Lack of association between blood-based detection of *Trypanosoma cruzi* DNA and cardiac involvement in a non-endemic area

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Received 11 November 2010, Revised 20 July 2011,

Accepted 27 July 2011

Cases of chronic Chagas disease have been increasing in non-endemic areas due to the growth in immigration. This study examined the association between positive *Trypanosoma cruzi*-DNA detection in blood by PCR and presence of chagasic cardiac involvement in a cohort of immigrants in a European city. No association was found in this study between the positive *T. cruzi* blood PCR and cardiac involvement.

INTRODUCTION

Chagas disease, caused by the protozoan *Trypanosoma cruzi*, has an unpredictable clinical course and only 20–30% of infected patients will develop clinical manifestations with mainly cardiac or gastrointestinal tract involvement (World Health Organization, 2002). Cases have been increasing in Europe, a non-endemic area, and especially in Spain, due to the recent growth in immigration from Latin America. The majority of patients in Europe are in the indeterminate phase of the illness and are asymptomatic when diagnosed. At this stage, diagnosis of infection relies on finding positive serology (using at least two different serological methods). *T. cruzi*-DNA

detection by PCR currently remains a sensitive parasitological test but may show a variable degree of efficiency as parasitemia is scarce in the chronic phase and even tissue parasitism is low and restricted to few anatomical sites (Gomes *et al.*, 1999; Freitas *et al.*, 2005; Piron *et al.*, 2007). Studies have analysed possible predictors of mortality in chronic Chagas disease and the role in pathogenesis and disease progression of parasite DNA in affected tissues, but few studies have examined markers such as blood parasite DNA (Basquiera *et al.*, 2003; Elias *et al.*, 2003; Schijman *et al.*, 2004; Rassi, Jr *et al.*, 2007). This study examined the association between positive *T. cruzi*-DNA detection in blood by PCR and presence of chagasic cardiac involvement in a cohort of immigrants in Europe.

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MATERIALS AND METHODS

An analysis of Chagas disease patients at the Tropical Medicine Unit, Ramon y Cajal

Hospital (Madrid, Spain) during the period January 2003–November 2009 was performed. *T. cruzi* diagnostic tests were performed at the Parasitology Department, National Microbiology Centre, Instituto de Salud Carlos III (Madrid, Spain). Anti-*T. cruzi* antibodies were detected by the indirect immunofluorescent antibody test and by ELISA (Flores-Chavez *et al.*, 2008). *T. cruzi* DNA was detected in blood by PCR using 121–122 oligonucleotides which detect kDNA common to all *T. cruzi* lineages (I–VI) as described below.

Blood Collection, DNA Extraction and PCR

In order to obtain DNA for PCR, 4 ml of blood were collected and mixed with 4 ml of 6 M guanidine-HCl and 0.2 M EDTA, pH 8.0. This sample mixed in a shaker overnight at room temperature after which it was boiled at 100 °C for 15 minutes. Two aliquots of 200 µl were mixed with 300 µl of 100 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 8, 50 µl of 10% SDS and 2 µl of 50 mg/ml proteinase K, and incubated at 56 °C for 1 hour. After incubation, 150 µl of 10% Chellex 100 (BioRad) were added in the same microtube and incubated at 60 °C for 30 minutes, and then at 100 °C for 10 minutes. The supernatant containing DNA was separated by centrifugation at 13 000g for 10 minutes at room temperature. DNA was precipitated with 650 µl of isopropanol and 2 µl of 20 mg/ml glycogen by centrifugation at 13 000g for 5 minutes at 4 °C. The sediment was washed by centrifugation in 70% ethanol as previously. Positive and negative controls in duplicate were included for each batch of 10 samples to monitor the risk of contamination. The limit of detection with this procedure is 1–0.1 parasites/ml (when the volume of the blood sample mixed with guanidine buffer is 10 ml). kDNA-PCR amplification was performed in a total volume of 75 µl containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM MgCl₂ and 0.2 mM of dNTPs, 0.5 µl of Taq DNA

