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Case Report

Evaluation of *Trypanosoma cruzi* parasitic load by real-time PCR and blood culture in long-term kidney transplant recipients

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Abstract

Introduction: Acute Chagas disease involving reactivation can occur after organ transplant, and follow-up by direct parasitological or molecular methods is essential for monitoring the parasitic load in such patients. In contrast, there is a little data on the parasitic load in long-term organ recipients. In this study, we examined the parasitic load in long-term kidney transplant patients and assessed the possibility of late Chagas disease reactivation.

Methodology: Blood cultures and real-time PCR were used to assess the parasitic load in four immunosuppressed patients who underwent kidney transplants (between 1996 and 2014) and were also treated for parasites.

Results: There were no positive blood culture or real-time PCR results in Chagas disease patients who received kidney transplants. The real-time PCR presented detection limit of 0.1 parasite equivalent/mL. The time interval between the transplant and sample collection varied from one to 19 years.

Conclusions: No parasites were detected in the evaluated patients. The use of benznidazole and immunosuppressive therapy may have contributed to control the *T. cruzi* infection. In transplanted patients with Chagas disease, the use of methods such real-time PCR and blood culture can monitor the parasitic load and prevent disease reactivation.

Key words: Chagas disease; Kidney transplant; real-time PCR.

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Introduction

Chagas disease is a neglected disease that affects 6-8 million people worldwide and is considered endemic in 21 countries of Central and South America [1]. Chagas disease is caused by the flagellate protozoan *Trypanosoma cruzi* and is transmitted by contaminated feces of triatomine bugs. Other forms of transmission currently recognized include congenital transmission, blood transfusions, organ transplants, infected food, and accidental infection in laboratories [2]. The transmission of Chagas disease through the donation of infected organs (liver, heart, lung, and kidney) had been described in various countries and is apparently associated with the migration of people from endemic to non-endemic areas.[3–8]. In Brazil, mandatory pre-transplant screening of organ donors and recipients has been implemented to minimize such transmission and reactivation of the disease [9].

An additional complication involves the reactivation of Chagas disease in individuals on immunosuppressive therapy that is commonly used to avoid transplant rejection. The reactivation of Chagas disease is usually more serious than the primary acute phase of the disease since it can cause the resurgence or worsening of cardiological and/or neurological symptoms.

Most cases of reactivation involve subjects HIV-seropositive subjects or heart transplant recipients and reports of Chagas disease reactivation have been reported in kidney transplant recipients, with most of them involving case reports. This reduced number of reports may reflect the fact that kidney injury is a comorbidity that is not generally associated with the pathology of Chagas disease. The most extensive study of Chagas disease patients who underwent kidney transplants detected *T. cruzi* infection in 17.22% of

donors and recipients in a series of 238 patients in Argentina; Chagas disease reactivation was observed in 21.7% of organ recipients [10]. Specialists recommend that organ recipients be monitored for Chagas disease reactivation for at least two years after transplants and at any interval after the intensification of immunosuppressive therapy [11,12].

In Brazil, there are approximately 5,700 kidney transplants each year [13], with a progressive increase for this intervention in elderly patients because of renal disease [14]. This scenario favors reactivation of the disease since most patients with chronic Chagas disease are elderly and present a variety of comorbidities [15–17].

Since the use of immunosuppressive therapy decreases with the length of time since organ transplant, it is expected that the parasitic load will also decrease correspondingly. However, factors such as age and other comorbidities may increase the parasitic load. In view of the limited amount of data available in the relationship between Chagas disease, parasitic load, and the length of time since renal transplants, in this study we used real-time PCR and blood culture to assess the parasitic load in these patients.

Methodology

This study involved a retrospective analysis of the medical records from the Nephrology Clinic at the University Hospital of the State University of Campinas (UNICAMP), Campinas São Paulo state, southeastern Brazil. The criteria for inclusion were: having had a kidney transplant, having a positive Chagas disease serology, having received immunosuppressive therapy, and remaining a patient of the Nephrology Clinic. The patients´ physical and/or electronic medical records were reviewed to collect personal and epidemiological data related to kidney transplants and Chagas disease. Thirty milliliters of venous blood was collected from each participant for blood cultures (24 mL) and real-time PCR (6 mL).

