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Short Communication

Molecular investigation of *Toxoplasma gondii* in oysters (*Crassostrea* spp.) sold on beaches in the State of Pará, Brazil

Investigação molecular de *Toxoplasma gondii* em ostras (*Crassostrea* spp.) comercializadas em praias do estado do Pará, Brasil

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Abstract

The aim of this study was to detect *Toxoplasma gondii* DNA in oysters (*Crassostrea* spp.) sold on seven beaches in the State of Pará, Brazil. According to the National Program for Hygiene and Sanitary Control of Bivalve Mollusks, 100 g of the edible part of mollusks is required to analyze contaminating microorganisms. In this study, 12 oysters were assumed to be equivalent to 100 g of edible parts when preparing each pooled sample. In total, 360 oysters were purchased from 30 vendors. From groups of 12 oysters purchased per vendor, 60 pooled samples were obtained, comprising 30 gill tissues and 30 gastrointestinal tracts. For molecular analysis, *nested*-PCR was conducted to amplify a 155-base-pair product of the *B1* gene from *T. gondii*. All analyzed samples were negative for *T. gondii*. Our findings indicate that the oyster samples sold on the beaches in the State of Pará were not contaminated by *T. gondii*.

Keywords: Food safety, molecular biology, oysters, toxoplasmosis.

Resumo

O objetivo deste estudo foi detectar DNA de *Toxoplasma gondii* em ostras (*Crassostrea* spp.) comercializadas em sete praias do Estado do Pará, Brasil. De acordo com o Programa Nacional de Controle Higiênico Sanitário de Moluscos Bivalves, 100 g da parte comestível dos moluscos são necessários para a análise de microrganismos contaminantes. Neste estudo, 12 ostras foram consideradas equivalentes a 100 g de partes comestíveis na preparação de cada amostra agrupada. No total, 360 ostras foram compradas de 30 vendedores. De grupos de 12 ostras adquiridas por vendedor, foram obtidas 60 amostras agrupadas, compreendendo 30 tecidos branquiais e 30 tratos gastrointestinais. Para a análise molecular, a *nested* PCR foi realizada para amplificar um produto de 155 pares de bases do gene B1 de *T. gondii*. Todas as amostras analisadas foram negativas para *T. gondii*. Os resultados indicam que as amostras de ostras comercializadas em praias do Estado do Pará não foram contaminadas por *T. gondii*.

Palavras-chave: Segurança alimentar, biologia molecular, ostras, toxoplasmose.

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Introduction

Toxoplasma gondii is a protozoan capable of infecting several species of homeothermic animals, including humans (Tenter et al., 2000). This parasite affects up to one-third of the world's population, and 60% of toxoplasmosis infections are foodborne (Djurković-Djaković et al., 2019). The cultural practice of consuming raw or undercooked food is the main cause of infection; thus, toxoplasmosis has been classified as a foodborne disease with the greatest impact on public health (Hussain et al., 2017).

Toxoplasmosis is a neglected and endemic disease in several countries (Torgerson & Mastroiacovo, 2013), and epidemiological studies on the pathogen are crucial for reducing potential health risks. In humans, there is a high seroprevalence of *T. gondii*, and 80% of infected individuals remain asymptomatic for life. However, the development of the disease in immunocompromised individuals and pregnant women can cause serious morbidity, including ocular and neurological complications, as well as fatal outcomes (Shen et al., 2016).

Contamination of marine environments by *T. gondii* has been observed in several studies based on its presence in aquatic species known as "environmental sentinels." Studies on the sea otter (*Enhydra lutris kenyoni*) (Verma et al., 2018) and the Amazon River dolphin (*Inia geoffrensis*) (Santos et al., 2011) have suggested the contamination of the aquatic environment by *T. gondii* oocysts. Oocysts are eliminated through felid feces and transported by runoff to estuaries and coastal waters (Fayer et al., 2004), where they can infect marine animals. Santos et al. (2011) detected anti-*T. gondii* antibodies in the Amazon River dolphin (*I. geoffrensis*), demonstrating that the aquatic environment favors the propagation of this pathogen. Infections of marine animals may also result from their consumption of invertebrate species (Cole et al., 2000).

