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Short Communication

Trypanosoma cruzi: Evaluation of PCR as a Laboratory Tool to Follow up the Evolution of Parasite Load

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Abstract

Background: The study evaluated qualitative PCR, primers 121-122 as a tool to follow up evolution parasite load of *Trypanosoma cruzi*.

Methods: The study was conducted at the State University of Maringa, in 2015. Step 1, dilutions 1/10 were performed from *T. cruzi*-Y strain to obtain preparations of 50,000-0.05 parasites/mL from which DNA were extracted, quantified, and amplified. Step 2, the extracted DNA in the dilutions 5-0.05 parasites/mL was re-diluted 1/10, 1/100, 1/1000, quantified, and amplified. Polyacrylamide gels were photographed and thicknesses of the 330 bp kDNA fragments were measured.

Results: Step 1, in the dilutions 50,000-50 parasites/mL kDNA fragments had same thickness and, dilutions 5-0.05 parasites/mL showed progressive decrease in thicknesses and staining intensity of the 330 bp fragments. Step 2, demonstrated that dilutions of five (re-dilutions 1/10 and 1/100) and 0.5 (1/10) parasites/mL produced similar thicknesses of the 330 bp fragments obtained in Step 1. However, very dilute DNA samples make difficult to reproduce the fragments thicknesses.

Conclusion: PCR, despite its limitations, was able to detect progressive decrease in thicknesses/staining intensity of kDNA fragments in the dilutions 5-0.05 parasites/mL. Hence, has the potential to be used to follow-up evolution of parasite load, not by quantifying the number of parasites, but by dynamic evolution of the fragments thicknesses during etiological treatment.

Introduction

Chagas disease (ChD) is caused by the protozoan *Trypanosoma cruzi*. In Brazil and Latin America, ChD is considered one of the most important endemic diseases, with an estimated 6-7 million people infected worldwide. However, due to the increase in population migration, it has been spread worldwide and is now considered a health issue in endemic countries (1).

The implementation of quantitative PCR assays to determine parasite load in the blood of ChD patients and follow their evolution during treatment is useful, mainly as a response indicator for infected organisms that undergo therapeutic regimens (2,3). Although real-time PCR has shown good results in the detection and quantification of *T. cruzi* load in blood samples from patients (4-6), it is still considered an expensive technique, relatively new and available in only a few research laboratories (7).

In our routine of the Chagas Disease Laboratory/State University of Maringa, we observed that follow-up of ChD patients receiving etiological treatment, using qualitative PCR (primers 121 and 122, protocol more currently used to detect *T. cruzi*), demonstrated a progressive decrease in the thicknesses of the 330 base pair (bp) kDNA fragments, probably indicating a decrease in the number of circulating parasites (8). Therefore, the conventional qualitative PCR technique, standardized and validated in the laboratory and is available in many diagnostic centers, may be tested to assess the evolution of parasite load (before, during, and after etiological treatment).

Hence, the aim of the present study was to evaluate experimentally the qualitative PCR (primers 121 and 122) as a laboratory tool to follow up the evolution parasite load of *T. cruzi*. For this purpose, we assessed the thicknesses of the 330 bp *T. cruzi* kDNA specific fragment for different parasite amounts from axenic cultures obtained by serial dilutions.

Materials and Methods

The study was performed at the State University of Maringa, Sector of Parasitology, in 2015, using the Y strain of *T. cruzi* (9), maintained in axenic culture with Liver Infusion Tryptose (LIT) medium at 28 °C. This strains belongs to the discrete typing units (DTU) *T. cruzi* II, DTU most frequently isolated from ChD patients studied in endemic areas of Brazil (10,11) and Southern Cone countries (12).

The number of parasites was quantified using the method of Brener (13). Successive dilutions, 1/10, were performed from cultures of *T. cruzi*-Y strain in LIT medium to obtain aliquots 50,000, 5,000, 500, 50, 5, 0.5, and 0.05 parasites/mL. The dilutions were centrifuged at 2,500 rpm/4 °C/20 min. The supernatants were discarded, and the pellets were centrifuged in 1 mL Krebs-Ringer-Tris (KRT). This procedure was performed three times. The pellets were re-suspended in a 100 µL solution of 6 M guanidine HCL and 0.2 M ethylenediamine tetraacetic acid (EDTA) and 100 µL of KRT. The dilutions were stored at room temperature and after 1 week boiled at 100 °C for 7 min (14). The samples were stored at 4 °C until DNA extraction. This was considered the first step of the study (Step 1).

