Cryptosporidium parvum in Oysters from Commercial Harvesting Sites in the Chesapeake Bay

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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.

Oocysts of *Cryptosporidium parvum* from human feces can enter surface waters through wastewater, leaky septic tanks, or recreational activities. Oocysts from other mammals, including wildlife, pets, and livestock (especially neonatal ruminants) can enter surface waters either directly or through runoff. Oysters can remove *C. parvum* oocysts from artificially contaminated water and retain them in hemocytes, on gills, and within the body for at least 1 month (1). Oocysts retained for 1 week by oysters were still infectious, as determined by testing in mice (1). Oocysts of *C. parvum* were found in oysters collected from tributaries of the Chesapeake Bay, at six sites selected for proximity to wastewater outfalls and cattle farms where high levels of fecal contamination might be expected (2). We examined oysters at sites where oysters are harvested for human consumption to determine if *C. parvum* oocysts were present. Oocysts recovered from these oysters were examined to determine the possible sources of contamination through oocyst genotyping and to determine if the oocysts were infectious.

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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	Bay location or river system	Fall 1997			Winter 1998			Fall 1998			
			IFA	PCR[a]	Mice infectivity	IFA	PCR[a]	Mice infectivity	IFA	Cp11	Mice infectivity	Water[b]
A	Mt. Vernon Wharf	Wicomico	28[c]	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 Mark	Fishing Bay	29	HT	Neg	0	BT & HT	Neg	1	Pos	Neg	ND
D	Beacon	Potomac	26	BT	ND	ND	ND	ND	2	Pos	Pos	10
E	Holland Point	Patuxent	28	BT	Pos	ND	ND	ND	1	Pos	Neg	31
F	Back Cove	Tangier Sound	ND	ND	ND	2	BT	Neg	6	ND	Neg	8
G	Old Woman's Leg	Tangier Sound	ND	ND	ND	0	BT	Neg	0	ND	Neg	ND

[a]Polymerase chain reaction and restriction fragment-length polymorphism (PCR-RFLP) on small subunit rRNA gene, 18s.
[b]Number of oocysts recovered per liter of filtered bay water.
[c]Number of oysters found positive for oocysts out of 30 oysters examined from each site.
[d]Neg 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

