

High seroconversion rates in *Trypanosoma cruzi* chronic infection treated with benznidazole in people under 16 years in Guatemala

Lucia Brum-Soares^[1], Juan-Carlos Cubides^[1], Iris Burgos^[1], Carlota Monroy^[2],
Leticia Castillo^[3], Selene González^[3], Pedro Albajar Viñas^[4]
and Pedro Pablo Palma Urrutia^[5]

- [1]. Unidade Médica Brasileira, Médicos Sem Fronteiras, Rio de Janeiro, Rio de Janeiro, Brasil.
[2]. Laboratory of Medical and Parasitological Entomology, San Carlos University, Guatemala City, Guatemala.
[3]. Parasitology Department, National Laboratory of Public Health, Guatemala City, Guatemala.
[4]. Department of Control of Neglected Tropical Diseases, World Health Organization, Geneva, Switzerland.
[5]. Medical Department, Doctors Without Borders, Barcelona, Spain.

Abstract

Introduction: Geographical, epidemiological, and environmental differences associated with therapeutic response to Chagas etiological treatment have been previously discussed. This study describes high seroconversion rates 72 months after benznidazole treatment in patients under 16 years from a project implemented by Doctors without Borders in Guatemala. **Methods:** An enzyme-linked immunosorbent assay was used to detect *Trypanosoma cruzi* IgG antibodies in capillary blood samples from patients 72 months after treatment. Fisher's exact test was used to establish association between characteristics, such as sex, age, and origin of patients, and final seroconversion. Kappa index determined concordance between laboratory tests. The level of significance was set to 5%. **Results:** Ninety-eight patients, aged 6 months to 16 years, were available for follow-up. Sex and origin were not associated with seroconversion. Individuals older than 13 were more prone to maintain a positive result 72 months after treatment, although results were not highly significant. Laboratory tests presented elevated Kappa concordance (95% CI) = 0.8290 (0.4955-1), as well as high (97%) seroconversion rates. **Conclusions:** The high seroconversion rate found in this study emphasizes the importance of access to diagnosis, treatment, and follow-up of individuals affected by Chagas disease. Moreover, it contradicts the idea that it is not possible to achieve a cure with the currently available drugs. This study strongly supports expanding programs for patients infected with *T. cruzi* in endemic and non-endemic countries.

Keywords: Benznidazole. Chagas. Chronic infection. Guatemala. Seroconversion.

INTRODUCTION

Chagas disease, also known as American trypanosomiasis, is a neglected tropical disease caused by *Trypanosoma cruzi* and represents one of the biggest burdens for health systems, societies, and economies in Latin America⁽¹⁾. It is transmitted mainly by insects of the *Triatominae* subfamily, although other forms of transmission, such as oral (by food contaminated with feces/urine of vectors), congenital, transfusion of infected blood, organ transplants, and even laboratory accidents have been reported. According to the World Health Organization (WHO), this pathology affects approximately six million people, causing more than 12,000 deaths annually, and endangering more than

70 million people living in risk areas of the 21 countries considered to be endemic⁽²⁾.

Chagas disease, originally restricted to jungle and rural areas in the Americas, has spread in the last decades to non-endemic territories, first through urbanization and then by people migrating to other continents. A challenge that hampers access to diagnosis and treatment is the lack of knowledge by medical staff and affected individuals with regard to infection by *T. cruzi* in non-endemic territories. The two available drugs, benznidazole (BZN) and nifurtimox, present high cure rates in the acute phase of the infection and even at the beginning of the chronic phase, with effectiveness decreasing over time. The benefits of etiological treatment at the infection's primary level and at the disease's secondary level are known, but vary geographically, both in terms of therapeutic response and time to seroconversion detection using available serological markers.

Since 1999, Doctors without Borders [*Médicos Sem Fronteiras* (MSF)] has developed projects for diagnosis and

Corresponding author: MSc. Juan-Carlos Cubides.
e-mail: juan.cubides@rio.msf.org
Received 11 October 2016
Accepted 23 November 2016

treatment of patients affected by Chagas disease. These have provided direct (with field projects) or indirect (through technical support and training) assistance in endemic and non-endemic countries, such as Bolivia, Brazil, Colombia, Guatemala, Honduras, Mexico, Nicaragua, and Paraguay⁽³⁾⁽⁴⁾. Overall the organization has tested more than 114,000 people, diagnosed 11,000 and effectively treated more than 9,000, making this group of patients one of the largest in the world.

