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Detection of *Toxoplasma gondii* DNA in Brazilian oysters (*Crassostrea rhizophorae*)

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ABSTRACT. The aim of this study was to detect evidence of *Toxoplasma gondii* using polymerase chain reaction (PCR)-based techniques in oysters (*Crassostrea rhizophorae*) obtained from the southern coastal region of Bahia, Brazil. A total of 624 oysters were collected, and the gills and digestive glands were dissected. Each tissue sample was separated into pools containing tissues (of the same type) from three animals, leading to a total of 416 experimental samples for analysis (208 samples each from the gills and digestive glands). Molecular analysis using PCR-based detection of the *T. gondii* AF 146527 repetitive fragment yielded negative results for all samples. However, when nested-PCR was used for detection of the *T. gondii*

SAG-1 gene, 17 samples were positive, with the gills being the tissue with maximal detection of the parasite. These positive results were confirmed by sample sequencing. It is therefore suggested that *C. rhizophorae* oysters are capable of filtering and retaining *T. gondii* oocysts in their tissue. This represents a risk to public health because they are traditionally ingested *in natura*.

Key words: Bivalve mollusks; Oyster contamination; *SAG-1*; Toxoplasmosis

INTRODUCTION

Toxoplasma gondii, a protozoan of high zoonotic importance, utilizes felids as its definitive host. Felids are the only animals capable of eliminating oocysts through their feces, whereas other homeothermic animals are considered to be intermediate hosts (Dubey, 2010). For immunologically healthy humans, the parasite infection is generally asymptomatic and presents influenza-like symptoms (Frenkel, 1990; Tenter et al., 2000). The most serious outcome of infection is seen in women who are infected for the first time during pregnancy, where infection can trigger fetal abnormalities and spontaneous abortion (Dubey, 2010), and in immunosuppressed individuals, where it may be lethal (Dubey, 2004).

Homeothermic animals that can be infected by the parasite include several species of marine mammals such as seals, sea lions, dolphins (Dubey et al., 2003), and sea otters (Conrad et al., 2005). It is believed that infection at sea occurs through the ingestion of sporulated oocysts (Conrad et al., 2005); these oocysts are transported in water from sewer sand storm drains and via rainwater runoff (Cole et al., 2000; Miller et al., 2002; Fayer et al., 2004; Conrad et al., 2005). Shoals and bays near urban centers are the areas that are primarily exposed to the parasite (Miller et al., 2002), due to the increased fecal pollution in these areas.

Oocysts are considered to be resistant to the environment, are small (11-13 μm), and possess the ability to sporulate in water and become infectious to their hosts, surviving for a period of up to 6 months in seawater (Lindsay et al., 2003). Ingestion of a single oocyst is sufficient for the infection of animals such as pigs and mice (Dubey, 2004). Human infection caused by oocyst ingestion is usually more severe than that caused by the ingestion of tissue cysts, regardless of the dose (Jones and Dubey, 2010).

Bivalve mollusks, although not homeothermic, can act as a host carrier for *T. gondii* oocysts (Lindsay et al., 2004; Conrad et al., 2005). These animals utilize a filter-feeding process (Fayer et al., 2003, 2004), leading to the ingestion of waterborne pathogens from the environment; these pathogens are then stored in their tissues (Robertson, 2007; Leal and Franco, 2008).

T. gondii was detected in *Mytilus* shellfish under natural conditions in California (Miller et al., 2008) and in oysters (*Crassostrea gigas*) in a cultivation system in Lago di Varano, Southern Italy (Putignani et al., 2011). In Brazil, the parasite was identified in *C. rhizophorae* oysters that were destined for sale and consumption in the fish markets in São Paulo (Esmerini et al., 2010).

The Brazilian coastal region is characterized by the presence of extensive mangroves, and, in these areas, oysters are highly regarded both for their taste as well as their nutritional value (Nascimento and Pereira, 2004), with the *C. rhizophorae* mollusk being of greatest eco-

nomic interest, especially in Bahia (Nascimento et al., 1986; Boehs et al., 2010). The principal threat that these oysters pose is via ingestion because these are traditionally ingested *in natura*, representing a risk to public health. In this study, the presence of *T. gondii* DNA was investigated in *C. rhizophorae* oysters from the southern coast of Bahia, Brazil, using polymerase chain reaction (PCR) and nested-PCR techniques.

MATERIAL AND METHODS

Sample collection

The oyster (*C. rhizophorae*) samples used in this study were acquired on a monthly basis from two cities on the southern coast of Bahia: Ilhéus, with oysters harvested from the natural beds of the Cachoeira River estuary; and from the Porto Campo region in Camamu Bay, where samples were obtained from the longline cultivation system. Three hundred and twelve oysters were collected from each city (624 unique tissue samples in total). In the laboratory, the outer surfaces of the shells were washed with distilled water, and the gills and digestive glands were removed and randomly separated into pools of samples (of the same type) from three animals. Each pool was analyzed as a single experimental sample; in total, there were 416 such samples, with 208 samples of each tissue type. The samples were placed in cryotubes and frozen at -80°C until the DNA extraction process.

