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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.

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### 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 worldwide 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 rhizophorae*) 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 <sup>a</sup>	G1	0	$0.5 \times 10^4$	5
	G2	0	$0.5 \times 10^4$	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	$0.5 \times 10^4$	5
	G2	15	$0.5 \times 10^4$	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	$0.5 \times 10^4$	5
	G2	10	$0.5 \times 10^4$	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	$0.5 \times 10^4$	5
	G9	5	$10^3$	5
	G10	5	$10^2$	5
	G11	5	$10^1$	5
	G12	5	0	5

<sup>a</sup> Only *T. gondii* oocysts.

and filtered through cheesecloth. Filtered material was pelleted by centrifugation ( $1000 \times g$ ,  $4^\circ\text{C}$ , 10 min) and then washed twice with TE buffer (10 mM Tris–HCl pH 8.0; 1 mM EDTA pH 8.0). The homogenates from each group of shellfish were then used for the bioassay in mice and the molecular analysis. Five aliquots of 1.5 ml were stored at  $-70^\circ\text{C}$  until DNA extraction.

For standardisation, tissues were contaminated with different concentrations of *Toxoplasma gondii* oocysts (Table 1), before homogenisation.

## 2.2. Standardisation phase

Sensitivity of the bioassay in mice, PCR protocols and efficacy of two DNA extraction protocols were first studied.

### 2.2.1. *T. gondii* oocysts

Two 2-month-old cats that tested seronegative for *T. gondii* by indirect fluorescent antibody test (IFAT  $<1:16$ ) were inoculated with *T. gondii* tissue cysts by ingestion of one infected mouse carcass for each cat. The mice had been infected 2 months earlier by inoculating them with oocysts of the ME 49 strain of *T. gondii*. Faecal samples from inoculated cats were daily examined for *T. gondii* oocysts, using a standard sucrose flotation technique. Oocysts were excreted from 5 to 9 days post-inoculation (PI). For sporulation, faecal samples containing oocysts were mixed with 2%  $\text{K}_2\text{Cr}_2\text{O}_7$  in Petri dishes for 5 days at  $25^\circ\text{C}$ . Sporulated oocysts in faecal material were sieved through a series of strainers (60, 100, 200 and 400 mesh) using tap water. After 24 h, the supernatant of the filtrate was discarded, and the sediment was distributed into 50 ml tubes and washed twice with distilled water by centrifugation ( $1000 \times g$ , 10 min). The number of oocysts was counted using the Neubauer chamber and concentrations of  $0.5 \times 10^4$ ,  $10^3$ ,  $10^2$  and  $10^1$  oocysts were calculated as well as used to test the sensitivity of the bioassay and molecular detection methods.

### 2.2.2. Bioassay in mice

Contaminated tissue homogenates from shellfish were orally inoculated (500  $\mu\text{l}$  per mouse) into five mice per group, using an animal feeding needle (Table 1). Swiss, outbred mice from the breeding colony at the Faculty of Veterinary Medicine, University of São Paulo were used. Imprints of lungs and brains from mice that died were examined for *T. gondii* tachyzoites or tissue cysts (Dubey and Beattie, 1988). Survivor mice were bled from the retroorbital plexus 8 weeks after being fed shellfish tissue. The sera were examined at dilutions of 1:16 in the IFAT for *T. gondii*. Mice were euthanised by cervical dislocation after bleeding and their brains were examined microscopically for tissue cysts (Dubey and Beattie, 1988).

### 2.2.3. DNA extraction from shellfish sieved tissue homogenates

To avoid amplicon contamination, PCR, nested-PCR and electrophoresis were each performed in a different room. Non-disposable materials were sterilised.

For oysters and mussels, 500  $\mu\text{l}$  samples of all stored aliquots were used for DNA extraction for each of the two

protocols tested. First, samples were washed three times in TE buffer ( $15,000 \times g$ , 5 min) and submitted to five freeze ( $-192^\circ\text{C}$ , 1 min) thaw ( $65^\circ\text{C}$ , 2 min) cycles.

The phenol–chloroform (PC) method used as described by Pena et al. (2006) and was based on Ausubel et al. (1999) protocols. Briefly, samples were digested by overnight incubation at  $37^\circ\text{C}$  in lysis buffer (10 mM Tris–HCl, pH 8.0; 100 mM NaCl; 25 mM EDTA; 1% SDS; 400  $\mu\text{g}/\text{ml}$  proteinase K). DNA was extracted via phenol, phenol–chloroform and chloroform steps, and then precipitated with two volumes of 100% ethanol. Following overnight incubation at  $-20^\circ\text{C}$  and centrifugation, each pellet was resuspended in 30  $\mu\text{l}$  of TE buffer, incubated for 10 min at  $56^\circ\text{C}$  and stored at  $-20^\circ\text{C}$  until amplification.

