ORIGINAL ARTICLE

Protocol optimization for the detection of *Trypanosoma cruzi* DNA in açai (*Euterpe oleraceae*) pulp

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ABSTRACT

Chagas disease, caused by the protozoan *Trypanosoma cruzi*, has often been linked to oral transmission through açai consumption. Molecular methods that allow fast and accurate identification of the pathogen are important for the detection of the presence of the parasite in this food. This study aimed to optimize polymerase chain reaction (PCR)-based detection of *T. cruzi* DNA in açai pulp. Several dilutions of *T. cruzi* DTU TcI trypomastigote forms were cultured in liver infusion tryptose (LIT) medium. *Trypanosoma cruzi* DNA was extracted from the cells and subjected to PCR. Subsequently, culture dilutions were added to açai pulp to evaluate the detection threshold of the optimized PCR assay. We demonstrate that our assay can detect *T. cruzi* DNA in açai pulp at a concentration of 1.08×10^{-10} ng μL^{-1}. We conclude that our optimized protocol is effective and can be used as an important tool for the detection of *T. cruzi* contamination in açai.

KEYWORDS: detection method; foodborne disease; food pathogen; molecular detection; Chagas disease

Otimização de protocolo para detecção de DNA de *Trypanosoma cruzi* em polpa de açaí (*Euterpe oleracea*)

RESUMO

A doença de Chagas, enfermidade causada pelo protozoário *Trypanosoma cruzi*, tem sido relacionada com frequência à transmissão oral pelo consumo de açaí. Métodos moleculares que fornecem uma identificação rápida e precisa do patógeno para a detecção da presença do parasita são de extrema importância para a detecção da presença do parasita neste alimento. Este estudo teve como objetivo otimizar a detecção de DNA de *T. cruzi* em polpa de açaí por meio da reação em cadeia da polimerase (PCR). Foram preparadas várias diluições das formas tripomastigotas de *T. cruzi* DTU TcI cultivadas em meio de cultivo *Liver Infusion Tryptose*. O DNA de *T. cruzi* foi extraído das células e submetido à PCR. Posteriormente, as diluições da cultura foram adicionadas às polpas de açaí para avaliar o limite de detecção do novo ensaio de PCR otimizado. Mostramos que nosso ensaio pode detectar DNA de *T. cruzi* em polpas de açaí na concentração de 1.08×10^{-10} ng μL^{-1}. Concluímos que a metodologia desenvolvida se mostra eficaz e pode ser uma ferramenta importante para a detecção de contaminação por *T. cruzi* em açaí.

PALAVRAS-CHAVE: métodos de detecção; doenças transmitidas por alimentos; patógenos alimentares; detecção molecular; doença de Chagas

INTRODUCTION

Trypanosoma cruzi Chagas, 1909 is a unicellular eukaryote that belongs to the kingdom Protozoa, order Kinetoplastida, and family Trypanosomatidae (Adl *et al.* 2012). This protozoan was described by Carlos Chagas in 1909 as the etiological agent of Chagas disease (CD) (Chagas 1909) and is characterized by a complex biological cycle that includes an intermediate host (triatomine arthropods), a definitive host (human), and various classes of mammalian hosts that act as domestic, peridomiciliary, and wild reservoirs (WHO 2017).

Oral transmission of CD was experimentally demonstrated for the first time, when trypomastigotes of the parasite were orally administered to laboratory animals (Nathan-Larrier 1921). In 2008, the route taken by orally inoculated metacyclic trypomastigotes of *T. cruzi* was described, revealing that these forms were capable to avoid gastric digestion by protecting themselves in the mucin layer and crossing the gastric mucosa (Yoshida 2008). Recently it was demonstrated that genetic factors can influence oral infectivity and that the high mortality rates in cases of human oral transmission can be explained by factors such as size of the inoculum, parasite genetics and lack of previous exposure to *T. cruzi* (Lewis *et al.* 2018). However, factors such as poor hygiene conditions during fruit handling and transport also affect the oral transmission of the pathogen (Xavier *et al.* 2014). Therefore, studies on the importance of these factors in the oral transmission of *T. cruzi* are necessary.

