PCR OF HEMOCULTURE AS STRATEGY TO IMPROVE THE DETECTION OF *Trypanosoma cruzi* IN THE CHRONIC PHASE OF CHAGAS DISEASE

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ABSTRACT

In order to associate the specificity of hemoculture and the sensitivity of PCR, we have proposed to perform the PCR from samples obtained from hemoculture (PCR-HC) of 79 patients in the chronic phase of Chagas disease. In addition to the PCR-HC, the methods of hemoculture and PCR of blood (PCR-BL) were performed. To carry out the PCR-HC, hemoculture and PCR-BL were used 2.4 mL of hemoculture sediment, 30 mL and 10 mL of blood, respectively. The DNA was extracted of each sample and the fragment of 330 pb of k-DNA from *Trypanosoma cruzi* minicircle was amplified. The PCR-HC (p=0.00005) and the PCR-BL (p=0.00121) were significantly more positive than the hemoculture. The positivity of the PCR-HC was also significantly higher (p=0.00032) than the PCR-BL. We have concluded that the PCR-HC is a valid alternative method to increase the detection of the parasite, improving the parasitological diagnosis and identification of etiologic treatment failure.

KEYWORDS: Diagnosis; Chagas disease; PCR of blood; PCR of hemoculture; hemoculture.

1. INTRODUCTION

Currently, 5 to 6 million people are infected with *Trypanosoma cruzi* all around the world, especially in Latin American countries, where Chagas disease is endemic[1]. During the chronic phase of infection, which is characteristic of low parasitemia, methods of parasitological diagnosis become limited. Thus, the association between a highly specific method such as hemoculture (100%)[2,3] and the high sensitivity of the PCR (76.4 to 100%)[2,4] could be an interesting approach to increase the detection capability of *T. cruzi* in patients in the chronic phase of infection. In this context, a pilot study was proposed to perform PCR from hemoculture samples obtained from patients in the chronic phase of Chagas disease.

2. MATERIAL AND METHODS

Seventy-nine patients with reagent ELISA method Chagas Test Elisa III (Bioschile® Ingenieria Genética S.A, Chile), who were attended in 2012 in the Chagas Disease Laboratory of the Universidade Estadual de Maringá (UEM) and ambulatory of the Hospital Universitário de Londrina, were recruited for this study. The age of these patients ranged from 35 to 89 years, with an average of 60.5 ± 9.9. Females were predominant, corresponding to 55.7% of the studied population. The patients signed a free and informed consent form approved by Permanent Committee of Ethics in Research Involving Human Beings (COPEP) of UEM, under protocol number 012/2010.

The hemoculture (HC) was performed with 30 mL of blood distributed in six heparinized conical tubes (15 mL). The tubes were centrifuged at 4 °C, 209.44 rad/s for 30 minutes to remove the plasma and add Liver Infusion Tryptose medium as previously described[5], with modifications. Once a week, the tubes were homogenized and the pellet aliquot was analyzed every 30 days for a total of 180 days. In this period, 0.4 mL of the pellet of each one of the six tubes, totaling 2.4 mL, was added to an equal volume of Guanidine-HCl 6M / EDTA 0.2M for PCR analysis of hemoculture (PCR-HC). After one week at room temperature, these samples were boiled at 100 °C for 15 min and stored at 4 °C until use[6]. From this mixture, 2.4 mL of material was used for DNA extraction.

The PCR of blood (PCR-BL) was performed with 10 mL collected, in the same time of hemoculture, in a conical tube (50 mL) containing an equal volume of Guanidine-HCl 6M / EDTA 0.2M[7] to extraction and amplification of DNA.

The DNA extraction and the conditions of PCR reaction and revelation of the amplified products were as

previously described[8]. The DNA was amplified in an automatic thermocycler (Techne® TC - 512 Staffordshire, England). The extraction and amplification of DNA were monitored using negative controls (non-infected individuals from non-endemic areas) and positive controls (individuals infected with *T. cruzi*). For amplification step, DNA of parasites obtained from culture was also used as positive control. The amplified products were visualized on 4% polyacrylamide gels, revealed by silver salts and digitally stored.

Data analysis was performed using *Software SAS 9.1*. The *Chi-square test* was used to investigate possible associations between variables. The significance level was 5% ($p < 0.05$).

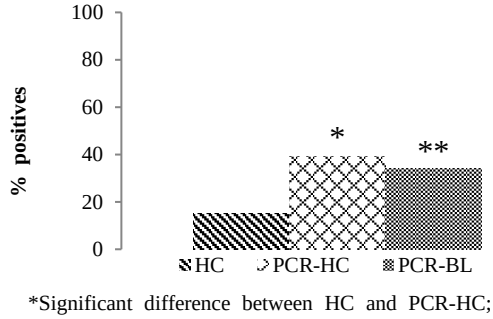
3. RESULTS

The hemoculture was positive in 15.2% (12/79) of the patients and the PCR-HC detected 39.2% (31/79), being the difference between these methods significant (p=0.00005). Twenty-seven (34.2%) patients were positive by PCR-BL, with an also significant difference (p=0.00121) in relation to hemoculture (Table 1, Figure 1).

