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Chagas disease in Latin American migrants: a Spanish challenge

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Abstract

Chagas' disease affects millions in Latin America and is the leading cause of cardiomyopathy and death due to cardiovascular disease in patients aged 30–50 years. As a consequence of immigration it has settled in several European countries, where besides imported cases, autochthonous infections arise through vertical transmission and blood/organ donation. All Latin American immigrants who attended our Unit were screened for *T. cruzi* infection (ELISA and IFAT ± PCR). An ECG and echocardiogram were requested for all positive patients, and oesophageal manometry, barium swallow and barium enema were requested according to patient symptoms. All patients under 50 years without severe cardiac involvement and who had not received correct treatment previously were treated with benznidazole 5 mg/kg/day for 60 days. Patients were followed-up with serology and PCR 1 month after treatment ended and every 6 months thereafter. A total of 1146 Latin Americans were screened for *T. cruzi* (357 positive serology results). The typical patient profile was a Bolivian female, of rural origin, in her fourth decade of life, without evidence of visceral involvement. Treatment tolerance was poor, with 29.7% discontinuing treatment due to adverse reactions. Among those with adverse reactions (52%), the most frequent were cutaneous hypersensitivity (68.7%), gastrointestinal upset (20%) and nervous system disturbances (16.2%). *T. cruzi* infection is no longer limited to Latin America. Poor treatment tolerance can limit current treatment options. More epidemiological data are necessary to estimate the magnitude of a problem of great relevance for public health and health resource planning.

Keywords: Chagas disease, immigrants, Spain, *Trypanosoma cruzi*
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Introduction

One hundred years after the discovery of Chagas' disease, it still affects millions in Latin America and is the leading cause of cardiomyopathy and death due to cardiovascular disease in patients aged 30–50 years [1]. Although the burden of the disease has decreased in the last years due to different control measures, thousands of new cases are being diagnosed each year [2] and many challenges still need to be overcome before its successful elimination. One of the main components of a good strategy would be better monitoring and estimation of disease burden worldwide [3].

In endemic areas, vectorial transmission accounts for over 80% of cases. However, with the recent increase in migration from Latin America to western countries, other modes of transmission are becoming increasingly more important. Those who migrate seeking work opportunities and to improve their quality of life are usually healthy, and younger than the general population. Most Latin Americans will have been infected during childhood and therefore, based on the natural course of the disease, these migrants would now be at an age when the first manifestations of cardiac involvement may be expected to appear.

Imported Chagas' disease has already been recognized as an emerging problem in the USA [4] and this has now reached Europe and, in particular, Spain [5–7]. In many European countries, Chagas' disease has now become a frequent reason for consultation, especially at specialized tropical medicine units, whilst previously this disease was practically unknown in these areas. The potential severity of the disease in certain patients, even if initially asymptomatic, should not be dismissed [8,9].

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The objective of this study was to describe the clinical-epidemiological characteristics of the largest cohort of Latin American immigrants with chronic *T. cruzi* infection described in Europe to date, as well as providing preliminary data on response to and tolerance of treatment with benznidazole.

Materials and Methods

Study design

This was a prospective, observational study performed at the Tropical Medicine Unit (TMU), Infectious Diseases Department, of the Ramón y Cajal Hospital in Madrid, Spain during a 7-year period (2003–2009). All immigrants from Central or South America who attended our Unit were screened for *T. cruzi* infection.

Diagnosis of Chagas disease

Chagas' disease was diagnosed in patients with two positive serological tests enzyme-linked immunosorbent assay and indirect immunofluorescence assay (ELISA and IFAT) against different parasite antigens. Polymerase chain reaction PCR was requested for all patients with positive serology (in-house PCR using 121-122 and Tczl-Tcz2 oligonucleotides) [10]. A baseline ECG and echocardiogram (ECC) were requested for all patients, and oesophageal manometry, barium swallow and barium enema were requested according to individual patient symptoms.