In northern Brazil, oral transmission of *T. cruzi* is frequent, especially in the Amazon region, where outbreaks of CD are associated with the consumption of contaminated pulp of açaí berries (*Euterpe oleracea* Martius) (Filigheddu *et al.* 2017; Shikanai-Yasuda *et al.* 2018; Brazil 2019). Contaminated Brazilian açaí berries have the potential to spread CD worldwide, as they are exported to many countries. Therefore, the detection of contaminants of açaí berries is important for both public health and compliance with sanitary rules for commercial export.

Food parasites are generally detected using microscopic examination, which is not a highly sensitive technique (Godoi *et al.* 2017). Therefore, PCR-based molecular methods have been used to detect pathogenic contamination in various foods (Alves *et al.* 2018; Ferreira *et al.* 2018; Oliveira *et al.* 2018).

Although the prevention of CD from oral transmission is of great relevance to public health, current methods of detection of *T. cruzi* in açaí pulp are ineffective due to the dark color of the fruit, high levels of organic matter (vegetable fibers) and characteristic physical-chemical properties of the pulp (Costa *et al.* 2018). Currently, there is no official method for the direct detection of *T. cruzi* in food (Ferreira *et al.* 2016) and the polymerase chain reaction (PCR), which has already been used for the diagnosis of infectious diseases (Cássia-Pires *et al.*, 2017; Silveira *et al.* 2018; Cardoso *et al.* 2020), can

potentially be used for this purpose due to its advantage in the fast and accurate identification of the pathogen (Cardoso *et al.* 2020).

Several studies have tested the use of PCR-based techniques for the detection of T. cruzi DNA in food, including açaí (Ferreira *et al.* 2016; Mattos *et al.* 2017; Godoi *et al.* 2017), but the efficacy of the technique still needs to be improved. The quality of the extracted DNA is of paramount importance, because an effective DNA extraction method that provides sufficient inhibitor-free DNA is crucial for high-sensitivity molecular techniques (Santos *et al.* 2009). Commercial DNA extraction kits offer the advantage of maintaining a quality standard for the extracted material. A wide variety of commercial DNA extraction kits are currently available, but only a few can be used for DNA purification from food products (Branquinho *et al.* 2012).

Thus, the aim of the present study was to present an optimized protocol of high efficiency and sensitivity for PCR detection of *T. cruzi* DNA extracted directly from açaí pulp through the modification of a commercial kit for DNA extraction from tissue samples, including the evaluation of the detection limit of the technique.

MATERIAL AND METHODS

DNA was extracted from 3.5×10^{4} $^{-1}$ *T. cruzi* discrete typing unit (DTU) TcIV isolate obtained from the protozoology collection of Fundação Instituto Oswaldo Cruz, Rio de Janeiro, Brazil. Genomic DNA was extracted using the Illustra™ blood genomicPrep Mini Spin Kit (GE Healthcare, Buckinghamshire, UK), according to the manufacturer's recommendations.

The extracted DNA was quantified using a spectrophotometer (NanoDrop™ Lite Spectrophotometer, ThermoFisher, Waltham, Massachusetts, EUA). Absorbance was measured at 260 nm (A260), 280 nm (A280), and 230 nm (A230) to assess the concentration of nucleic acids, proteins, and organic contaminants, respectively. The extracted DNA was subsequently electrophoresed on a 1% agarose gel in Tris-Borate-EDTA (TBE) buffer. The gel was stained with 6× GelRed™ (1 μL dye / 5 μL sample) and the results were analyzed using a gel documentation system (Gel Doc Xr+™, Bio-Rad®, USA).

For PCR, two primers previously described by Ochs *et al.* (1996), TCZ3 (5'-TGCTGCASTCGGCTGATCGTT TTCGA-3') and TCZ4 (5'-CARGSTTGTTTGGTGTCC AGTGGTTGTG-3'), were eluted in TE buffer (pH 8.0) to a concentration of 10 pmol μL^{-1}, to amplify the sequence of approximately 149 bp.

The PCR mix comprised 0.5 IU Taq DNA polymerase, 100 mM Tris-HCl (pH 8.5), 500 mM KCl, 50 mM MgCl$_2$,

0.2 mM of each dNTP, 5 pmol of each primer, and 133.6 ng µL^{-1} template DNA in a final volume of 25 µL.

The PCR reaction was performed in a thermocycler (Veriti 96 Well Thermal Cycler, Applied Biosystems®, Foster City, California, USA). The cycling parameters were as follows: initial denaturation at 94°C for 5 min; followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C/56 °C/57 °C for 20 s, and extension at 72 °C for 20 s; and final extension at 72 °C for 7 min. The amplified samples were stored at 4 °C for further analysis.

