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SURVIVAL OF *TOXOPLASMA GONDII* OOCYSTS IN EASTERN OYSTERS (*CRASSOSTREA VIRGINICA*)

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ABSTRACT: *Toxoplasma gondii* has recently been recognized to be widely prevalent in the marine environment. It has previously been determined that Eastern oysters (*Crassostrea virginica*) can remove sporulated *T. gondii* oocysts from seawater and that oocysts retain their infectivity for mice. This study examined the long-term survival of *T. gondii* oocysts in oysters and examined how efficient oysters were at removing oocysts from seawater. Oysters in 76-L aquaria (15 oysters per aquarium) were exposed to 1×10^6 oocysts for 24 hr and examined at intervals up to 85 days postexposure (PE). Ninety percent (9 of 10) of these oysters were positive on day 1 PE using mouse bioassay. Tissue cysts were observed in 1 of 2 mice fed tissue from oysters exposed 21 days previously. *Toxoplasma gondii* antibodies were found in 2 of 3 mice fed oysters that had been exposed 85 days previously. In another study, groups of 10 oysters in 76-L aquaria were exposed to 1×10^5, 5×10^4, or 1×10^4 sporulated *T. gondii* oocysts for 24 hr and then processed for bioassay in mice. All oysters exposed to 1×10^5 oocysts were infected, and 60% of oysters exposed to 5×10^4 oocysts were positive when fed to mice. The studies with exposure to 1×10^4 oocysts were repeated twice, and 10 and 25% of oysters were positive when fed to mice. These studies indicate that *T. gondii* can survive for several months in oysters and that oysters can readily remove *T. gondii* oocysts from seawater. Infected filter feeders may serve as a source of *T. gondii* for marine mammals and possibly humans.

Toxoplasmic encephalitis has recently been recognized as a primary disease of sea otters and other coastal-dwelling marine mammals (Cole et al., 2000; Lindsay, Thomas et al., 2001; Miller et al., 2001, 2002; Dubey, Zarnke et al., 2003; Kreuder et al., 2003). Cole et al. (2000) suggested that *Toxoplasma gondii* oocysts could enter the marine environment from domestic cat feces and that marine invertebrates may serve as transport hosts. Miller et al. (2002) found a positive correlation between the presence of coastal storm runoff water and the presence of antibodies to *T. gondii* in sea otters. Lindsay, Phelps et al. (2001) demonstrated that Eastern oysters (*Crassostrea virginica*) could remove sporulated *T. gondii* oocysts from seawater and that they were orally infectious for mice. Arkush et al. (2003) conducted similar studies with mussels (*Mytilus galloprovincialis*) and determined that sporulated *T. gondii* oocysts were filtered out of seawater by mussels and that they remained infectious for mice. Lindsay et al. (2003) recently demonstrated that *T. gondii* oocysts could sporulate in seawater and that they remained viable for at least 6 mo in seawater. This suggests that once in the marine environment, *T. gondii* oocysts can survive for extended periods of time and be available to a variety of potential transport hosts. The present study was conducted to examine the long-term survival of sporulated *T. gondii* oocysts in Eastern oysters and to examine how efficient these oysters were at removing *T. gondii* oocysts.

MATERIALS AND METHODS

Toxoplasma gondii oocysts

A *T. gondii*–naive cat was fed tissues from a naturally infected chicken from New England containing tissue cysts of *T. gondii* (Dubey, Graham et al., 2003). The cat was housed and infected in a cat colony at the United States Department of Agriculture, Animal Parasitic Diseases Laboratory, Beltsville, Maryland (Dubey, 1995). Feces containing unsporulated oocysts were collected, sporulated, purified using Sheather's sugar solution, and then stored in 2% (v/v) sulfuric acid at 4 C. The suspension of purified oocysts was sent on cool packs to the Center for Molecular Medicine and Infectious Diseases, Department of Biomedical Sciences and Pathobiology, Virginia–Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, Virginia, for use in the present study. Oocysts were washed free of sulfuric acid by repeated centrifugation and stored at 4 C in Hanks balanced salt solution (HBSS) until used to infect aquaria in the present study. The appropriate volume of stock oocyst suspension was diluted in 10 ml of seawater and used to expose oysters in 76-L aquaria.