The following symptoms were considered suggestive of Chagas' disease once other causes were excluded. *Cardiac* [11]: palpitations, syncope, fatigue, dyspnoea on exertion, oedema or atypical chest pain. *Gastrointestinal* [12]: dysphagia with odynophagia, epigastric pain, regurgitation, ptyalism and malnutrition in severe cases of mega-oesophagus and prolonged constipation in megacolon.

The following criteria were used to diagnose visceral involvement due to Chagas' disease. *ECG* [2]: complete right bundle branch block (RBBB) in isolation or associated with left anterior hemiblock, complex ventricular arrhythmias and sustained ventricular tachycardia, supraventricular tachyarrhythmias, type II second degree and complete A–V blocks, sinus bradycardia <50 beats per minute and presence of a permanent pacemaker. *Echocardiogram* [11]: left ventricular (LV) dysfunction with decreased ejection fraction (LVEF) and increased LV diastolic diameter, wall movement abnormalities, apical aneurysm or presence of thrombus within a cardiac cavity, with no other justifiable cause. *Oesophageal manometry* [13]: aperistalsis, non-relaxing or partially relaxing lower oesophageal sphincter (LEE). *Barium swallow* [13]: slow transit, retention of contrast, oesophageal dilatation or dolicomegaesophagus. *Barium enema* [13]: dolichocolon or megacolon.

Treatment and follow-up

All patients under 50 years, without severe cardiac involvement and who had not received correct treatment previously in their country of origin were treated with benznidazole 5 mg/kg in two to three divided doses daily for 60 days [14,15]. We also offered treatment to those above 50 years without severe cardiac involvement, after explaining the risks and benefits of therapy. From September 2009, those patients who stopped treatment early due to side-effects were treated with nifurtimox with gradually increasing doses to reach a maintenance dose of 8–10 mg/kg/day for 90 days. Patients were considered to have been adequately treated when the drug was taken for at least 1 month.

Once treatment was started, patients were seen after 2 weeks and at the end of treatment in order to ascertain treatment adherence, tolerance and toxicity. Full blood count and biochemistry tests were also performed at these times. *T. cruzi* serology and PCR were performed 1 month after treatment ended and every 6 months thereafter. An ECG was performed annually in the absence of new symptoms suggesting cardiac involvement. Treatment was considered successful when a disappearance of antibodies using serological tests was observed. Patients with persisting antibodies following treatment were considered to have an as yet undetermined response to treatment (due to the short duration of follow-up in some patients). Treatment was considered to have failed when persistence of the parasite was detected by PCR [2].

Adverse effects were classified according to the Cancer Therapy Evaluation Program, Common Toxicity Criteria, Version 2.0 [16]. In the case of milder adverse effects the discontinuation of treatment was discouraged if possible but benznidazole was stopped in cases of clear intolerance or severe adverse events, particularly significant leucopoenia (<2500 cells/mm^3), peripheral neuropathy or severe allergic dermopathy.

Baseline characteristics were analysed using descriptive statistics. Qualitative variables were expressed as absolute frequencies and percentages, and quantitative variables as the median and interquartile range (IQR). Categorical variables were compared using the chi-square or Fisher exact test, and continuous variables by the *t*-test or Mann–Whitney *U*-test. Survival curves were calculated using the Kaplan–Meier method and differences were evaluated using the log-rank test. A p value <0.05 was considered significant. SPSS (Chicago, IL, USA) software package version 15.0 was used for statistical analysis.

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The local ethics committee approved the study protocol and database registry. Patients were included after an informed consent form was signed.

Results

A total of 2125 immigrants from all over the world were seen during the study period. A significant increase in the number of patients screened for *T. cruzi* was observed from 2003 (18 patients) to 2009 (408 patients) (β = 73; IC 95%: 31–114; p 0.007). *T. cruzi* serology was performed for 1146 Latin American patients; this was positive in 357 (31%). Out of 337 PCR tests (20 patients did not have baseline PCR results but for some patients more than one PCR was performed), 212 patients (63%) had one or both positive PCRs. Serology and PCR results for the total number of individuals screened is shown in Table 1.