DNA extraction

The Easy-DNA extraction kit (Invitrogen, Carlsbad, CA, USA) was used for DNA extraction according to manufacturer guidelines, with some adaptations made to the process for breaking the oocyst wall.

Approximately 1 g sample from each tissue was used for DNA extraction. Samples were mixed with a solution containing 400 µL TE buffer (Tris-HCl EDTA, provided in the kit) and 1 g glass beads, and homogenized in a vortex for 2 min. Subsequently, samples were centrifuged for 10 min at 21,913 *g*. The resulting supernatant was transferred to a 2.0-mL microtube. For maximal extraction, an additional 150 µL TE buffer was added to the original microtube containing the glass beads and homogenized and centrifuged again as previously described. Next, 350 µL Solution A from the Easy-DNA extraction kit (Invitrogen) was added; the mixture was homogenized, and thermal shocks were administered (five cycles of freezing in liquid nitrogen for 15 s and thawing at 92°C for 2 min). Proteinase K (5 µL) was added and the tubes were incubated in a water bath at 65°C for 1 h, followed by addition of 150 µL Solution B. For positive control samples, oocysts from *T. gondii* were added to the oyster tissues and the extraction was performed as described above.

The extracted DNA was quantified using a NanoDrop (Thermo Scientific, Walther, MA, USA) spectrophotometer, and the purity was checked by estimating the A_{260}/A_{280} ratio. The samples that showed concentrations above 1000 ng/µL were diluted to 100 ng/µL for further analysis.

PCR amplification

PCR was performed using primers T4 (5'-CGC TGC AGG GAG GAA GAC GAA

AGT TG-3') and T5 (5'-CGC TGC AGA CAC AGT GCA TCT GGA TT-3') that amplified a 529-bp *T. gondii* repetitive sequence (GenBank accession No. AF 146527) (Homan et al., 2000). The reaction mixture included 5 µL extracted DNA as the template, and a 20-µL mixture [0.2 mM of each primer, 0.2 mM dNTPs (Invitrogen), 1X PCR buffer, 2.5 mM $MgCl_2$, and 2 U Taq DNA polymerase (Invitrogen)]. The PCR program for amplification of parasite DNA was as follows: 5 min at 94°C for initial denaturation; 33 cycles each of 1 min at 94°C for denaturation, 1 min at 60°C for annealing, and 1 min at 72°C for extension; and extension for 10 min at 72°C. The PCR products were subjected to electrophoresis on a 1% agarose gel and stained with SYBR® Safe DNA Gel Stain (Life Technologies, Carlsbad, CA, USA). DNA extracted from oyster tissue to which had been added *T. gondii* RH strain tachysoites was used as a positive control. For the negative control, ultrapure water was used. Positive and negative controls were included in each test.

Nested PCR amplification

Nested PCR for the amplification of a 329-bp region of the *SAG-1* gene of *T. gondii* was performed using the primary primers F1: 5'-GTT CTA ACC ACG CAC CCT GAG-3' and R1: 5'-AAG AGT GGG AGG CTC TGT GA-3'; and the secondary primers F2: 5'-CAA TGT GCA CCT GTA GGA AGC-3' and R2: 5'-GTG GTT CTC CGT CGG TGT GAG-3'. The first round of PCR was performed with 5 µL extracted DNA and a 20-µL reaction mixture [0.25 mM of each primer, 0.2 mM dNTP (Invitrogen), 1X PCR buffer, 3.0 mM $MgCl_2$, and 1.25 U Taq DNA polymerase (Invitrogen)]. Parasite DNA amplification was performed under the following conditions: 5 min at 94°C for denaturation; 35 cycles each of 1 min at 94°C for denaturation, 1 min at 58°C for annealing, and 1 min at 72°C for extension; and a final extension at 72°C for 10 min. A 2-µL aliquot of the PCR product was used in the nested PCR performed using the secondary primers. The second round of PCR was carried out under the same cycling conditions as the first. The PCR products were subjected to electrophoresis on a 1% agarose gel, and stained with SYBR® Safe DNA Gel Stain.

Sequencing

Samples considered positive on the basis of the *SAG-1* nested PCR results were subjected to a purification process using the PureLink®-Quick Gel Extraction kit and PCR Purification kit (Invitrogen) and were sent to the Ludwig Biotec Company (Porto Alegre, RS, Brazil) for sequencing in both directions using internal primers. The sequencing results were analyzed and subjected to a BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for identification.