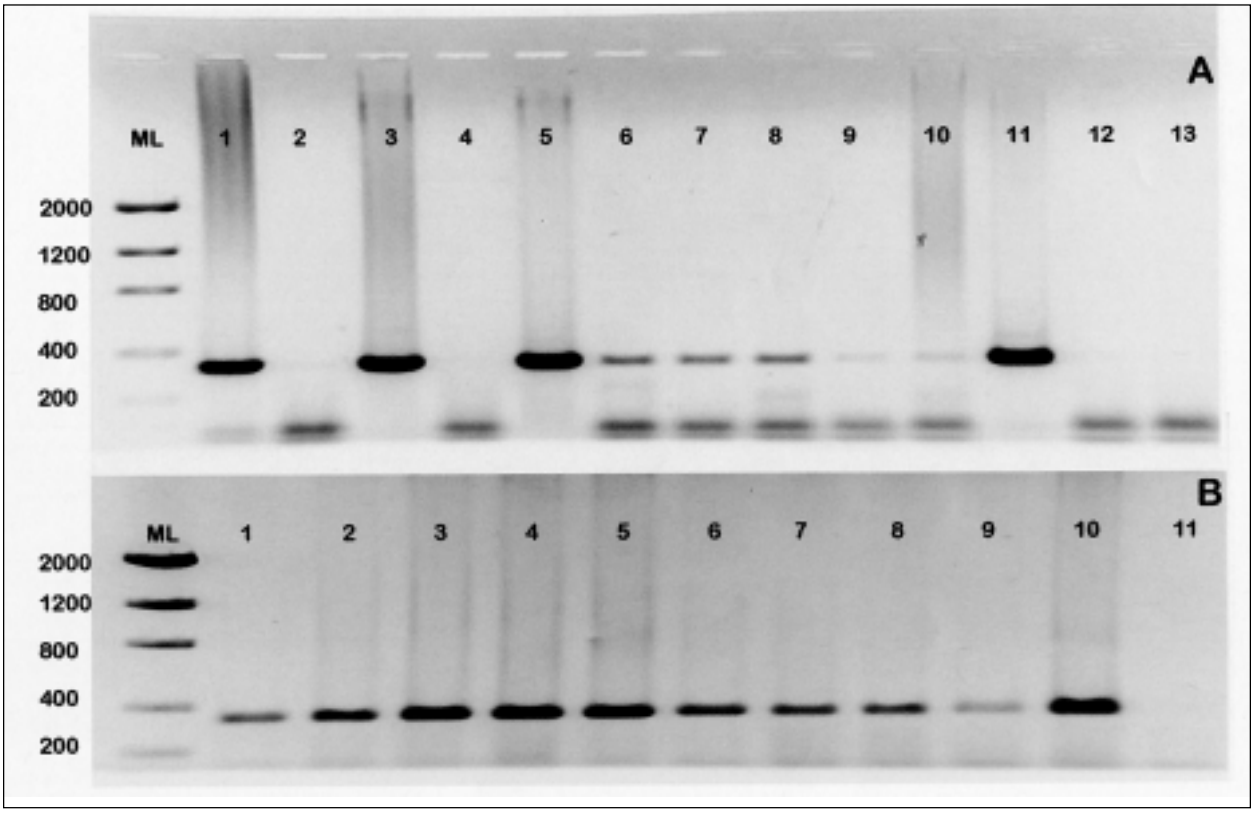

Figure.[1,2] Panel A. Results of nested *Cryptosporidium parvum* CP11 gene PCR performed on pooled oyster hemolymph and gill tissues. Expected PCR product size is 344 bp. Samples analyzed were collected from Maryland Department of Natural Resources oyster harvesting sites at Mt Vernon Wharf (lanes 1-5), 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.

[1]A PCR product of approximately 1,325 bp was amplified first in primary PCR, by using primers 5'-TTCTAGAGCTAATACATGCG-3' and 5'-CCCTAATCCTTCGAAACAGGA-3'. The PCR reaction contained 10 µl of Perkin-Elmer 10X PCR buffer, 6 mM of $MgCl_2$, 200 µM of each dNTP, 100 nM of each primer, 2.5 U Taq polymerase, and 0.25 µl of DNA template, for a total of 100 µl reaction. Thirty-five cycles each consisted of 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 1 minute, with an initial hot start at 94°C for 3 minutes and a final extension at 72°C for 7 minutes. A secondary PCR product of 826 or 864 bp (depending on species) was then amplified from 2 µl of the primary PCR reaction, using primers 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' and 5'-AAGGAGTAAGGAACAACCTCCA-3'. The PCR and cycling conditions were identical to primary PCR, except that 3 mm of $MgCl_2$ was used in the PCR reaction. For restriction fragment analysis, 20 µl of the secondary PCR products were digested in a total of 50 µl reaction mix consisting of 20 units of *Ssp* I (New England BioLabs, Beverly, MA; for species diagnosis), or *Vsp* I (Gibco BRL, Grand Island, NY; for genotyping of *C. parvum*) and 5 µl of restriction buffer at 37°C for 1 hour. The digested products were fractionated on 2.0% agarose gel and stained with ethidium bromide.

[2]PCR products were purified first by the Wizard PCR Preps DNA purification system (Promega, Madison, WI), then sequenced by fluorescent cycle sequencing by using dye terminator chemistry on an ABI 377 Automated Sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA). Sequences obtained from oyster samples were then aligned with sequences obtained from known *Cryptosporidium* spp. (*C. parvum, C. baileyi, C. muris, C. serpentis, C. meleagridis, C. wrairi*, and *C. felis*) and various *C. parvum* genotypes (human, bovine, monkey, dog, pig, mouse, ferret, marsupial, and desert monitor). Isolates corresponding to those transmissible to humans are reported as human genotype or genotype 1, and isolates corresponding to those transmissible among cattle, mice, and humans are reported as bovine genotype or genotype 2.