The PCR amplicons were subjected to electrophoresis on a 2% agarose gel in 0.5% TBE buffer. The gel was stained with GelRed® non-mutagenic dye and the results were analyzed using an ultraviolet light transilluminator with an image capture system (Gel Doc XR+ Gel Documentation System, Hercules, California, USA).

To assess the detection limit of the methodology, after optimizing the extraction method and defining the parameters of the PCR assay, the *T. cruzi* DTU TcIV DNA was serially diluted from an initial concentration of 133.6 ng µL^{-1} until the maximum dilution where it was still possible to detect the parasite. PCR amplification of DNA samples was performed in quintuple to determine the reproducibility of the assay.

The detection limit for the parasite DNA extracted from the açai pulp was determined by experimental contamination with the same isolate used for PCR optimization (*T. cruzi* DTU TcIV trypomastigote). In this procedure, the parasite was cultured in liver infusion trypsin (LIT) medium in a greenhouse at 28 °C. Subsequently, 3.0×10^4 parasite cells were diluted to 10^{-10} mL^{-1} cells, and 1 mL of the suspension of each dilution was added to 9 ml of pre-bleached açai pulp (80 °C for 10 s, followed by cooling to 7 °C). The contamination was performed with 10 dilutions in quintuple, totalling 50 samples.

The parasite's DNA was extracted from each of the 50 samples using the Illustra™ Tissue and Cells genomicPrep Mini Spin Kit (GE Healthcare, Buckinghamshire, UK), according to the manufacturer's recommendations and with modifications, aiming at determining the most appropriate protocol. The modifications consisted of diluting 200 µL of the contaminated açai sample in 100 mL PBS (phosphate-buffered saline) buffer followed by centrifuging at 14,000 ×g for 2 min. The pellet was suspended in 100 mL PBS. The cells were lysed, and DNA was purified in 50 µL of the solution supplied by the manufacturer. The extracted DNA was kept at 37 °C for 5 min, then stored at 4 °C.

The extracted DNA was quantified using a Nanodrop Lite® spectrophotometer, using 260-nm, 280-nm and 230-nm filters. The DNA quality was assessed by electrophoresis on a 1% agarose gel in TBE buffer, and the gel was stained with the 6× GelRed ™ dye (1 µL dye / 5 µL sample). The extraction protocol that provided the most adequate DNA samples in terms of purity was considered for later analyses.

Subsequently, these DNA samples were subjected to PCR in triplicate, according to the PCR protocol previously described, to evaluate the possibility of amplifying the DNA extracted from the açai pulp. Following PCR, one of the amplicons obtained was sequenced by ACTGene Análises Moleculares Ltda. using an automated sequencer (ABI PRISM 3130 Genetic Analyzer XL, Applied Biosystems®), following the ABI PRISM Big Dye Terminator Cycle protocol. Consensus sequences obtained from the GenBank database were subjected to a BLASTn search (http://www.ncbi.nlm.nih.gov) for comparison. The significance of the alignments was defined based on the e-value analysis.

