Oocysts of Cryptosporidium parvum, a zoonotic waterborne pathogen, can be removed by bivalve molluscs from contaminated water and retained on gills and in hemolymph. We identified oocysts of C. parvum in oysters from seven sites in the Chesapeake Bay area. These findings document the presence of C. parvum infectious for humans in oysters intended for human consumption.

The Study

From 43 commercial oyster harvesting sites where the Maryland Department of Natural Resources makes routine annual collections, seven were selected to test for the presence of C. parvum oocysts (Table). Approximately 30 oysters were examined from each site on three occasions (Table). From each oyster, 3 to 5 ml of hemolymph was aspirated from the adductor muscle. All gill tissue from each oyster was excised and washed in 5 ml of PBS. For examination by immunofluorescence microscopy, 200 µl of hemolymph and gill washing from each oyster was air dried overnight. Slides were stained with Merifluor fluorescein-labeled anti-Giardia and anti-Cryptosporidium monoclonal antibodies (Merifluor; Meridian Diagnostics, Cincinnati, OH) and examined with an epifluorescence microscope equipped with a fluorescein isothiocyanate-Texas Red dual wavelength filter. Specimens were considered positive when round bodies 4.5 to 5.5 µm in diameter with distinct green fluorescing walls were identified.

Hemolymph and gill washings from six oysters were pooled, resulting in five aliquots from each collection site. Pooled aliquots were tested for infectivity in mice and examined by polymerase chain reaction (PCR) for the presence of C. parvum-specific DNA.
Table. Identification of Cryptosporidium parvum oocysts recovered from oysters in the Chesapeake Bay

| Site     | Location or river system | Fall 1997 | Winter 1998 | Fall 1998 |
|----------|--------------------------|-----------|-------------|-----------|
|          | Bias Location             | IFA PCR*  | Mice infectivity | IFA PCR*  | Mice infectivity | IFA Cp11 infectivity | Water^b |
| A        | Mt. Vernon Wharf          | 28^e ND   | Neg^d        | 15 BT     | Pos            | 4 Pos Neg        | ND       |
| B        | Wetipquin Nanticoke      | 29 BT     | Neg          | 3 Neg     | Neg            | 8 Pos Neg        | 79       |
| C        | Halfway Fishing Mark Bay  | 29 HT     | Neg          | 0 BT & HT | Neg            | 1 Pos Neg        | ND       |
| D        | Beacon Potomac Point      | 26 BT     | ND           | ND ND     | ND             | 2 Pos Pos        | 10       |
| E        | Back Cove Tangier Sound Leg | ND ND     | ND           | ND ND     | ND             | 1 Pos Neg        | 31       |
| F        | Old Woman's Tangier Sound | ND ND     | ND           | 2 BT      | Neg            | 6 ND Neg         | 8        |
| G        |                          | ND ND     | 0 BT         | Neg       |                | 0 ND Neg         | ND       |

*Polymerase chain reaction and restriction fragment-length polymorphism (PCR-RFLP) on small subunit rRNA gene, 18s.
^bNumber of oocysts recovered per liter of filtered bay water.
^cNumber of oysters found positive for oocysts out of 30 oysters examined from each site.
^dNeg indicates that PCR using Cp11 primers failed to detect Cryptosporidium DNA in the DNA extracted from the ilea of mice that were intubated with pooled hemolymph and gill washings from oysters.

HT, human genotype; BT, bovine genotype; ND, not done; IFA, immunofluorescent assay; PCR, polymerase chain reaction.

Three hundred to 400 µl of each of the five aliquots of pooled hemolymph and gill washings from each site was administered to each of four 7- to 10-day-old BALB/c mice by gastric intubation. Mice were necropsied 96 hours postinoculation, and 1 cm of terminal ileum was placed in DNA extraction buffer to obtain total DNA as described (3). Mouse ileum DNA (100-1,000 ng/reaction) was analyzed for Cryptosporidium DNA by PCR, using CP11-P5 and CP11-P6 primers (3). The PCR products were analyzed by polyacrylamide gel electrophoresis and ethidium bromide staining, followed by image capture on a charge coupled device camera.

Pooled hemolymph and gill washings shipped to the Centers for Disease Control and Prevention within 1 week of collection were rinsed three times by repeatedly suspending in 10 ml sterile distilled water and centrifuging at 1,500 X g for 10 min. Supernate was decanted, and pelleted specimens were stored at 4°C until subjected to five freeze-thaw cycles, followed by phenol-chloroform extraction to extract DNA. Purified DNA was dissolved in 50 µl distilled water and stored at -20°C until PCR analysis.

