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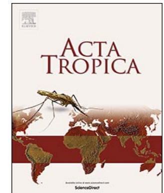

Molecular detection of *Trypanosoma cruzi* in acai pulp and sugarcane juice

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ABSTRACT

Chagas disease, caused by *Trypanosoma cruzi* affects about 6–8 million people worldwide. Although transmission by triatomine insects has been controlled, other means of transmission maintain the infection. These forms of transmission are responsible for introducing Chagas disease in other non-endemic countries of the world. Thus, Chagas disease, nowadays is a worldwide health problem. In Brazil, acai pulp and sugarcane juice have been associated with Chagas disease outbreaks. The difficulties in isolation of the parasite from foods are hampering source tracking which could allow the confirmation of an implicated food commodity in these outbreak investigations. To address this scientific gap, we evaluated the performance of real-time PCR (qPCR) for detecting *T. cruzi* in acai pulp and sugarcane juice. All experiments were performed with acai pulp and sugarcane juice samples contaminated with different concentrations of *T. cruzi*. In assays with qPCR, the results showed that the ideal procedure for *T. cruzi* identification in acai pulp and sugarcane juice consisted of: i. centrifugation; ii. DNA extraction with a commercial kit for stool matrix; and iii. qPCR using a specific molecular marker for *T. cruzi*. The seeding in LIT medium of experimentally contaminated foods was effective in detecting the parasitic load by qPCR. The efficacy of qPCR was also verified testing food samples crushed with infected Triatomines. In conclusion, this methodology can be used to perform rapid diagnosis in outbreaks, facilitating measures in disease control.

1. Introduction

Chagas disease is endemic in 21 countries in Americas and, migration of infected people can bring the disease to areas in the world that has no *Trypanosoma* cruzi transmission. Chagas disease affects approximately 8 million people and causes approximately 12,000 deaths/year (Schmunis and Yadon, 2010; PAHO/WHO, 2016).

The transmission by triatomine insects have been controlled in endemic countries such as Brazil, Venezuela, Chile, Uruguay, and some areas of Argentina and Bolivia. However, controlling transmission modes other than the vector-borne represents a challenge or similar verbiage. Other means of transmission maintain the infection. These forms of transmission are responsible for introducing Chagas disease in European countries, Japan, Australia, and North America (Teixeira et al., 2011; Coura et al., 2014). In addition to vectorial transmission, parasites are also transmitted by blood transfusion, congenital means, organ transplants, laboratory accidents, and ingestion of contaminated food (Moraes-Souza and Ferreira-Silva, 2011; Shikanai-Yasuda and Carvalho, 2012; Alkmim-Oliveira et al., 2013; Márquez et al., 2013; Coura et al., 2014; Ferreira et al., 2014).

Oral transmission occurs by ingestion of contaminated foods and is commonly shown in wild mammals since they can feed on triatomine bugs and other infected mammals (Dias, 2006; Dias et al., 2011; ANVISA, 2016). *T. cruzi* infection in foods *in nature* is accidental and can occur during harvesting, storage, transport, or preparation (Fregonesi et al., 2010). In humans, this event occurs accidentally after ingesting contaminated cane juice, acai pulp, soups, homemade food, milk, and semi-raw meat (Toso et al., 2011; Marques et al., 2013).

Acai pulps have nutritional properties and are popular throughout Brazil and abroad (Fregonesi et al., 2010). However, the consumption of contaminated acai has been linked to several outbreaks of acute Chagas disease in Venezuela, Brazil, and Colombia (Nobreba et al.,

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2009; Ferreira et al., 2014). Palm trees are very dense and infested with triatomines with an intense sylvatic cycle (Dias et al., 2014).

Brazil has the highest incidence of oral transmission responsible for increased morbidity and mortality. Thus, the oral transmission of Chagas disease has become one of the most important modes of transmission from the public health perspective. Between 2000 and 2011, 1252 cases of acute Chagas disease were reported, and of these, 70% were attributed to oral transmission (Dias, 2006; Dias et al., 2011; Coura et al., 2014; Ferreira et al., 2014). In Amazon region a number of outbreaks have been reported and normally these outbreaks are characterized by clinical symptoms of acute Chagas disease. Normally they were characterized as a group of individuals gathered in one place, having ingested the same type of food, becoming sick almost simultaneously with fever and general manifestations of a systemic infection (Alarcón de Noya et al., 2010; Ferreira et al., 2014). Thus, several outbreaks of the disease were related to consumption of foods and beverages, highlighting the importance of this transmission route in humans (Nóbrega et al., 2009; Alarcón de Noya et al., 2010; Shikanai and Carvalho, 2012; Marques et al., 2013). Difficulties in clarifying these outbreaks included the small number of confirmed cases and difficulty of isolating parasites in food (Nobrega et al., 2009; Passos et al., 2012).

Sugarcane juice in Brazil is produced largely without criteria of good manufacturing practices. In addition, sugarcane juice constitutes an optimal substrate for the growth of many microorganisms with many nutrients (Cardoso et al., 2006).

Molecular methods have contributed to the ability to detect and quantify pathogens in food and water (Palomino-Camargo and González-Muñoz, 2014; Ferreira et al., 2016). However, no laboratorial method has been validated for detecting parasites in the foods related to outbreaks (ANVISA, 2016). To address this scientific gap, we evaluated the performance of real-time PCR (qPCR) for detecting *T. cruzi* in acai pulp and sugarcane juice. All experiments were performed with acai pulp and sugarcane juice samples contaminated with different *T. cruzi* concentrations.