polymerase (Roche), 200 ng of 121 (5'-AAATAATGTACGGGKGAGATGCATG A-3') and 122 (5'-GGTTCGATTGGG GTTGGTGTAATATA-3') primers for *T. cruzi* detection, 5.6 pmol of REV (5'-GACGGTATCTGATCGTCTTC-3') and 1.1 pmol of HUF (5'-GAGCCGCCTGG-ATACCGC-3') primers for inhibition monitoring, and 10 µl of DNA of each sample or controls. The temperature profile was as follows: 95 °C for 1 minute of denaturation with a longer initial time of 5 minutes at 95 °C, 64 °C for 1 minute for primer annealing and 72 °C for 1 minute for extension (35 cycles), with a final incubation at 72 °C for 10 minutes to extend the annealed primers. PCR products of 330 bp were analysed by electrophoresis on a 2% agarose gel. Positive and negative controls, in duplicate, were included for each amplification protocol. The analytical sensitivity is 0.01–0.001 parasites/reaction. The PCR was valid when the controls were amplified correctly. DNA extraction was also valid when extraction controls were amplified correctly. The result of each sample was valid when both duplicates yielded the same result and when these were in accordance with the results of serological tests. When any of these criteria failed or the inhibition became evident, the whole procedure and serology were repeated. Tracking of blood and serum samples from the patient was also analysed in order to monitor the risk of misidentification.

More than one PCR result (≤ 3) was available for some patients using this technique as repeated blood samples were obtained over a period of several months.

The presence of cardiac involvement was evaluated as per clinical protocol for each patient and compared with PCR result. As some patients had discrepant PCR results (both positive and negative PCR results), two situations were considered. For the first analysis, patients with at least one positive PCR were classified as 'positive' and for the second more restrictive analysis, only patients with all positive PCRs were classified as 'positive' (patients with discrepant results

were excluded from the positive PCR group for this second analysis). Patients with only one PCR performed which was positive were considered positive in both.

Patients were considered to have cardiac involvement by Chagas disease on finding an abnormality on ECG or echocardiogram. The following were considered ECG abnormalities suggestive of cardiac involvement due to Chagas disease: complete RBBB with/without left anterior fascicular block, complex ventricular arrhythmias, sustained ventricular tachycardia, supraventricular tachyarrhythmias (atrial fibrillation, atrial flutter and atrial tachycardia), second degree type II and complete atrioventricular block, sinus bradycardia <50 beats/min and the presence of a permanent pacemaker (Sosa-Estani *et al.*, 2009). In the absence of other causes, the following were considered suggestive findings on echocardiography: left ventricular dysfunction, increased left ventricular end-diastolic diameter, wall-motion abnormalities, apical aneurysm or intracavitary thrombus (Gascon *et al.*, 2007). Patients for which PCR and/or examination results to determine cardiac involvement were not available were excluded from the final analysis.

Significance of baseline differences was determined by the chi-squared test, Fisher's exact test or the unpaired *t*-test, as appropriate. The association between PCR and cardiac involvement was described by means of the odds ratio and its 95% confidence interval. A two-sided *P* value <0.05 was considered statistically significant.

RESULTS

During the study period, 359 Chagas disease patients were diagnosed by serology. For 102 patients, it was not possible to ascertain the presence of cardiac involvement and 11 patients did not have a PCR result. Data were available for analysis for 246 patients. There were 169 females (68.7%) and 77 males with a mean age of

37.1 years (range: 16–69 years). All patients were originally from endemic countries: the majority of patients (239/246, 97.2%) were from Bolivia, with two patients from Paraguay and one each from Argentina, Brazil, Chile, Ecuador and Honduras. Most patients (190/246, 77.2%) came from rural areas; 211 (85.8%) recalled having seen the vector; 28 (11.4%) had received a blood transfusion many years previously in their country of origin and in at least 41 (16.7%), vertical transmission was a possibility (mother with known positive Chagas serology). Only 21/246 patients (8.5%) had received previous specific treatment for the infection in their country. In total, 43 patients (17.5%) were considered to have cardiac involvement (four of these patients had cardiac and gastrointestinal involvement). In total, 146 patients had more than one PCR result available.

Baseline characteristics (age, gender, country of origin, place of residence, previous blood transfusion and previous treatment) for patients with any positive or all positive PCRs compared to those with negative PCRs were not different with the exception of previous treatment. Previous history of treatment with anti-*T. cruzi* drugs (benznidazole and/or nifurtimox) in their country of origin was significantly more frequently associated with a negative PCR (*P*<0.05).

There was no association between PCR and cardiac involvement when any positive PCR was classified as positive (*n*=157) and no association was found when all PCRs performed had to be positive (*n*=137). When a logistic regression analysis was performed, the presence of previous treatment was found to be a possible confounding factor, but this did not change the lack of association between PCR and cardiac involvement both for any positive PCR or when all positive PCRs were considered (Table). No association was found between cardiac involvement and rural versus urban area of residence (36/190, 18.9% versus 7/56, 12.5%; *P*=0.26).