A small subunit rRNA gene-based, nested PCR and restriction fragment-length polymorphism (RFLP) technique developed for species- and genotype-specific diagnosis of Cryptosporidium (4,5) was used to characterize oocysts from oysters.

To confirm PCR-RFLP results, all positive secondary PCR products were sequenced. Samples collected in the fall of 1998 were also assayed by nested CP11 PCR (Figure).

At least 50 L of water from each site was filtered by the membrane disk (393-mm diameter, 3-µm pore size, white SSWP [Millipore Corp., Bedford, MA]) method (7). After filtration, an elution protocol (Method 1622) was followed (7). To test the recovery efficiency of this method, 10-L samples of bay water were processed as above, except that four samples were spiked with 10^5 and four others with 10^6 purified C. parvum oocysts. The concentration of oocysts in Chesapeake Bay water (Table) was adjusted for the recovery efficiency of the membrane disk method.

Findings

During three collection periods, oocysts corresponding in size and shape to those of C. parvum and labeled with fluoresceinated anti-Cryptosporidium antibody were detected in oysters collected at six of seven sites (Table). These findings were confirmed in all but one case by positive PCR results for the 18s rRNA gene. Specimens from the one site at which oocysts were not detected by microscopy were found positive by PCR. The presence of oocysts in oysters obtained at the last collection period was confirmed by PCR for the CP11 gene sequence (GenBank accession no. AF124243).

PCR-RFLP testing for the 18s rRNA gene identified two genotypes of C. parvum in hemolymph and gill washings from oysters. All
Figure 1. Panel A. Results of nested Cryptosporidium parvum CP11 gene PCR performed on pooled oyster hemolymph and gill tissues. Expected PCR product size is 344 bp. Samples analyzed were collected from Maryland Department of Natural Resources oyster harvesting sites at Mt Vernon Wharf (lanes 1-5), Wetipquin (lanes 6-8), Beacon (lane 9), and Holland Point (lane 10). Lane 11: C. parvum positive control. Lanes 12 and 13 are 1° and 2° no template controls, respectively. Panel B: Results of oyster (Crassostrea virginica) small subunit ribosomal RNA PCR performed on the same oyster tissues analyzed in Panel A, lanes 1-10. Lane 11: no template control. Expected PCR product size is 340 bp.

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As an adjunct to the 18S rRNA assay, a nested PCR was also performed by using primers derived from an 11 kDa protein, extracted from C. parvum oocysts. The sequence of outer forward primer P5 is: 5' AAC ATC CAT CGA GTT TAG TA 3' and of outer reverse primer P6 is: 5' GCA AGA GCG CAT TGG TGA AT 3'; the expected PCR product size is 541 bp. The sequence of inner forward primer Cp 11/F is: 5' GTC TAG AAC CGT TAC TGT TAC TGG 3', and of outer reverse primer CP11/R is: 5' CAA CTC CTG GAA GCA TCT TAA CAG 3'; the expected PCR product size is 334 bp.

For PCR, pooled oyster gill washings and hemolymph were subjected to nucleic acid extraction by using the Ambion Totally RNA kit, followed by isopropanol precipitation of nucleic acids and a 70% ethanol wash. The pellet was resuspended in 30-50 µl of molecular biology grade water, and 5-10 µl used as template for PCR. These reactions were performed in 50-µl volumes containing 1.5 mM MgCl₂, 1U Taq polymerase, 1X PCR buffer, 50 pmol each primer, and 10 mM dNTP mix (Life Technologies, Gaithersburg, MD). Cycling parameters for all reactions were 2 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. Three microliters of primary PCR product was used as template for secondary PCR. PCR products were purified first by the Wizard PCR Prep DNA purification system (Promega, Madison, WI), then sequenced by fluorescent cycle sequencing on an ABI 373 Automated Sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA).

The predicted size of the PCR product is 540 bp. For PCR, pooled oyster gill washings and hemolymph were subjected to nucleic acid extraction by using the Ambion Totally RNA kit, followed by isopropanol precipitation of nucleic acids and a 70% ethanol wash. The pellet was resuspended in 30-50 µl of molecular biology grade water, and 5-10 µl used as template for PCR. These reactions were performed in 50-µl volumes containing 1.5 mM MgCl₂, 1U Taq polymerase, 1X PCR buffer, 50 pmol each primer, and 10 mM dNTP mix (Life Technologies, Gaithersburg, MD). Cycling parameters for all reactions were 2 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. Three microliters of primary PCR product was used as template for secondary PCR. PCR products were purified first by the Wizard PCR Prep DNA purification system (Promega, Madison, WI), then sequenced by fluorescent cycle sequencing on an ABI 373 Automated Sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA).

