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Maximizing Recovery and Detection of *Cryptosporidium parvum* Oocysts from Spiked Eastern Oyster (*Crassostrea virginica*) Tissue Samples[▽]

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Numerous studies have documented the presence of *Cryptosporidium parvum*, an anthropozoonotic enteric parasite, in molluscan shellfish harvested for commercial purposes. Getting accurate estimates of *Cryptosporidium* contamination levels in molluscan shellfish is difficult because recovery efficiencies are dependent on the isolation method used. Such estimates are important for determining the human health risks posed by consumption of contaminated shellfish. In the present study, oocyst recovery was compared for multiple methods used to isolate *Cryptosporidium parvum* oocysts from oysters (*Crassostrea virginica*) after exposure to contaminated water for 24 h. The immunomagnetic separation (IMS) and immunofluorescent antibody procedures from Environmental Protection Agency method 1623 were adapted for these purposes. Recovery efficiencies for the different methods were also determined using oyster tissue homogenate and hemolymph spiked with oocysts. There were significant differences in recovery efficiency among the different treatment groups ($P < 0.05$). We observed the highest recovery efficiency (i.e., 51%) from spiked samples when hemolymph was kept separate during the homogenization of the whole oyster meat but was then added to the pellet following diethyl ether extraction of the homogenate, prior to IMS. Using this processing method, as few as 10 oocysts could be detected in a spiked homogenate sample by nested PCR. In the absence of water quality indicators that correlate with *Cryptosporidium* contamination levels, assessment of shellfish safety may rely on accurate quantification of oocyst loads, necessitating the use of processing methods that maximize oocyst recovery. The results from this study have important implications for regulatory agencies charged with determining the safety of molluscan shellfish for human consumption.

Cryptosporidium parvum is an anthropozoonotic protozoan parasite that causes an acute self-limiting gastrointestinal illness in immunocompetent individuals but can contribute significantly to morbidity and mortality of the immunocompromised, due in part to the lack of reliably effective therapeutics (2, 20). *Cryptosporidium* is transmitted fecal-orally by ingestion of the sporulated oocysts, and most human cryptosporidiosis outbreaks have been associated with waterborne routes of transmission (22). Contamination of surface waters used for drinking water, recreational activities, and shellfish production with *Cryptosporidium* can occur via agricultural runoff, wastewater discharges, storm water runoff, and fecal contamination by wildlife (12).

Oysters, as filter feeders, are capable of bioaccumulating water contaminants, including human pathogens. The ability of the Eastern oyster (*Crassostrea virginica*) to remove *C. parvum* oocysts from artificially contaminated water and retain them in hemolymph, on gills, and in the oyster body for periods up to 1 month after exposure has been demonstrated previously (3). Numerous studies report finding *C. parvum* oocysts in oysters and other bivalve mollusks collected from commercial shellfish harvesting sites (5, 7, 9, 10, 14, 16, 18, 21), indicating a risk of human cryptosporidiosis from the consumption of contaminated bivalves, especially those that are consumed raw such as oysters. Commercial shellfish harvesting sites are monitored for the presence of fecal coliforms as an indicator of water quality and shellfish safety; however, *Cryptosporidium* oocyst levels may not correlate with fecal coliform counts (1, 10).

The isolation of *Cryptosporidium* oocysts from naturally contaminated oysters is technologically complex, and low recovery efficiencies resulting from the tissue processing steps present a major challenge for detection of *Cryptosporidium* in environmental samples. Several methods for processing oyster tissues to recover *Cryptosporidium* oocysts can be found in the literature (4, 8, 10, 15, 19), but no data are available on the relative recovery efficiencies of the different methods. The purpose of the present study was to determine the recovery efficiency of different oyster processing methods and to establish an optimal method for detection of commercial oysters naturally contaminated with *Cryptosporidium*. The results from this study will be used to determine the best method for quantifying *Cryptosporidium* levels in native oysters collected from the Chesapeake Bay but also have broad applicability to all oyster species harvested commercially.

