 14]. In our study, congenital infection was also confirmed by culture of the parasites from the blood of 3 of the 5 infants with infection diagnosed at 0–2 months of age, and from 2 of the 3 infants with diagnosis at 6 months. In a previous study on early diagnosis of congenital CD, the sensitivity of hemoculture was similar to that in our study (62.1% vs 66.7%) [29]. The typing of 5 parasite isolates showed the predominance of DTU V in the congenitally infected newborns, in agreement with other findings of Bolivian mothers and their offspring [30, 31]. Whether or not congenital infection is associated with some T. cruzi lineages is not clear [30].

Treatment with the available trypanocidal drugs is recommended for congenital cases [13]. In common with other observers, cure in patients with congenital CD was demonstrated by both serology and PCR negativity [14, 32]. In our study, PCR results became negative earlier or at the same time as serological results, probably reflecting the effectiveness of the treatment in clearing the parasites. PCR also confirmed therapeutic failure in 1 case, with results shifting to positive during the follow-up period. As we reported in a previous study, PCR is a sensitive and specific tool for the early detection of the parasite’s susceptibility to treatment [21].

An important limitation of these antiparasitic agents is the high incidence of adverse reactions [33, 34]. The incidence and tolerance of side effects seems to be associated with the age of the patient [33]. We observed good tolerance to benznidazole without side effects in all congenital cases treated during the first year of life. Early treatment increases the possibility of success, and so treatment must be administered as early as possible [14, 15]. In our study, cure was achieved in 100% of the congenital cases, a result that reinforces the need for early diagnosis and treatment of the congenital infection.

We found a statistically significant relationship between vertical transmission and a positive PCR result during gestation. Indeed, all transmitting mothers were PCR positive. Parasitemia detected by PCR should be a risk factor for the vertical transmission of CD. Consequently, PCR screening should be carried out in pregnant infected women to identify a priori those mothers who have a high probability of transmitting the infection to their offspring.

Regarding the prevention of vertical transmission, recent reports support the view that the treatment of infected women in early life is a good way to avoid congenital CD [16]. We detected a PCR-negative conversion in all mothers who

---

<table>
<thead>
<tr>
<th>Infected mothers</th>
<th>Pregnancies</th>
<th>PCR results during pregnancies</th>
<th>Newborns</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=59</td>
<td>54 pregnancies in untreated mothers</td>
<td>9 infected</td>
<td>n=29</td>
</tr>
<tr>
<td></td>
<td>10 pregnancies in previously treated mothers</td>
<td>0 infected</td>
<td>n=26</td>
</tr>
</tbody>
</table>

**Figure 1.** Comparison of vertical transmission and the polymerase chain reaction (PCR) results in treated and untreated infected mothers. One mother was pregnant twice, before and after treatment; she transmitted the infection before being treated. One of the 28 untreated mothers with positive PCR results gave birth to twins, both of whom were infected. Abbreviation: PCR, polymerase chain reaction.
became pregnant between 0 and 4 years after treatment, again indicating the effectiveness of benznidazole treatment for clearing parasites [21]. Our data suggest that although treatment of chronically infected women does not guarantee cure, the likelihood of congenital transmission is decreased by treating infected women before pregnancy. This sample size was too small for us to evaluate this finding statistically. The vertical transmission rate found in our study suggests that the real burden of congenital CD in nonendemic countries may be higher than that reported to date [12, 26, 27]. This highlights the need for systematic PCR screening for T. cruzi among infected pregnant women and their offspring in nonendemic countries. A direct relation between therapy in infected mothers and the prevention of vertical transmission was observed, suggesting that treatment of women before pregnancy prevents congenital CD transmission.

Notes

Acknowledgments. We thank A. Lopez-Barajas and C. Marañón (Instituto de Parasitología y Biomedicina López Neyra, Consejo Superior de Investigaciones Científicas) for their technical assistance in the parasite culture and genomic DNA purification.

Financial support. This study was supported by the Network of Tropical Diseases Research RICET (grants RD06/0021/1007 and RD06/0021/0014) and the Project of Research in Health (grant PS09/01956). M. C. T. and M. C. L. were also supported by the Junta de Andalucía (grant P08-CVI-04037), I + D + I National Plan (MEC-Spain), and FEDER (grant BFU2010-1670).

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References