Of those with positive *T. cruzi* serology, 146 (41%) were referred to the TMU from other health centres (other hospitals, primary care centres or transfusion centres), 103 (29%) were recruited following specific campaigns, 99 (28%) were self-referred and 9 (2%) attended due to clinical symptoms. At the time of the first visit the median duration of residence in Spain was 3.4 years (interquartile range 2.3–5.2).

The median age was 36 years (interquartile range 29–44), 346 (97%) were from Bolivia, three from Paraguay, two from Argentina, two from Brazil, two from Ecuador, one from Honduras and one was from Chile, and 241 (67.5%) were female. In total, 83.5% recalled having seen the vector in their homes, and 78.7% had lived in rural areas. Two hundred and nine (59%) patients had a relative with Chagas disease and for 64 of them vertical transmission was a possibility (mother with Chagas' disease). Thirty-six (10%) patients had received a previous blood transfusion.

A total of 168 (47%) patients had previous positive serology (mostly performed at transfusion centres in Bolivia), but only 33 of them had received previous anti-trypanosomal treatment and none appeared to have received a complete course. Five of them (15.2%) had a positive PCR result before starting therapy with benznidazole in our Unit.

Clinical data were available for 252 patients (27 were lost to follow-up and for 78 patients tests were still being completed at the end of the study period). Visceral involvement was diagnosed in 56/252 patients (22.2%): 43 (17.1%) had cardiac involvement, nine (3.5%) had gastrointestinal involvement and four (1.6%) had both. There were no significant differences in visceral involvement by sex (p 0.21).

TABLE 1. Serology and PCR results for the total number of individuals screened

	n	Number of positives (%)
Individuals screened by serology	1146	357 (31)
Individuals with PCR performed	337	212 (63)

Out of 47 patients with cardiomyopathy, one died due to an acute myocardial infarction, one was a heart transplant recipient (due to dilated cardiomyopathy), two were awaiting cardiac transplantation (dilated cardiomyopathy), two had pacemakers (due to complete A-V blocks), 12 had evidence of cardiomyopathy on ECC and 29 had ECG abnormalities suggestive of Chagas' disease (mainly complete RBBB in isolation or associated with left anterior hemiblock). Of the 13 patients with gastrointestinal involvement, one had megacolon and megasigma, three had abnormalities on oesophageal manometry suggestive of Chagas' disease, four had dolichomegacolon and five had dolichosigma.

Eleven women were pregnant and there were no cases of vertical transmission. Four patients were infected with HIV, all asymptomatic, with an adequate immunological status and with no documented reactivation of Chagas' disease. Three of them had a negative PCR result and one a positive one.

Out of those 252 patients for whom clinical data were available, 195 patients received treatment with benznidazole (12 of them were above 50 years old and preferred to take the treatment) and the remaining 57 were pending initiation of treatment at subsequent visits. One hundred and four completed the treatment course without significant adverse events, or with mild adverse events that did not lead to treatment discontinuation. Eleven discontinued benznidazole due to adverse events but were considered to have received a complete course as they took the treatment for at least 1 month. Of the remaining 80 patients: 30 received <1 month of treatment (discontinued due to side-effects) and 50 were still completing the treatment at the end of the study period.

A safety and tolerability analysis was performed on 148 out of 195 patients (those patients who had completed treatment or had already discontinued it due to adverse reactions at the end of the study period were included in the analysis). In total, 77/148 (52%) patients had documented adverse reactions (Table 2). Of these, 44/148 (29.7%) stopped treatment even though side-effects were mild. Median time to interruption was 22 days (95% CI 9–34). Age, sex and HIV infection were not significantly associated with the occurrence of adverse reactions or treatment interruptions. Among the 77 patients who developed toxicity, the most common side-effects were cutaneous toxicity (morbilliform rash, pruritus and occasional mucosal involvement) in 68.7%, gastrointestinal symptoms (nausea, vomiting, gastric pain and anorexia) in 20%, and nervous system disturbances

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TABLE 2. Adverse reactions related to benznidazole treatment

Patients who could be evaluated in the safety analysis	148
Patients with any adverse reaction (%)	77 (52.0)
Grade 1	36 (24.3)
Grade 2	33 (22.3)
Grade 3	7 (4.7)
Grade 4	1 (0.7)
Treatment discontinuation due to adverse reactions (%)	44 (29.7)
Treatment discontinuation due to adverse reactions <30 days of therapy[a] (%)	33 (22.3)

[a]Treatment considered incomplete.