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MATERIALS AND METHODS

Source of *C. parvum* oocysts. The oocysts were obtained from experimental infection of a female Holstein calf, extracted from the feces using continuous

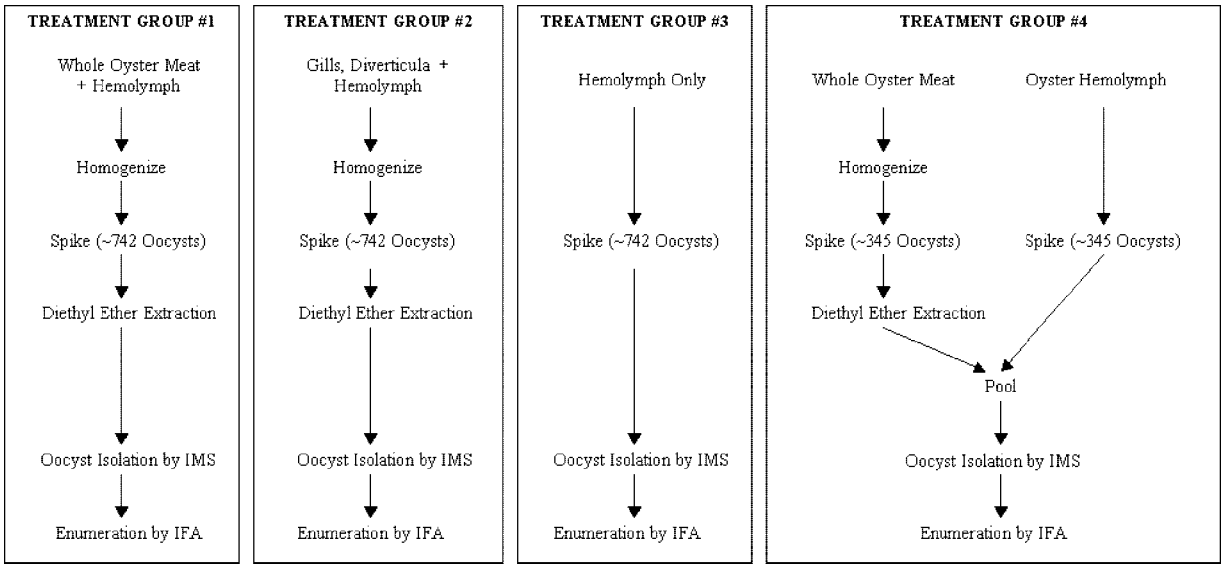

FIG. 1. Diagram presenting four treatment methods of Eastern oyster (*Crassostrea virginica*) tissue spiked with *Cryptosporidium parvum* oocysts.

flow centrifugation, purified by cesium chloride gradient centrifugation, and stored at 4°C in phosphate-buffered saline (PBS) (pH 7.4).

Processing of *C. parvum* spiked oyster tissue samples. One hundred *C. virginica* oysters were obtained from Oyster Ranchers Association Inc., Ridge, MD; placed into 208 liters of artificial seawater (salinity, 12 ppt); and left for 2 days to equilibrate. The recovery of oocysts was compared using four different oyster tissue processing methods (groups 1 to 4) (Fig. 1). For treatment group 1, the entire oyster tissue from 11 oysters, including hemolymph aspirated from the adductor muscle prior to shucking, was pooled, added to 60 ml of PBS, and homogenized by 5 pulses of 30 s each, using an Omni Mixer homogenizer (Omni International, Marietta, GA). The homogenate was divided evenly among 11 50-ml conical tubes. For group 2, hemolymph was collected from 11 oysters, which were then shucked and dissected to remove the gills and digestive diverticula. These tissues were added to the hemolymph along with 140 ml of PBS, homogenized, and divided evenly among 11 tubes. For group 3, only hemolymph was collected from 11 oysters, which was then pooled and distributed evenly among 11 tubes. For group 4, the meat was processed separately from the hemolymph. The whole meat from 11 oysters was pooled, added to 120 ml of PBS, and homogenized as described above, while hemolymph from those oysters was pooled and kept separately. Again, both homogenate and hemolymph were divided equally among 11 tubes.