A phylogenetic tree was built with the aid of the Blast Tree View® program (https://www.ncbi.nlm.nih.gov/blast/treeview/treeView.cgi), by the Fast Minimum Evolution method, that calculated an alignment between the pairs of the query and the sequences of the NCBI database for all parasites belonging to the genera *Leishmania* or other species of *Trypanosoma* registered in the database.
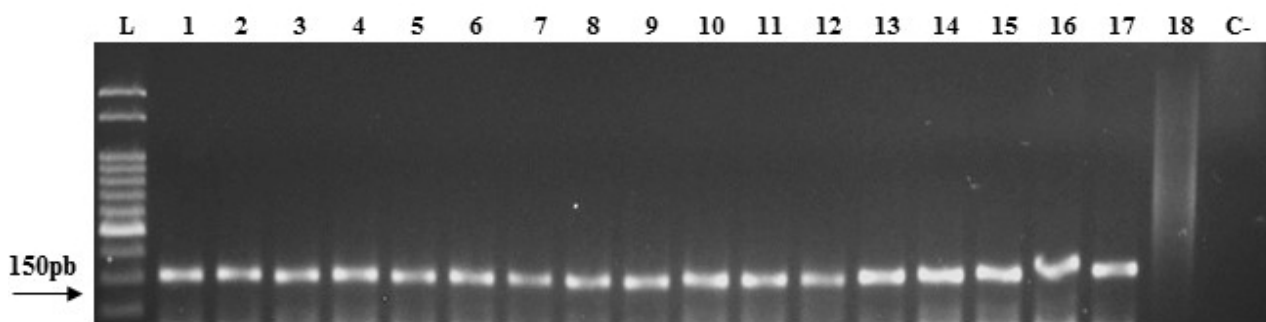
Figure 1. A 2% agarose gel showing *T. cruzi* DNA fragments (approximately 149 bp). L: 50-bp marker; lane 1: dilution of DNA 10^0 (equivalent to a concentration of 133.6 ng µL^{-1}); lane 2: DNA 10^{-1} (1.336×10^{-3} ng µL^{-1}); lane 3: DNA 10^{-2} (1.336×10^{-4} ng µL^{-1}); lane 4: DNA 10^{-3} (1.336×10^{-5} ng µL^{-1}); lane 5: DNA 10^{-4} (1.336×10^{-6} ng µL^{-1}); lane 6: DNA 10^{-5} (1.336×10^{-7} ng µL^{-1}); lane 7: DNA 10^{-6} (1.336×10^{-8} ng µL^{-1}); lane 8: DNA 10^{-7} (1.336×10^{-9} ng µL^{-1}); lane 9: DNA 10^{-8} (1.336×10^{-10} ng µL^{-1}); lane 10: DNA 10^{-9} (1.336×10^{-11} ng µL^{-1}); lane 11: DNA 10^{-10} (1.336×10^{-12} ng µL^{-1}); lane 12: DNA 10^{-11} (1.336×10^{-13} ng µL^{-1}); lane 13: DNA 10^{-12} (1.336×10^{-14} ng µL^{-1}); lane 14: DNA 10^{-13} (1.336×10^{-15} ng µL^{-1}); lane 15: DNA 10^{-14} (1.336×10^{-16} ng µL^{-1}); lane 16: DNA 10^{-15} (1.336×10^{-17} ng µL^{-1}); lane 17: DNA 10^{-16} (1.336×10^{-18} ng µL^{-1}) - detection threshold; lane 18: DNA 10^{-17} (1.336×10^{-19} ng µL^{-1}); C-: negative control.

RESULTS

The optimized PCR assay detected *T. cruzi* DNA with good accuracy and precision when 57 °C for 30 s was used in the annealing step. The optimized PCR showed a detection threshold of 1.336×10^{-18} ng µL^{-1} for *T. cruzi* DNA (Figure 1).

With regard to the experimental contamination of açai pulp, the average concentration of DNA extracted from all 50 analyzed samples was 10.8 ng µL^{-1}. The extracted DNA samples usingthe modifications of the original protocol had a clear appearance, indicating that the pigments present in açaí that could inhibit the reaction were eliminated. The mean absorbance (A260/280) and (A260/230) values were 1.9 ± 1.0 and 2.0 ± 1.4 D.O., respectively. The detection limit of *T. cruzi* DNA in the dilutions of contaminated pulp was 3×10^{-6} cells mL^{-1} of *T. cruzi,* which corresponded to 1.08×10^{-10} ng µL^{-1} of DNA in the sample (Figure 2).

Analysis of the fragment sequenced in this study revealed significant identity with *T. cruzi* DNA. The sequenced fragment showed 100% identity and 97% coverage with the subject sequence DQ914506.1 (*T. cruzi* clone VL10_1_1 satellite sequence) accessed from GenBank®. Moreover, no significant similarity was found between the *T. cruzi* DNA sequence and DNA sequences of parasites belonging to *Leishmania* or other species of *Trypanosoma* (Figure 3).

DISCUSSION

Ferreira *et al.* (2016) proposed a benchtop protocol for extracting *T. cruzi* DNA from foods using detergent CTAB (cetyltrimethylammonium bromide). However, they could only achieve PCR amplification of the parasite DNA with a minimum concentration of 2.5 cells mL^{-1} of açaí. We achieved amplification of *T. cruzi* DNA from a much lower inoculum concentration (3×10^{-6} cells mL^{-1}), demonstrating that our method is highly effective.