In Guatemala, vectors, such as *Triatoma dimidiata* and *Rhodnius prolixus*, are found in 18 of the country's 22 departments, with an infestation rate of 12-35%. According to a national survey carried out in 2000 in the five most endemic departments (Zacapa, Chiquimula, Jalapa, Jutiapa, and Santa Rosa), the infection rate among school children was 4.9%⁽⁵⁾. These facts motivated MSF to launch a Chagas disease diagnosis and management project in the country.

The present article describes the results derived from the evaluation of seroconversion rates in a population enrolled in one such MSF project between 2005 and 2006. Its objective was to bring medical assistance to patients under 16 years of age in the municipality of Olopa, Chiquimula Department

(**Figure 1**). As the region had a history of high house infestation by *R. prolixus*, project activities were only possible after a complete vector control campaign had been carried out by the Ministry of Health between 2000 and 2004. A final certification of Interruption of transmission of *T. cruzi* by *R. prolixus* was issued in 2005. Actions included the implementation of a community vigilance system, diagnosis, and treatment⁽⁴⁾.

METHODS

Area and population studied

According to the national census, the majority of the population lived in rural areas (74%), in conditions of poverty (62.7%) or extreme poverty (28.3%), and with an illiteracy rate of 27.7%⁽⁶⁾. An estimated 8,919 people in the municipality were under the age of 16.

During the course of the project, a total of 8,129 individuals, aged 9 months to 15 years, were tested, amounting to a 91.1% screening coverage. Peripheral blood samples were collected on Whatman n° 1 filter paper and diagnosis of Chagas infection was established by serological positivity using a conventional