DNA extraction was performed according to Wincker et al. (15) modified by Gomes et al. (16). The extracted DNA was re-suspended in 20 µL of sterile ultrapure water. The purity and concentration of total DNA of *T. cruzi* (ng/µL; purity DNA ratio: 260/280 nm ≥ 1.8) of each dilution (2 µL) were determined in the NanoDrop spectrophotometer 2000 (Thermo Scientific, West Palm Beach, FL, USA). The DNA (two µL of each dilution) was amplified according to Gomes et al. (16) with the specific primers: 121 (5'-AAATAATGTACGGGTGAGATGCATGA-3') and 122 (5'-

GGTTCGATTGGGGTTGGTGTAATATA-3'), which detect 330 bp *T. cruzi* kDNA specific fragment. The amplification products were subjected to 4.5% polyacrylamide gel electrophoresis and stained with silver salts (17).

In this first step of the study (Step 1), we obtained dilutions of 50,000, 5,000, 500, 50, 5, 0.5, and 0.05 parasites/mL, followed by DNA extraction, quantification, amplification and, electrophoresis. To facilitate interpretation of the results, we used two µL of the total volume of 20 µL extracted DNA for amplification. Therefore, the respective thicknesses observed of the 330 bp specific fragments of *T. cruzi* kDNA corresponded to an estimated number of 5,000, 500, 50, 5, 0.5, 0.05, and 0.005 parasites for the dilutions mentioned above, respectively.

In the second step (Step 2), the DNA extracted in the Step 1 of the dilutions 5, 0.5, and 0.05 parasites/mL underwent three additional dilutions, 1/10, 1/100, and 1/1000 in sterile ultrapure water, followed by DNA quantification, amplification and, electrophoresis. For DNA amplification was used two µL of the total solution of 20 µL DNA re-diluted. Therefore, the respective thicknesses observed of the 330 bp specific fragments of *T. cruzi* kDNA corresponded to an estimated number of 0.05, 0.005, and 0.0005 parasites (dilution: 5 parasites/mL; re-dilution: 1/10, 1/100, and 1/1000); 0.005, 0.0005, and 0.00005 parasites (dilution: 0.5 parasites/mL; re-dilution: 1/10, 1/100, and 1/1000); and 0.0005, 0.00005, and 0.000005 parasites (dilution: 0.05 parasites/mL; re-dilution: 1/10, 1/100, and 1/1000).

After electrophoresis and staining with silver salts, the 4.5% polyacrylamide gels were photographed with a digital camera (Sony Cyber-Shot 7.2 megapixel) at a distance of 30 cm. The images were printed on photographic paper (15 × 21 cm), and the edge thicknesses of the 330 bp *T. cruzi* kDNA specific fragments were measured in millimeters (mm) using an analog caliper. As a reference, for the measurements were used the positive controls fragments.

The Steps 1 and 2 were performed three times at different moments and one result representative of the steps was presented.

Results

Fig. 1 and Table 1 show the results of the Step 1. The evaluation of the kDNA specific fragments demonstrated that the thicknesses of the 330 bp fragments were equal for the 5,000-50 parasites/mL dilutions (DNA corresponded to an estimated number of 500-5 parasites and 65-59 ng/µL concentration of total DNA). For the 50,000 parasites/mL dilution (DNA corresponded to an estimated number of 5,000 parasites and 155 ng/µL concentration of total DNA) the 330 bp specific fragment was not visualized. However, in others experiments the thicknesses of the fragments obtained were equal to the 5,000-50 parasites/mL dilutions. The positive/negative extraction and amplification controls worked, and the extracted samples of DNA showed adequate purity (ratio: 260/280 nm ≥ 1.8). Moreover, the thicknesses analysis of *T. cruzi* kDNA specific fragments for the 5-0.05 parasites/mL dilutions (DNA corresponded to an estimated number of 0.5-0.005 parasites and 49-18 ng/µL concentration of total DNA) revealed a progressive decrease of the thicknesses and staining intensity of the 330 bp fragments.

Fig. 2 and Table 2 present the results of the Step 2. The thicknesses analysis of *T. cruzi* kDNA specific fragments showed that only dilution of 5 parasites/mL (DNA corresponded to the estimated 0.5 parasites and 49 ng/µL concentration of total DNA) presented re-dilutions with a progressive decrease in thicknesses and staining intensity of the specific fragments.