Bivalve mollusks can serve as hosts for transporting parasites. These aquatic organisms are a potential source of contamination as they can filter large volumes of water, increasing the likelihood of exposure to chemical and biological contaminants including bacteria, viruses, and other parasites, which can be accumulated and retained in bivalve tissues, making them useful bioindicators of environmental contamination. Furthermore, significant health risks may be incurred by individuals who consume raw or undercooked bivalve mollusk specimens (Evangelista-Barreto et al., 2008).

The presence of protozoans in oysters has been reported in several studies. In Southern Italy, protozoans were detected in oysters farmed under natural conditions (Putignani et al., 2011). Likewise, there have been reports of protozoan contamination in oysters farmed in Brazil, in southern Bahia (Ribeiro et al., 2015), Pará (Monteiro et al., 2019), and Maranhão (Silva et al., 2020), as well as in oysters sold in the fish market in São Paulo (Esmerini et al., 2010). However, no studies have focused on screening for *T. gondii* in oysters sold on the beaches in the Amazon region.

It is necessary to investigate the presence of pathogenic agents in oysters, owing to the increased farming of these animals in Brazil, representing 10.3% of mollusks farmed and destined for human consumption (Pereira & Rocha, 2015). The State of Pará has propitious features for oyster farming because it is surrounded by extensive mangrove areas where oysters are both farmed and subjected to extractive farming (Sampaio et al., 2017) for human consumption.

Because of the consumer preference for *in natura* oysters and the current lack of standardized methods for detecting the parasite in bivalve mollusks, control measures should be implemented to monitor the presence of these protozoans throughout the food chain. This would facilitate the accurate assessment of risks and ensure the safety of food for human consumption. The present study was conducted to screen for the presence of *T. gondii* DNA in oysters sold on beaches in the State of Pará.

Material and Methods

Study area

The study was carried out between February 2017 and July 2018 on the beaches belonging to the municipalities of Bragança, Marapanim, and Salinópolis located in the Mesoregion of Northeastern Pará and Barcarena, Mosqueiro, and Belém in the Metropolitan Mesoregion of Belém in the State of Pará, Brazil (Figure 1).