Ten of 11 tubes from each group were spiked with *C. parvum* oocysts, and the last tube was treated as an unspiked negative control. Each tube was spiked with $\sim 1.0 \times 10^3$ oocysts except tubes from group 4, where meats and hemolymph were kept separate. For this group, each tube was spiked with $\sim 5 \times 10^2$ oocysts. All tubes were vortexed and stored overnight at 4°C. To verify the actual number of oocysts spiked into each sample, the same volume of oocyst suspension was added directly to three slide wells, allowed to dry, and fixed with methanol and then oocysts were counted using immunofluorescent antibodies (IFA). The average of the replicates was used as the actual number of oocysts spiked into each sample.

Ten milliliters of diethyl ether was added to each tube of oyster homogenate, resulting in a total volume of 30 ml. The tubes were vortexed for 30 s and then centrifuged (1,000 × *g*; 10 min) to pellet oocysts. The supernatant, including the tissue suspended between the aqueous and organic layers, was removed, and the pellet was washed twice, once with 20 ml of PBS and again with 20 ml of distilled water. For group 4, where hemolymph was not homogenized with the meat, the hemolymph was added to the distilled water during the second wash. The pellets were resuspended in 10 ml distilled water. For group 3, where only the hemolymph was collected, distilled water was added to the hemolymph to a 10-ml total volume.

Processing of oysters exposed in *C. parvum*-contaminated water. A 208-liter tank filled with artificial seawater at 12-ppt salinity and holding 55 oysters was spiked with 1.0×10^5 *C. parvum* oocysts per oyster for a total inoculum of 5.5×10^6 *C. parvum* oocysts. To induce filter feeding, 1 ml of Instant Algae shellfish food (Reed Mariculture Inc., Campbell, CA) was added to the tank. Oysters were allowed to filter the water for 24 h. The recovery of oocysts was compared using four different processing methods, with 10 oysters processed separately for each group (i.e., I to IV) (Fig. 2). For treatment group I, whole oyster tissue and hemolymph from each oyster were homogenized together after 1× PBS was added to a total volume of 20 ml. For treatment group II, the gills and digestive diverticula were added to the hemolymph along with 1× PBS to a total volume of 20 ml and then homogenized. For treatment group III, the hemolymph was drawn and each oyster was dissected to remove the gills and digestive diverticula. The meat and hemolymph were kept in separate tubes. After 1× PBS was added to the gills and digestive diverticula to a total volume of 20 ml, the meat was homogenized. The hemolymph samples were brought up to a 10-ml total volume with 1× PBS. For treatment group IV, oocyst recovery from meat and that from hemolymph were characterized separately in order to determine the distribution of oocysts between meat and hemolymph after natural uptake. Hemolymph was drawn from the oysters, and gills and digestive diverticula were dissected from the meat and stored separately. Gills and diverticula were homogenized after 1× PBS was added to a total volume of 20 ml. Hemolymph samples were brought up to a total volume of 10 ml with 1× PBS. To determine whether the distribution of oocysts between oyster meat and hemolymph is related to exposure time, 10 oysters were harvested from the tank 1 week after water contamination and processed as described for group IV.

Ten milliliters of diethyl ether was added to each tube of oyster homogenate, resulting in a total volume of 30 ml. The tubes were vortexed for 30 s and then centrifuged at 1,000 × *g* for 10 min to pellet *C. parvum* oocysts. The supernatant was removed, and the pellet was washed twice, once with 20 ml of PBS and again with 20 ml of distilled water. For group III, where hemolymph was not homogenized with the meat, the hemolymph was added to 10 ml of distilled water during the second wash. The pellets were resuspended in 10 ml distilled water. For group IV, immunomagnetic separation (IMS) was performed separately on the hemolymph and resuspended homogenate pellets.

