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Molecular analysis of *Toxoplasma gondii* in samples of açaí (*Euterpe oleracea*) ice cream purchased from commercial establishments

Beatriz Januário ALENCAR[1], Lívia Vieira GARDI[1], Laís Pereira da SILVA[1], Bruna dos Santos LEITE[1], Juliano Gonçalves PEREIRA[1], Antonio Carlos PAES[1], Marcos Vinícius RANGEL[2], Isabella Neves AIRES[2], Thainá Valente BERTOZZO[3], Vera Cláudia Lorenzetti Magalhães CURCI[4], Michel dos Santos PINTO[5], Kátia Denise Saraiva BRESCIANI[5], Simone Baldini LUCHEIS[1,2]

Abstract

The purpose of this study was to analyze the occurrence of *Toxoplasma gondii* in the samples of ice cream made with açai (*Euterpe oleracea*) pulp. Fifty samples of ice cream made with açaí pulp, purchased from 11 municipalities in the state of São Paulo, were analyzed. Conventional polymerase chain reaction (PCR) was performed with the primers TOX4/TOX5, and nested PCR (nPCR) was carried out with the primers TgNP1/TgNP2. Of the 50 samples evaluated, 1 (2%) was positive according to nPCR. The molecular detection of *T. gondii* in the samples of ice cream made with açai pulp in this study serves as an alert of the need for continuing epidemiological vigilance to detect this zoonosis, whose occurrence is increasing due to the widespread and expanding consumption of food products made from açai pulp.

Keywords: *Euterpe oleracea*; açai; toxoplasmosis; diagnosis; spatial analysis.

Practical Application: This study highlights the need for food safety measures against *Toxoplasma gondii* in açaí ice cream.

1. INTRODUCTION

Toxoplasmosis is a zoonosis of human and veterinary medical importance caused by *Toxoplasma gondii*, an obligate intracellular unicellular parasite (Kochanowski & Konshy, 2018; Attias et al., 2020; Zhao & Ewald, 2020). It can be transmitted via various pathways, including contaminated food and/or water, as well as by transplacental infection.

According to the World Health Organization (WHO), in 2015, acquired toxoplasmosis and ascariasis were ranked as the parasitic diseases with the largest total number of people infected and the greatest number of symptomatic cases attributed to contaminated foods (*apud* Symeonidou et al., 2023).

The symptoms of severe cases include fever, lymphadenopathy, lymphocytosis, and muscle aches, lasting from a few days to several weeks. The ability of *T. gondii* to form cysts and persist in the host for years is a key to its widespread dissemination in humans and animals around the world (Jeffers et al., 2018). Although the majority of infected people are asymptomatic or only exhibit mild symptoms, *T. gondii* can have a severe negative neurological impact on the fetus when acquired during pregnancy. It is estimated that globally between 170,000 and 200,000 cases of congenital toxoplasmosis occur annually, involving more than 5000 cases of miscarriages or neonatal deaths, plus 24,000 cases of chorioretinitis in the first year of life and 9300 cases of hydrocephalus and other neurological abnormalities (Milne et al., 2020).

The symptoms are usually more severe in immunosuppressed individuals (Dumètre & Dardè, 2003), such as transplant recipients, who undergo long-term immunosuppression therapy (Zhang et al., 2022). The parasite is an important cause of neurological and psychiatric diseases (Montazer et al., 2023).

Studies have associated severe outbreaks of human toxoplasmosis with the ingestion of oocysts, with plants having been identified as the possible vehicles of infection, probably contaminated by irrigation water (Marín-García et al., 2022). Indeed, outbreaks associated with contaminated water are more common than those originating in the soil, fruits, or vegetables (Dumètre & Dardè, 2003).

