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Analysis of marine bivalve shellfish from the fish market in Santos city, São Paulo state, Brazil, for *Toxoplasma gondii*

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ABSTRACT

The aim of this study was to determine if *Toxoplasma gondii* are present in oysters (*Crassostrea rhizophorae*) and mussels (*Mytella guyanensis*) under natural conditions using a bioassay in mice and molecular detection methods. We first compared two standard protocols for DNA extraction, phenol–chloroform (PC) and guanidine-thiocyanate (GT), for both molluscs. A total of 300 oysters and 300 mussels were then acquired from the fish market in Santos city, São Paulo state, Brazil, between March and August of 2008 and divided into 60 groups of 5 oysters and 20 groups of 15 mussels. To isolate the parasite, five mice were orally inoculated with sieved tissue homogenates from each group of oysters or mussels. For molecular detection of *T. gondii*, DNA from mussels was extracted using the PC method and DNA from oysters was extracted using the GT method. A nested-PCR (Polymerase Chain Reaction) based on the amplification of a 155 bp fragment from the B1 gene of *T. gondii* was then performed. Eleven PCR–RFLP (Restriction Fragment Length Polymorphism) markers, SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, CS3 and Apico, were used to genotype positive samples. There was no isolation of the parasite by bioassay in mice. *T. gondii* was not detected in any of the groups of mussels by nested-PCR. DNA of *T. gondii* was apparently detected by nested-PCR in 2 groups of oysters (3.3%). Genotyping of these two positive samples was not successful. The results suggest that oysters of the species *C. rhizophorae*, the most common species from the coast of São Paulo, can filter and retain *T. gondii* oocysts from the marine environment. Ingestion of raw oysters as a potential transmission source of *T. gondii* to humans and marine mammals should be further investigated.

1. Introduction

Toxoplasmosis is one of the most common parasitic infections of man and other warm-blooded animals (Dubey and Beattie, 1988; Remington et al., 1995). Felids are both intermediate and definitive hosts for this parasite because they excrete oocysts of *Toxoplasma gondii* in the faeces (Dubey and Beattie, 1988). Sporulated oocysts of *T. gondii* can retain infectivity for at least 18 months in soil (Frenkel et al., 1975). Humans and terrestrial animals have been infected after exposure to sporulated oocysts in contaminated soil or fresh water (Bowie et al., 1997; Aramini et al., 1999; Tenter et al., 2000) and this type of transmission has been documented in Brazil (Bahia-Oliveira et al., 2003; De Moura et al., 2006).

T. gondii also infects marine mammals. Seroprevalence of the parasite in different groups of marine mammals, such as cetaceans, pinnipeds and sirenians, including the southern sea otter (*Enhydra lutris nereis*), suggests world-wide contamination of the marine environment, as reviewed by Fayer et al. (2004). In Brazil, reports of *T. gondii* seroprevalence in marine mammals are very limited but also indicate this agent is circulating in Brazilian marine waters. Silva et al. (personal communication)

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examined 12 Brazilian antillean manatees (*Trichechus manatus manatus*) and found one seropositive animal (8.3%). This was the first report of anti-*T. gondii* antibodies in this animal species. Toxoplasmic encephalitis has been recognised as a primary disease of sea otters and other marine mammals (Cole et al., 2000; Miller et al., 2001; Kreuder et al., 2003).

Infected marine animals rarely consume recognised intermediate hosts. This suggests an exposure to *T. gondii* oocysts. Cole et al. (2000) suggested that marine mammals could be infected by ingesting invertebrates which could act as phoretic agents for *T. gondii* oocysts. Oocysts would enter the marine environment through storm runoff (Miller et al., 2002a) or sewage (Fayer et al., 2004) and would be concentrated by filter-feeding invertebrates, such as bivalve shellfish, consequently serving as a source of infection for marine animals when consumed as food items (Cole et al., 2000; Lindsay et al., 2001; Arkush et al., 2003).

Experimental studies have shown that eastern oysters (*Crassostrea virginica*) can remove *T. gondii* oocysts from seawater and that the oocysts retain their infectivity in mice for at least 6 days after capture by the oysters (Lindsay et al., 2001). Later, it was observed that oocysts may remain viable for up to 85 days in these oysters (Lindsay et al., 2004). Lindsay et al. (2003) observed that *T. gondii* oocysts can sporulate in seawater and subsequently infect intermediate hosts. They also reported that oocysts can survive for at least 6 months in seawater. The specific small subunit ribosomal RNA (ssrRNA) of *T. gondii* could be detected for up to 21 days after exposure in artificially exposed mussels (*Mytilus galloprovincialis*), but viable oocysts were detected for only 3 days (Arkush et al., 2003).

In this study, we attempted to isolate and molecularly detect *T. gondii* in bivalve marine molluscs (oysters and mussels) from a commercial source in Santos city, São Paulo state, Brazil.

2. Materials and methods

2.1. Marine bivalve shellfish

Oysters (*Crassostrea rhiziphorae*) and mussels (*Mytella guyanensis*) were acquired each week from a fish market in Santos city, São Paulo state. The bivalves at this market originated from Cananéia region, in the same state. Initially, groups of 15 mussels and 10 oysters were used, but this was later modified to groups of 5 oysters due to the thickness and volume of the oyster tissue. For the standardisation phase of the experiment, which is described below, shellfish were collected from 01/Jan/2007 to 29/Feb/2008 and for the isolation and detection experiments, shellfish were collected from 01/Mar/2008 to 29/Aug/2008.

Bivalve outer shell surfaces were washed with distilled water. Oyster or mussel tissues and the enclosed liquid from each group were removed and placed in 500 ml beakers, homogenised with distilled water using a mixer

Table 1
Distribution of mussel, oyster and control groups according to oocyst concentration of *Toxoplasma gondii* used to contaminate shellfish tissue homogenates during the standardisation phase.

Groups	Identification	Number of shellfish	Oocyst concentration used	Number of mice inoculated
Control[a]	G1	0	0.5×10^4	5
	G2	0	0.5×10^4	5
	G3	0	10^3	5
	G4	0	10^3	5
	G5	0	10^2	5
	G6	0	10^2	5
	G7	0	10^1	5
	G8	0	10^1	5
Mussel	G1	15	0.5×10^4	5
	G2	15	0.5×10^4	5
	G3	15	10^3	5
	G4	15	10^3	5
	G5	15	10^2	5
	G6	15	10^2	5
	G7	15	10^1	5
	G8	15	10^1	5
	G9	15	0	5
Oyster	G1	10	0.5×10^4	5
	G2	10	0.5×10^4	5
	G3	10	10^3	5
	G4	10	10^3	5
	G5	10	10^2	5
	G6	10	10^2	5
	G7	10	10^1	5
	G8	5	0.5×10^4	5
	G9	5	10^3	5
	G10	5	10^2	5
	G11	5	10^1	5
	G12	5	0	5

[a] Only *T. gondii* oocysts.