IMS and IFA procedures. To recover *C. parvum* oocysts from the homogenate pellets or hemolymph, each 10-ml sample was processed by IMS according to the manufacturer's instructions (Dynabeads anti-*Cryptosporidium* and anti-*Giardia* kit; Dynal) except for the addition of a second wash step using 1 ml of 1× SL-Buffer A and a second elution that was combined with the first elution for 100 μl total eluant on each slide well. Negative controls consisted of 10 ml of distilled water, and for the positive control, 10 ml of distilled water was spiked with $\sim 1.0 \times 10^3$ *C. parvum* oocysts. Slides were fixed with methanol and air dried, and oocysts were enumerated by IFA using a commercially available test kit (Meri-Fluor Cryptosporidium/Giardia; Meridian Bioscience Inc.) per the manufacturer's instructions.

PCR detection of *C. parvum* oocysts from oyster homogenates. To determine the limit of detection using the optimized processing method for recovery, a nested PCR procedure was performed on DNA extracted from *C. parvum* oocysts recovered from spiked oyster homogenates. Five unexposed oysters were processed as described for treatment group 4 from the homogenate spiking

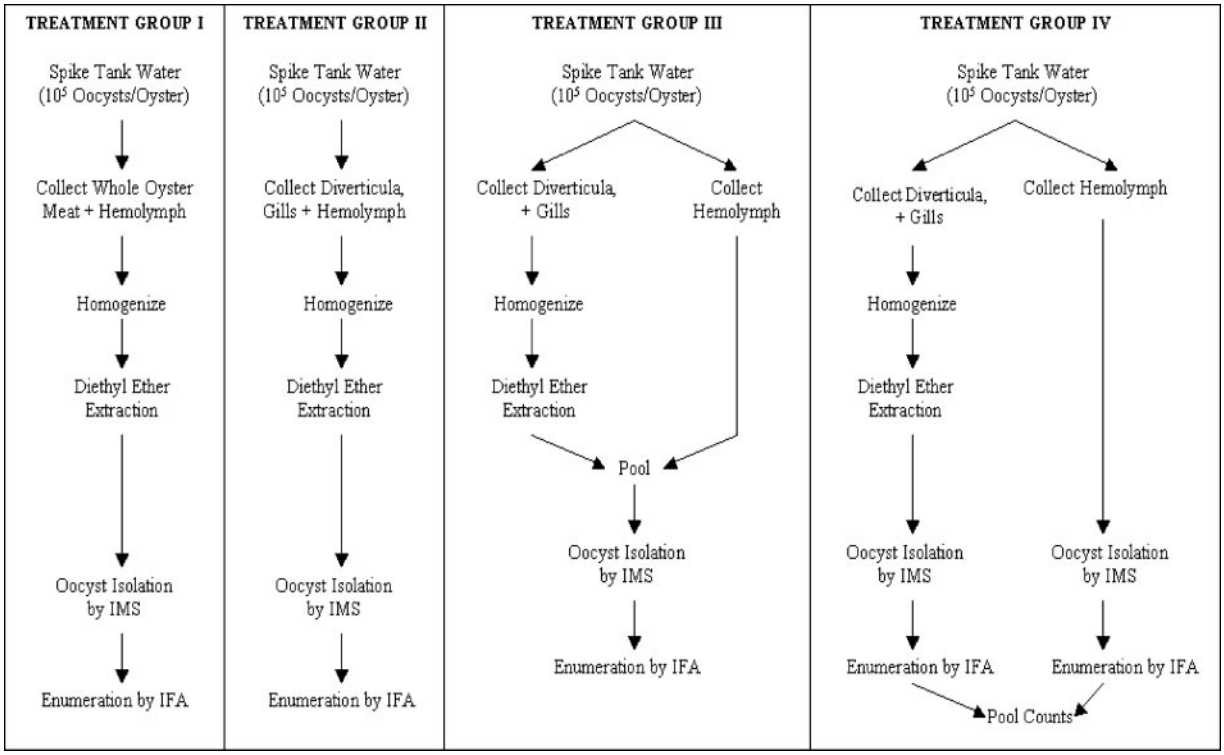

FIG. 2. Diagram presenting four treatment methods applied to Eastern oysters (*Crassostrea virginica*) exposed to *Cryptosporidium parvum* in contaminated water.

