DETECTION OF TRYPANOSOMA CRUZI AND TREATMENT MONITORING BY PCR FROM DRIED BLOOD SPOT SAMPLES IN CHILDREN

DETECCIÓN DE TRYPANOSOMA CRUZI Y MONITOREO DEL TRATAMIENTO CON PCR EN GOTAS DE SANGRE EN PAPEL.

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Abstract

Background: Parasitic infections by Trypanosoma cruzi (T. cruzi) are frequent in children from endemic areas. Specific therapies have been successfully used in pediatric populations to treat this disease. T. cruzi diagnosis should be optimized and become available for any clinical environment.

Objective: To study T. cruzi prevalence in children from an area of active transmission and carry out a post-treatment follow-up. To verify the feasibility of detecting DNA of
T. cruzi from dried blood spot.

Methods: We analyzed presence of T. cruzi in 78 Aboriginal children (Toba community) that attended to a rural school of Chaco province, Argentina. Serum and whole blood (dried blood spot) were assessed by means of serological techniques and PCR. Positive children received Benznidazole. Diagnosis and post treatment follow-up of T. cruzi infection were performed.

Results: The serology assay showed infection in 34 of 78 (43.5%) children studied; PCR was positive in 5/34, displaying parasitemia. Serology remained positive in 28/28 children 120 days post-treatment, while PCR was positive in 18/28 (6/34 children were lost in follow-up). No adverse effects during the treatment were reported.

Conclusions: We were able to establish T. cruzi prevalence in the studied population and also to prove the usefulness of dried blood spot for T. cruzi detection using PCR in isolated areas. This method allowed us to verify early treatment failure. Possible causes of this failure are discussed below.

Resumen

Antecedentes: Las infecciones por Trypanosoma cruzi (T.cruzi) en niños son frecuentes en áreas endémicas. Las terapias específicas contra el parásito son exitosas especialmente en edad pediátrica. El diagnóstico de infección por T.cruzi debería estar optimizado y disponible para ser utilizado indistintamente en áreas urbanas o rurales.

Objetivos: Estimar la prevalencia de T.cruzi en una población pediátrica de un área endémica, además de realizar un seguimiento post tratamiento. Comprobar la factibilidad de detección de ADN de T.cruzi en gotas de sangre en papel.

Métodos: Se estudió la presencia de T.cruzi en 78 niños aborígenes (Comunidad Toba) de una escuela rural de la Provincia de Chaco, Argentina. Se utilizaron suero y sangre entera (gotas en papel nucleico) para estudios serológicos y PCR respectivamente. Los niños con infección confirmada recibieron tratamiento con Benznidazole.

Resultados: Los ensayos serológicos demostraron la infección debida a T.cruzi en 34 de 78 (43.5%) niños estu-

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Supported by Hospital Privado Universitario de Córdoba
The authors have no conflicts of interest to disclose

diados; en tanto que la PCR fue positiva en 5/34, confirmando parasitemia. Luego de 120 días post tratamiento, la serología siguió positiva en 28/28 niños, en tanto que la PCR fue positiva en 18/28 (6/34 niños no pudieron incluirse en el seguimiento). No se reportaron efectos adversos durante el tratamiento.
Conclusiones: Se estableció la prevalencia de T.cruzi en la población estudiada y se demostró la utilidad de gotas de sangre en papel nucleico para la detección del parásito mediante PCR en áreas inhóspitas. Esta metodología nos permitió verificar una falla temprana en el tratamiento. Posibles causas de esta falla son discutidas en este trabajo.

Palabras claves: T. cruzi - PCR – gotas de sangre disecadas en papel
Key words: T. cruzi - PCR - dried blood spot