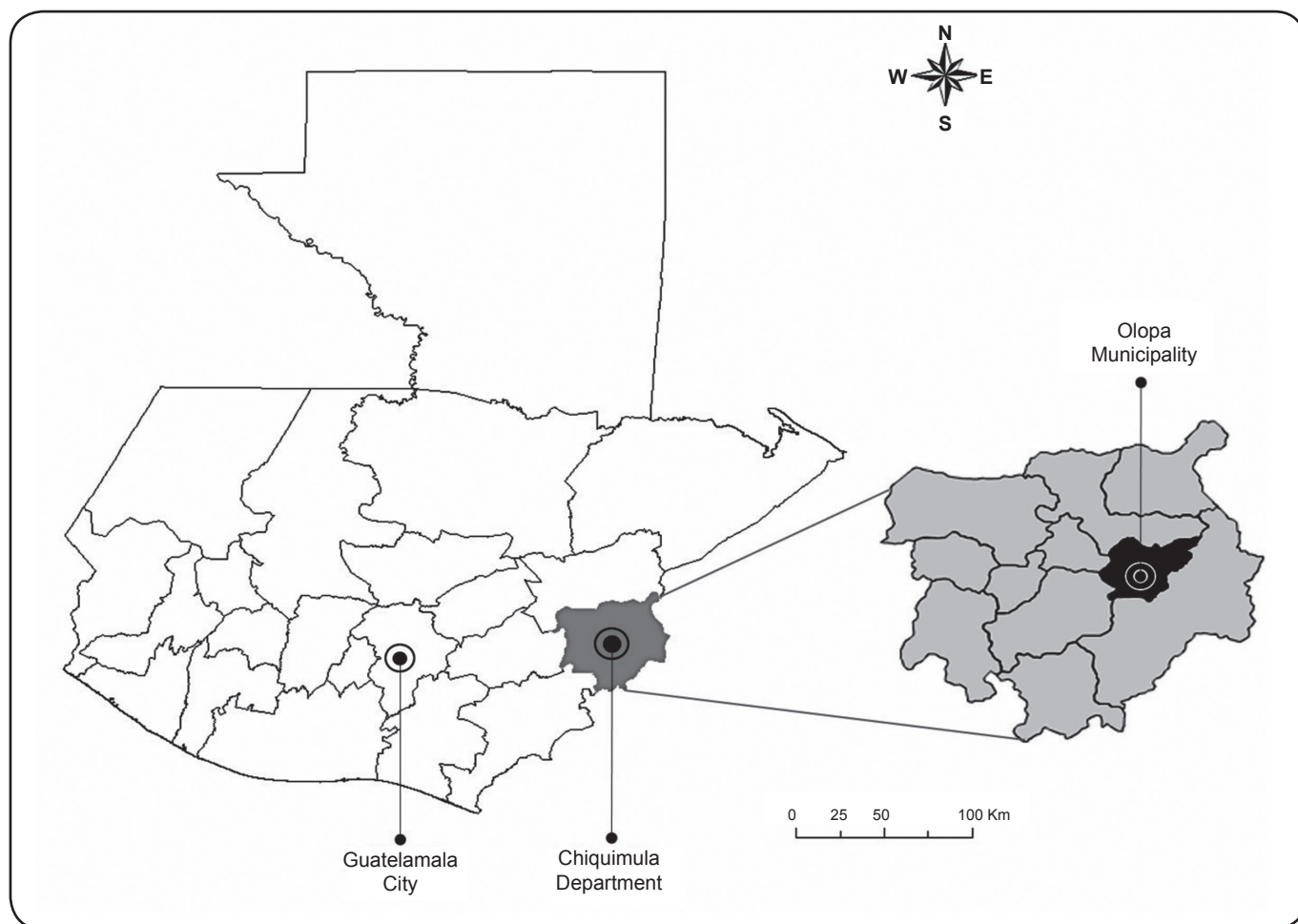


FIGURE 1. Geographical location of the Chagas project supported by MSF-OCBA. Olopa, Guatemala, 2005-2006. **MSF-OCBA:** *Médicos Sem Fronteiras—Operational Centre Barcelona*

enzyme-linked immunosorbent assay (ELISA) for the detection of immunoglobulin G (IgG) antibodies against *T. cruzi*. Positive results were confirmed by indirect hemagglutinin assay (IHA); inconclusive results were verified by recombinant ELISA.

Diagnosis was confirmed in 121 individuals serologically screened between March 2005 and June 2006 (1.5% seroprevalence). Of those diagnosed with Chagas disease, 118 successfully completed the proposed treatment scheme with 5mg of BZN/kg/day for at least 56 days. This represented a 97.5% treatment rate among confirmed cases. The patients were clustered into five groups based on their geographical location inside Olopa municipality. Treatment was carried out over a year and a half (**Figure 2**).

Prior to the project's completion in Olopa, an inter-institutional agreement was signed with local representatives of Guatemala's Ministry of Health. This determined follow-up and therapeutic response verification of patients that had completed treatment. To verify cure levels, the agreement specified at least two serological controls during each patient's follow-up, to be conducted at 18 and 36 months post-treatment. Unfortunately, this agreement could not be fulfilled within the stipulated timetable. To trace and find individuals in need of follow-up, MSF was urged to resume programmatic monitoring of treated patients using the initial treatment group lists and corresponding contact information.

In 2011, MSF approached Guatemalan local authorities to search for the 118 patients treated during the Chagas project

