

Anti-serum validation for use in immunohistochemistry for *Trypanosoma cruzi* detection

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Abstract

Introduction: The detection of *Trypanosoma cruzi* in tissue samples is important in many situations, such as testing of the reactivation of the infection. The detection of *T. cruzi* nests in endomyocardial biopsies (EMB) may be useful to evaluate graft rejection. Given their scarcity, such nests are not routinely identified. To increase the diagnosis sensitivity, immunohistochemistry (IHC) may serve as a promising strategy. Here, we validate an antiserum for the detection of *T. cruzi* infection by IHC. **Methods:** We used 1) positive controls (PCs) – 13 EMB, 12 skin biopsies, and 1 heart with *T. cruzi* nests as sections stained with hematoxylin and eosin (HE); 2) negative controls – a) 10 explant hearts and 10 EMB with no amastigote nests or clinical/laboratory signs of chagasic infection; and b) eight samples with leishmaniasis, toxoplasmosis, or histoplasmosis; and 3) Cases – 31 EMB of chagasic patients with no parasite nests in HE sections but detected positive for *T. cruzi* DNA by polymerase chain reaction. As a primary antibody, a hyperimmune serum from *T. cruzi*-infected rabbits was used. **Results:** IHC results were positive for 21 of 26 PCs (80.8%) and one case of cutaneous leishmaniasis. In 4 of 31 cases, IHC revealed nests (12.9%), which were undetected by conventional histological examination. **Conclusions:** This study shows that IHC with the tested antiserum increases the sensitivity of the diagnosis and may be recommended for routine use in EMB analyses of cardiac transplant patients with Chagas disease.

Keywords: Chagas disease. Heart transplantation. Immunohistochemistry. *Trypanosoma cruzi*.

INTRODUCTION

Chagas disease, a chronic systemic parasitic infection caused by the protozoan *Trypanosoma cruzi*, was discovered by Carlos Justiniano Ribeiro Chagas in 1909¹. According to the World Health Organization (WHO), it is one of the most neglected diseases in the world². An estimated 6 to 7 million people are infected worldwide, especially in Latin America³. Despite the successful programs to control the transmission of the etiological agent through vector and transfusion routes in Brazil in the last decade⁴, a considerable Brazilian population (about 3 to 4 million individuals) is infected by the parasite or is at the risk to develop serious lesions of the disease. Furthermore, the uncontrolled migration of people infected with *T. cruzi* poses a challenge to control new cases in non-endemic countries⁵⁻⁷ and urban areas of endemic countries⁸.

The majority of infected individuals (60% to 70%) fail to develop clinically apparent disease (indeterminate form). The remaining 30% to 40% present an anatomical clinical form of chronic cardiac, digestive (megaesophagus and/or megacolon), or cardio-digestive disease usually 10 to 30 years after the initial infection.

Chronic Chagas heart disease is the most serious and frequent manifestation of the disease. It affects 20%-30% of infected individuals and is an important cause of morbidity and mortality, as many individuals are unable to perform their usual activities and die of progressive heart failure and related complications⁹.

About 10% of patients with heart failure secondary to Chagas disease progress to New York Heart Association (NYHA) functional class IV¹⁰. In the last two decades, cardiac transplantation has become one of the therapeutic modalities and serves as the only strategy that may modify the natural progression of the disease¹¹.

In addition to the complications seen in solid organ transplants, Chagas disease is characterized with the reactivation of chagasic infection, owing to the immunosuppressive treatment

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that patients receive to avoid graft rejection¹². Reactivation of Chagas disease in patients with depressed cellular immunity has been observed since the 1960s¹³.

There are several ways to recognize the reactivation of *T. cruzi* infection in transplanted individuals. In some cases, the clinical suspicion is related to the appearance of skin lesions that are suggestive of reactivation. Common manifestations of reactivation of chagasic infection are panniculitis, myocarditis with signs of heart failure, fever, fatigue, anorexia, diarrhea, meningitis, encephalitis, and stroke¹⁴⁻¹⁷.