Two important outbreaks have occurred in Brazil: one in the municipality of Santa Isabel do Ivaí in the state of Paraná and the other in the municipality of Ponta de Pedras in the state of Pará. The latter outbreak (Morais et al., 2016) involved 73

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[1]*Universidade Estadual Paulista "Júlio de Mesquita Filho", School of Veterinary Medicine and Animal Sciences, Department of Animal Production and Preventive Veterinary Medicine, Botucatu, SP, Brazil.*
[2]*Universidade Estadual Paulista "Júlio de Mesquita Filho", Medical College, Department of Tropical Diseases and Image Diagnosis, Botucatu, SP, Brazil.*
[3]*Centro Universitário Sagrado Coração, Bauru, SP, Brazil.*
[4]*Biological Institute, Araçatuba, SP, Brazil.*
[5]*Universidade Estadual Paulista "Júlio de Mesquita Filho", Faculdade de Medicina Veterinária, Araçatuba, SP, Brazil.*
*Corresponding author: simone.b.lucheis@unesp.br
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cases with clinical findings, in which açaí (*Euterpe oleracea*) was identified as the origin of the infection. The present study was inspired by this incident. Ponta de Pedras is one of the major producers of açaí in the country, but the outbreak happened when this production was almost nil. Outbreaks like this, related to plants, generally occur due to contamination during production, including sowing, growth, harvesting, transportation, and processing for consumption. To pin down the outbreak's origin, mapping was performed of the residences where the patients lived, making them probable points of interest to the outbreak. This analysis indicated a spatial connection between the concentration of toxoplasmosis cases and the distribution of the main points of the sale of açai juice and other products during the outbreak, through the reports of patients.

Preventive research involving *T. gondii* in açaí samples is important because toxoplasmosis is generally asymptomatic or has mild symptoms. However, it can severely afflict the nervous system, even causing fetal deaths, when the infection is acquired by the mother during pregnancy. Besides this, it is a global public health challenge, particularly in view of undernotification and the possibility of infection through many routes. This study was inspired by the importance of this zoonosis and the lack of published works regarding infections by *T. gondii* due to the consumption of açaí, a practice that is growing in popularity.

The purpose of this study was to analyze the occurrence of *T. gondii* in the samples of açai pulp and identify the risk of contamination of the various food products made with açai pulp.

2. MATERIAL AND METHODS

2.1. Ethical approval

This study was approved by the Ethical Committee on the Use of Animals (CEUA) of the Botucatu School of Medicine, under number 267/2022.

2.2. Samples of açai ice cream from various retail sources in São Paulo State

We analyzed 50 samples of açai in the form of ice cream, purchased from different points of sale (supermarkets and self-service dispensers), located in 11 municipalities in the state of São Paulo: Avaré, Botucatu, São Manuel, Bauru, Barra Bonita, Agudos, Pardinho, Pederneiras, Lençóis Paulista, Itatinga, and Bofete. As soon as purchased, the samples were placed in identified containers and carried to the laboratory in an ice chest (with recyclable ice), where they were immediately stored in a freezer at -20°C.

2.3. Molecular tests

2.3.1. Extraction of Toxoplasma gondii *DNA*

We used a GE Healthcare® kit (Ilustra Tissue and Cells GenomicPrep Mini Spin kit) for DNA extraction, according to the manufacturer's protocol.

2.3.2. Conventional PCR

Conventional PCR (cPCR) was carried out with the following conditions: each reaction tube with a capacity of 0.2 mL received PCR buffer (50 mM of KCl, 20 mM of Tris-HCl), 1.6 mM of $MgCl_2$, 0.2 mM of dNTPs, 1 U of Taq-polymerase (Platinum® Taq DNA Polymerase, Invitrogen®), 0.2 µM of each primer, 1 µL of the sample, and 8.3 µL of ultrapure water (MIX-PCR). Therefore, each tube contained 11 µL of MIX-PCR and 1 µL of the DNA extraction product. We then performed cPCR for the amplification of the 529 bp repeat fragment, consisting of 200–300 copies of the *T. gondii* genome, to assure the quality of the amplification of the DNA extracted using the primers TOX4 and TOX5, whose cycling profile was determined according to Homan et al. (2000), with modifications:

TOX4 – 5'(CGCTGCAGGGAGGAAGACGAAAGTTG) 3'

TOX5 – 5' (CGCTGCAGACACAGTGCATCTGGATT) 3'.

The amplification cycle followed a denaturing period of 7 min at 94°C and an annealing period with 40 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, with a final extension cycle of 5 min at 72°C. After this, the mixture was refrigerated at 4°C.