Table 1. Comparison of the hemoculture (HC) results with the PCR of hemoculture (PCR-HC) and the PCR of blood (PCR-BL) in patients infected with *Trypanosoma cruzi* (n = 79).

Variables	HC				*p*
	P		N		
	n	%	n	%	
PCR-HC					
P	11	13.9	20	25.3	0.00005*
N	1	1.3	47	59.5	
PCR-BL					
P	9	11.4	18	22.8	0.00121**
N	3	3.8	49	62.0	

*Significant difference between HC and PCR-HC;**Significant difference between HC and PCR-BL; $p \leq 0.05$; P = Positive; N = Negative.



*Significant difference between HC and PCR-HC;

Figure 1. Positivity of the hemoculture (HC), PCR of hemoculture (PCR-HC) and PCR of blood (PCR-BL) in patients infected with *Trypanosoma cruzi* (n=79). Após o ";" No pé dessa figura falta a seguinte informação: **Significant difference between HC and PCR-BL; $p \leq 0.05$.

For 31 patients with PCR-HC positive, 18 (58.1%) were also positive by PCR-BL. For 48 patients with PCR-HC negative, 9 (18.7%) were positive by PCR-BL. Even so, the number of patients with PCR-HC positive (31/79) was significantly higher (p=0.00032) when compared to those detected by PCR-BL (27/79) (Table 2).

Table 2. Comparison of the PCR of hemoculture (PCR-HC) with the PCR of blood (PCR-BL) in samples from patients infected with *T. cruzi* (n = 79)

Variables	PCR-HC				*p*
	P		N		
	n	%	n	%	
PCR-BL					
P	18	22.8	9	11.4	0.00032*
N	13	16.5	39	49.4	

*Significant difference between the methods: $p \leq 0.05$; P = Positive; N = Negative.

4. DISCUSSION

In order to increase the detection of *T. cruzi* in patients in the chronic phase of Chagas disease, the specificity of hemoculture was associated with the sensitivity of PCR and the PCR-HC was carried out. This method compared with the PCR-BL and with the hemoculture showed higher detection capability of the parasite.

The performance of the PCR-HC carried out in samples collected at 180 days (period of higher positivity of the hemoculture) was significantly better ($p<0.05$), when compared with PCR-BL and the HC. The PCR-BL also showed a higher parasite detection capability than the HC, with a significant difference between these two methods, in both treated and untreated patients agreeing with other authors[2,4,7,9,10,11]. The hemoculture was the method with the lowest detection capability of the parasite, however, it is important to consider its fundamental role for the diagnosis of infection by *T. cruzi*. It is also important to emphasize that the detection of the parasite was higher only when the HC was associated with the PCR (PCR-HC). It should be noted that even in the absence of the parasite multiplication, PCR-HC can detect parasites independent of Discrete Typing Unit (DTU) that belong, differing from HC that can select a DTU over another, depending on the time required for growth and development of the parasite. Other authors have also associated a molecular method (conventional PCR) with a parasitological method (xenodiagnosis - PCR-XD) and have observed an increase in *T. cruzi* detection capability when compared to the parasitological method carried out singly[9,12].

The association between the PCR and the HC (PCR-HC) was important to increase the parasite detection capability, because this method was also significantly better when compared to PCR-BL. This is the first study

reporting a significant difference when two methods with distinct approaches (HC and PCR) were associated. Another study[5] that associated a molecular method (PCR) and another parasitological method (xenodiagnosis - XD) resulting in PCR-XD, showed no significant difference when compared to PCR-BL. The highest positivity of the PCR-HC can be explained by the larger volume of blood used in the hemoculture and by the parasite multiplication in the culture medium, which does not occur with the PCR-BL. Even the PCR-HC detecting the parasite in a significantly higher number of samples compared to PCR-BL, 9 samples were only positive by PCR-BL and 13 were only positive by PCR-HC. These results suggest that the combination of these two methods can be important to increase the number of individuals diagnosed. The DNA degradation or presence of inhibitors in the samples may explain this discrepancy in results.

5. CONCLUSION

We concluded that the PCR-HC is a valid alternative and choice to increase the detection of *T. cruzi* in patients in the chronic phase of infection. The association of this method with the PCR-BL increases the number of individuals with positive results and improves the diagnosis of the chronic phase and the identification of therapeutic failure. Despite the long time required for obtaining results by PCR-HC, this study opens possibilities for a more systematic analysis, in order to decrease the amount of time and verify if the association of real-time PCR with hemoculture can further increase the detection of *T. cruzi*.

Ethical Approval: This study was approved by Permanent Committee of Ethics in Research Involving Human Beings (COPEP) of UEM, under protocol number 012/2010.

Conflicts of interest: The authors declare that they have no conflicts of interest.

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