Circulating *T. cruzi* is considered as a parasitological criterion for the diagnosis of acute phase or reactivation of chagasic infection by the II Brazilian Consensus on Chagas Disease and may be detected by several methods¹⁸. The parasitic deoxyribonucleic acid (DNA) may be effectively detected by molecular biology techniques, especially polymerase chain reaction (PCR) from blood samples or endomyocardial biopsies (EMB)¹⁹. PCR becomes positive a few days or weeks before the traditional parasitological tests and prior to clinical signs of reactivation^{20,21}. Given its high sensitivity, PCR results from blood samples should be interpreted with caution in subjects with chronic Chagas disease, as the test may be positive in immunocompetent or immunocompromised individuals without clinical signs of reactivation^{20,22}.

Endomyocardial biopsies, routinely performed after transplantation, aim to evaluate the immune rejection of grafts and determine its intensity. A pathologist is asked to identify and grade cellular rejection (absent, mild, moderate, or severe cell rejection). This graduation is based on histopathological aspects seen in EMB, such as the number of foci and pattern of inflammatory infiltrate, myocardiocytic lesion, edema, hemorrhage, and vasculitis²³. Conventional histopathological evaluation can identify the immunological rejection as well as amastigote nests of *T. cruzi* to confirm the reactivation of the infection in the implanted organ^{24,25}. As such nests are small and very scarce, these are rarely identified in routine exams. To increase the sensitivity of the morphological method, immunohistochemistry (IHC) may serve as a promising technique, as it allows more precise identification of sparse and very small nests.

The morphological evaluation of EMB after cardiac transplantation is a part of the protocol in these patients and has enormous practical importance because it guides immunosuppressive treatments in cases of cellular rejection and detects possible reactivation of the chagasic infection, which needs specific antiparasitic treatment. In these two scenarios with different clinical behaviors, the pathologist may contribute to the diagnosis of reactivation. However, it is difficult to make the differential diagnosis between moderate/severe acute cell rejection and reactivation of the chagasic infection, as the inflammatory infiltrate in both situations is morphologically indistinguishable.

Immunohistochemistry method has been used for the detection of *T. cruzi* antigens for more than 30 years²⁶. Since then, it has been shown to facilitate the identification of parasites, especially small nests, as compared to conventional pathological examination²⁷.

Previous studies have shown that the anti-*T. cruzi* polyclonal antiserum can detect the parasite in both human and other animal tissues. The highest detection rates were observed during the acute phase of the disease and in cases of chronic phase with active inflammation²⁸⁻³⁰. The detection rate is higher in heart samples than in brain samples³¹. Greater detection of the parasite was found in cases with moderate to severe inflammatory process³².

Despite the potential advantages of IHC in the detection of the reactivation of chagasic infection, there are no available commercial antibodies. This situation is probably associated with the lack of interest of technology companies in providing such an antibody, as Chagas disease diagnosis seems important only in countries with high prevalence of this disease.

The aim of this study is to validate an antiserum (polyclonal antibody) produced at a scientific research institution for the immunohistochemical diagnosis of *T. cruzi* infection.

METHODS

Selection, inclusion, and exclusion of samples

The Hospital das Clínicas da Universidade Federal de Minas Gerais (HC-UFGM) Heart Transplant Service has been assisting patients with end-stage heart failure since 2006. Up to 2016, 81 patients with chagasic cardiopathy were submitted to heart transplantation. In this study, all the anatomopathological reports performed at the HC-UFGM Pathological Anatomy Laboratory from these patients after transplantation were reviewed and comprised 81 surgical specimens of cardiac explantation, 823 EMB, 18 skin/subcutaneous biopsies, and 1 autopsy of a chagasic patient who died after transplantation in order to select positive controls. Samples of EMB with insufficient material to acquire new histological sections and/or to extract DNA for PCR were excluded from the study.

Ethical considerations

The project followed all research standards involving human beings established in the Resolution 196 of the National Health Council to safeguard the rights and well-being of the people studied. The project was approved by the Research Ethics Committee (COEP) of UFGM.

Endomyocardial biopsies

Biopsies were performed at pre-established times and according to standard procedures at the HC-UFGM, following the recommendations of the Second Brazilian Guideline on Cardiac Transplantation³³. From each patient, four or five endomyocardial fragments were obtained; these fragments were fixed in 10% formalin, processed for inclusion in paraffin, and cut into sections (5µm thickness). From each sample, at least 15 staggered cuts were obtained and these were stained with hematoxylin and eosin (HE). All technical processing of the samples was performed in the Pathology Laboratory of the HC-UFGM.