(headache, anxiety, dizziness, disesthesias or paresthesias) in 16.2%. Five patients (6.5%) developed mild leucopoenia and two had taste disturbances (2.6%).

We investigated whether benznidazole dosing was related to the incidence of adverse reactions or treatment discontinuation in two ways: according to whether dose was ≤ or >300 mg daily, and according to total daily dose. There was no significant association between benznidazole dose (≤ or >300 mg daily) and the occurrence of adverse reactions (χ^2 = 0.018; p 0.89), the severity of reactions (χ^2 for trend = 1.46; p 0.69), or treatment discontinuation (χ^2 = 0.67; p 0.41). Total daily dose was not associated with the occurrence of adverse reactions (p 0.73), the severity of reactions (p 0.98), or treatment discontinuation (p 0.18). For the three patients who received nifurtimox as second-line treatment, this was well tolerated in all cases.

Median follow-up after treatment was 1 year (IQR 0.3–2). For eight patients, *T. cruzi* antibody levels decreased following treatment, but none of the serologies became negative during the study period. In 70 cases, there was no documented fall in antibody levels. In the 65 patients with a positive pre-treatment PCR this became negative following treatment in all of them.

Discussion

There has been a recent increase in the number of patients with Chagas' disease outside areas classically considered endemic for the disease, particularly in Spain where, until recently, the disease was hardly ever diagnosed [17]. A seroprevalence of 31% was found in our study population. This high prevalence may have been partly due to selection bias, as TMU is a specialized referral centre with screening programmes for Chagas' disease, which also include testing patients' relatives. However, studies performed at other Spanish referral centres have described similar seroprevalence rates (34% [8] and 41% [9]). The patients included in this study may well be at higher risk for the infection as most of them were from Bolivia (currently the Latin American country with the highest prevalence rates), from rural areas, where the majority were exposed to the vector and had affected family members.

A significant increase in the number of *T. cruzi* serologies performed has also been documented during the period 2003–2009. This trend will probably be maintained in the future irrespective of migration fluxes, as only a minority of immigrants residing in our country have so far been tested for Chagas' disease. In fact, the majority of patients in the cohort had been residing in Spain for several years (median duration of residence, 3.4 years).

The rates of visceral involvement were slightly lower than those observed in the literature: 18.6% cardiac involvement and 5.1% gastrointestinal involvement in our study, vs. 20–30% and 10%, respectively [18]. These figures could increase in the future, taking into account that the median age of the patients was 36 years (similar to that described in other Spanish studies) [8] and that the majority come from rural areas, were probably infected during childhood and that visceral damage develops 20–30 years after the infection. The rates of visceral involvement found in immigrants, therefore, are not very different to those found in patients in endemic areas.

Benznidazole is the most widely used drug for treatment, but, although consensus of expert opinion maintains that aetiological treatment should be offered to all patients under 50 years with reactive *T. cruzi* serology and without evidence of advanced cardiomyopathy [14,19], there is weak evidence supporting its use in the chronic phase of the infection [15]. In our study all patients who met these two criteria were treated and we also offered treatment to those older than 50 years. Tolerance, in general, was poor, with a frequency of adverse reactions equivalent to that described in other studies (52% vs. 50%). Nevertheless, the proportion of adverse effects due to cutaneous hypersensitivity was higher than previously reported (68.7% vs. 20–25%) [20]. Even though most adverse effects were not severe, a large proportion of our patients decided to stop treatment (29.7%), mainly due to pruritus/cutaneous lesions, thus limiting treatment options [21]. Even though some authors postulate that benznidazole dose may be associated with the appearance of adverse effects, we found no statistically significant differences when different doses were considered.