experiment, except that IMS eluants were stored in microcentrifuge tubes instead of being fixed onto slides. Each oyster sample was spiked with 10, 100, 1,000, or 10,000 *C. parvum* oocysts. Tris-EDTA (pH 8) was added to the IMS eluants to a final volume of 500 μl. Twenty microliters of 10% sodium dodecyl sulfate and 5 μl 20-mg/ml proteinase K were added to each tube and then incubated overnight at 45°C. DNA was purified by phenol-chloroform extraction and ethanol precipitation. To determine if PCR inhibitors were preventing detection of spiked homogenates, genomic DNA was added to the DNA extracted from the oyster sample spiked with 1.0×10^4 oocysts just prior to PCR amplification. A 434-bp fragment of the 18S rRNA gene was amplified using methods modified from the work of Jellison et al. (17). Briefly, a 1,056-bp fragment was amplified using the forward primer KLJ1 (5′-CCACATCTAAGGAAGGCAG C-3′) and reverse primer KLJ2 (5′-ATGGATGCATCAGTGTAGCG-3′). For the secondary PCR, the forward primer CPB-DIAGF (5′-AAGCTCGTAGTT GGATTTCTG-3′) and the reverse primer CPB-DIAGR (5′-TAAGGTGCTGA AGGAGTAAGG-3′) were used to amplify the 434-bp fragment. The initial amplification reaction was performed with 15 μl of DNA template, and 3 μl of the primary amplification product was used as a template for the secondary PCR. Each PCR consisted of 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 60 s; an initial denaturation step consisting of incubation at 94°C for 4 min and a final extension step consisting of incubation at 72°C for 10 min were also included. PCR products were visualized on a 1% agarose gel stained with ethidium bromide.

Statistical analysis. The statistical analysis was performed using STATA 8.0 (Stata Corporation, College Station, TX). A Kruskal-Wallis nonparametric test of equality was used to determine if there were any significant differences in recovery between any of the oyster processing method groups. Pairwise comparisons using a nonparametric two-sample Mann-Whitney test were used to determine which groups had significantly different oocyst recoveries. Results were considered to be significant at $P < 0.05$.

RESULTS

Recovery of *C. parvum* oocysts from spiked tissue samples. *C. virginica* homogenates were spiked with $\sim 1.0 \times 10^3$ or $\sim 5.0 \times 10^2$ *C. parvum* oocysts, depending on processing treatment group. Based on direct IFA counts of the spiking suspension the actual numbers of oocysts spiked into the homogenates were on average 742 and 345 oocysts, respectively. The average numbers of oocysts recovered and the recovery percentages for each treatment group are presented in Table 1. There were significant differences in recovery among the treatment groups (Kruskal-Wallis, $P = 0.0001$). Pairwise comparisons using a two-sample Mann-Whitney test showed that oo-

TABLE 1. IFA results obtained from oyster homogenates spiked with *Cryptosporidium parvum* oocysts and oysters exposed for 24 h to *C. parvum*-contaminated water

C. parvum exposure	Oyster processing method	Mean oocyst count ± SD	Recovery (%)[a]
Spiked homogenate	1	82 ± 26	11.0
	2	150 ± 54	20.1
	3	306 ± 43	41.3
	4	351 ± 38	50.9
Exposure for 24 h	I	2,735 ± 2,164	
	II	1,026 ± 940	
	III	1,000 ± 641	
	IV	829 ± 1,309[b]	

[a] For treatment groups 1 to 3 the denominator (spiked oocyst number) was 742 oocysts, but for treatment group 4, where hemolymph and tissue homogenate were spiked separately with 345 oocysts each, a denominator of 690 oocysts was used.

[b] Values for treatment group IV are the sum of oocyst recoveries from hemolymph and tissue homogenate, which were processed and counted separately.