The guanidine-thiocyanate (GT) method was performed according to a modification (Sangioni et al., 2005) of a previously described protocol (Chomkzynski, 1993). Briefly, samples were digested with GT (5 mol/l) for 10 min at room temperature. DNA was then extracted via a chloroform step and, after centrifugation, the supernatant was precipitated with 600  $\mu\text{l}$  of 100% isopropanol. Following overnight incubation at  $-20^\circ\text{C}$  and centrifugation, each pellet was resuspended in 800  $\mu\text{l}$  of ethanol 70% and centrifuged again. Supernatant was discarded and the pellet was dried at room temperature, resuspended with 30  $\mu\text{l}$  of TE buffer, incubated for 15 min at  $56^\circ\text{C}$  and stored at  $-20^\circ\text{C}$  until used.

### 2.2.4. PCR and nested-PCR

Nested-PCR targeted the amplification of a 155 bp fragment from the B1 gene of *T. gondii* (Burg et al., 1989). The primers used for PCR were T1-Fext, AGCGTCTCTT-CAAGCAGCGTA and T2-Rext TCCGACGGACTTCTATC-TCTGT; the primers used for nested-PCR were T3-Fint, TGGGAATGAAAGAGACGCTAATGTG and T4-Rint, TTAAAGCGTTCGTGGTCAACTATCG (Yai et al., 2003).

Reactions consisted of 10 mM Tris–HCl, 50 mM KCl, 50 mM  $\text{MgCl}_2$ , 10 pmol/ $\mu\text{l}$  of each primer, 200  $\mu\text{M}$  of each deoxynucleotide triphosphate and 1.5 U Taq DNA polymerase. As a template, 5  $\mu\text{l}$  of extracted DNA for the first reaction and 5  $\mu\text{l}$  of the amplified product for the second reaction were added in a final volume of 50  $\mu\text{l}$ . All the amplifications were performed in an Eppendorf Mastercycler<sup>®</sup> gradient thermal cycler. The cycling conditions were an initial denaturation at  $94^\circ\text{C}$  for 3 min, followed by 25 cycles of  $94^\circ\text{C}$  for 45 s,  $55^\circ\text{C}$  for 60 s,  $72^\circ\text{C}$  for 90 s and a final extension at  $72^\circ\text{C}$  for 10 min; for nested-PCR, initial denaturation was followed by 35 cycles. The final amplified DNA was analysed by electrophoresis in 2% agarose gels and stained with ethidium bromide followed by visualisation under ultraviolet light. Each batch of assays included, as a positive control template, DNA extracted from the RH strain of *T. gondii* and at least two water negative control templates.

## 2.3. Experimental phase

### 2.3.1. Marine bivalve shellfish

A total of 300 oysters were distributed into 60 groups of 5 oysters and 300 mussels were distributed into 20 groups of 15 mussels. Samples were calculated assuming a *T.*

*gondii* prevalence of 1% (95% confidence interval) and oyster or mussel populations of >100,000 (Thrusfield, 1995).

### 2.3.2. Bioassay in mice

Experimental design was the same as the one used during the standardisation phase.

### 2.3.3. DNA extraction from shellfish sieved tissue homogenates

The PC method was used to extract DNA from mussel tissue homogenates and the GT method was used to extract DNA from oyster tissue homogenates. Both protocols were as described above.

### 2.3.4. PCR and nested-PCR

PCR and nested-PCR protocols used were the same described above in the standardisation phase.

### 2.3.5. Sequencing

Positive nested-PCR products were eluted from agarose gel using a clean-up system (Illustra™ GFX<sup>®</sup>, GE Healthcare, UK). The purified PCR products were sequenced in both directions using the internal primers and the ABI PRISM<sup>®</sup> Big Dye<sup>®</sup> Terminator v3.1 sequencing kit (Applied Biosystems, USA). Sequencing products were analysed on an ABI377 automated sequencer. Derived sequences were aligned using the BioEdit v7.0.9.0 (Hall, 1999) and submitted to a BLAST search (BLASTN, www.ncbi.nlm.nih.gov/BLAST).

### 2.3.6. Genotyping of *T. gondii*

Genotyping of positive samples was attempted using PCR-RFLP genetic markers SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico, as previously described (Su et al., 2006; Dubey et al., 2007). DNA previously extracted was used together with new DNA extractions to confirm positive results.