Statistical analysis

The relative frequencies of positive samples from the cities from which the samples were obtained (Ilhéus and Camamu) were compared with the χ^2 Pearson test using the Epi-Info 7 software (Dean et al., 2011), and the incidence of detection of *T. gondii* from the oyster organs (gills and digestive glands) was compared with the McNemar exact test using the BioEstat 5 software (Ayres et al., 2007). In all analyzes, significant P values were considered when less than 0.05 (Ayres et al., 2007).

RESULTS

The method for extracting DNA from oyster tissue, as described in this study, was effective for the detection of *T. gondii*, as described below. On average, DNA concentrations of 1736.2 ng/μL with an A_{260}/A_{280} ratio ranging from 1.80 to 2.08 were obtained.

All 624 oyster samples from the southern coast of Bahia (Ilhéus and Camamu) were considered negative by molecular analysis for the *T. gondii* repetitive sequence AF 146527. However, using nested PCR for the *SAG-1* gene, 17 (8.1%) samples yielded positive results, showing a corresponding amplicon at the expected size of 392 bp for DNA of tissue samples from both the gills and digestive glands.

For Ilhéus, seven (6.7%) samples yielded positive results with nested PCR; four (57.1%) of these were from pools of gill tissue and three (42.9%) were from the digestive glands. For Camamu Bay, 10 (9.6%) samples were positive; nine (90%) were obtained from the gills and only one was from the digestive gland (Figure 1). There was no significant difference between the cities in terms of incidence (P = 0.31). However, the incidence of detection differed with respect to the tissue studied (P = 0.02), with a higher frequency of positive results obtained for gill samples.

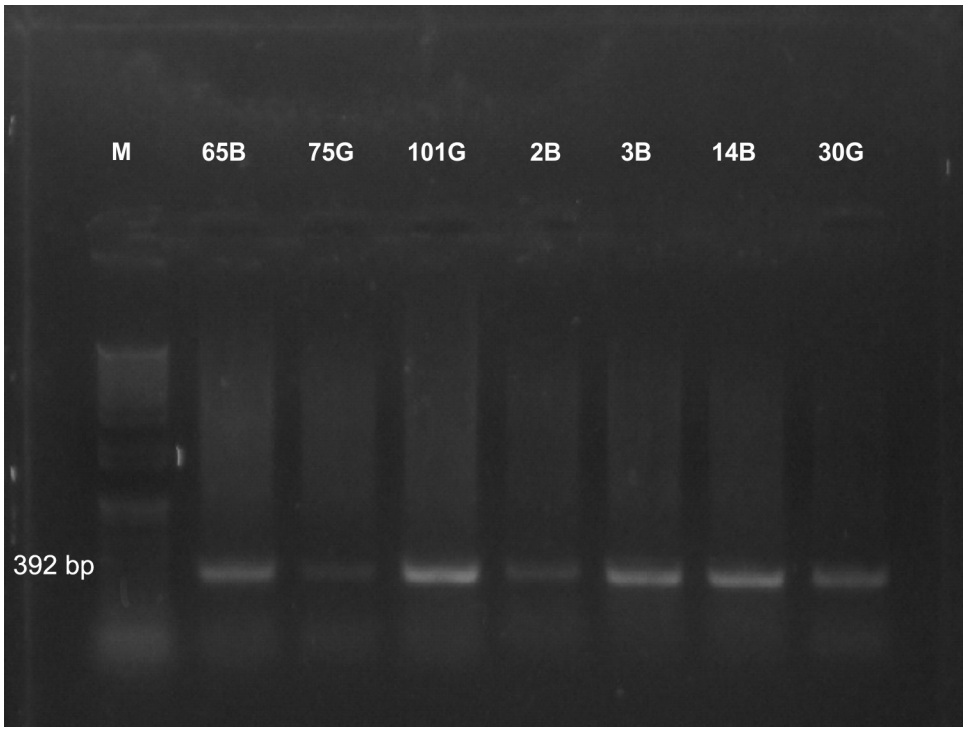

Figure 1. Electropherogram showing amplification of the *Toxoplasma gondii SAG-1* gene using nested polymerase chain reaction (PCR). The amplified product is shown at 329 bp. *Lane M* = molecular weight marker; *lane 65B* = Ilhéus's gills samples; *lane 75G* and *101G* = Ilhéus's digestive gland samples; *lane 2B*, *3B* and *14B* = Camamu's gills samples; *lane 30G* = Camamu's digestive gland samples. Resolution of PCR products was performed on a 1% agarose gel.

The 17 samples identified as positive using the nested PCR technique were confirmed by sequencing and showed between 99 and 100% identity with the *SAG-1* gene sequences of *T. gondii* available in the National Center for Biotechnology Information database (accession Nos. GQ253073.1, GQ253086.1, GQ253075.1, and GQ253074.1).