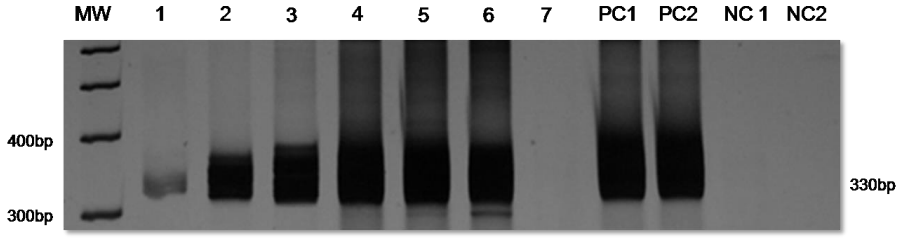

Fig. 1: Polyacrylamide gel (4.5%) that show 330 bp *Trypanosoma cruzi* kDNA specific fragments for the following dilutions (parasites/mL) of the Step 1: (1) 0.05, (2) 0.5, (3) 5, (4) 50, (5) 500, (6) 5,000, (7) 50,000. MW - 100 bp, molecular weight DNA ladder; PC1, positive control for PCR (DNA from *T. cruzi* strain PR-379); PC2, positive control for extraction; NC1, no DNA in the reaction mixture for PCR amplification; NC2, negative control for extraction; bp, base pairs

Table 1: Estimated number of parasites, concentration of total DNA, and thicknesses of the 330 bp *Trypanosoma cruzi* kDNA specific fragments for different parasite dilutions in the Step 1

Dilution (parasites/mL)	Line in the Polyacrylamide gel	[a]Estimated number of parasites	Concentration of total DNA (ng/µL)	Thicknesses of the 330 bp fragments (mm)
50,000	7	5,000	155	0.0
5,000	6	500	65	9.0
500	5	50	61	9.0
50	4	5	59	9.0
5	3	0.5	49	8.6
0.5	2	0.05	37	6.8
0.05	1	0.005	18	4.0

a To perform the amplification reaction, we used 2 µL of the original solution of 20 µL of DNA extracted for each dilution. Thus, the thicknesses of the 330 bp *T. cruzi* kDNA specific fragments corresponded to DNA of the following estimated number of: 5,000, 500, 50, 0.5, 0.05, 0.005 parasites

Moreover, demonstrated that only dilutions of 5 (re-dilutions 1/10 and 1/100) and 0.5 (re-dilutions 1/10) parasites/mL (DNA corresponded to the estimated number of 0.05, 0.005 and 0.005 parasites respectively) produced similar thicknesses of the 330 bp fragments of the Step 1.

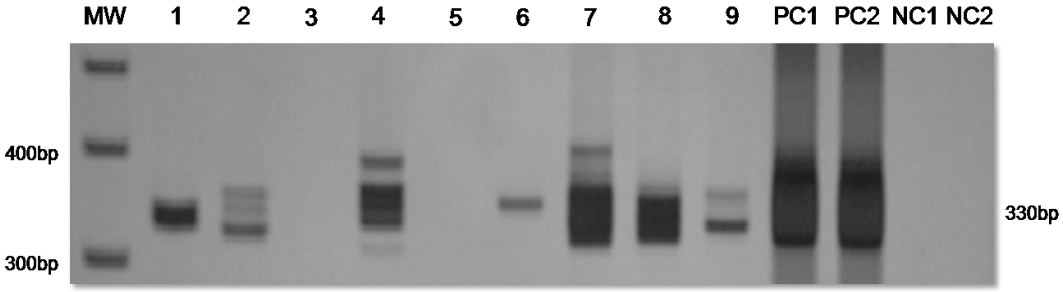

Fig. 2: Polyacrylamide gel (4.5%) that show 330 bp *Trypanosoma cruzi* kDNA specific fragments for the following dilutions of the Step 2: 0.05 parasites/mL, (1) 1/10, (2) 1/100, (3) 1/1000; 0.5 parasites/mL, (4) 1/10, (5) 1/100, (6) 1/1000; 5 parasites/mL, (7) 1/10, (8) 1/100, (9) 1/1000. MW - 100 bp, molecular weight DNA ladder; PC1, positive control for PCR (DNA from *T. cruzi* strain PR-379); PC2, positive control for extraction; NC1, no DNA in the reaction mixture for PCR amplification; NC2, negative control for extraction; bp, base pairs