This study was approved by an institutional Ethics Committee (approval number CAAE 45940515.5.0000.5404). All patients enrolled in this investigation were informed about the study prior to participation and provided informed consent form that allowed access to their data.

Blood Culture

Blood cultures were obtained as described by Chiari *et al*. [18] and Luz *et al*. [19]. Twenty-four milliliters of venous blood was collected in four vacuum tubes with EDTA followed by centrifugation (1,903 g, 10 minutes, 4 ºC). The plasma was removed and the erythrocyte pellet was washed with liver infusion tryptose (LIT) culture medium containing 10% fetal bovine serum and 10% penicillin/streptomycin. The supernatant was discarded and the erythrocyte pellet was resuspended in 6 mL of LIT followed by incubation at 28 ºC. The cultures were examined microscopically (40× magnification) every 15 days until a positive result was obtained or until 120 days had elapsed.

Isolation of genomic DNA

Genomic DNA was isolated from leukocytes immediately after collecting blood into EDTA-containing vacuum tubes. The blood was centrifuged twice (752 g, 4 ºC, 10 minutes each) with 12 mL of red cell lysis buffer (0.0114 M NH_4Cl and 0.01 M NH_4HCO_3). After the second centrifugation, the supernatant was discarded, and the pellet was resuspended in 5 mL of TKM1 buffer (10 mm Tris-HCl pH 7.6, 10 mM KCl, 10 mM $MgCl_2$, and 20 mM EDTA) and 1 mL of Triton X-100®. After further centrifugation (52 g, 15 minutes, 4° C), the supernatant was again discarded, and the pellet was resuspended in 1 mL of TKM1 buffer and centrifuged a final time (8,000 g, 5 minutes, room temperature). The supernatant was discarded, leaving the leucocyte pellet. After cell lysis, DNA was extracted using a High Pure PCR template preparation (Roche®, Basel, Switzerland), according to the manufacturer´s recommendations, followed by elution in 200 µL of elution buffer. Aliquots were stored at -20 ºC.

Quantitative PCR

Standard curve

Epimastigote forms of *T. cruzi* (DTU TCII) were obtained from positive blood culture of a patient with Chagas disease and cultured in LIT medium. When the culture was in the logarithmic phase, the parasite number was determined in a Neubauer chamber and found to be 1.25×10^5 parasite equivalents/mL. DNA was extracted from this culture, and serial dilutions were prepared in water (1.25×10^5 to 1.25×10^{-3} parasite equivalents/mL). Using these dilutions, the standard curve was build and used as a basis to compare the results obtained from patients.

real-time PCR

Sample quantification was done as described by Piron *et al*. [20]. Primers Cruzi 1 forward (5' ASTCGGCTGATCGTTTTCGA 3') and Cruzi 2 reverse (3' AATTCCTCCAAGCAGCGGATA 5') amplified a 166 base pair sequence of *T. cruzi* satellite

DNA. The sequence Cruzi 3 (FAM-CACACACTGGACACCAA) was used as the probe. Each reaction consisted of 1x Taqman Universal Master Mix with UNG (Applied Biosystems), RNase P 0.1x (Applied Biosystems), 500 nM of each primer (Cruzi 1 and Cruzi 2), 200 nM of probe (Cruzi 3), 3 µL of DNA and deionized water to complete 30 µL. The RNAse P gene (Applied Biosystems®) was used to confirm DNA intactness, monitor the reproducibility and yield of DNA, and confirm the absence of PCR inhibitors. A Rotor-Gene 6000 real-time PCR cycler (Corbett® Life Science, Australia) was used for quantification. The cycle of reactions consisted of one cycle at 50 ºC for 2 minutes, one at 95 ºC for 10 minutes, and 45 cycles at 95 ºC for 15 seconds and 58 ºC for 1 minutes. The results were quantified using Rotor-Gene software v.1.7.87 and were considered valid when the samples showed RNAse P gene amplification and were positive when the cycle threshold (CT) was < 40. Samples with amplification signals ≥ 0.1 parasite equivalents/mL were considered quantifiable. Samples without DNA (no template control) were included in each run to confirm the absence of contaminants during sample preparation. Samples that showed no amplification signal were considered negative.