Positive controls

Twenty-six HE-stained samples containing *T. cruzi* amastigote nests were selected as positive controls. These included 13 EMB, 12 skin biopsies, and 1 heart (autopsy) containing sections of left ventricle and interventricular septum.

Negative controls

Two groups of samples included as negative controls were as follows: 1) Samples without evidence of *T. cruzi* infection. Twenty anatomopathological specimens without amastigote nests of *T. cruzi* were selected; the patients had no clinical manifestations or laboratory tests indicative of chagasic infection. 2) Eight samples with a parasite morphologically similar to *T. cruzi* and potentially capable of showing cross-reaction. As *T. cruzi* and other microorganisms have some morphological similarities, leishmaniasis, toxoplasmosis, and histoplasmosis samples were included. These parasites or fungi were histologically confirmed by IHC analysis using their respective antibodies. In total, negative controls were represented by 10 explanted hearts, 10 EMB, 6 leishmaniasis, 1 toxoplasma, and 1 histoplasma samples.

Cases

Aside from the four or five tissue fragments from EMB for histopathological examination, one additional fragment was collected from all chagasic heart transplant patients in ethanol to evaluate for the presence of *T. cruzi* DNA by PCR, according to the current research protocol on Chagas disease carried out at HC-UFGM. From these samples, we selected 31 patients that were negative for parasite nests in conventional examination (HE) but positive for *T. cruzi* DNA in PCR test.

Histopathological study

All slides routinely examined for the evaluation of graft rejection were reviewed by two authors (Azevedo PHR and Brasileiro Filho G) for the validation of the histopathological findings, especially for the confirmation or exclusion of *T. cruzi* nests.

Immunohistochemical study

Antiserum production

For the anti-*T. cruzi* polyclonal antibody (antiserum) production, three New Zealand rabbits, approximately 60 days old, were kept in individual cages at the Animal Science Center of the Federal University of Ouro Preto [*Universidade Federal de Ouro Preto* (UFOP)] and provided with food and water *ad libitum*. The use, maintenance, and manipulation of the animals followed the standards recommended by the Brazilian College of Animal Experimentation (COBEA).

The animals were intraperitoneally inoculated with 10^7 blood trypomastigote form of *T. cruzi* Y strain obtained from mice previously infected with this strain. Six months after inoculation, the animals were submitted to general anesthesia and whole blood was collected by cardiac puncture with subsequent euthanasia.

The blood was left at room temperature (20-25°C) in 10-mL glass tubes for 1h to induce clot formation and then centrifuged at 1,372g for 10 min. The serum obtained was homogenized, aliquoted, identified, and stored at -80°C until testing.

The antiserum was assessed in immunohistochemical assays on paraffin-embedded section from heart, spleen, and intestine of mice and dogs uninfected or infected by different strains of *T. cruzi* and proved to be effective in these animal samples.

The antiserum preparation was carried out at the Nucleus of Research in Biological Sciences of UFOP.

Immunohistochemical reaction

The histological sections were overnight oven dewaxed at 56°C, rehydrated with xylol baths (thrice for 5 min), 100% and 80% ethyl alcohol (thrice each, for 5 min), and running water for 5 min. Heat-induced antigen retrieval was performed with 0.01M sodium citrate solution (pH = 6.0) at 90°C for 20 min. The sections were blocked with an endogenous peroxidase in a methanol/hydrogen peroxide mixture (100mL of methanol and 3.5mL of 30% hydrogen peroxide) for 30 min at room temperature, followed by two washes in 10% phosphate buffered saline (PBS) solution (5 min each). The sections were subsequently blocked with skimmed milk powder diluted in 10% PBS (6g of skimmed milk powder in 100mL of PBS) for 30 min at room temperature to block nonspecific binding. The sections were incubated with the primary antibody (1:400,000 dilution) in PBS/1% bovine albumin (1870, Inlab, Diadema, Brazil) in a humid chamber at room temperature for 1h, followed by two washes with 10% PBS solution (5 min each). Next, the sections were incubated in a humid chamber for 30 min at room temperature with anti-immunoglobulin G (anti-IgG) secondary antibody (Novolink, Newcastle, UK), followed by two washes in 10% PBS solution (5 min each). Streptavidin-peroxidase complex (Kit Dako, LSAB, K0675, Carpinteria, USA) was subsequently applied and the preparation was incubated for 30 min in a humid chamber at 37°C. The peroxidase reaction was developed by incubation of the samples in DAB solution (50mg of 3,3'-diaminobenzidine tetrahydrochloride in 200mL of PBS and 400µL of 30% hydrogen peroxide) for 5 min. To stop the reaction, the sections were washed in PBS for 5 min and counter stained with Harris hematoxylin for 3s. The samples were washed in running water for 5 min, dehydrated, diaphanized in xylol, and mounted in Entellan (Merck, Darmstadt, Germany).