Nifurtimox has a similar efficacy profile to benznidazole, but is associated with a higher incidence of side-effects (40–97%) [22]. Nevertheless, if one drug is ineffective or must be discontinued, the other can be used as an alternative [12]. In our cohort only a few patients were treated

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after benznidazole intolerance with nifurtimox and even though no hypersensitivity reactions were documented more data would be needed in order to draw any significant conclusion. Promising studies with new molecules are currently under way [23,24], but for the time being treatment options for *T. cruzi* are very limited.

During the study period, no patients could be considered to have been cured as antibody titres did not decrease significantly or become negative [25]. However, the fall in antibody titres has been described to occur 5 years following treatment [2], and this period may vary depending on age and the phase of the illness, so a greater decrease in antibody levels may be expected during the next few years of follow-up. On the other hand, PCR became negative following treatment in all those patients who had a positive PCR pretreatment, so none of the treatments could be considered to have failed either. The value of PCR relies mainly on positive results, and it may be useful as an early marker of resistance to chemotherapy years before serology [26]. It is important to remember that a negative PCR does not guarantee parasitological cure, as parasitaemia may fluctuate in the chronic phase of the infection so it may become positive after a longer time of follow-up [25,26]. Although not currently available, flow cytometry may be a useful tool to demonstrate cure in the near future [27].

T. cruzi infection is no longer limited to the Latin American continent and has currently emerged in certain non-endemic areas such as the USA and western Europe. In Spain the majority of patients are female Bolivian immigrants in the fourth decade of their lives, who can transmit the infection through blood transfusion, organ transplantation or congenital transmission. Treatment tolerance is poor, which can further limit current treatment options. Sound epidemiological data are necessary in order to estimate the magnitude of a problem of great relevance for public health and health resource planning.

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Transparency Declaration

The authors declare that they have no conflicts of interest.