Results

Four subjects who were carriers of Chagas disease and underwent kidney transplants were evaluated from August 2015 to August 2016. The patients were analyzed individually, with the small total number (n = 4) precluding statistical analysis of the results. The mean age was 55.2 years (range: 35-66 years), with three of the patients being female; all patients were born in areas endemic for Chagas disease. Three patients had the cardiac form and one an undetermined form of the disease. Analysis of these clinical parameters were based on II Brazilian Consensus on Chagas disease [2]. The earliest transplant was done in 1996 (n = 1), with the remainder being done in 2010 (1), 2011 (1), and 2014 (1). Antiparasitic treatment was administered in all cases, before or at the time of the transplant. The time interval between the transplant and sample collection varied from one to 19 years. No parasitic load was detected in any of the patients, whether by blood culture or real-time PCR (Table 1). The real-time PCR runs showed good parameters up to 1.25×10^{-1} parasite equivalent/mL (Efficiency = 0.96; $R^2 = 0.99$; Slope = -3.42) .

Case 1

A female patient, 66 years old, from the state of Paraíba in northeastern Brazil, was diagnosed with indeterminate Chagas disease in May 1995, based on Machado-Guerreiro reaction (1/4) and indirect immunofluorescence (IFI - 1/40). In January 1996 the patient had positive xenodiagnoses. The mode of Chagas disease transmission was uncertain because even though the patient was from a region where the disease was endemic, she had also received five blood transfusions during her life. The basal disease was chronic renal failure secondary to pyelonephritis. In June 1996, the patient received benznidazole for 60 days followed by a kidney transplant from a live donor the following July, there were no complications. Immunosuppressive therapy at the time of transplant consisted of cyclosporine 350 mg, azathioprine 150 mg, and prednisone 100 mg. Despite the absence of symptoms associated with acute Chagas disease after the transplant, there was an increase in serum titers for Chagas disease so antiparasitic treatment was restarted for an additional two months. Blood samples for blood culture and real-time PCR were obtained in December 2015. Immunosuppressive therapy at this point

Table 1. Profile of patients enrolled in this study.

Characteristics	P01	P02	P03	P04	Range / Mean
Sex	Female	Female	Male	Female	
Age	71	39	67	44	39-71 / 55.2
Clinical form of CD	Und	Cardiac	Cardiac	Cardiac	
Geographic region	Paraíba	Minas Gerais	Minas Gerais	Minas Gerais	
Underlying disease	CRI - Pyelonephritis	Chronic GN	Chronic GN	Und CRI	
Transplant date	1996	2010	2011	2014	
Donor type	Related living	Deceased	Deceased	Deceased	
Antiparasitic treatment (year)	1996	2013	2010	2011	
Period between transplant and sample collect (years)	19	6	5	1	1-19
Blood culture	Negative	Negative	Negative	Negative	
Real-time PCR	Negative	Negative	Negative	Negative	

CRI: Chronic Renal Insufficiency; GN: Glomerulonephritis; Und: Undetermined, Mycophenolate precursors.

consisted of cyclosporine 100 mg, prednisone 5 mg, and allopurinol 100 mg. The last medical evaluation was in March 2020, and the medicines in use were cyclosporine 100 mg, prednisone 5 mg, omeprazole 40 mg, folic acid 5 mg, simvastatin 20 mg, and vitamin D 20,000 U/week. The patient had comorbidities that included hypertension, hepatitis C, thalassemia trait, repetitive urinary tract infections, gastric neoplasm, and condylomatosis.

Case 2

A female patient, 40 years old, from the state of Minas Gerais in southeastern Brazil, had a chronic renal failure of undetermined cause and received a kidney from a deceased donor in May 2010. Chagas disease was detected after the transplant, based on the results of routine tests, by ELISA and IFI (1/640) tests. Blood forms trypomastigotes were not detected in direct tests. At that time, immunosuppressive therapy consisted of tacrolimus 10 mg, sodium mycophenolate 1080 mg, basiliximab 20 mg, and methylprednisolone 500 mg. The patient received antiparasitic treatment in June 2013, because she showed progression from the undetermined form of Chagas disease to the cardiac form, developing block of the anterosuperior division of the left bundle branch. Blood samples for blood culture and real-time PCR were collected in January 2016. At that time, immunosuppressive therapy consisted of tacrolimus 2 mg, sodium mycophenolate 1,440 mg, and prednisone 2.5 mg. The last medical evaluation was in January 2020, when the patient present the following comorbities: hypertension, persistent hyperparathyroidism, dyslipidemia, and repetitive urinary tract infections. The medications in use were tacrolimus 2 mg, sodium mycophenolate 1,440 mg, prednisone 2.5 mg, omeprazole 40 mg, nitrofurantoin 100 mg, atorvastatin 20 mg, furosemide 40 mg, cinacalcet 60 mg, atenolol 50 mg, and amiodarone 200 mg.