All sections were carefully examined under a microscope, initially under a panoramic view and then under 100 and 400× magnifications, to detect for signals indicative of target antigens of the antibody tested. Each positive sign of *T. cruzi* was topographed and subsequently compared to the sections stained with HE.

Polymerase chain reaction

As an alternative to detect chagasic infection in the samples studied, two PCR methods were used to test the fragments of heart biopsies for *T. cruzi* DNA as follows: 1) kinetoplast deoxyribonucleic acid polymerase chain reaction (kDNA PCR), specific amplification of a 330-bp fragment corresponding to the variable region of the mini-circle of the kDNA performed using primers S35 and S36, as described elsewhere^{21,34}; 2) real-time PCR of the 24SαrDNA gene, according to the method of Costa et al³⁵. These proposed protocols based on the PCR directed to mitochondrial (kDNA) and nuclear (rDNA 24Sα) markers have been useful for the diagnosis of Chagas disease, owing to good diagnostic sensitivity and specificity³⁵. For control groups (positive and negative), only kDNA PCR was performed; DNA samples were obtained from histological sections of paraffin-embedded tissues. In the group cases, both PCR methods were

used and DNA was extracted from fresh EMB collected in ethanol. Samples that were positive for at least one of the two PCR methods were considered as positive.

RESULTS

The results of the conventional histological (HE), DNA amplification (PCR), and IHC tests of all the cases examined in this study are summarized in **Figure 1**.

Immunohistochemical analysis of the 26 samples used as positive controls (13 EMB, 12 skin biopsies, and 1 necropsy of chagasic patient that died after transplantation) revealed

amastigote nests in eight EMB (**Figure 2**), 12 skin biopsies, and the autopsy heart (21 cases, 80.8%) (**Figure 1**). In five EMB (19.2% of total positive cases), the amastigote nests identified with HE staining were not seen in the IHC reactions (**Figure 1**). Eleven of the 26 positive controls (42.3%) were positive for the parasite DNA in PCR.

The IHC reaction with the anti-*T. cruzi* serum was negative in 27 of 28 negative control cases (96.4%). For one sample of cutaneous leishmaniasis (3.8%), the positive reaction with anti-*T. cruzi* antibody showed parasites dispersed in the interstitium (**Figure 3**). Both toxoplasma and histoplasma

Positive controls					Negative controls					Cases				
Nº case	HE	PCR	IHC	Sample	Nº case	HE	PCR	IHC	Sample	Nº case	HE	PCR	IHC	Sample
1	+	-	+	Skin biopsies	27	-	-	-	Hearts of non-chagasic patients	55	-	+	+	EMB of chagasic heart-transplanted patients
2	+	+	+		28	-	-	-		56	-	+	+	
3	+	-	+		29	-	-	-		57	-	+	+	
4	+	-	+		30	-	-	-		58	-	+	+	
5	+	+	+		31	-	-	-		59	-	+	-	
6	+	+	+		32	-	-	-		60	-	+	-	
7	+	-	+		33	-	-	-		61	-	+	-	
8	+	-	+		34	-	-	-		62	-	+	-	
9	+	-	+		35	-	-	-		63	-	+	-	
10	+	+	+		36	-	-	-		64	-	+	-	
11	+	+	+	37	-	-	-	65	-	+	-			
12	+	+	+	38	-	-	-	66	-	+	-			
13	+	-	+	39	-	-	-	67	-	+	-			
14	+	-	-	40	-	-	-	68	-	+	-			
15	+	+	+	41	-	-	-	69	-	+	-			
16	+	+	-	42	-	-	-	70	-	+	-			
17	+	-	-	43	-	-	-	71	-	+	-			
18	+	-	-	44	-	-	-	72	-	+	-			
19	+	-	+	45	-	-	-	73	-	+	-			
20	+	-	+	46	-	-	-	74	-	+	-			
21	+	-	+	47	-	-	-	75	-	+	-			
22	+	-	+	48	-	-	-	76	-	+	-			
23	+	+	+	49	-	-	-	77	-	+	-			
24	+	-	-	50	-	-	+	78	-	+	-			
25	+	+	+	51	-	-	-	79	-	+	-			
26	+	+	+	52	-	-	-	80	-	+	-			
				Autopsy	53	-	ND	-	Toxoplasmosis	81	-	+	-	
					54	-	ND	-	Histoplasmosis	82	-	+	-	
										83	-	+	-	
										84	-	+	-	
										85	-	+	-	