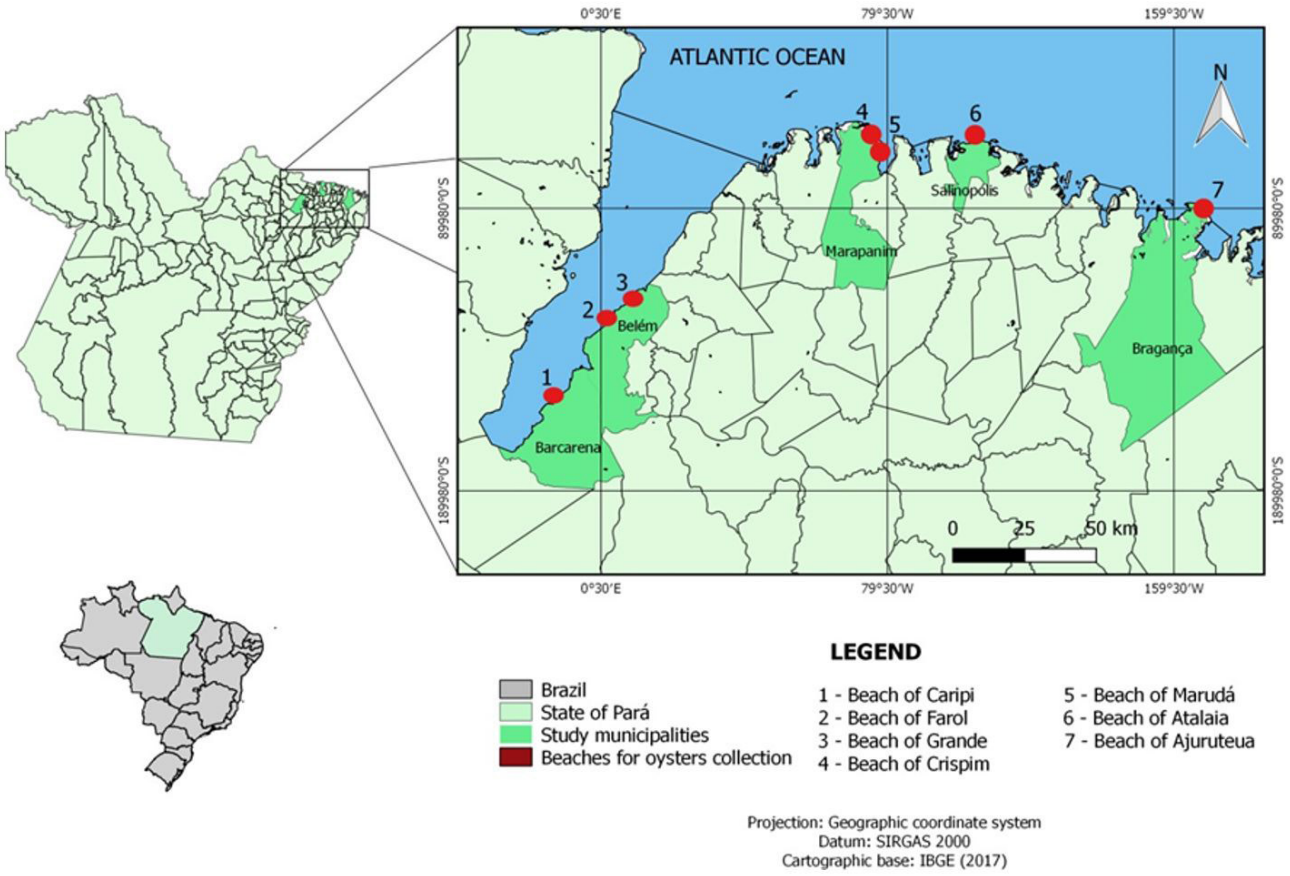

Figure 1. Map of beaches in the State of Pará, Brazil where oysters are sold. Points 1–3 are estuary channels (river beaches), and points 4–7 are along the coast of the Atlantic Ocean (sea beaches).

Sample

The number of vendors and oyster samples varied according to the location, as shown in Table 1. There were no official records of street vendors in terms of health surveillance at each collection point. Twelve oysters were purchased from each vendor, providing a pool of gill tissues and gastrointestinal tracts. In total, 360 oysters were purchased from 30 vendors. The samples were identified, stored in plastic bags, and transported on ice in an isothermal box to the Zoonoses and Public Health Laboratory of the Federal University of Pará for further processing.

Table 1. Number of oysters collected per beach, according to the origin, and tissues used for *T. gondii* DNA search.

Study area (Beaches)	Nº Vendors	Origin	Nº Oysters	Pool		
				Gills	GIT	Nº
Caripi	1	Farming	12	1	1	2
Ajuruteua	3	Farming	36	3	3	6
Atalaia	11	Farming/Extractive farming	132	11	11	22
Crispim	2	Farming	24	2	2	4
Marudá	4	Farming	48	4	4	8
Farol	3	Farming	36	3	3	6
Praia Grande	6	Farming	72	6	6	12
Total	**30**		**360**	**30**	**30**	**60**

Sample processing

The oyster specimens were removed from their bags, washed with running water to remove any dirt adhering to the outside, and then opened with a knife. Using sterile forceps and scissors, the soft part of the oyster was removed. A mass of 100 g is necessary for testing according to methods used for microbiological analyses of bivalve mollusks (Brasil, 2012). In the present study, based on the fact that 12 oysters were found to be equivalent to 100 g of edible parts, the gill tissues and gastrointestinal tracts were collected and pooled separately from groups of 12 oysters, resulting in 30 pooled samples of each tissue type and 60 pooled samples in total (Table 1). The samples were then placed in sterile microtubes and stored in a freezer at -20 °C until DNA extraction.

DNA extraction

The Norgen® stool extraction kit (Biotek Corp.) and IllustraTM Tissue and Cell Prep Mini Spin kit (GE Healthcare, Little Chalfont, UK) were used to extract DNA from the samples as described by Monteiro et al. (2019).