Case 3

A male patient, 63 years old, from the state of Paraná in southern Brazil, was diagnosed with the undetermined form of Chagas disease in 2010 based on immunological results (ELISA and IFI 1/1280), he later developed the cardiac form with left branch block and received benznidazole for 60 days starting in February 2010. His basal disease was diabetic nephropathy he received a kidney from a deceased donor in June 2011. At the time, immunosuppressive therapy consisted of tacrolimus 10 mg, sodium mycophenolate 360 mg, methylprednisolone 500 mg, and basiliximab 20 mg. There where no symptoms of Chagas disease reactivation and *T. cruzi* blood forms were not detected after transplantation. Blood samples were collected for blood culture and real-time PCR in January 2016. At this point, immunosuppressive therapy was consisted of azathioprine 100 mg and prednisone 10 mg. The patient had several comorbidities that included diabetes mellitus type 2, hypertension, osteoporosis, urethra stenosis, repetitive urinary tract infections, and subclinical hypothyroidism. The last medical evaluation occurred in January 2020, at which time the medicines in use were azathioprine 50 mg, prednisone 5 mg, losartan 50 mg, clonidine 0.15 mg, metformin 500 mg, hydralazine 50 mg, furosemide 40 mg, alendronate 70 mg/week, folic acid 5 mg, ferrous sulfate 40 mg, omeprazole 20 mg, aspirin 100 mg, simvastatin 20 mg, calcium carbonate 500 mg, cephalexin 500 mg, nitrofurantoin 100 mg and regular insulin.

Case 4

A female patient, 34 years old, from the state of Minas Gerais State in southeastern Brazil. She was diagnosed with the undetermined form of Chagas disease in 2010 (ELISA and IFI 1/160). Approximately one year later, the disease evolved to the cardiac form with a block of the anterosuperior division of the left bundle branch. In 2011, she received benznidazole for 60 days. The patient had chronic glomerulonephritis and received a kidney from a deceased donor in January 2014. The immunosuppressive therapy at the time consisted of tacrolimus 5 mg, methylprednisone 500 mg, sodium mycophenolate 360 mg and anti-thymocyte immunoglobulin 5 mg. Blood samples were collected for blood culture and real-time PCR in December 2015. At this point, immunosuppressive therapy consisted of tacrolimus 5 mg, sodium mycophenolate 720 mg and prednisone 10 mg. The patient underwent a medical evaluation in January 2020 and presented comorbidities that included hypertension, hypothyroidism, hepatic steatosis and discrete cholelithiasis. The medicines in use were prednisone 5 mg, tacrolimus 2 mg, sodium mycophenolate 360 mg, atorvastatin 20 mg, aspirin 100 mg, omeprazole 20 mg, carvedilol 12.5 mg, puran 62.5 mg and nortriptyline 25 mg.

Discussion

Serological screening before blood transfusion and organ transplants is an efficient means for minimizing the transmission of Chagas disease. According to the II Brazilian Consensus for Chagas disease [2], transplant recipients who test positive for the disease must be

followed-up to monitor any increase in parasitic load. Screening tests should be performed weekly for the first two months post-transplant, every two weeks in the third month, and then monthly for at least six months [21]. The methods commonly used in these cases comprise direct methods, such as blood smear or concentration techniques, e.g., microhematocrit or the Strout method [2].

Patients who undergo kidney transplants receive immunosuppressive drugs to minimize rejection of the new organ. However, immunosuppression may result in the reactivation of latent infections such as Chagas disease. Immunological tests to detect anti-*T. cruzi* antibodies are usually applied for identifying chronic Chagas disease. Since kidney transplant patients on immunosuppressive therapy commonly have low levels of IgG isotypes, serology should not be used to assess Chagas disease reactivation during immunosuppression [2]. Approximately 22% of Chagas disease patients who undergo kidney transplants without prior antiparasitic treatment show reactivation of the disease [10]. However, the rate of reactivation may vary among different centers and in relation to the organ received [11,22]; this rate may also be influenced by undiagnosed and unpublished cases, especially from non-endemic countries [23].