FIGURE 1: Results of *Trypanosoma cruzi* detection PCR, conventional histology using HE, and IHC of positive controls, negative controls, and cases. **HE:** hematoxylin and eosin; **PCR:** polymerase chain reaction; **IHC:** immunohistochemical reaction; **EMB:** endomyocardial biopsies; **ND:** not done.

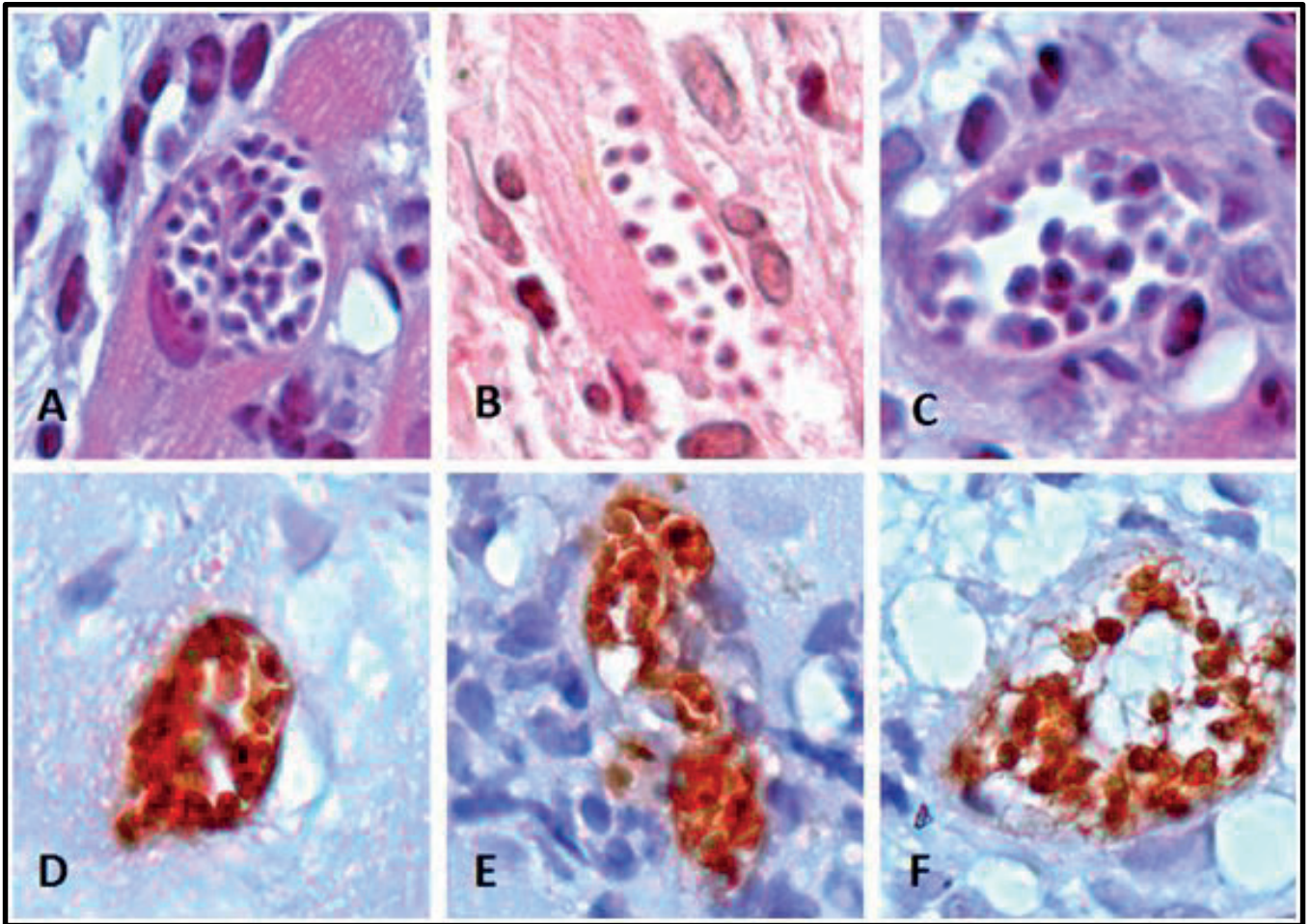


FIGURE 2: A, B, and C. Endomyocardial biopsies showing nests of amastigote forms of *T. cruzi* in the cytoplasm of myocytes. Hematoxylin and eosin (1,000× magnification). D, E, and F. *T. cruzi* amastigote forms stained by the immunohistochemical reaction with anti-*T. cruzi* polyclonal antibody (1,000× magnification). *T.*: *Trypanosoma*.