## 3. Results

### 3.1. Standardisation phase

The sensitivity of the bioassay in mice to isolate *Toxoplasma gondii* in shellfish tissue homogenates experimentally contaminated with oocysts is shown in Table 2. Parasites were isolated from mussel groups at  $0.5 \times 10^4$ ,  $10^3$  and  $10^2$  oocyst concentrations and from oyster groups at  $0.5 \times 10^4$  and  $10^3$  oocyst concentrations. *T. gondii* was isolated at  $0.5 \times 10^4$ ,  $10^3$ ,  $10^2$  and  $10^1$  concentrations from control groups.

*T. gondii* DNA could be detected by nested-PCR from mussel groups at  $0.5 \times 10^4$  and  $10^3$  oocyst concentrations when the PC protocol was used and could not be detected using the GT protocol. Parasite DNA could be detected from oyster groups only at  $0.5 \times 10^4$  oocyst concentration when the PC protocol was used and was also detected at  $0.5 \times 10^4$ ,  $10^3$  and  $10^2$  using the GT protocol. In the control groups, *T. gondii* DNA was detected at  $0.5 \times 10^4$ ,  $10^3$  and  $10^2$  with both extraction protocols (Table 3).

**Table 2**

Isolation of *Toxoplasma gondii* from shellfish tissue homogenates experimentally contaminated with oocysts by bioassay in mice.

Oocyst concentration	Group (G) <sup>a</sup>		
	Mussel	Oyster	Control <sup>b</sup>
$0.5 \times 10^4$	4 (G1)	3 (G1)	5 (G1)
	4 (G2)	1 (G2) 5 (G8)	5 (G2)
$10^3$	1 (G3)	0 (G3)	5 (G3)
	3 (G4)	1 (G4) 5 (G9)	5 (G4)
$10^2$	0 (G5)	0 (G5)	4 (G5)
	3 (G6)	0 (G6) 0 (G10)	4 (G6)
$10^1$	0 (G7)	0 (G11)	4 (G7)
	0 (G8)		0 (G8)
0	0 (G9)	0 (G12)	nd

Nd: not done.

<sup>a</sup> No. of mice *T. gondii* positive of five mice inoculated.

<sup>b</sup> Only oocysts.

**Table 3**

*Toxoplasma gondii* DNA detection by nested-PCR from shellfish tissue homogenates experimentally contaminated with oocysts.

Oocyst concentration	Group <sup>a</sup>					
	Mussel		Oyster		Control <sup>b</sup>	
	PC <sup>c</sup>	GT <sup>d</sup>	PC	GT	PC	GT
$0.5 \times 10^4$	5/8	0/8	1/11	7/11	2/2	2/2
$10^3$	2/8	0/8	0/11	5/11	2/2	2/2
$10^2$	0/5	0/5	0/8	3/8	2/2	2/2
$10^1$	0/5	0/5	0/2	0/2	0/2	0/2
0	0/5	0/5	0/5	0/5	nd	nd

Nd: not done.

<sup>a</sup> Number of 500  $\mu$ l positive aliquots/number of examined aliquots.

<sup>b</sup> Only oocysts.

<sup>c</sup> Phenol–chloroform DNA extraction protocol.

<sup>d</sup> Guanidine–thiocyanate DNA extraction protocol.

### 3.2. Experimental phase

*T. gondii* was not isolated from the 60 groups of oysters or the 20 groups of mussels by bioassay in mice. Three mice from different shellfish groups died at 20, 32 and 33 days, respectively PI from unidentified causes. All survivor mice were negative at IFAT (titers <1:16) and no parasites were found in their tissues.

*T. gondii* DNA was not detected in any of the 20 groups of mussels using nested-PCR. Five 500  $\mu$ l aliquots from each group were examined.

*T. gondii* DNA was detected from 2 of the 60 groups of oysters examined, corresponding to 3.3% of the oyster groups. Five 500  $\mu$ l aliquots from each group were examined. In these positive samples, only one of the five aliquots examined was found to be positive.

Sequencing was only successful with one of the two positive samples. The nested-PCR-B1 sequences from this sample revealed 100% identity with a homologous sequence of *T. gondii* (they were identical to sequence gbAF179871, Burg et al., 1989). Examination of different aliquots and changes in the protocol, using high volumes of

DNA, were attempted with the other samples, but a sequence of good quality was not obtained.

