APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1997, p. 2086–2088 Vol. 63, No. 5
0099-2240/97/$04.00+0

Potential Role of the Eastern Oyster, *Crassostrea virginica*, in the Epidemiology of *Cryptosporidium parvum*

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Received 20 December 1996/Accepted 13 February 1997

Oysters were placed in an aquarium containing artificial seawater, and *Cryptosporidium parvum* oocysts were added. Oocysts were later found in the gill washings, hemocytes, and gut contents of the oysters. Hemocytes containing oocysts were intubated into four mice. *C. parvum* stages developed in the ileal epithelia of all of the mice, indicating that the oocysts in the hemocytes remained infective.

Cryptosporidium parvum oocysts have been found in surface waters throughout North America (13), and outbreaks of cryptosporidiosis have been associated with filtered and chlorinated water from an unnamed lake, Lake Michigan, and Lake Mead (12). Many surface waters empty into the marine environment in localities where dumping of raw sewage is a common practice (4). Although attempts to survey marine waters for *Cryptosporidium* oocysts are rare, oocysts were detected in marine water in Hawaii near a sewage discharge site (10). Oysters that filter enteric pathogens from contaminated seawater can become vectors of disease if eaten raw. From 1988 through 1995 consumption of raw oysters was linked to 136 of 302 human *Vibrio* infections (1). The present study was conducted to determine if oysters could be artificially infected with *Cryptosporidium* oocysts (14) and if they could also serve as reservoirs of infection.

Two hundred forty cultured Eastern oysters (*Crassostrea virginica*) from the Chesapeake Bay were evenly divided between aquaria designated A and B. Each aquarium contained 190 liters of artificial seawater (Forty Fathoms Bio-Crystals Marine Mix; Marine Enterprises, Baltimore, Md.) with 11 ppt of salt at 21°C. The oysters, acclimated for 2 weeks before testing, were fed 1 teaspoon of algal paste three times each week. Feedings were withheld for 1 week preceding exposure to oocysts. Water in the aquaria was circulated with a power filter (300 gal/h) without the filter cartridge.

Oocysts of *C. parvum* AUCP-1 purified from calf feces (3, 11) were stored for less than 2 weeks in distilled water at 4°C before 1.2×10^8 oocysts were placed in aquarium A (concentration = 630 oocysts/ml). No oocysts were added to aquarium B.

To determine the rate at which the oysters removed oocysts from the water, 250-ml water samples were taken from aquarium A at 1, 2, 3, 4, 24, and 168 h after oocysts were added. The samples were centrifuged at 1,200 × *g* for 15 min, the pellets were resuspended in distilled water, and the oocysts were counted with a hemacytometer. The samples taken at the times indicated above contained ca. 300, 200, 100, 100, 0, and 0 oocysts per ml, respectively. All appeared normal when viewed by bright-field and interference-contrast microscopy.

Examination of 20 oysters in aquarium A at four time points after exposure indicated that oocysts accumulated on the gills and within hemocytes in the hemolymph (Table 1). At 3 h, oocysts were aspirated from the gills of 15 of 20 oysters, and 4 of 20 oysters had oocysts within the hemocytes (Fig. 1). At 1 month, oocysts in both sites still appeared morphologically normal. Immunofluorescence microscopy of Merifluor (Meridian Diagnostics, Cincinnati, Ohio)-stained specimens was superior to that of acid-fast-stained specimens in terms of sensitivity of staining and ability to recognize oocysts. Oocysts were not found in gill washings or hemolymph from control oysters.

Histologic sections from 20 oysters each were prepared as previously described (9) and stained with Merifluor and acid-fast reagents at 24, 168, and 720 h after exposure to oocysts, which were detected in 19, 10, and 11 oysters, respectively (Table 2). Most were in the lumens of the stomachs and intestines (Fig. 2), with as many as 12 oocysts within one bolus of digesta. Usually only one to four oocysts were seen in the gills, diverticula, and gonadal tissue of each oyster. The oocysts appeared to be free, but some may have been within cells. None were observed in tissues from control oysters.

To determine if oocysts ingested by oysters remained infective for mammals, 1.0×10^6 oocysts per oyster were placed in aquarium C. One week later 5 ml of hemolymph was aspirated from each of 25 oysters and pooled. Hemolymph obtained from 25 unexposed oysters in aquarium D was pooled separately. The total number of hemocytes in each pool was determined by a hemacytometer. The percentage of hemocytes containing oocysts was determined from droplets of hemolymph

TABLE 1. Presence of oocysts of *C. parvum* AUCP-1 in gill washings and within hemocytes of Eastern oysters (*Crassostrea virginica*)

Time after exposure[a] (h)	No. of oysters[b] with oocysts in:	
	Gill washings	Hemocytes
3	15	4
24	11	4
168	13	6
720	7	6

[a] Oysters were exposed to 1.2×10^8 oocysts in 190 liters of artificial seawater.
[b] Of 20 oysters examined.