Oysters

Eastern oysters, *C. virginica*, were obtained from commercial sources and originated from the Chesapeake Bay. Oysters were acclimated in 76-L aquaria containing artificial seawater (32 ppt NaCl) for at least 2 days before use. The oysters were fed 15 ml of algae paste thrice a week (microalgae culture, cell count 3.1×10^9 cells/ml, dry weight 6%; Isochrysis 1200 Premium Fresh Instant Algae, Reed Mariculture/Instant Algae Products, Inc., San Jose, California).

As a control for natural exposure to *T. gondii*, 2 oysters were taken from the source oysters, not exposed to *T. gondii*, and processed for feeding to mice. Two groups of 3 mice (1 group per oyster) each were fed the processed oyster tissue and examined as described below.

Oyster bioassay for *Toxoplasma gondii*

Selected oysters were removed from the aquaria and their outer shell surfaces thoroughly washed in tap water from a hose. Individual oyster tissue and enclosed liquid were then removed, placed in individual 50-ml plastic tubes, and processed for feeding to mice. The oyster material was homoge-

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TABLE I. Survival of *Toxoplasma gondii* oocysts* in Eastern oysters exposed for 24 hr and examined at various times after exposure.

Day	Number oysters examined/ number positive by mouse bioassay†	*T. gondii* tissue cysts in mice‡
1	10/9	Yes
2	2/1	Yes
4	2/2	Yes
6	2/0	No
8	2/1	Yes
10	2/2	Yes
12	2/0	No
14	2/2	Yes
16	2/1	Yes
21	2/1	Yes
28	2/0	No
85	3/2	No

* Aquaria containing 15 oysters each were contaminated with 1×10^6 *T. gondii* oocysts.
† Number mice positive by MAT for antibodies to *T. gondii*.
‡ *Toxoplasma gondii* tissue cysts observed in mice by light microscopy.

nized on the high setting in a stomacher machine (Laboratory Blender Stomacher 80, Seward Medical, London, U.K.) at room temperature for 2 min and filtered through cheesecloth to remove particulate material. Collected material was pelleted by centrifugation and resuspended in approximately 2–3 ml of HBSS. The processed material from each oyster was fed to 3 female, 25–30 g, CD-1 mice using an animal feeding needle. We did not examine processed material microscopically for oocysts. There is no good way to determine viability of oocysts using light microscopy, and we based our conclusions solely on results of the mouse bioassay.

Mice were bled from the retroorbital plexus 6–8 wk after being fed oyster tissue. The sera were examined at dilutions of 1:25 in the modified direct agglutination test (MAT) (Dubey and Desmonts, 1987) for *T. gondii*. Mice were killed after bleeding, and 2 portions of cerebral cortex from each mouse were examined as unstained squash preparations for tissue cysts.

Validation of mouse bioassay

To determine the sensitivity of our mouse bioassay, oysters were removed from their shells and the oyster tissue and liquid individually placed in 50-ml plastic tubes. One milliliter of HBSS containing 1,000, 100, or 10 *T. gondii* oocysts was placed on each of 3 oysters per treatment separately, and the tubes were inverted repeatedly to mix the liquid and the oyster tissue. Oysters were then individually processed as above, and processed material was fed to a group of 3 mice/oyster. Three groups of 3 mice each were fed 100, 50, or 10 *T. gondii* oocysts and used as positive controls.

Long-term survival of *Toxoplasma gondii* in oysters

Three aquaria containing 15 oysters each were infected with 1×10^6 *T. gondii* oocysts. After 24 hr of exposure, oysters were removed, and their outer shell surfaces washed with tap water. Ten oysters were examined 24 hr after exposure (Table I), and the remaining 35 oysters were placed in clean aquaria and used to determine long-term survival of *T. gondii* oocysts in oysters. Two oysters were removed and individually processed for feeding to mice at 2, 4, 6, 8, 10, 12, 14, 16, 18, 21, and 28 days after exposure. Three oysters were examined 85 days after exposure. The remaining 10 oysters were not examined.

TABLE II. Recovery of *Toxoplasma gondii* in mice fed Eastern oysters exposed to various doses of oocysts for 24 hr.

Dose of oocysts per aquaria	Number of oysters examined/ numbers of oysters positive in mouse bioassay*
1×10^5	10/10
5×10^4	10/6
1×10^4	10/1
1×10^4	8/2†

* Oysters were considered positive if a mouse in the group fed its tissue was positive for antibodies in the MAT or by brain smear.
† All mice fed oyster tissue from 2 oysters died because of complications from oral gavage; therefore, results from only 8 oysters are reported.