2.3.3. Nested PCR

The *T. gondii* DNA was also detected by nested PCR (nPCR) employing the external primers TgNP1/TgNP2, which hybridize the ITS1 region common to *T. gondii* and *Neospora caninum* (Hurtado et al., 2001), along with the internal primers TgNP1/TgNP2, which amplify a 227 bp fragment of the ITS1 region of *T. gondii*. The tachyzoite DNA of the RH strain of *T. gondii* and ultrapure water were used as positive and negative controls, respectively, and were included in all the PCR runs (Santos Silva et al., 2020). The following are the primers used and the amplification conditions in a MasterCycler Pro Gradient (Eppendorf®) thermocycler:

NN1 – 5'-CCTTTGAATCCCAAGCAAAACATGAG-3'

NN2 – 5'-GCGAGCCAAGACATCCATTGCTGA-3'

TgNP1 – 5'-TGATAGTATCGAAAGGTAT-3'

TgNP2 – 5'-ACTCTCTCTCAAATGTTCCT-3'.

The DNA was amplified by 3 min of denaturing at 94°C, followed by 15 cycles at 94°C for 30 s, 65°C for 45 s, 72°C for 1 min, and a final extension at 72°C for 5 min. For the external primers, the amplification was at 94°C for 3 min, followed by 35 cycles at 94°C for 20 s, 53°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 5 min (Kochanowski & Konshy, 2018).

2.3.4. Agarose gel electrophoresis

Aliquots of 10 µL of the amplified samples were homogenized with 2 µL of the running buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol, and 70% ultrapure water) and were submitted to horizontal electrophoresis containing TBE 1X (0.1 M of Tris, 0.09 M of boric acid, and 0.001 M of

EDTA). The identification of the amplified products was carried out by 1.5% agarose gel electrophoresis, stained with SYBR Safe® (Invitrogen) at 0.1 µL/mL, with a voltage of 100 V for 90 min. The molecular weight standard was LowRanger 100 bp DNA Ladder (Norgen), and the sizes of the amplified fragments were compared visually with the molecular weight standards of the strains used as positive controls, with the use of ultraviolet light. The images were captured with a Major Science™ system and documented using the Vision Works LS software.

2.3.5. Controls

The positive controls used were the RH strains of *T. gondii,* and the negative control was sterile ultrapure water.

2.3.6. Statistical analysis

The data were submitted to the χ^2 test and Fisher's exact test at a 5% significance level. The Kappa concordance coefficient was calculated to evaluate the real concordance of cPCR and nPCR (Daguer et al., 2004). The results indicated moderate concordance between the techniques (a coefficient of 0.5) based on the comparison of the nominal results of these two metrics with the positive and negative terms according to Landis and Koch (1997).

2.3.7. Spatial analysis

We performed spatial analysis to verify the origin of all the açaí ice cream samples collected in the region studied, in particular, to identify the municipality of origin of the positive sample, using the geographic information system (GIS) as the instrument, with the QGIS 3.16.8 software. Furthermore, we used the QGIS software of the Canva platform to assemble and organize the maps.

3. RESULTS

The two molecular techniques used to analyze the data were cPCR employing the primers TOX4/TOX5 and nPCR using the primers NN1/NN2 — TgNP1/TgNP2. The latter method indicated that one of the 50 samples (2%) was positive, originating from the municipality of São Manuel.

The gel image in Figure 1 represents the results of the molecular analysis of the açaí samples with the primers TgNP1/TgNP2. Note the amplification of the sample circled in red (column 8).

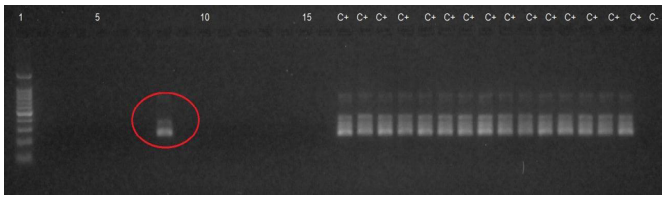

Figure 1. 2% agarose gel stained with Syber Safe. 100 bp molecular weight marker - Line 1. Primers TgNP1/TgNP2: *Toxoplasma gondii* contamination of açai samples. Positive controls (L17–L31): RH strain of *Toxoplasma gondii*. Negative control (L32): ultrapure water.

The development of PCR is a great advance in the diagnosis of infection by *T. gondii*. Various studies have demonstrated the better accuracy, sensitivity, and specificity of PCR in comparison with traditional methods (Dehkordi et al., 2013), by increasing the possibility of detecting small quantities of the target DNA. Furthermore, the diagnosis can be confirmed in a single day (Sah et al., 2019). A previous Brazilian study detected *T. gondii* by molecular biology, indicating the existence of environmental contamination and the circulation of the parasite in the region (Santos Silva et al., 2020).