Genes were submitted to GenBank with the following accession numbers: C. parvum CP11 gene (AF202082), and C. baileyi 11 kDa protein (AF202081).
18s PCR-positive specimens with the exception of those collected from Fishing Bay were bovine genotype. Of two specimens from that location, one contained human genotype alone and the other contained both genotypes.

Eighty aliquots of pooled hemolymph and gill washings were tested for infectivity in mice by PCR of mouse ileum (Table). Oocysts from sites E (2 of 5 aliquots), A (2 of 5 aliquots), and D (5 of 5 aliquots) were found to be infectious at all three collection periods. No other aliquots had positive PCR findings.

The CP11 gene nested PCR was performed on pooled samples, collected in October 1998 from five oyster beds; although no amplicons were observed for the outer primer set P5/P6, all beds were positive by nested PCR (Figure). The nested amplicons from specimens from sites A and C were sequenced and found to have a 99% homology with the C. parvum CP11 sequence (data not shown). As a control for the quality of DNA extracted from the oyster tissues, we used a PCR assay for the small subunit of the ribosomal RNA gene of Crossostrea virginica (Figure, Panel B). The amplification of this gene fragment from the oysters negative for Cryptosporidium by CP11 gene PCR (e.g., Figure, Panel A, sample 2) indicates that PCR-inhibitory substances were successfully removed by using our nucleic acid purification protocol. Accordingly, sample 2 can be considered a true negative for the presence of Cryptosporidium oocysts.

The mean recovery efficiency of the membrane disk filtration method was 71.1%, CV=13.3%. Oocysts were detected in water samples collected in fall 1998 from sites B, D, E, and F. The concentration of oocysts at each site ranged from 8 to 79 oocysts/L (Table), with a mean of 32 oocysts/L.

Conclusions

C. parvum oocysts were found in oysters collected from all seven commercial oyster harvesting sites sampled in the Chesapeake Bay. These findings confirm those of previous studies, in which oysters (1) and clams (8) acquired Cryptosporidium oocysts from artificially contaminated aquarium water, and oysters (2) and mussels (9) acquired oocysts in nature. Collectively, these findings establish that bivalve molluscs can effectively remove and retain oocysts of Cryptosporidium from feces-contaminated estuarine waters.

PCR-RFLP testing for the 18s rRNA gene identified two genotypes of C. parvum in hemolymph and gill washings from oysters. Although many species of migratory and residential waterfowl, as well as amphibians, reptiles, and numerous mammals, inhabit the drainage area of sites from which oysters were collected, only the human and bovine genotypes of C. parvum were recovered from the oysters.

Results from infectivity studies indicate that only three sites of 16 tested over three collection periods yielded oocysts that produced detectable infections in mice. Based on positive IFA and PCR findings, 16 collections contained C. parvum bovine genotype oocysts. The low rate of infectivity for mice may reflect the small number of oocysts that were administered to each mouse of a lack of infectivity due to age or unknown environmental effects.

Neither the age of the oocysts nor how long they may have been on land, in the water, or retained by the oysters could be determined. In a previous study, oysters retained oocysts for at least 1 month after exposure and the oocysts infected mice when tested 1 week after exposure (1). In this study, salinity values and water temperatures during the three successive collection periods (based on data recorded at site A) were 9.0, 6.0, and 15.0 ppt and 12.0, 9.0, and 16.0°C, respectively. Oocysts suspended in 10 and 20 ppt artificial seawater at 20°C retained infectivity for mice when held for 12 and 8 weeks, respectively (2). Therefore, freshly deposited oocysts at these sites could have retained infectivity for 2 to 3 months.

At all sites sampled, examination of gill washings and hemolymph by both IFA microscopy and PCR revealed the presence of C. parvum oocysts. This finding indicates that water at these sites contained human or animal feces when oysters were filtering and that oocysts excreted in those feces were acquired by the oysters. Because oocysts of this species are infectious for humans but can be rendered noninfectious by heating to temperatures above 72°C (10), we recommend that oysters be cooked before being eaten, especially by persons with any type of immunodeficiency. Oocysts can also be rendered noninfectious by freezing at -20°C for 24 hours (11), but because viral or bacterial pathogens might also be acquired by oysters from water contaminated with feces and can survive freezing, we recommend cooking rather than freezing.
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