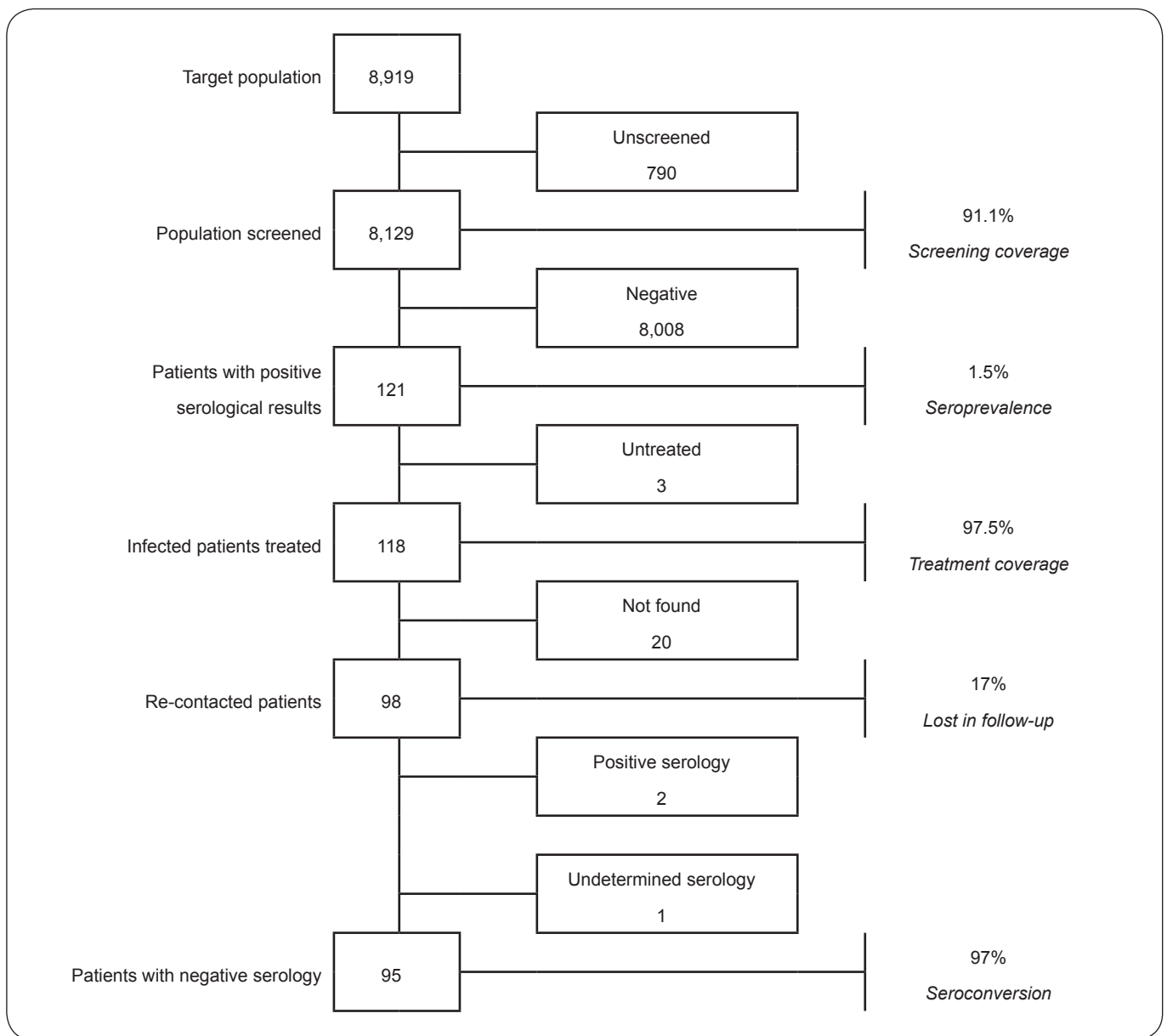


FIGURE 2. Diagram of diagnosis and treatment of *Trypanosoma cruzi* infection during the implementation of the MSF-OCBA project in Olopa, Department of Chiquimula, Guatemala. **MSF-OCBA:** Médicos Sem Fronteiras-Operational Centre Barcelona.

in Olopa. The objective was to verify effectiveness of the therapeutic response to BZN at a primary health care level. Additionally, the initiative sought to document, for the first time, seroconversion among Guatemalan patients following a period of approximately 72 months after treatment. Activities were carried out through an inter-institutional collaboration with San Carlos University and the *Laboratorio Nacional de Salud (LNS)*.

Identification of patients for follow-up

Active search for patients was performed through direct supervision by local health professionals with knowledge of Olopa municipality communities and with previous work experience in the same MSF project. A total of 118 patients treated with BZN were contacted using maps drawn during the original project, visits to patients' residences were scheduled, and contact information was adequately updated. Following identification and localization of children available for follow-up, letters of individual and collective call to families and community leaders were distributed in order to inform about sampling and testing activities.

Data and sample collection

Upon receipt of informed consent by participants or their tutors, data registration and capillary blood sample collection began. The procedure followed a regional protocol adopted by the previous MSF project. Specimens were delivered to the team at San Carlos University, from where they were transported to the LNS for processing of serological tests.