Introduction

Chagas's disease or American Trypanosomiasis is a parasitic disease caused by the protozoa T. cruzi; the primary factor associated with its transmission is the presence of the triatomine insect vector in the households. Other forms of transmission include blood transfusion, occupational accidents, organ transplantation, as well as oral and congenital transmission. This last pathway and vector-borne are the main transmission routes in the pediatric population. Infected live-born infants can be classified into three clinical categories: a- critical disease at birth with high risk of death, b- perceptibly healthy child at birth with progression to symptoms in the first weeks to months and c- asymptomatic during childhood, but with risk of cardiac or gastrointestinal involvement decades later. Most infected newborns are asymptomatic or have subtle findings and fall into categories b or c [(1)]. The acute phase is followed by an asymptomatic chronic state that ends producing irreversible cardiac injury in 30% of the cases [(2)].
It is estimated that 16 to 18 million persons are infected by T. cruzi worldwide; 50,000 of them die each year. In Argentina, during the last decade, improvements were made for control of vector transmission. Nevertheless, T. cruzi infection is more stressed in aboriginal communities of Chaco province, Argentina, with a seroprevalence of 23-70% [(3,4,5)]. However, the Technical Report of the Chagas Disease National Control Program in Argentina presented in July 2011 at the XVIII Intergovernmental Meeting INCOSUR/Chagas reported a serological prevalence of 14% in children aged 5 to 14 years. In addition, it should be taken into account that in areas of active transmission and without vector control, new infections occur mainly before the children reach the age of 10-14 years [(6)] .
Nowadays, Benznidazole (Bz) and Nifurtimox are the drugs accepted for treatment of Chagas' disease [(7)]. Bz has been successfully used in pediatric populations with favorable reports regarding efficacy and toxicity [(8,9,10)].
The goal of our study was to estimate the prevalence of T. cruzi infection in children that attended to the rural school of an aboriginal community living in an endemic area. For this purpose, we used serological studies and qualitative Polymerase Chain Reaction (PCR) testing blood samples preserved in filter paper (dried blood spot). Short-term efficacy of the treatment was evaluated.

Methods

Study population and serological assay

A prospective study of 78 aboriginal children that attended a rural school of a Toba community (approximately 500 people) located in area number 60 of San Martin Department, Chaco province, Argentina, was performed from 2006 until 2011. The cohort consisted of 42 girls and 36 boys, aged 5 to 16 years-old, mean ± SD: 10 ± 2 years old. The serological diagnosis of Chagas disease was performed according to WHO guidelines [(11)]. Indirect hemagglutination (IHA, Chagatest, Wiener lab, Rosario, Argentina), Enzyme-linked immunosorbent assay (ELISA, Chagatek, Buenos Aires, Biomeriux Argentina) and Indirect immnunofluorescence (IFI, Ififluor Parasitest Chagas, Laboratorio IFI, Buenos Aires, Argentina) were used according to the manufacturer's specifications.
Serum was obtained from capillary blood (2 mL) by means of puncture with digital lancet (BD, Franklin Lakes, NJ USA), collected in sterile tubes with polypropylene gel (BD, Franklin Lakes, NJ USA) which were centrifuged and then stored at 4°C until they were processed.
A child was considered positive for T. cruzi infection when two serological techniques yielded positive results. Only these children were subjected to a specific PCR to detect the parasite in peripheral blood. All tests were performed at the Molecular Diagnostics Laboratory of Hospital Privado Universitario de Cordoba, Argentina.

Nucleic acid preparation
Samples for dried blood spot cards (Biodynamics, Buenos Aires, Argentina) were collected from capillary blood and processed as follows: a piece of filter paper (approximately 3 mm in diameter) was placed in a 500 µL tube; 40 µL of 0.04 M NaOH were added. Next, the filter paper was boiled for 30 minutes and subsequently neutralized with 60 µL of pH 7.5, 0.05 mM Tris-hydrochloride. An aliquot of 10 µL per reaction tube was used as template DNA for PCR.

PCR technique
PCR was performed in 50 µL reaction mixtures containing DNA extracted from dried blood spot, deoxynucleotides triphosphate and Taq polymerase (Promega, U.S.A.), specific oligonucleotides TCZ1 (CGAGCTCTTGCCCACACGGGTGCT) and TCZ2 (CCTCCAAGCAGCGGATAGTTCAGG) (Ruralex, Buenos Aires, Argentina) designed to amplify a 188 bp fragment of repetitive nuclear DNA region of T. cruzi, reaction buffer (Promega, U.S.A), and water to a final volume. Positive and negative controls were processed both in parallel with the patient's samples and included in each run.
A fragment of TGF- β human gene was amplified by PCR from each sample to guarantee DNA quality. Both determinations included a no template control with nuclease-free water as template. The PCR products were visualized in non-denaturing silver stained 8% polyacrylamide gel electrophoresis.