Modifications to the DNA extraction protocol of the commercial kit manufacturer were necessary to increase the purity and concentration of the DNA extracted from the açai pulp, as results obtained from previous tests without these modifications demonstrated a large amount of organic contaminants in the extracted DNA, which inhibited PCR (data not shown).

The physicochemical characteristics of açaí, along with the pigments present in the fruit, may interfere with DNA extraction and molecular analysis techniques (Yamaguchi *et al.* 2015; Ferreira *et al.* 2018; Lucas *et al.* 2018), and pose a challenge for the detection of *T. cruzi* in açaí pulps. The method described here is highly effective due to its advantageous ability to obtain purified DNA, which is not possible using alternative techniques (Godoi *et al.* 2017). Purified DNA can be obtained using a commercial kit, which contains a purification column with silica gel to which DNA binds. The bound DNA is subsequently washed until a high degree of purity is achieved. This also allows rapid and uniform extraction of DNA samples and eliminates the contaminants and inhibitors which are present in food matrices.

Desjardins and Conklin (2010) used microvolume NanoDrop quantification protocols to estimate the concentration of nucleic acids, and showed that the A260/280 ratio for pure DNA should be between 1.8 and 2.0, and A260/280 ratios outside this range may indicate the presence of proteins, phenols, or other contaminants. They also suggested that the A260/230 ratio for a "pure" nucleic acid should range from 1.8 to 2.2, and lower A260/230 ratios may indicate contamination by carbohydrates, peptides, phenols, and aromatic compounds. Based on this, our DNA samples obtained through the proposed extraction method were of adequate purity, as they fit the parameters suggested by Desjardins and Conklin (2010) for pure DNA samples without contaminants.