Laboratory procedures

Serological analysis relied on ELISA (Wiener®) tests with different antigenic preparations (conventional and recombinant) following manufacturer's instructions and standard laboratory protocols. The selected tests offered good sensitivity and specificity, and were considered as the most appropriate given the absence of other tests, such as immunofluorescence (IFI) or IHA, at the LNS. Moreover, this methodology had been used successfully to verify post-treatment cure rates in another MSF Chagas project in Honduras and thus allowed for easy comparison between the two⁽³⁾.

In accordance with WHO recommendations, cure was defined as two negative test results performed on the same sample in the same day. Cut-off values were calculated by summing up absorbance values from all negative controls and then adding a constant factor (0.200 for the conventional test and 0.300 for the recombinant one). Samples with absorbance above the cut-off value plus 10% were considered positive and samples below the cut-off value minus 10% were considered negative. Discordant results, such as when an ELISA test presented one positive and one negative result, indicated the need for a new follow-up control. If both tests were positive and the possibility of reinfection was discarded, the patient was considered not cured. Results were sent to the Brazilian Medical Unit, Chiquimula's Health Area Coordination, Chagas National Subprogram, San Carlos University and Valle University for review. They were then delivered to patients and their families.

Statistical analysis

Demographic variables and laboratory test results were used for a descriptive analysis of the cohort of patients treated with BZN and available for follow-up. Fisher's exact test was used to establish association between variables, such as sex, age, and origin, and seroconversion results. Concordance between laboratory tests was measured using the Kappa index. The level of significance was set at 5%.

Ethical considerations

All information and samples from Chagas patients were collected after activities were properly explained and signed informed consent was obtained. Procedures were in accordance with the ethical standards of MSF the participant local institutions (LNS and San Carlos University), and the Helsinki Declaration of 1964, as revised in 1975, 1983, 1989, 1996, and 2000.

RESULTS

After checking names and addresses of the 118 patients treated during the implementation of the 2005-2006 Chagas project, 20 patients could not be found mainly due to population movement (loss in follow-up 17%). Thus, seroconversion verification was performed on 98 patients (**Table 1**).

The group of patients available for follow-up included a slightly greater number of females than males (57% vs 43%), aged between 6 and 16 years. Most belonged to the 10 to 14 years range (75% of follow-up cohort, average age 11 years, standard deviation \pm 2.5 years) and came from locations, such as La Prensa (31%) and Las Palmas (15%).

When establishing association patterns between demographic variables and post-treatment seroconversion results, characteristics, such as sex and origin, were found not to be significant (Fisher's exact test = 0.323 and 0.881, respectively). In contrast, age at which treatment had begun appeared associated with seroconversion. Accordingly individuals older than 13 presented a higher tendency to maintain a positive result 72 months after treatment (Fisher's exact test = 0.050), even though results were not highly significant. A concordance of 98.98% was found among laboratory tests, with Kappa [95% confidence interval (95% CI)] = 0.8290 (0.4955-1).

Post-treatment effectiveness was measured as the proportion of patients with negative results in both tests. In this study 97% of patients seroconverted 72 months after etiological treatment with BZN, 2% remained positive according to the tests, and 1% presented discordant results. The findings indicate a tendency for patients to be disease-free; however, long-term trends will require further follow-up.

DISCUSSION

During the first 10 years of MSF interventions aimed at controlling Chagas disease, there was substantial uncertainty regarding the real benefits and risks of treating patients in the chronic phase. MSF confirmed the feasibility and benefits of treating patients during such phase, first in children and then in adults⁽³⁾. In 2010, the resolution *Chagas disease*:

TABLE 1

Demographic data and test results of patients available for follow-up. Project supported by MSF-OCBA. Olopa, Guatemala, 2005-2006.