References

1. Rassi A Jr, Rassi A, Marin-Neto JA. Chagas heart disease: pathophysiologic mechanisms, prognostic factors and risk stratification. *Mem Inst Oswaldo Cruz* 2009; 104 (suppl 1): 152–158.
2. Sosa-Estani S, Viotti R, Segura EL. Therapy, diagnosis and prognosis of chronic Chagas disease: insight gained in Argentina. *Mem Inst Oswaldo Cruz* 2009; 104 (suppl 1): 167–180.
3. Reithinger R, Tarleton RL, Urbina JA, Kitron U, Gurtler RE. Eliminating Chagas disease: challenges and a roadmap. *BMJ* 2009; 338: b1283.
4. Bern C, Montgomery SP. An estimate of the burden of Chagas disease in the United States. *Clin Infect Dis* 2009; 49: e52–e54.
5. Jackson Y, Myers C, Diana A *et al.* Congenital transmission of Chagas disease in Latin American immigrants in Switzerland. *Emerg Infect Dis* 2009; 15: 601–603.
6. Lescure FX, Canestri A, Melliez H *et al.* Chagas disease, France. *Emerg Infect Dis* 2008; 14: 644–646.
7. Perez de Ayala A, Perez-Molina JA, Norman F, Lopez-Velez R. Chagasic cardiomyopathy in immigrants from Latin America to Spain. *Emerg Infect Dis* 2009; 15: 607–608.
8. Manzardo C, Trevino B, Gomez i Prat J *et al.* Communicable diseases in the immigrant population attended to in a tropical medicine unit: epidemiological aspects and public health issues. *Travel Med Infect Dis* 2008; 6: 4–11.
9. Munoz J, Gomez i Prat J, Gallego M *et al.* Clinical profile of *Trypanosoma cruzi* infection in a non-endemic setting: immigration and Chagas disease in Barcelona (Spain). *Acta Trop* 2009; 111: 51–55.
10. Flores-Chavez M, Fernandez B, Puente S *et al.* Transfusional chagas disease: parasitological and serological monitoring of an infected recipient and blood donor. *Clin Infect Dis* 2008; 46: e44–e47.
11. Gascon J, Albajar P, Canas E *et al.* Diagnosis, management and treatment of chronic Chagas' heart disease in areas where *Trypanosoma cruzi* infection is not endemic. *Rev Esp Cardiol* 2007; 60: 285–293.
12. Rassi A Jr, Dias JC, Marin-Neto JA, Rassi A. Challenges and opportunities for primary, secondary, and tertiary prevention of Chagas' disease. *Heart* 2009; 95: 524–534.
13. de Oliveira RB, Troncon LE, Dantas RO, Menghelli UG. Gastrointestinal manifestations of Chagas' disease. *Am J Gastroenterol* 1998; 93: 884–889.
14. Bern C, Montgomery SP, Herwaldt BL *et al.* Evaluation and treatment of chagas disease in the United States: a systematic review. *JAMA* 2007; 18: 2171–2181.
15. Perez-Molina JA, Perez-Ayala A, Moreno S, Fernandez-Gonzalez MC, Zamora J, Lopez-Velez R. Use of benznidazole to treat chronic Chagas' disease: a systematic review with a meta-analysis. *J Antimicrob Chemother* 2009; 64: 1139–1147.
16. Cancer Therapy Evaluation Program. Common Toxicity Criteria (CTC). Version 2.0. 1999. Available at: http://ctep.cancer.gov/protocoldevelopment/electronic_applications/docs/ctcv20_4-30-992.pdf (last accessed 9 February 2010).
17. Guerri-Guttenberg RA, Grana DR, Ambrosio G, Milei J. Chagas cardiomyopathy: Europe is not spared! *Eur Heart J* 2008; 29: 2587–2591.
18. Rassi A Jr, Rassi A, Marin-Neto JA. Chagas disease. *Lancet* 2010; 9723: 1388–1402.
19. Sosa-Estani S, Segura EL. Etiological treatment in patients infected by *Trypanosoma cruzi*: experiences in Argentina. *Curr Opin Infect Dis* 2006; 19: 583–587.
20. Viotti R, Vigliano C, Lococo B *et al.* Side effects of benznidazole as treatment in chronic Chagas disease: fears and realities. *Expert Rev Anti Infect Ther* 2009; 7: 157–163.
21. Marin-Neto JA, Rassi A Jr, Morillo CA *et al.* Rationale and design of a randomized placebo-controlled trial assessing the effects of etiologic treatment in Chagas' cardiomyopathy: the BENznidazole Evaluation

©2010 The Authors

For Interrupting Trypanosomiasis (BENEFIT). *Am Heart J* 2008; 156: 37–43.
22. Rodrigues Coura José, de Castro Solange L. A critical review on Chagas disease chemotherapy. *Mem Inst Oswaldo Cruz* 2002; 97: 3–24.
23. Urbina JA, Payares G, Sanoja C, Lira R, Romanha AJ. *In vitro* and *in vivo* activities of ravuconazole on *Trypanosoma cruzi*, the causative agent of Chagas disease. *Int J Antimicrob Agents* 2003; 21: 27–38.
24. Urbina JA. Ergosterol biosynthesis and drug development for Chagas disease. *Mem Inst Oswaldo Cruz* 2009; 104 (Suppl 1): 311–318.
25. Gomes YM, Lorena VM, Luquetti AO. Diagnosis of Chagas disease: what has been achieved? What remains to be done with regard to diagnosis and follow up studies? *Mem Inst Oswaldo Cruz* 2009; 104 (Suppl 1): 115–121.
26. Murcia L, Carrilero B, Muñoz MJ, Iborra MA, Segovia M. Usefulness of PCR for monitoring benznidazole response in patients with chronic Chagas'disease: a prospective study in a non-disease-endemic country. *J Antimicrob Chemother* 2010; 65: 1759–1764.
27. Vitelli-Avelar DM, Sathler-Avelar R, Wendling AP *et al.* Non-conventional flow cytometry approaches to detect anti-*Trypanosoma cruzi* immunoglobulin G in the clinical laboratory. *J Immunol Methods* 2007; 2: 102–112.

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