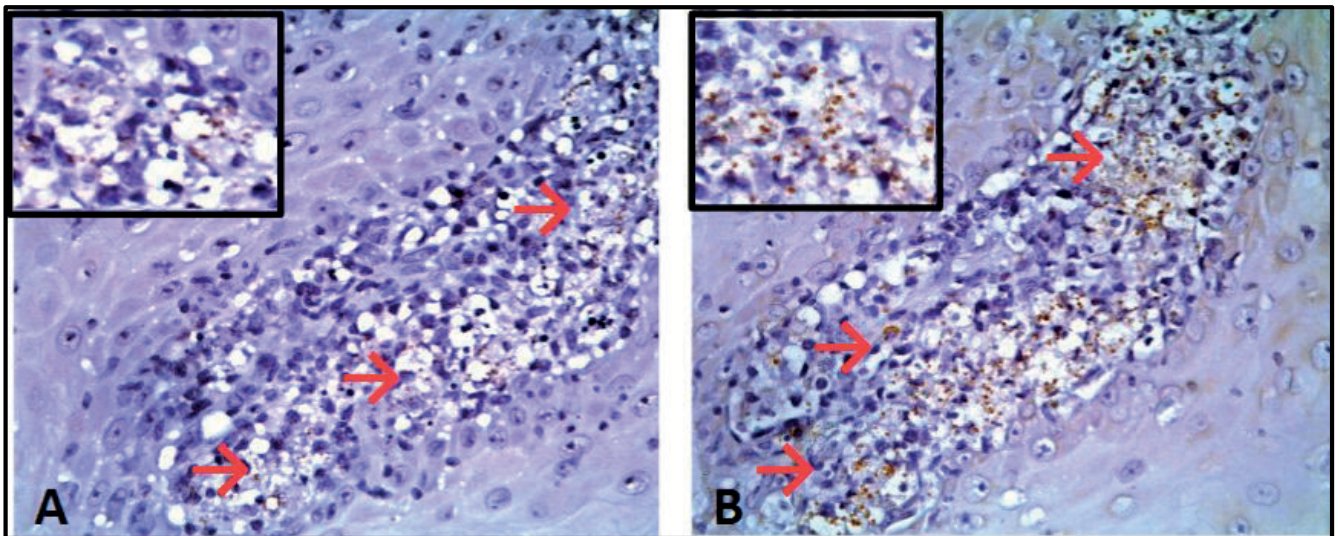


FIGURE 3: A. Skin biopsy of a patient with clinical and immunohistochemical diagnosis of cutaneous leishmaniasis showing parasitic amastigote forms dispersed in the interstitium, as stained with anti-*T. cruzi* antibody (arrows; 400× magnification). In detail, cross-reactive leishmania amastigote forms (1,000× magnification). B. Same biopsy and topography of the case shown in A revealing many amastigote forms of leishmania stained with anti-leishmania antibody. *T.*: *Trypanosoma*.