Variable	Category	Percentage	Test result (<i>relative frequency</i>)			P
			negative	indeterminate	positive	
Sex	male	43.0	(97.6)	(0.0)	(2.4)	0.323
	female	57.0	(96.4)	(3.6)	(0.0)	
Age (years)	< 2	2.0	(100.0)	(0.0)	(0.0)	0.698
	5-9	27.0	(100.0)	(0.0)	(0.0)	
	10-14	52.0	(96.2)	(1.9)	(1.9)	
	> 14	17.0	(94.2)	(5.8)	(0.0)	
Origin	Prensa	31.0	(93.6)	(3.2)	(3.2)	0.881
	Palmas	15.0	(100.0)	(0.0)	(0.0)	
	Cumbre	8.0	(100.0)	(0.0)	(0.0)	
	Paternino	7.0	(100.0)	(0.0)	(0.0)	
	Valle Nuevo	6.0	(100.0)	(0.0)	(0.0)	
	Amatillo	5.0	(100.0)	(0.0)	(0.0)	
	Pomas	5.0	(100.0)	(0.0)	(0.0)	
	Tituque Abajo	5.0	(80.0)	(20.0)	(0.0)	
	Cerrón	3.0	(100.0)	(0.0)	(0.0)	
	Roblarcito	3.0	(100.0)	(0.0)	(0.0)	
	Tuticopote	3.0	(100.0)	(0.0)	(0.0)	
Otras*	9.0	(100.0)	(0.0)	(0.0)		

MSF-OCBA: *Médicos Sem Fronteiras-Operational Centre Barcelona*. *Corresponding to individual (2) urban and (9) rural locations.

control and elimination was approved by the World Health Assembly [(WHA), resolution 63.20]. This constituted a great achievement for the affected populations, because it recommended for the first time that countries' primary health care systems offer diagnosis and treatment to patients in both phases (acute and chronic) of the disease.

The results of the present study strongly support the implementation of programs to expand primary health care access to patients infected with *T. cruzi* in endemic and non-endemic countries, in compliance with WHA resolution 63.20. Results show high parasitic cure rates (97% of treated patients) and their publication represents an important tool in promoting diagnosis and treatment of this neglected disease. This is particularly important, given that more than 90% of infected people in the world have never had access to diagnosis or treatment.

The benefits of an etiological treatment for Chagas disease, such as high percentage of parasitic cure, prevention of disease progression due to a decreased parasitic load⁽⁷⁾⁽⁸⁾⁽⁹⁾, and primary inhibition or interruption of congenital transmission⁽¹⁰⁾, have been broadly documented in the literature. One of the main barriers for the widespread use of BZN is the high reported incidence of adverse drug reactions (ADRs), documented in up to 50% of treated patients⁽¹¹⁾. Whereas only a minor number

of ADRs are severe (1%)⁽¹²⁾, about a third of patients interrupt BZN treatment due to ADRs or other reasons⁽¹¹⁾⁽¹²⁾⁽¹³⁾.

We emphasize the importance of maintaining appropriate and close follow-up of treated patients as part of the integral handling program for Chagas disease. As mentioned by Yun et al.⁽¹³⁾, the success of MSF projects derived mostly from weekly assessment of patients during treatment. This increased adherence enabled the early detection of any adverse reaction, reduced the proportion of moderate and severe reactions, and maintained a 0% mortality rate.

In most acute and chronic infections, the result of treatment is not evaluated and it is assumed that the expected benefit was achieved. In contrast to other infectious diseases, in Chagas follow-up helps prevent transmission, since those not cured cannot donate blood or organs, and women of childbearing age will need to perform a screening of the newly born for the possibility — even though remote after treatment — of congenital transmission.

The results of this study are limited by the diagnostic procedures. To confirm results, it is recommended that two different laboratory techniques be used. In our case only ELISA tests, with different antigenic preparations (conventional and recombinant), were performed due to the absence of other tests, such as IFI or IHA, at the LNS. It is also suggested that at least

two serological controls be conducted at 18 and 36 months post-treatment to verify its efficacy. Unfortunately, this control was only performed after 72 months, making it difficult to estimate an average time to seroconversion.

Despite the limiting circumstances, the findings from this study are consistent with previously documented initiatives in the region^{(3) (4) (13)}. The closest and more comparable results correspond to those published on a cohort of 231 Honduran children treated with BZN in 2009; whereby 93.9% seroconverted (negative serological results) 36 months after antiparasitic treatment⁽³⁾. In our cohort, no differences relating to sex or age were found to correlate with seroconversion results.