Ethics statement
A written informed consent was obtained from parents or legal guardians of all the minors. The protocol was approved by the Bioethical committee of Hospital Privado Universitario de Cordoba, Argentina.

Results
Serological diagnosis and PCR
Serological evidences of T. cruzi infection were found in 34 of the 78 (43.5 %) children studied. This positive serological group was studied with PCR and only 5 (15%) yielded positive results (Figure 1).

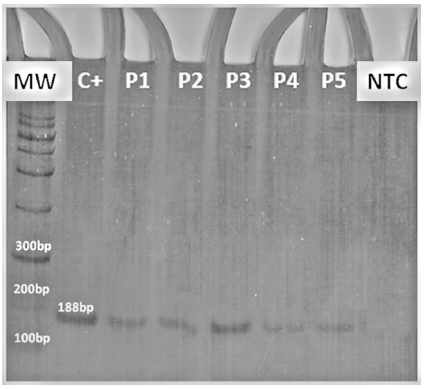

Figure 1: Specific PCR for T. cruzi.

At the time of performing the mentioned tests, none of the children presented clinical symptoms of disease.
Polyacrylamide gel electrophoresis (silver staining) of PCR products amplified from DNA extracted from dried blood spot. MW: molecular weight marker. C +: positive control (188bp). Positive children: P1 to P5. NTC: no template control. PCR: polymerase chain reaction

Post-treatment follow-up
One hundred and twenty days after the end of therapy, 28 of the 34 children with positive serology were studied (the other six had finished their education in that school). All of them (n= 28) had positive IHA and ELISA, whereas PCR was positive in 64.2% (n= 18). There were no children with double negative results (Serology and PCR), but 10 of them showed positive serology and negative PCR after treatment. No adverse effects during the treatment period were reported.

Discussion
The study was carried out in an isolated area, endemic for Chagas disease. The pediatric population diagnosed with this disease received the appropriate medication. No side effects were reported during therapy with Bz.
It was possible to detect the parasite from a small portion of dried blood spots with a simple, fast and inexpensive method. We decided to use silver-stained polyacrylamide gel since this process allows detecting minimal quantities of DNA, which led to improve the sensibility in this method. The mentioned procedure allowed us to perform the sampling in the field of study without deterioration of the specimens, in order to reach reliable results.
Currently, the success of the treatments is limited by the lack of reliable criterion of cure and limited availability of methods for direct parasitological detection. For example, we can mention the low sensibility of the Strout parasite detection test and hemoculture or the complications and slowness of xenodiagnosis. Besides, these analyses are burdensome and require high biosecurity conditions [12,13]. On the other hand, the presence of specific antibodies for T. cruzi several years after the treatment constitutes a great challenge to interpret the therapeutic awaited achievement [14]. Searching for more sensitive methods, PCR constitutes a very good option for parasite detection in infected persons and post treatment follow-up [15,16,17,18,19].
We cannot affirm that a single negative PCR result is indicative of cure; especially considering that intermittent parasitemia in infected people exists. However, negative results from serological and PCR techniques after treatment are likely indicative of a cure [20].
In our study, we verified failures of the treatment, since 64.2% of treated children persisted with positive parasitemia evidenced by PCR, even 120 days after end of therapy. To achieve a more accurate conclusion about this finding, the absence of compliance control and the

high rate of reinfection in that environment should be taken into consideration. Furthermore, there is a lack of information about the completion of disinfection programs (house spraying, surrounding home, animal control).

An upcoming challenge would be to amplify the parasite from the same kind of sample, but using real-time PCR, since the use of silver-stained polyacrylamide gel is time-consuming and more expensive than the proposed technique.

Standards must be established for data collection (records treatment and zone disinfection) in endemic areas to obtain conclusions that transfer the true efficacy of PCR methods for diagnosis and monitoring of Chagas disease. Nevertheless, we verified the high rate of T. cruzi infection in the studied population by the proposed techniques and were able to assess the ability of PCR for detecting treatment failure from dried blood spot.

Acknowledgments

The authors are thankful to the School Campo Bermejo Díaz de Nieto, Chaco; the Classroom Teacher Nelly Ortiz and the Nurse in charge of the Toba community, Eugenio Hilario. We also want to thank Estela Ambrosioni and Elisa Grasso for their assistance with the writing and edition of this paper.

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