The use of the two molecular techniques (cPCR and nPCR) was important to optimize the detection of *T. gondii*, with the nPCR employing the primers TgNP1/TgNP2 being more efficient in its detection than cPCR with the primers Tox4/Tox5.

The use of the primers TOX4 and TOX5 is well described in the literature, including for the detection of *T. gondii* by cPCR. However, the use of nPCR with the primers TgNP1 and TgNP2, which amplify a 227 bp fragment in the RH region of ITS1 of *T. gondii,* yielded better results (more sensitive and specific to detect the parasite's DNA) than the use of cPCR with the primers TOX4 and TOX5, as also reported by Hurtado et al. (2001).

Figure 2 presents the state of São Paulo, highlighting the 11 municipalities from where the açai ice cream samples were obtained.

4. DISCUSSION

Outbreaks of acute toxoplasmosis have been described in several countries, including Brazil, related both to water and food contamination (de Moura et al., 2006; Ekman et al., 2012; Meireles et al., 2015).

This study was inspired by a report of acute toxoplasmosis in five individuals, whose symptoms included lymphadenopathic fever. All of them were residents of the municipality of Ponta de Pedras, Pará State, where the local Health Secretariat also indicated that other similar cases had occurred (Morais et al., 2016). Therefore, we conducted a study in that region and confirmed the existence of 73 cases with clinical and laboratory profiles compatible with acute toxoplasmosis. The clinical, laboratory, and epidemiology results revealed that the outbreak of the acute disease was caused by the consumption of açaí juice contaminated with *T. gondii* oocysts, probably acquired during the collection and transport of the berries or the processing of the juice.

The detection of a sample of açai ice cream positive for *T. gondii* in this study highlights the importance of seeking the probable sources of food contamination. Food-borne diseases, especially those caused by protozoa such as *T. gondii*, are often not recognized. Purees, ice creams, sherbets, and juices can be contaminated by the water used in their preparation, which is, in turn, contaminated by the feces of cats infected with sporulated *T. gondii* oocysts (Pereira et al., 2010).

The molecular detection of a contaminated açai ice cream sample obtained from a self-service dispenser in São Manuel serves as an alert of the need for the ongoing epidemiological vigilance of this zoonosis in food products made from açaí berries, whose popularity is high and still growing. Also important are educational campaigns on health and good hygiene practices by food preparers.