The concentration of *T. cruzi* DNA extracted from açai was lower than that of the positive control DNA extracted from the culture medium. However, the concentration and quality of the extracted *T. cruzi* DNA were adequate for PCR amplification, suggesting its applicability for commercial açai samples. Ferreira *et al.* (2018) proposed the use of PCR for *T.*
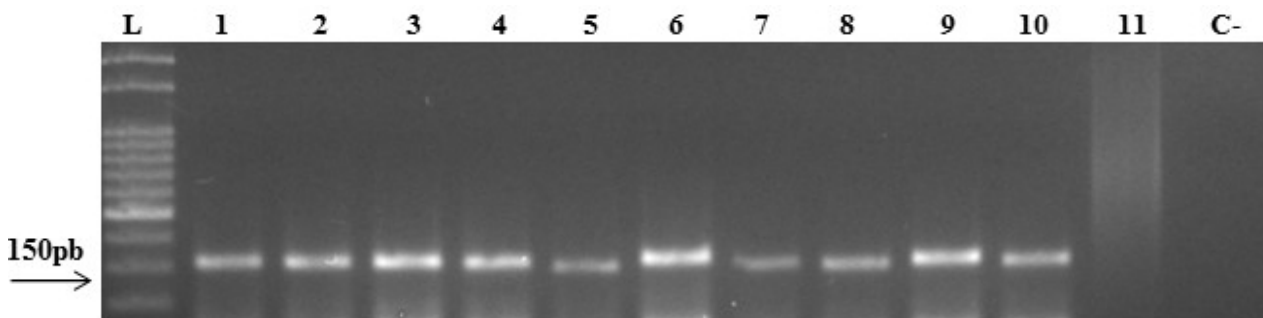
Figure 2. A 2% agarose gel showing PCR-amplified *T. cruzi* DNA fragments (149 bp) at different dilutions of *T. cruzi* inoculated in açaí pulp. L: 50 bp marker; lane 1: diluition of DNA 10^{-0} (equivalent to a concentration 10.8 ng µL^{-1}); lane 2: DNA 10^{-1} (1.08 x 10^{-2} ng µL^{-1}); lane 3: DNA 10^{-2} (1.08 x 10^{-3} ng µL^{-1}); lane 4: DNA 10^{-3} (1.08 x 10^{-4} ng µL^{-1}); lane 5: DNA 10^{-4} (1.08 x 10^{-5} ng µL^{-1}); lane 6: DNA10^{-5} (1.08 x 10^{-6} ng µL^{-1}); lane 7: DNA 10^{-6} (1.08 x 10^{-7} ng µL^{-1}); lane 8: DNA 10^{-7} (1.08 x 10^{-8} ng µL^{-1}); lane 9: DNA 10^{-8} (1.08 x 10^{-9} ng µL^{-1}); lane 10: DNA 10^{-9} (1.08 x 10^{-10} ng µL^{-1}) - detection threshold; lane 11: DNA 10^{-10} (1.08 x 10^{-11} ng µL^{-1}); C-: negative control.
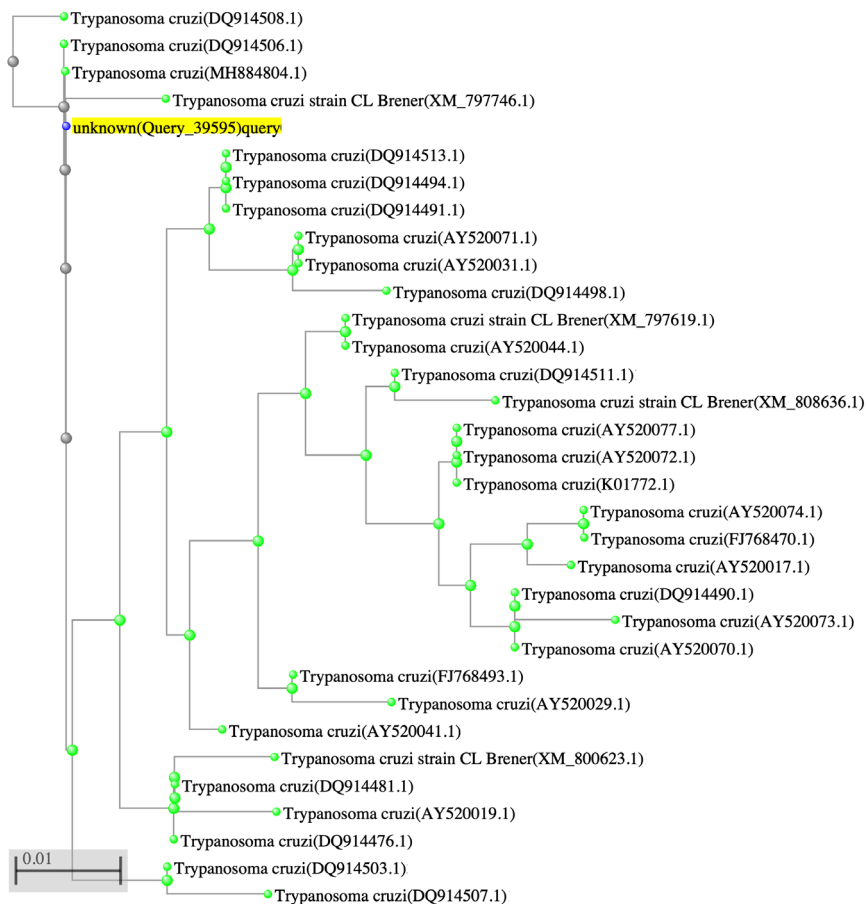
Figure 3. Phylogenetic tree showing the relationship of alignment between sequence pairs presented by the NCBI database query produced from the Blast Tree View® program and containing the name of similar paired species and their identification code. "Unknown (Query_39595)" corresponds to the sample used in this study (*Trypanosoma cruzi* TcIV). This figure is in color in the electronic version.

cruzi detection in açaí samples, tested only with mixtures of DNA, in which the detection limit was 3 pg μL^{-1}. However, our method represented an improvement over theirs because it had a lower detection threshold (1.36 pg μL^{-1}).

The sequencing results also confirmed that standard PCR optimized in the present study using the primers described by Ochs *et al.* (1996) is a robust technique for the detection of *T. cruzi* without nonspecific amplification of other microorganisms. Primers that bind to *T. cruzi* kinetoplastic minicircle DNA (kDNA) or nuclear satellite DNA (satDNA) might demonstrate variations in the amplified fragments, and may even bind to kDNA or satDNA from other species, such as *T. rangeli* (Seiringer *et al.* 2017; Ferreira *et al.* 2018). However, DNA sequence analysis with other sequences accessed from GenBank did not reveal similarity with genes of other species of the genus *Leishmania* or other species of *Trypanosoma*.

CONCLUSIONS

We conclude that the proposed PCR method is an effective technique for the detection of *T. cruzi* DNA in açaí pulp at concentrations of 1.08×10^{-10} ng μL^{-1} and above, and could be an important tool for the prevention of Chagas disease caused by the consumption of contaminated pulp of this fruit.

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