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.

As a control for the quality of DNA extracted from the oyster tissues, PCR was performed by using primers derived from the small subunit ribosomal RNA sequence of the oyster, *Crassostrea virginica* (6). The OyF forward primer sequence is: 5' GTC TCA AAG ATT AAG CCA TGC ATG 3' (corresponding to nucleotides 34-57), and the OyR reverse sequence is: 5' TGA TTC CCC GTT ACC CGT TAC AA 3' (nucleotides 354-376). The predicted size of the PCR product is 340 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 $MgCl_2$, 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 (13-15 µl) were electrophoresed on 1% agarose gels and visualized with ethidium bromide and UV illumination.

For cycle sequencing, the amplicon was cut from a 1% agarose gel, extracted by using the QIAquick Gel Extraction kit (Qiagen, Valencia, CA) and sequenced by using dye-terminator chemistry on the ABI 373 and 377 models fluorescence automated sequencers (Perkin-Elmer Applied Biosystems, Foster City, CA). Sequences were analyzed with AutoAssembler® (Perkin-Elmer Applied Biosystems, Foster City, CA, and Genetics Computer Group, Madison, WI) software.

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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References

1. Fayer R, Farley CA, Lewis EJ, Trout JM, Graczyk TK. Potential role of the Eastern oyster, *Crassostrea virginica*, in the epidemiology of *Cryptosporidium parvum*. Appl Environ Microbiol 1997;63:2086-8.
2. Fayer R, Graczyk TK, Lewis EJ, Trout JM, Farley CA. Survival of infectious *Cryptosporidium parvum* oocysts in seawater and Eastern oysters (*Crassostrea virginica*) in the Chesapeake Bay. Appl Environ Microbiol 1998;64:1070-4.
3. Jenkins MC, Trout J, Fayer R. Development and application of an improved semiquantitative technique for detecting low-level *Cryptosporidium parvum* infections in mouse tissue using polymerase chain reaction. J Parasitol 1998;84:182-6.
4. Xiao L, Escalante L, Yang C, Sulaiman I, Escalante AA, Montali J, et al. Phylogenetic analysis of *Cryptosporidium* parasites based on the small subunit ribosomal RNA gene locus. Appl Environ Microbiol 1999;65:111578-83.
5. Xiao L, Morgan U, Limor J, Escalante AA, Arrowood M, Shulaw W, et al. Genetic diversity within *Cryptosporidium parvum* and related *Cryptosporidium* species. Appl Environ Microbiol 1999;65:3386-96.
6. Littlewood DT, Ford SE, Fong D. Small subunit rRNA gene sequence of *Crassostrea virginica* (Gmelin) and a comparison with similar sequences from other bivalve molluscs. Nucleic Acids Res 1991;19:6048.
7. U.S. Environmental Protection Agency. Method 1622: *Cryptosporidium* in water by filtration/IM/FA. Washington: The Agency; 1997. EPA 821-R-97-023:51.
8. Graczyk TK, Fayer R, Cranfield MR, Conn DB. Recovery of waterborne *Cryptosporidium parvum* oocysts by freshwater benthic clams (*Corbicula fluminea*). Appl Environ Microbiol 1998;64:427-30.
9. Graczyk TK, Fayer R, Lewis EJ, Trout JM, Farley CA. *Cryptosporidium* oocysts in Bent mussels (*Ischadium recurvum*) in the Chesapeake Bay. Parasitol Res 1999;85:30-4.
10. Harp JA, Fayer R, Pesch BA, Jackkson GJ. Effect of pasteurization on infectivity of *Cryptosporidium parvum* oocysts in water and milk. Appl Environ Microbiol 1996;62:2866-8.
11. Fayer R, Nerad T. Effects of low temperatures on viability of *Cryptosporidium parvum* oocysts. Appl Environ Microbiol 1996;62:1431-3.