DISCUSSION

To our knowledge, this is the first study to describe the detection of *T. gondii* DNA in tissue samples of oysters obtained from the northeastern coast of Brazil.

Detecting oocysts in environmental samples is considered to be a scientific challenge because of the technical limitations of parasite detection. Although the nested PCR technique is sufficiently sensitive to identify *T. gondii*, the resistant form of this parasite (oocysts) in the environment is difficult to detect, since it has an extremely tough wall that can interfere with DNA extraction.

According to Dumètre and Dardé (2003), various techniques can be used to break the oocyst wall, including *in vitro* encystation, digestion with proteinase K, grinding with glass beads, and the use of thermal shocks, but the protocols are not standardized with respect to the temperature and the number of freeze/thaw cycles to be used. The current study employed glass beads, thermal shock, and proteinase K addition, which is similar to the process used by Esmerini et al. (2010), except that glass beads were not used in that study. Putignani et al. (2011) opted to use only thermal shock in their study. These differences in DNA extraction procedures may influence the results obtained by different researchers.

In this study, an 8.1% positive detection rate was obtained for *T. gondii*, a result higher than that obtained in the study performed in Brazil by Esmerini et al. (2010), who reported a 3.3% positive rate. Several factors might be related to this higher detection rate, including the tissue used and the sites from where the samples were collected. In this experiment, the oysters were dissected and only the gills and digestive glands were used, because these are the sites from which parasites are detected most often (Robertson, 2007; Leal and Franco, 2008). In the study by Esmerini et al. (2010), the entire oyster was used, and this might have lowered the sensitivity of detection. The environment in Southern Bahia is characterized by the Atlantic Forest, with a high relative humidity that is favorable for oocysts in the environment. The climate on the southern coast of Bahia is tropically humid, and characterized by high rainfall of up to 60 mm in the dry months, and of around 1400 mm annually (Klumpp et al., 2002). This, together with the low levels of basic sanitation in the region, may be predisposing factors for increased parasite incidence. Another important factor is the presence of various species of wild felids (Moura, 2003), together with the large quantity of domestic cats wandering throughout the region.

Positive results were obtained for the oysters in the months of April, August, and September in Ilhéus and in April, June, and August in Camamu. This can be explained by the higher rainfall at that time of the year, with rainfall of nearly 300 mm in August in Ilhéus and rainfall greater than 150 mm in April in Camamu, reaching 300 mm in June 2012 (INMET). It is believed that the spread of oocyst contamination from land to the marine coast arises mainly as a result of rain water runoff, which transports waste and the feces of infected cats, as described by Cole et al. (2000) and Conrad et al. (2005). According to Miller et al. (2002), shoals and bays close to urban centers are the areas that are most exposed to *T. gondii* because of the greater fecal pollution in these areas. This study identified *T. gondii* in oysters from

bays (estuaries), which are found close to the coastal populations, where all the waste (human/animal) is dumped into the environment without any kind of treatment.

The highest prevalence of positive detection of *T. gondii* was found in samples from gill tissues (76.5%), which is similar to the results from studies by Esmerini et al. (2010) and Putignani et al. (2011). There is evidence that these tissues are ideal for the detection of the parasite (Potasman et al., 2002; Arkush et al., 2003; Putignani et al. 2011), because oysters, by means of their filter-feeding mechanism, can ingest approximately five liters of water per hour and therefore light particles like *T. gondii* oocysts are retained in the gills (Ruppert and Barnes, 1996; Robertson, 2007; Leal and Franco, 2008).

According to Sande et al. (2010) and Brandão et al. (2013), both the Cachoeira River estuary and Camamu Bay are considered suitable areas for extracting mollusks, which are used as a source of food for the coastal population, as well as for sale during the high season, as summer. Bivalve mollusks reflect the conditions in which they live and are considered bio-indicators of environmental contamination (Miller et al., 2002). The estuarine environments in the cities of Ilhéus and Camamu were considered to be contaminated with *T. gondii* oocysts because oysters from both these environments were positive for the parasite.

The parasite *T. gondii* has a large worldwide distribution, and in Brazil there is also a high prevalence of infection by the parasite in humans and animals. The reported cases of toxoplasmosis in Brazil are primarily associated with the consumption of undercooked or raw meat and through ingestion of oocysts in contaminated food and water (Dubey et al., 2012). Dubey (2004) suggested that oocyst infections, regardless of the dose, are clinically more serious in humans than infections acquired through tissue cysts. In the current study, we could not determine the viability of the parasite, but it is widely known that the oocysts can sporulate in seawater and become infectious for a period of up to 6 months in water (Lindsay et al., 2003). In the region of this study, oysters are generally eaten *in natura*, and it is known that even a single oocyst of *T. gondii* can cause infection. Thus, it can be assumed that the coastal population and individuals who ingest oysters purchased from the fish market are at risk of acquiring toxoplasmosis.

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