Molecular diagnosis

Nested-PCR analysis was performed for each sample using the following sets of primers to amplify an internal 155-base-pair DNA fragment of the *B1* gene in *T. gondii* (Yai et al., 2003): Toxo1 (5′-AGC GTC TCT CTT CAA GCA GCG TA-3′), Toxo 2 (5′- TCC GCA GCG ACT TCT ATC TCT GT-3′), Toxo 3 (5′-TGG GAA TGA AAG AGA CGC TAA TGT G-3′), and Toxo 4 (5′-TTA AAG CGT TCG TGG TCA ACT ATC G-3′). Feces containing *T. gondii* oocysts were used as the positive control and were provided by the Laboratory of Parasitic Diseases, USP Faculty of Veterinary Medicine and Zootechnics. Ultrapure water was used as the negative control. The molecular analyses were performed as per the methods described by Monteiro et al. (2019).

Results and Discussion

Analysis of the 60 pooled oysters revealed that *T. gondii* DNA was absent in all the samples: both from gill tissues and gastrointestinal tracts. The results of this study are similar to those of Tei et al. (2016) on bivalve mollusks collected from a beach in Orlando (USA). These findings suggest the absence of aquatic environment contamination by *T. gondii* oocysts. Although the oysters used in this study were sourced from different farms and extraction areas, most samples were obtained from estuaries in the municipality of Curuçá. In this area, oyster farmers received technical assistance and guidance concerning the production of bivalve mollusks (Hoshino, 2009), and were made aware of the sanitary measures for the cultivation of oysters suitable for human consumption.

Several factors influence the contamination of bivalve mollusks by *T. gondii*, and these may be attributable for the absence of parasite DNA in the present study. These include the presence of felids in the environment, the impact of human activity on the natural habitat, as well as rains, which increase the movement of contaminants. All these factors have been reported to affect the prevalence and dissemination of the protozoan across rivers and seas (Silva et al., 2020).

The environment in which oysters are grown reflects the quality of the water in which these animals are produced, as mollusks are considered as bioindicators of environmental contamination (Evangelista-Barreto et al., 2008). Therefore, the absence of protozoan DNA in the study samples indicates that *T. gondii* oocyst contamination of estuaries in coastal areas of Pará is low. This may be due to the geographical distance of this estuarine area from surrounding urban areas. Such estuarine areas have negligible human and feline interference, with no major impacts from human actions and absence of felines on the estuary margins, which consequently reduces the possibility of environmental contamination. This suggests that the oysters sold on the beaches of Pará are free from *T. gondii* contamination, making them harmless and fit for consumption.

Factors related to the size of the analyzed samples can affect the results obtained (Ribeiro et al., 2015). Considering that there is no specific manual for the analysis of protozoa in mollusks, the present study was adapted from the National Program for Hygienic-Sanitary Control of Bivalve Mollusks (Brasil, 2012), which aims to guarantee the safety and quality of mollusks intended for human consumption. Thus, 100 g of the edible part of the oysters collected from vendors was sampled, which differs from other studies in which samples were directly collected from the crop.

Studies on bivalve mollusks are relevant in terms of aquatic environment contamination considering that these organisms obtain nutrients from filtration. Some studies have reported a low occurrrence of *T. gondii* DNA

in oysters (Esmerini et al., 2010; Ribeiro et al., 2015; Monteiro et al., 2019; Silva et al., 2020). Monteiro et al. (2019) detected *T. gondii* in oysters from northeastern Pará. As this region is still poorly studied, the absence of *T. gondii* as described in the present study is a valuable addition to the epidemiological data of this protozoan concerning oyster farming in the Amazon region.

In Brazil, the National Program for Hygiene and Sanitary Control of Bivalve Mollusks evaluates only microbiological parameters and does not include research on protozoans. Considering the growth of oyster farming in the State of Pará, through the production of cultivated oysters and extractive farming (Pereira & Rocha, 2015; Sampaio et al., 2017), as well as the results showing the identification of *T. gondii* in oysters in northeastern Pará (Monteiro et al., 2019), we recommend testing for *T. gondii* as a standard practice. Further research and analyses are essential to formulate guidelines regarding the risks of consuming *in natura* oysters.

The samples evaluated in this study were not contaminated by *T. gondii*; however, considering the lack of standardized tests for the detection of *T. gondii* in bivalve mollusks, to reduce or eliminate the risks of consuming oysters *in natura*, further studies are needed to investigate the role of oysters in the transmission of this protozoan.

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