Table 2: Estimated number of parasites, concentration of total DNA, and thicknesses of 330 bp *Trypanosoma cruzi* kDNA specific fragment for the re-dilutions in the Step 2

Dilution (parasites/mL)	Re-dilution	Line in the Poly-acrylamide gel	[a] Estimated number of parasites	Concentration of total DNA (ng/µL)	Thicknesses of the 330 bp fragments (mm)
5	1/10	7	0.05	4.9	6.6
	1/100	8	0.005	0.7	4.8
	1/1000	9	0.0005	0.2	4.4
0.5	1/10	4	0.005	3.7	4.7
	1/100	5	0.0005	0.5	0.0
	1/1000	6	0.00005	0.1	1.0
0.05	1/10	1	0.0005	1.8	2.5
	1/100	2	0.00005	0.2	5.2
	1/1000	3	0.000005	0.0	0.0

a To perform the amplification reaction, we used 2 µL of the original solution of 20 µL for each dilution. Thus, the thickness of the 330 bp T. cruzi kDNA specific fragments corresponded to DNA of the following estimated number: 0.05, 0.005, and 0.0005 parasites (dilution: 5 parasites/mL; re-dilution: 1/10, 1/100, 1/1000); 0.005, 0.0005, and 0.00005 parasites (dilution: 0.5 parasites/mL; re-dilution: 1/10, 1/100, 1/1000); 0.0005, 0.00005, and 0.000005 parasites (dilution: 0.05 parasites/mL; re-dilution: 1/10, 1/100, 1/1000).

Discussion

The outcomes obtained in the Step 1, in all experiments, showed similar patterns, in which the thicknesses of the fragments were maintained in the dilutions of 5,000-50 parasites/mL (DNA corresponded to the estimated number of 500-5 parasites and 65-59 ng/µL concentration of total DNA), whereas the thicknesses/staining intensity of the 330 bp specific fragments decreased in the dilutions of 5-0.05 parasites/mL (DNA corresponded to the estimated number of 0.5-0.005 parasites and 49-18 ng/µL concentration of total DNA). Nevertheless, samples with excess DNA (50,000 parasites/mL) may show false negative results.

The lower reproducibility obtained in the Step 2 may be explained by the very low concentration of DNA in these re-dilutions, i.e., very diluted DNA samples make difficult to reproduce PCR amplifications, which may produce variation of the thicknesses/staining intensity of the 330 bp fragments or false negative results in samples with the same low DNA concentration. In practice, these outcomes may produce questionings on the follow-up of chronic ChD patients under etiological treatment with low parasite load using qualitative PCR. In the low parasite load may not be possible to observe a progressive decrease of the thicknesses/staining intensity of the 330 bp *T. cruzi* kDNA specific fragment, but the beneficial effect of the treatment will provide persistent negative results of the PCR during follow-up of chronic ChD patients.

In fact, the main aim of this study is not quantify the number of circulating parasites, but show that progressive decrease in the thicknesses of the kDNA fragments often detected in ChD patients under etiological treatment can indicate a decrease in the number of circulating parasites and consequently beneficial effect of chemotherapy performed. Thus, to assess evolution of the parasite load from ChD patients using qualitative PCR (primers 121 and 122), the ideal experimental design would be extract and amplify DNA in a same reaction battery the serial samples collected at different moments (before, during, and after etiological treatment) (8), to enable

comparisons of the thicknesses of the kDNA fragments.

Conclusion

Qualitative PCR, targets kDNA (primers 121 and 122), despite its limitations, was able to detect progressive decrease in the thicknesses/staining intensity of the 330 bp fragments in the dilutions 5-0.05 parasites/mL (DNA corresponded to an estimated number of 0.5-0.005 parasites). Hence, conventional qualitative PCR, as we observed in our laboratory routine, has the potential to be used to follow-up evolution parasite load of *T. cruzi*, in the absence of specific quantitative techniques. It may aid in the early detection of refractory cases to etiological treatment, e.g., when the thicknesses/staining intensity of the kDNA fragments remains constant, and detection of beneficial effect of the etiological treatment, e.g., when there is a decrease in the thicknesses/staining intensity of the kDNA fragments until constant negativity of results, indicating a decrease in the number of circulating parasites.

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