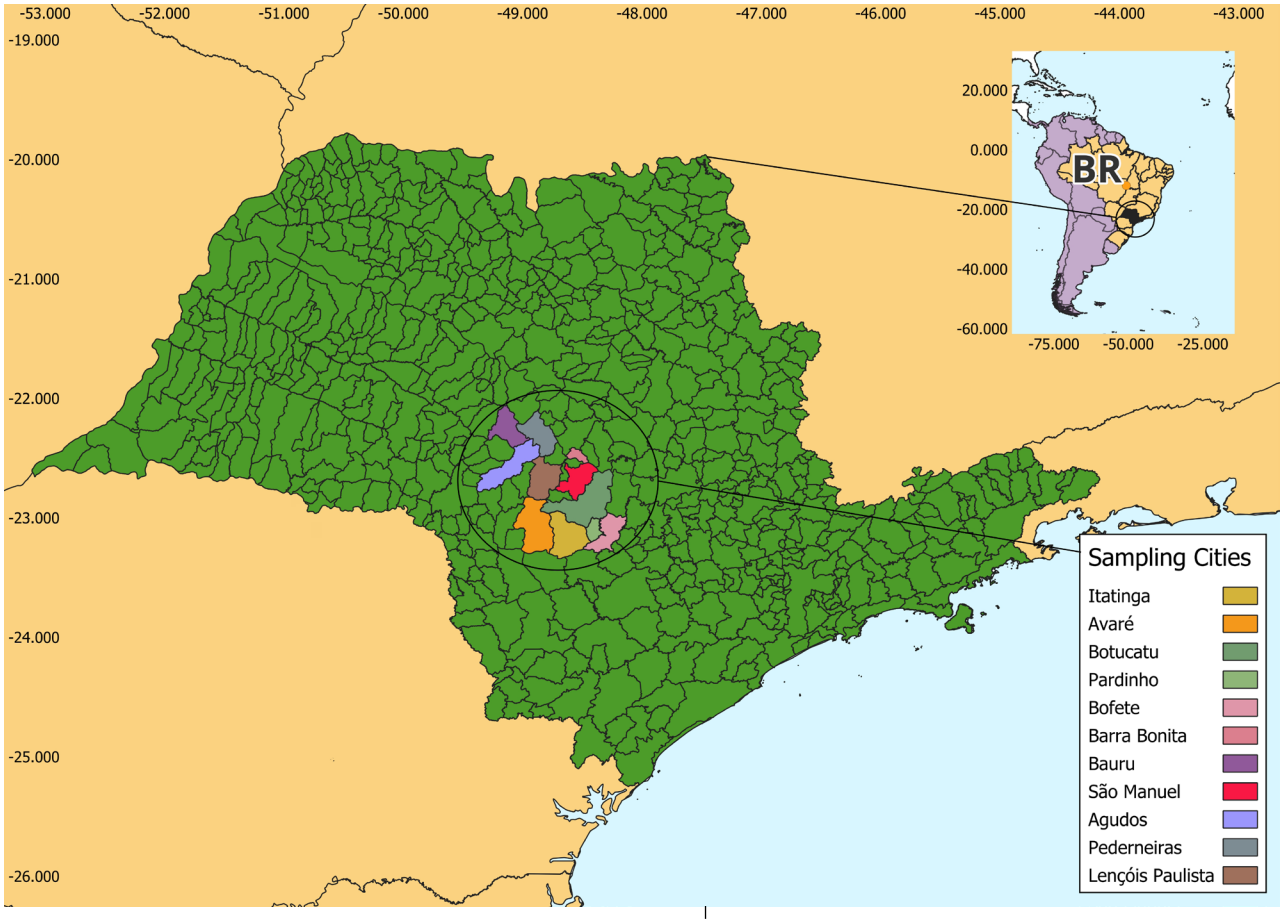

Figure 2. Map of the state of São Paulo, highlighting the 11 municipalities from where the açaí ice cream samples were purchased: Agudos, Avaré, Barra Bonita, Bauru, Bofete, Botucatu, Itatinga, Lençóis Paulista, Pardinho, Pederneiras, and São Manuel. The sample positive for *T. gondii* was acquired in São Manuel (marked in red).

There is insufficient information in the literature on the contamination of fresh fruits and vegetables by *T. gondii* oocytes. However, there is some evidence that these oocysts might be responsible for the contamination of foods and beverages, causing human infections (Hurtado et al., 2001; Dumètre & Dardè, 2003). A study conducted by Marchioro et al. (2016) detected *T. gondii* DNA in raw vegetables, more specifically 0.6% (1/62) in the samples of butterhead lettuce, 3.7% (4/106) in the samples of curly lettuce, 5.0% (2/40) in the samples of chicory, 14.3% (1/7) in the samples of arugula, and 20.0% (1/5) in the samples of parsley, indicating the consumption of fresh greens as an important source of human infection.

Toxoplasmosis has been recognized as an important threat to public health, due to the sequelae derived from parasitism, especially those associated with the ocular and congenital forms (Pereira et al., 2010).

The sale of açaí ice creams and other preparations in self-service dispensers is of particular concern because of the difficulty in determining the origin of the products and in inspecting commercial establishments by health authorities such as the Federal Inspection Service, and thus without the assurance of food safety.

The outbreaks of toxoplasmosis are common and require a careful analysis of the cases, their distribution and extension, to obtain clues about the source of infection. This knowledge is essential to formulate adequate preventive measures (Meireles et al., 2015). The molecular detection of *T. gondii* in an açai ice cream sample in this study serves as an alert of the need for further studies on this type of food, to elucidate the source of contamination.

5. CONCLUSION

The molecular detection of *T. gondii* was possible using the nPCR technique in a sample of açaí ice cream from a self-service shop in the municipality of São Manuel, which is a warning that epidemiological surveillance for this zoonosis should be continued, on this type of food, given that açaí ice cream is a widely consumed food by the population. The nPCR molecular technique, using the external primers TgNP1–TgNP2, was more efficient than cPCR with the Tox4/Tox5 primers in detecting the positive sample.

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