Efficacy of removal of *Toxoplasma gondii* from seawater by oysters

Groups of 10 oysters were placed in aquaria and exposed to 1×10^5, 5×10^4, or 1×10^4 oocysts for 24 hr. After 24 hr of exposure, oysters were removed and their surfaces washed with tap water; each was individually processed for feeding to mice.

RESULTS

Control oysters

None of the 6 mice fed processed oyster tissue from the 2 nonexposed source oysters was positive.

Validation of bioassay

All mice fed 100, 50, or 10 *T. gondii* oocysts were brain smear and MAT positive. Two of 3 oysters contaminated with 1,000 *T. gondii* oocysts and processed were detected as positive in mice, as were 2 of 3 oysters contaminated with 100 *T. gondii* oocysts. All 3 oysters contaminated with 10 *T. gondii* oocysts were positive by bioassay in mice.

Long-term survival of *Toxoplasma gondii* in oysters

Table I shows the results of bioassay of oysters exposed to *T. gondii* for 24 hr and then examined at various time intervals. Tissue cysts were seen in the brains of mice fed oyster tissue as long as 21 days after exposure. Tissue cysts were not seen in mice fed oysters exposed for 85 days, but MAT indicated that 2 of 3 oysters examined contained detectable oocysts. Thus, serological evidence indicates that oysters can retain *T. gondii*–infective oocysts for 85 days.

Efficacy of removal of *Toxoplasma gondii* from seawater by oysters

Table II shows the results of studies designed to determine how efficient oysters are at removing *T. gondii* oocysts. In the

second replicate of 1×10^4 oocysts, all the mice inoculated with tissues from 2 oysters died because of complications of oral gavage, and the results are based on only 8 oysters for this replicate.

DISCUSSION

Our study demonstrates that Eastern oysters readily remove *T. gondii* oocysts and that oocysts may remain viable for up to 85 days. This is longer than the 6 days previously reported by Lindsay, Phelps et al. (2001). Arkush et al. (2003) found that mussels contained *T. gondii* small-subunit ribosomal RNA for up to 21 days after exposure, but viable oocysts were detected for only 3 days. M. B. Lee and E. H. Lee (2003) used *Eimeria acervulina* as a surrogate for *T. gondii* and found that Eastern oysters retained *E. acervulina* infection for only 2 days. They concluded that coccidial oocysts pass through oysters in less than a day and that oysters were not likely to be a source of infection for humans. This is in contrast to the present study, which demonstrated that *T. gondii* oocysts survive for up to 85 days, and another study, which demonstrated they are able to survive in Eastern oysters for 6 days (Lindsay, Phelps et al., 2001).

Infective oocysts of *Cryptosporidium parvum* have been found in naturally infected Eastern oysters (Fayer et al., 1998, 1999, 2002) from the Chesapeake Bay. Graczyk et al. (2001) found oocysts of *C. hominis* (syns. *C. parvum* genotype 1; *C. parvum* genotype H; Morgan-Ryan et al., 2002) in naturally infected Zebra mussels (*Dreissena polymorpha*) from the St. Lawrence River, Quebec, Canada. Gomez-Bautista et al. (2000) reported infective *C. parvum* oocysts in mussels (*M. galloprovincialis*) and cockles (*Cerastoderma edule*) from a shellfish-producing region in Spain. Graczyk et al. (1999) found oocysts of *Cryptosporidium* sp. in Bent mussels (*Ischadium recurvum*) from the Chesapeake Bay. In a survey of shellfish from Spain and Italy, Freire-Santos et al. (2000) provided evidence of infection of clams (*Dosinia exoleta*, *Ruditapes philippinarum*, *Venerupis pullastra*, *V. rhomboideus*, *Venus verrucosa*), mussels (*M. galloprovincialis*), and oysters (*Ostrea edulis*) with *Cryptosporidium* sp. using immunofluorescent microscopy for oocysts. These studies indicate that shellfish can readily remove and retain coccidial oocysts from fresh and seawaters. Further work is needed to determine the prevalence of *T. gondii* in shellfish from areas with at-risk marine mammal populations.

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