2. Materials and methods

2.1. Food samples and *T. cruzi*

The experiments were performed using acai pulp and sugarcane juice (in aliquots). Samples of *in natura* acai pulp were collected in Para State (north, Brazil) and samples of *in natura* sugarcane juice were collected in São Paulo State (southeast, Brazil). All samples used in this study were not naturally infected with *T. cruzi*. They were previously tested by PCR and results were always negative.

T. cruzi (Y strain) trypomastigotes were grown and maintained in MK_2 cells at 37 °C, 5% CO_2, in RPMI medium containing 2.2 g/L sodium bicarbonate, 5 mg/mL gentamicin 10% fetal bovine serum (in Centro de Parasitologia e Micologia, Instituto Adolfo Lutz, SP, Brazil). After 4–5 days, parasites were collected from supernatants of cell cultures, washed twice, counted, suspended in phosphate-buffered saline, pH 7.2, at different concentrations for infecting food samples, for artificial xenodiagnosis and positive controls. A stock solution of 1×10^7 trypomastigotes was used to prepare the standard curve, after DNA extraction.

2.2. Food contamination with trypomastigotes and experiments

For qPCR, aliquots of 50 g of acai pulp and sugarcane juice were contaminated with 1×10^5, 1×10^4, 1×10^3, 1×10^2 and 10 trypomastigotes each. Then, samples were centrifuged for 10 min at 2500*g* after sample mixture. Next, 500 μL of pellet and supernatant were used for DNA extraction.

For cultures, aliquots of 50 g of acai pulp and sugarcane juice were contaminated with 6×10^3 trypomastigotes. Next, samples were seeded in 100 mL of Liver Infusion Tryptose medium (LIT) and incubated at 28 °C for 10 days. These experiments were performed with intention of increase the number of parasites, and consequently, increase the detection of parasite DNA in qPCR. Aliquots of 500 μL were collected each 2 days for DNA extraction. After 10 days the experiments were stopped, since the cultures presented yeast and bacteria contamination.

2.3. Food contamination with *Triatoma infestans* artificially infected with *T. cruzi*

In order to test the molecular protocol for *T. cruzi* detection in foods, aliquots (500 g) of acai pulp or sugarcane juice were crushed, in a blender for 2 min, with 2 third instar nymphs of *T. infestans* (Hemiptera, Reduviidae) with positive artificial xenodiagnosis. Aliquots of 45 mL from the mixture were used to perform qPCR. Artificial xenodiagnosis was performed at Laboratório de Doença de Chagas Elias Boainain, Instituto Dante Pazzanese de Cardiologia (SP, Brazil) as described before, (Araujo-Jorge and Castro, 2000).

2.4. DNA purification

Before performing the DNA extraction, trypomastigotes were crushed and digested in a lysis buffer (Tris-HCl, 10 mM, pH 8.0; EDTA 10 mM; SDS, 0,5%; N-laurilsarcozil, 0.01%; proteinase K, 100 μg/mL) by incubation at 95 °C for 5 min and mixed for 3 min. Then, DNA was extracted by QIAamp DNA Mini Kit (Qiagen), and DNA from food samples was extracted by DNA QIAamp DNA Stool Mini Kit (Qiagen). The extractions were done according to the manufacturer's instructions. DNA concentrations and purity were determined by the ratio of O.D. at 260 and 280 nm in a NanoDrop ND1000 (Thermo Scientific, Waltham, MA, USA.).

2.5. Real-time PCR

The reactions were previously standardized using serial dilutions of DNA from *T. cruzi (*Y strain) as template. The design of the primer set (Cruzi 32/148), which amplified a specific region of kDNA minicircles (Schijman et al., 2011), included the forward-32 (5′ TTTGGGAGGGG-CGTTCA-3′) and reverse-148 (5′ATATTACACCAACCCCAATCGAA-3′) primers and the MGB TaqMan probe71 (5′CATCTCACCCGTACATT3′), labeled with FAM and NFQ at the 5′ and 3′ ends, respectively (Qvarnstrom et al., 2012). Cruzi 32/148 primer set is successfully used in our laboratory as molecular diagnosis of Chagas disease in clinical samples. Thus, we decided to use the same molecular marker for food experiments.

PCR(s) were performed in a final volume of 20 μL containing 0.5 μL of each primer in a concentration of 18 μM, 0.25 μL of TaqMan probe in a concentration of 5 μM and 10 μL of 2X TaqMan Universal PCR Master Mix (Applied Biosystems). DNA extracted from acai pulp or sugarcane juice (approximately 100 ng/μL) was tested in duplicate using 3 μL and 1 μL volumes of the sample, DNA control (50 ng) or DNA for standard curve (3 μL for each point, in triplicate). PCR amplification was performed as previously described (Colombo et al., 2015), on an Applied Biosystems 7500 Real-time PCR System using the following cycling structure: 2 min, 50 °C, and 95 °C for 10 min holds followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

2.6. Data analysis

Standard curve and limit of quantification of Cruzi 32/148-qPCR primer set for qPCR were analyzed using GraphPad Prism 6.05 Software Inc. (San Diego, CA, USA). A standard curve was constructed as described before (Colombo et al., 2015), by using eight different concentrations of total DNA obtained from 1×10^6 trypomastigotes (corresponding to 35 ng/μL) in triplicate. The serial dilutions ranged up to 0.1 trypomastigote and the cycle threshold values (C_T) were plotted as

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