TABLE. *Chagas disease: association between cardiac involvement and T. cruzi-DNA detection in blood by PCR*

Crude and adjusted odds ratio for patients with at least one positive PCR*

	OR	95% CI
PCR+ versus PCR-	0.95	0.48-1.87
Adjusted by previous therapy†	0.80	0.40-1.60

Crude and adjusted odds ratio for patients with all positive PCRs

	OR	95% CI
PCR+ versus PCR-	1.01	0.52-1.95
Adjusted by previous therapy†	0.89	0.45-1.73

*PCR: *T. cruzi*-DNA detection in blood by PCR.†Previous treatment with anti-*T. cruzi* drugs (benznidazole and/or nifurtimox).

DISCUSSION

In the last decade, the Spanish health system has been forced to adapt to the emergence of a non-endemic infection with sufficient impact to warrant changes in national legislation with respect to the screening of blood donations (Ministerio de Sanidad y Consumo, 2005) and which has been predicted may become an important cause of cardiomyopathy in the near future (de Ayala *et al.*, 2009). Early detection of cases, especially those who may progress clinically with a poor prognosis, becomes crucial when dealing with an infection which may be expected to have significant public health repercussions. Currently, most patients are diagnosed in the indeterminate phase and there are no specific laboratory variables which may serve as markers of disease progression or outcome.

Serological methods are the most widely used for diagnosis of chronically infected patients in non-endemic areas, but the role of PCR has yet to be established. Based on the results presented here, there appears to be no association between a positive blood

PCR and the presence of cardiac involvement in chronically infected chagasic patients outside endemic areas. *T. cruzi* DNA may be difficult to detect in this phase even in specific tissues. Even though the prevalence of intact *T. cruzi* parasites in chronically inflamed tissues such as the heart appears to be low according to several studies, a positive correlation between parasite DNA in tissue lesions and disease progression and severity has been shown (Elias *et al.*, 2003; Schijman *et al.*, 2004). Transmission via chronically infected blood donors and reactivation in immunosuppressed individuals support the theory that at least some parasites persist in the host, but the natural biology of the parasite and its reservoirs in the human body have not been fully elucidated. Distinct evolutionary lineages of the parasite (mainly *T. cruzi* discrete typing units, DTUs, I-VI) (Zingales *et al.*, 2009) have also been identified. *T. cruzi* DTUs may be associated with different geographical distribution and transmission cycles. Multiple DTUs with different tissue tropisms may coexist in patients with Chagas disease, so parasites detected in blood by PCR may not be the same as those involved in cardiac lesions (Macedo and Pena, 1998; Freitas *et al.*, 2006; Valadares *et al.*, 2008). Specific parasite DTUs may be associated with cardiac involvement, as shown in a recent study where *T. cruzi* I was frequently associated with cardiac damage (Burgos *et al.*, 2010).

T. cruzi detection in blood by PCR appears to be dependant on level of parasitemia and its low level in the chronic phase may partly explain the intra-patient variability and discrepancy in PCR results observed for some patients in this study. The use of quantitative PCR, which is not currently available at our hospital, may provide useful information in future (Piron *et al.*, 2007; Duffy *et al.*, 2009). Positive PCR results in patients with negative serology have also been reported in the literature (Gomes *et al.*, 1999), but this was not observed in this study.

Although limitations of this study may not allow extrapolation of the findings to populations in endemic countries or even to other immigrant populations, until more is known about this disease and the parasite, blood PCR will probably remain just an adjunct in the diagnosis of chronic infection. In one cohort in an endemic country, patients with chronic Chagas disease (seropositive) and positive blood *T. cruzi* DNA detected by PCR were found to be at higher risk of progression to chronic Chagas cardiomyopathy (Basquiera *et al.*, 2003). In the current study, PCR result alone did not provide any additional information on which chronically infected patients may be expected to have cardiac involvement. Previous treatment was associated with a negative PCR and some authors have suggested it may be used to monitor response to treatment in the chronic phase (Gomes *et al.*, 1999), but if the above considerations are taken into account, certain limitations may be anticipated. However, detection of *T. cruzi* DNA by PCR may be of use as a surrogate marker of treatment failure as a positive post-treatment PCR would indicate a lack of parasitological response (Pérez-Ayala *et al.*, 2011). For these patients, PCR and additional studies may help determine whether treatment failure was due to the presence of resistant parasite strains and this may aid future therapeutic decisions.

Further research is needed and PCR studies in non-endemic areas may provide valuable information as patients will not ordinarily be re-infected unless they travel.

ACKNOWLEDGMENTS. We would like to thank Ms L. Moreno and the staff at the Tropical Medicine Unit and parasitology laboratory for technical assistance.

This study was supported by the Spanish Ministry of Science and Innovation and the Instituto de Salud Carlos III within the Network of Tropical Diseases Research (RICET RD06/0021/0020 and RD06/0021/0009).

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