The general distribution pattern of *T. cruzi* lineages and sublineages (*T. cruzi* I, IIa, IIb, IIc, IId, and IIe) in the American continent⁽¹⁴⁾ shows that in Central America *T. cruzi* I is the predominant type, whereas other countries present greater diversity. This fact helps explain the epidemiological complexity of the parasite, as well as the possible relationship between the range of clinical manifestations and diverse response to treatment observed in various studies⁽¹⁵⁾.

In our study, a possible association between onset of treatment at a younger age and seroconversion was detected. Individuals older than 13 years were more prone to maintain a positive result 72 months after treatment (Fisher's exact test = 0.050). It should be noted that results were not highly significant, which may be due to a small number of patients in the overall cohort or in this particular age group. Nevertheless, this finding constitutes an interesting point for future studies examining diversity of the therapeutic response, seroconversion times, and follow-up schedules.

In contrast, geographical differences, epidemiological and environmental aspects associated with clinical patterns of morbidity, and the therapeutic response to Chagas disease have been discussed previously^{(14) (15) (16)}. These differences are important at a practical level for implementing different therapeutic schemes according to patients' geographical origin. At a clinical level, previous studies have demonstrated for example that digestive forms are rare north of the Equator and that chagasic heart disease becomes more severe and evident in the central regions of South America compared to endemic areas in the south^{(5) (16)} or even in Central America. In terms of therapeutic response and when compared to findings from studies conducted in Bolivia⁽¹³⁾, patients from Central America require a shorter follow-up time after treatment to ascertain cure or detect a reduction in *T. cruzi* antibodies.

An analysis of the first ten years of MSF projects in Honduras, Guatemala, and Bolivia was published in 2009⁽¹³⁾. That study evaluated preliminary results for a group of 31 patients from Guatemala with available serology 18 months after treatment; 58.1% of them were already found to be negative. In our study, whereby 98 patients from Olopa were evaluated 72 months after treatment, 97% of them seroconverted. Such cure rate is similar to the one reported for the treatment cohort in Honduras (92.7%, 36 months after treatment) and substantially higher than in Bolivian patients (5.4%, 60 months after treatment). These results support expanding primary health care-provided

diagnosis and treatment options against the disease. Further investigation will deepen our understanding of the disease and its regional particularities, as well as substantiate new therapeutic schemes adapted to each reality.

Specifically, for undetermined results from serological tests (corresponding to just one patient in this study), it is important to note that absorbance readings were close to the cut-off point (reading = 0.333; cut-off point = 0.330), but still not classifiable as negative. It is possible that in this specific case, further follow-up time was required to reach seroconversion. The possibility of patient reinfection is considered minimal, given that between the project's completion and re-contact with patients, vigilance and vector control activities were carried out in the area. Moreover, a program implementing better housing facilities was also set in place and virtually eliminated intradomiciliary transmission of *T. cruzi*.

The lack of a better biomarker to detect cure is also a limiting factor in evaluating and understanding the therapeutic response to Chagas disease, as well as in the development of more effective drugs. Differences between seroconversion rates in Central and South America⁽¹³⁾ and the time to seroconversion, put into evidence the need for conducting detailed studies to confirm the results found in the present article with respect to seroconversion time. This would certainly improve etiological treatment protocols with doses, duration, and follow-up times adapted to the characteristics of Chagas disease in different geographical areas.

Such projects and their results highlight the importance of access to diagnosis, treatment, and follow-up of individuals affected by Chagas disease and contradicts the idea that it is not possible to achieve a cure with the currently available drugs. At the same time, the results offer information to patients and health professionals for planning drug administration and diagnostic tests, update monitoring protocols for those affected by *T. cruzi* infection, and for promoting activities directed towards disease control.

Acknowledgments

We express our gratitude for all the support and collaboration to the LNS, San Carlos University, and the population of Olopa municipality in Guatemala; as well to the field teams and to the Medical Department of the MSF Operational Center in Barcelona.

Conflict of interest

The authors declare that there are no conflicts of interest.

Financial support

This manuscript is the result of the analysis of programmatic data and activities regarding the management of Chagas disease developed by MSF in the municipality of Olopa, Chiquimula Department, Guatemala, between 2005 and 2006. All activity costs during project implementation as well as those derived from the production of this document were covered by the organization.