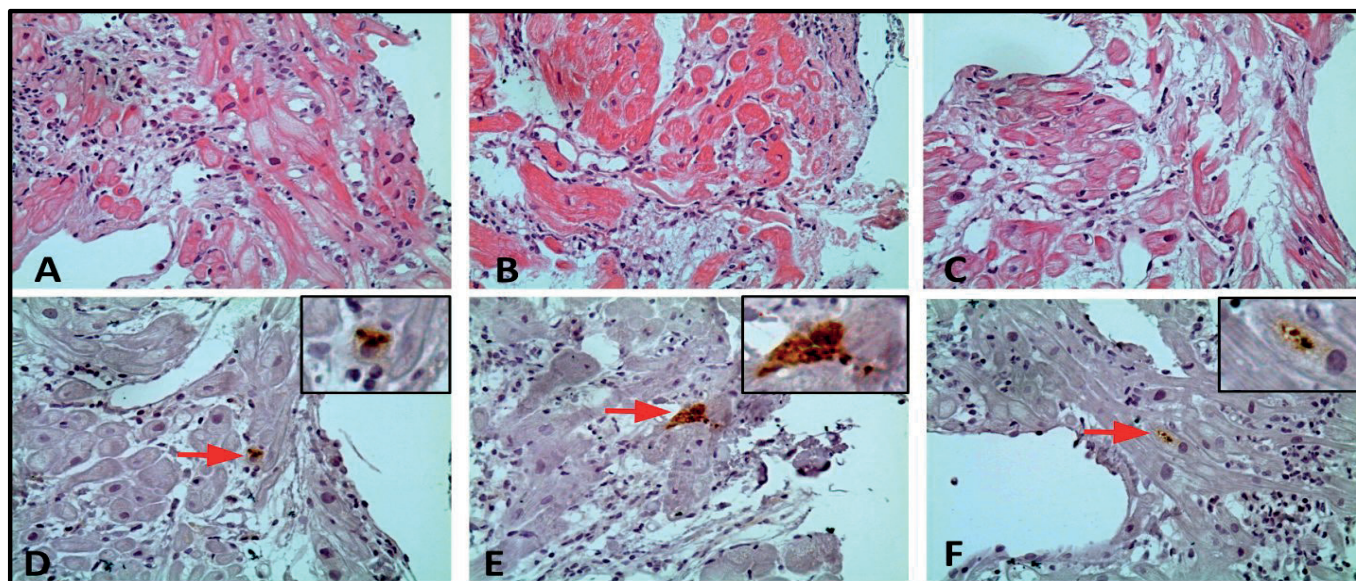


FIGURE 4: A, B, and C. Endomyocardial biopsies with no evidence of *T. cruzi* amastigote nests in histopathological examination using hematoxylin and eosin (200× magnification). D, E, and F. Same biopsies and topography of the cases shown in A, B, and C, respectively, with *T. cruzi* amastigote forms stained with anti-*T. cruzi* polyclonal antibody (arrows; 200× magnification). In detail, *T. cruzi* amastigote forms (1,000× magnification). *T.*: *Trypanosoma*.

samples showed negative results. None of the 26 negative controls were positive for *T. cruzi* DNA in the PCR test.

Four (12.9%) of the 31 samples of EMB selected as cases were positive for *T. cruzi* nests with IHC (Figure 4); parasite nests were undetected by conventional histological examination.

From the 85 samples analyzed, 26 (31.3%) had positive histopathology (*T. cruzi* amastigote nests), while the remaining 59 (69.4%) cases showed negative HE staining (absence of *T. cruzi* nests).

Twenty-one of the 26 (80.8%) samples selected as positive controls were positive for *T. cruzi* antigens in IHC test. Of the 28 negative controls, 27 (96.4%) showed no reaction with *T. cruzi* antigens.