Attempts to genotype the two positive samples were not successful for any of the genetic markers used. There was no DNA amplification. Changes in the protocol, such as using high volumes of DNA and performing PCR not as a multiplex, but using each marker individually, were attempted without positive results.

#### 4. Discussion

Faeces from humans and animals contaminated with encysted protozoan parasites can be carried into coastal waters. *Giardia* cysts and *Cryptosporidium* oocysts have been found in marine waters and shellfish worldwide as reviewed by Fayer et al. (2004). *Toxoplasma gondii* oocysts have not been reported in coastal waters, but studies showing uncommon infection of the threatened California southern Sea Otter provided evidence of contamination of the coastal waters with this pathogen. These studies also provided evidence for land-based surface runoff as a source of *T. gondii* infection for this marine mammal (Thomas and Cole, 1996; Miller et al., 2002a, 2002b; Kreuder et al., 2003).

Molluscan shellfish such as oysters, mussels, cockles and clams can concentrate pathogenic microorganisms as a result of filtering large volumes of water (Trollope, 1984). Examination of bivalves can demonstrate faecal pollution even when water testing produces negative results (Ayres et al., 1978). Laboratory studies have already shown that marine bivalves can concentrate *T. gondii* oocysts, but few surveys of *T. gondii* in wild invertebrates have been reported.

The investigated shellfish, *Crassostrea rhizophorae* and *Mytella guyanensis*, are both very common along the Brazilian marine coast and are commercially important. In the present study, we demonstrated for the first time that the oyster *C. rhizophorae* can harbour *T. gondii* in its tissues. Experimentally, the *C. virginica* had been reported to be capable of removing *T. gondii* oocysts from seawater (Lindsay et al., 2004). In the present investigation, 2 out of 60 groups of oysters were positive by nested-PCR but not by bioassay in mice. Only one of the five aliquots tested was positive in both cases and the signals observed in the electrophoresis were weak, which suggests low concentration of *T. gondii* DNA in the samples. The amount of material orally administered to each mouse was small and perhaps oocysts were not sporulated or viable in these positive samples. It is difficult to determine if the samples were non-infectious. Previous studies have indicated that *T. gondii* can sporulate and remain viable in seawater for several months (Lindsay et al., 2003). For oyster groups, it was observed that sensitivity of isolation by bioassay in mice was  $10^3$  oocysts and sensitivity of detection by nested-PCR was  $10^2$  oocysts.

In the present study, *T. gondii* was not isolated by bioassay in mice or molecularly detected from the 20 mussel groups. It was observed that sensitivity of isolation by bioassay in mice was  $10^2$  oocysts and sensitivity of detection by nested-PCR was  $10^3$  oocysts. These mussels were primarily obtained from the same region as the

oysters. There are no experimental studies about *Mytella* spp. as a phoretic agent for protozoan parasites. More comprehensive studies will be necessary to verify if this mussel can remove and retain *T. gondii* oocysts from seawater. Arkush et al. (2003) detected *T. gondii*-specific ssrRNA in experimentally exposed mussels (*Mytilus galloprovincialis*) and confirmed samples remained bioassay-positive for at least 3 days post-exposure. Recently, Miller et al. (2008) sampled 1396 marine and estuarine invertebrates and found a TaqMan positive bay mussel. Multilocus PCR-RFLP analyses and DNA sequencing performed at the B1 and SAG1 loci identified a Type X strain of *T. gondii* previously isolated from southern sea otters (Miller et al., 2004).

In this study, there was no success in genotyping the two positive oyster samples using the set of markers described by Su et al. (2006). Nested-PCR-B1 gel bands suggested a low concentration of DNA. Comparison between genotypes from the marine environment with terrestrial animals would clarify routes and mechanisms of land-to-sea transmission (Fayer et al., 2004). In Brazil, there are no reports of isolation of *T. gondii* from marine mammals and even the serologic data are very limited.

During the standardisation phase, it was observed that the phenol-chloroform extraction protocol was more efficient for mussel tissues and the guanidine-thiocyanate extraction protocol was more efficient for oyster tissues, probably because of specific inhibition factors in tissues of these shellfish that could be better investigated.

In this study, mussels and oysters were processed in groups, not individually, because there is very limited data about the presence of *T. gondii* in the Brazilian marine environment and also for economic reasons. Future studies will consider processing each shellfish separately, as a potential means of increasing detection sensitivity.

This preliminary study indicates 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 along the coast of São Paulo. Ingestion of raw oysters as a potential transmission source of *T. gondii* to humans and marine mammals should be further investigated.

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