REFERENCES

1. Lee BY, Bacon KM, Bottazzi ME, Hotez PJ. Global economic burden of Chagas disease: a computational simulation model. *Lancet Infect Dis* 2013; 13:342-348.
2. World Health Organization. Chagas disease (American Trypanosomiasis): Fact sheet. World Health Organization International; 2016. Updated March 2016. Available from: <http://www.who.int/mediacentre/factsheets/fs340/es/>
3. Escribà JM, Ponce E, Romero AD, Viñas PA, Marchiol A, Bassets G, et al. Treatment and seroconversion in a cohort of children suffering from recent chronic Chagas infection in Yoro, Honduras. *Mem Inst Oswaldo Cruz* 2009; 104:986-991.
4. Hashimoto K, Alvarez H, Nakagawa J, Juárez J, Monroy C, Córdón-Rosales C, et al. Vector control intervention towards interruption of transmission of Chagas disease by *Rhodnius prolixus*, main vector in Guatemala. *Mem Inst Oswaldo Cruz* 2012; 107:877-887.
5. Moncayo A, Silveira AC. Current epidemiological trends for Chagas disease in Latin America and future challenges in epidemiology, surveillance and health policy. *Mem Inst Oswaldo Cruz* 2009; 104 (supl 1):17-30.
6. Guatemalan National Institute of Statistics (INE). Chiquimula Department Characterization 2012. Guatemala: 2013. 74p. Available from: <https://www.ine.gov.gt/sistema/uploads/2013/12/09/IdAiQPEvPhCxVPz9sumuZmbPlyXvalDx.pdf>
7. Coura JR. Chagas disease: what is known and what is needed - A background article. *Mem Inst Oswaldo Cruz* 2007; 102 (supl 1): 113-122.
8. Marin-Neto JA, Rassi Jr A, Avezum Jr A, Mattos AC, Rassi A. The BENEFIT trial: testing the hypothesis that trypanocidal therapy is beneficial for patients with chronic Chagas heart disease. *Mem Inst Oswaldo Cruz* 2009; 104 (supl 1):319-324.
9. Viotti R, Vigliano C, Lococo B, Bertocchi G, Petti M, Alvarez MG, et al. Long-term cardiac outcomes in treating chronic Chagas disease with benznidazole versus no treatment: a nonrandomized trial. *Ann Intern Med* 2006; 144:724-734.
10. Moscatelli G, Moroni S, García-Bournissen F, Ballering G, Bisio M, Freilij H, et al. Prevention of congenital Chagas through treatment of girls and women of childbearing age. *Mem Inst Oswaldo Cruz* 2015; 110:507-509.
11. Morillo CA, Marin-Neto JA, Avezum A, Sosa-Estani S, Rassi Jr A, Rosas F, et al. Randomized trial of benznidazole for chronic Chagas' cardiomyopathy. *N Engl J Med* 2015; 373:1295-1306.
12. Hasslocher-Moreno AM, do Brasil PEAA, de Sousa AS, Xavier SS, Chambela MC, Sperandio da Silva GM. Safety of benznidazole use in the treatment of chronic Chagas' disease. *J Antimicrob Chemother* 2012; 67:1261-1266.
13. Yun O, Lima MA, Ellman T, Chambi W, Castillo S, Flevaud L, et al. Feasibility, Drug safety, and effectiveness of etiological treatment programs for Chagas disease in Honduras, Guatemala, and Bolivia: 10-year experience of Médecins Sans Fronrières. *PLoS Negl Trop Dis* 2009; 3:e488. doi: 10.1371/journal.pntd.0000488.
14. Macedo AM, Pena SD. Genetic variability of *Trypanosoma cruzi*: implications for the pathogenesis of Chagas disease. *Parasitol Today* 1998; 14:119-124.
15. Noireau F, Diosque P, Jansen AM. *Trypanosoma cruzi*: adaptation to its vectors and its hosts. *Vet Res* 2009; 40:26.
16. Pereira JB, Coura JR. Diferenças regionais na doença de Chagas: aspectos clínicos, epidemiológicos e laboratoriais de duas áreas endêmicas do Brasil. *Rev Soc Bras Med Trop* 1993; 26 (supl 2):70-72.