DISCUSSION

In comparison with the conventional HE staining, IHC performed with the antiserum used in this study showed good sensitivity for the detection of *T. cruzi*, indicative of the feasibility of this method for the detection of the parasite. Five EMB (19.2% of positive controls) samples were negative for the parasite antigen in IHC. The most likely explanation for this result is the scarcity of the parasite nests and their small size, which may explain their absence in many histological sections. *T. cruzi* nests seen in the histological sections (HE) may not have been included in the sections obtained in the second microtomy; the paraffin block adjustment in the microtome to obtain new sections for IHC may have greatly deepened the sample, which explains the absence of these small and few nests in the sections used for IHC. Hence, it is recommended that the tissue sections are collected at the same time on slides for both conventional histopathology and IHC to include amastigote nests in most histological sections obtained. Such a technique is already

performed in other contexts, wherein the target of analysis is scarce and may be lost in the acquisition of new sections, such as axillary sentinel node biopsies for the evaluation of patients with breast cancer³⁶.

A cutaneous leishmaniasis sample was detected positive with the anti-*T. cruzi* antiserum in IHC. No other negative control sample showed positive reaction, indicative of the good specificity of the method. Anti-*T. cruzi* polyclonal antibody used in the present study may cross-react with other parasites and should therefore be used in close correlation with the clinical and laboratory data of the patient as well as obvious morphological correspondence to the histopathological examination in HE. Cross-reaction with antigens of leishmaniasis was also observed by Barbosa et al.³⁷ using another antiserum against *T. cruzi*. Thus, it is recommended to use this method with large number of leishmaniasis cases to better understand the results and possible cross-reaction. Furthermore, it is necessary to test more specimens with anatomopathological diagnosis of other infectious diseases caused by parasites with morphologies similar to those of amastigote forms of *T. cruzi* (e.g., toxoplasmosis and histoplasmosis) to recognize other probable cross-reactions of the antibody under study.

The present study showed that IHC using anti-*T. cruzi* antiserum increases the chances of detection of the parasite in EMB. Four of 31 EMB harboring *T. cruzi* DNA, as detected by PCR, were positive for *T. cruzi* nests in IHC but were undetected by conventional histology. This increase in the sensitivity of the morphological diagnosis of the reactivation of chagasic infection supports the use of the method as a complementary evaluation technique in the EMB of transplanted chagasic patients.

The differential diagnosis between cell rejection and reactivation of chagasic infection has enormous importance in

the case of transplant patients because the clinical management is very different in both situations. Thus, it is essential that, in addition to the conventional histopathological examination (HE) for the detection of parasite amastigote nests in all EMB of cardiac transplant patients with Chagas disease, the morphological evaluation should be complemented with IHC study. We suggest that the transplant patient care groups should include IHC as a routine test in the histopathological evaluation of all EMB in the care protocols to diagnose the reactivation of *T. cruzi* infection, at least for samples where mononuclear cell infiltration is evident. Given the small size of the nests and their occasional suppression in serial histological sections, IHC should be performed for at least three sequential cuts to increase the chances of the parasite detection.

The negativity of *T. cruzi* DNA in the PCR method for 15 (57.7%) of the 26 positive controls may be attributed to some factors. The most likely reason is DNA degradation by formaldehyde used as fixative. Standard 10% formalin solution was used in all samples. Conventional (unbuffered) formaldehyde solutions have a wide pH range, which may cause DNA damage and impair DNA detection in PCR³⁸. It is, therefore, recommended that such samples be fixed in buffered formaldehyde (pH of 7.4) to increase the preservation of the parasite DNA and improve its detection chance.

As this study is at an initial stage, we were unable to adequately characterize the antibody studied. In fact, an antiserum with a large number and variety of antibodies was used in this study. The isolation and characterization of the antibody may provide further information on its properties and explain the possible cross-reactivity with leishmanial antigens.

The anti-*T. cruzi* polyclonal antibody (antiserum) used in this study proved to be adequate for the purpose of increasing the sensitivity of the morphological diagnosis of reactivation of chagasic infection. The increase in parasite detection observed in this study recommends the use of IHC with anti-*T. cruzi* antiserum tested herein as a complementary method in the early diagnosis of the reactivation of *T. cruzi* infection in chagasic patients submitted to heart transplantation.

It is also expected that commercial companies would get interested in providing antibodies for such purpose for the widespread clinical usage. We expect that the antiserum used herein could be an alternative to this.

Conflict of interest

The authors declare that there is no conflict of interest.

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