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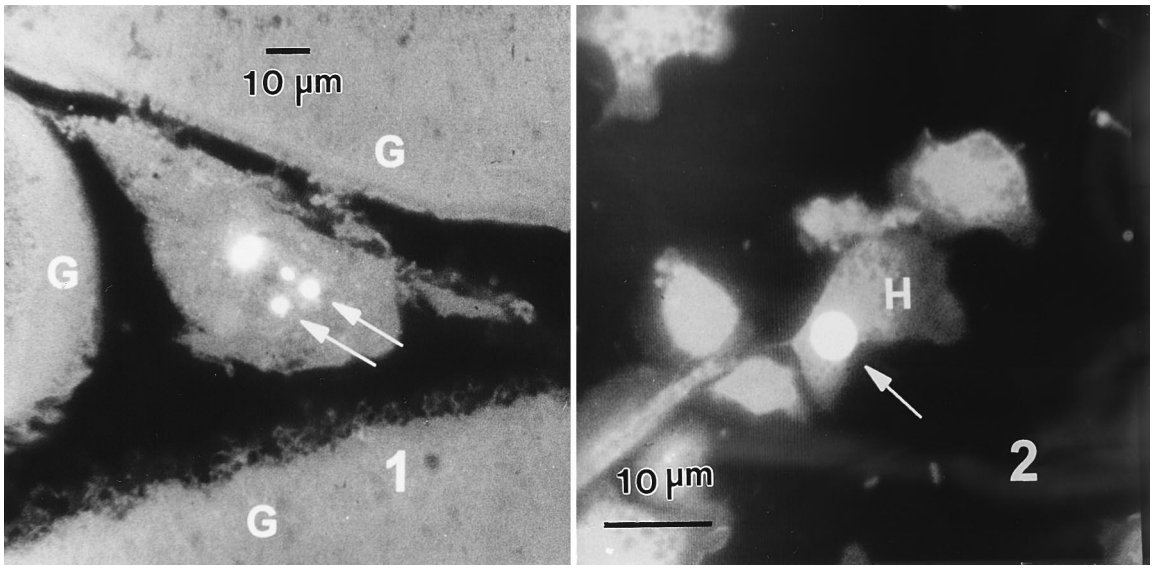

FIG. 1 and 2. Immunofluorescence photomicrographs of oocysts of *C. parvum* observed with a Zeiss Axioskop 20 microscope equipped with epifluorescence and a fluorescein isothiocyanate-Texas red dual-wavelength filter.
FIG. 1. Oocysts (indicated by arrows) within a fecal bolus in the lumen of the gut (G) in a histological section fixed 168 h after exposure.
FIG. 2. Oocyst (indicated by the arrow) within a hemocyte (H) aspirated with hemolymph 24 h after exposure.

placed on glass microscope slides and stained with Merifluor reagents. The concentration of hemocytes was adjusted to 10^8 per ml, and 100 µl of hemolymph containing approximately 250 oocysts within hemocytes was administered by gavage to each of four 9-day-old BALB/c mice. The same concentration of hemocytes from the unexposed oysters was administered to four additional mice. All mice were euthanized by overexposure to CO_2 96 h later, and the ilea were processed for histology. Hematoxylin- and eosin-stained sections revealed stages of *C. parvum* in all four of the mice that received hemocytes from oysters exposed to oocysts (Fig. 3), but none were observed in mice that received hemocytes from unexposed oysters.

For immunofluorescent staining, 2 ml of Merifluor monoclonal antibody mixed with 1 ml of counterstain was diluted with 3 ml of phosphate-buffered saline; 20 to 50 µl of this solution was pipetted over hemolymph smears or histologic sections on microscope slides. After 30 min at room temperature, the slides were rinsed three times with Merifluor wash buffer and coverslips were mounted with Merifluor reagent. Before the histologic sections were stained, paraffin was removed with xylene and the sections were hydrated through a series of alcohols to water.

The present study and collaborative in vitro data (7) suggest that if oocysts of *C. parvum* gain access to marine waters where oysters are harvested for human consumption, the oocysts could be taken up by oysters, potentially posing a threat to public health. However, no such link has yet emerged based on epidemiological data. With currently available commercial reagents, routine examination of oysters for oocysts of *C. parvum* is unlikely. It would be laborious and expensive and could possibly lead to false-positive identification. Reagents used in the present study to detect *C. parvum* also stain other *Cryptosporidium* species (6). Because species from marine environments have not been tested, it is not known if oocysts isolated from fish such as red drum (*Sciaenops ocellatus*) (2), barramundi (*Lates calcarifer*) (5), masked unicorn fish (*Naso lituratus*) (8), an unidentified species of tropical marine fish (8), or others would give false-positive reactions. However, based on present findings it appears prudent to survey oysters in natural waters to initially determine the presence of any species of *Cryptosporidium* oocysts.

TABLE 2. Detection of oocysts of *C. parvum* AUCP-1 in Eastern oysters (*Crassostrea virginica*) as determined by immunofluorescence microscopy of histologic sections

Time after exposure (h)	No. of tissue sections[a] with oocysts				Total no. of oysters[a] with oocysts
	Gills	Stomach and intestine	Digestive diverticula	Other areas	
24	2	19	0	1	19
168	4	7	1	5	10
720	3	3	4	3	11

[a] Of 20 oysters examined.

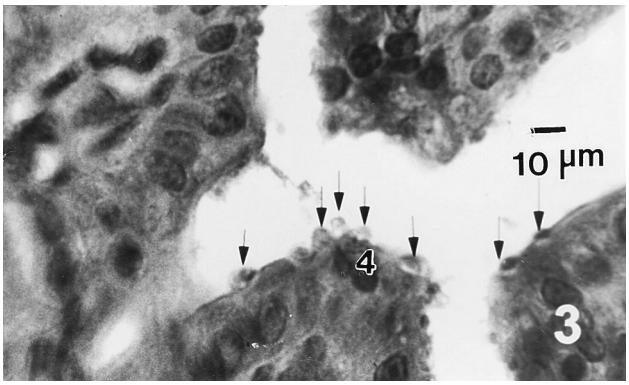

FIG. 3. Developmental stages of *C. parvum* (indicated by arrows) in the ileum of a mouse that received hemocytes containing oocysts of *C. parvum* from oysters 1 week after the oysters were exposed.

We thank Dorothy Howard and Andrew Cho for excellent technical support.

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