In this work, we examined the parasitic load in four long-term kidney recipients, several years after the transplant, based on screening with real-time PCR and blood culture tests. Blood culture provides an indirect, semi-quantitative assessment of parasitic load. This test, which has high specificity low sensitivity, allows the genotyping of *T. cruzi*, although the parasite lineage may interfere with the sensitivity of the test [24,25]. None of the patients screened here had a blood culture positive for *T. cruzi*.

Although direct methods provide a well-standardized means of detecting Chagas disease reactivation, conventional PCR has been used for the early detection of such reactivation in heart transplant patients [26]. PCR has also been used as a confirmatory method after kidney transplants [27–31]. In one case, an inconclusive diagnosis of Chagas disease was reached in a kidney recipient with cardiac manifestations considered to be indicative of disease reactivation; in this case, direct and indirect parasitological test results were negative whereas the nested-PCR was positive [32]. Although conventional PCR is more sensitive than direct methods, this test still has some limitations [33].

Quantitative PCR is a useful tool for analyzing the parasitic load during *T. cruzi* infection because of its ability to quantify DNA. This method has been widely used to monitor the parasitic load in HIV subjects and was also used to analyze a case of Chagas disease reactivation in a patient with follicular lymphoma [34,35]. In the present study, the detection limit for the parasitic load was 0.1 parasite equivalents/mL, a level similar to that obtained by Ramirez *et al*. (0.2 parasite equivalents/mL) [36].

The lack of a standardized protocol for real-time PCR among laboratories, led Schijman *et al*. (2011) [37] to analyze the real-time PCR protocols used to detect chronic infection; the parameters examined included the type of biologic samples, the method and efficiency of DNA extraction, the *T. cruzi* molecular target used, and reaction conditions. Two of the various protocols examined showed better sensitivity, one of which (described by Piron *et al*. [20] was used in the present study. Although the real-time PCR was able to detect 0.1 parasite equivalents/mL, none of the four patients examined showed any parasites DNA.

Immunosuppression is generally induced with high drug doses, while maintenance of the immunosuppression state involves the use of decreasing doses over time and a good outcome of the graft in the host [38]. The intensification of immunosuppression increases the risk of reactivation [22,39]. The type of organ transplanted may also influence the rate of reactivation. For example, few studies have examined Chagas disease reactivation after liver transplants, possibly because of the drug doses used in immunosuppressive therapy in such cases [11,12].

In this study, all patients were on maintenance immunosuppressive therapy at the time of sample collection. Despite variation in the triple therapy and doses used by the patients, all of them followed the recommendations of the Brazilian Ministry of Health [38]. Whereas the use of mycophenolate mofetil has been reported to increase the reactivation of Chagas disease in heart transplant patients [40,41], such reactivation was not seen here after renal transplants .

Antiparasitic treatment with benznidazole is more effective in the acute phase of Chagas disease compared to the chronic phase [2]. Although no randomized clinical trials have assessed the use of benznidazole in immunocompromised subjects [12], some authors have reported satisfactory outcomes with this drug in the long-term survival of patients [22,23,42]. In the present investigation, no symptoms of Chagas disease reactivation were observed immediately after transplantation, nor were there any side effects related to the use of benznidazole.

Several factors may influence the real-time PCR results in immunosuppressed subjects. A low parasitic

load is usually found in chronic Chagas patients > 50 years old [15] and was also observed in the patients in this study. Fluctuations in the sensitivity of the method may reflect transitory and intermittent parasitemia of *T. cruzi* in the host. Factors such as blood volume, sampling frequency and the time interval between sample collection and processing may also affect the sensitivity of the method [43]. One limitation of the present observational study was the small number of the patients analyzed, which precluded rigorous statistical analysis. This limitation could be overcome by undertaking a multicentric study.

Conclusions

No parasites were detected in the patients screened by blood culture or real-time PCR methods. The lack of detection of *T. cruzi* in this study may reflect the fact that the patients were evaluated in the chronic phase of Chagas disease. In addition, the prior use of benznidazole combined with maintenance immunosuppressive therapy may have contributed to control of the *T. cruzi* infection since the function of the immune system is close to normal in this condition. However, a follow-up of at least once a year must be used to avoid late reactivation of the disease.

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