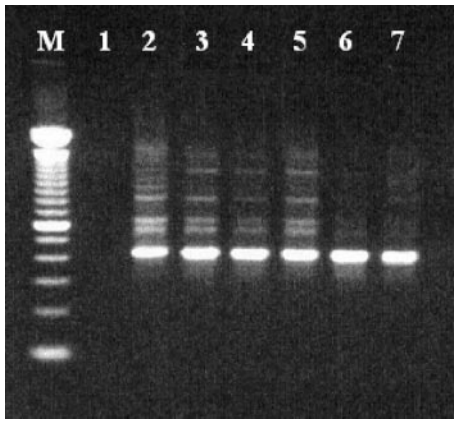

FIG. 3. Nested PCR detection of *Cryptosporidium parvum* oocysts spiked into Eastern oyster (*Crassostrea virginica*) tissue homogenate. Lanes: M, 100-bp molecular marker; 1, PCR negative control; 2, PCR positive control; 3, 10^4 oocysts plus *C. parvum* genomic DNA; 4, 10^4 oocysts; 5, 10^3 oocysts; 6, 10^2 oocysts; 7, 10 oocysts.

cyst recovery from treatment group 4 was significantly greater than the recovery from treatment group 3 ($P = 0.0143$), oocyst recovery from treatment group 3 was greater than recovery from group 2 ($P = 0.0002$), and oocyst recovery from treatment group 2 was greater than recovery from group 1 ($P = 0.0036$). The recovery efficiency of the IMS kit as determined by numbers of oocysts recovered from distilled water spiked with *C. parvum* oocysts was 90%. No oocysts were detected in any of the negative-control oysters by IFA.

Recovery of *C. parvum* oocysts from oysters exposed to *C. parvum*-contaminated water. After 24 h of exposure to *C. parvum*-contaminated water, all oysters tested positive for the oocysts by IFA, regardless of the processing method used to recover oocysts. The average number of oocysts observed by IFA for each treatment group is shown in Table 1. For treatment group IV, where hemolymph and tissue homogenates were processed and analyzed separately, the average recovery from hemolymph alone was 116 oocysts (14% of total recovery), while the average recovery from the tissue homogenate was 713 oocysts (86% of total recovery). A nonparametric Kruskal-Wallis test of equality showed that there were significant differences in oocyst recoveries depending on processing method used ($P = 0.0404$). Pairwise comparisons using a nonparametric two-sample Mann-Whitney test showed that recovery from treatment group I was greater than recovery from treatment group II ($P = 0.0494$) and treatment group IV ($P = 0.0082$) but not statistically different from recovery from treatment group III ($P = 0.0696$).

One week after exposure to *C. parvum*-contaminated water, average recoveries from oysters ($n = 10$) processed using the method for treatment group IV were 27 oocysts (i.e., 40% total recovery) from hemolymph samples and 40 oocysts from tissue homogenates (i.e., 60% of total recovery).

PCR detection of *C. parvum* oocysts from oyster homogenates. For oysters processed as described for treatment group 4 of the homogenate spiking experiment, as few as 10 spiked oocysts could be detected using a nested PCR procedure (Fig. 3). *Cryptosporidium* DNA was amplified for each of the spiked samples, including the DNA extract to which genomic DNA was added, indicating that PCR inhibition was not observed.

DISCUSSION

The considerable differences in *C. parvum* oocyst recovery among treatment groups indicate that the method used to process oysters and other bivalve mollusks collected from commercial harvesting sites has a large impact on the likelihood of detecting *Cryptosporidium* contamination and thus properly assessing the safety of shellfish for human consumption. The authors of one study (19) that compared recoveries from spiked whole-tissue homogenates, gill washings, and hemolymph concluded that processing whole tissue gave superior recoveries, but no data were provided. Though IMS is a standard procedure for isolating *Cryptosporidium* oocysts from water samples as described in Environmental Protection Agency method 1623 (23), few studies of *Cryptosporidium* contamination of shellfish have made use of this concentration step, which may increase sensitivity of detection. Incorporation of the IMS step allows the entire sample to be screened for the presence of oocysts, avoiding the need to dilute the tissue homogenate, hemolymph, or gill wash sample for visualization on the IFA slide. In the present study, the IMS protocol used for analyzing water samples was modified by the addition of an extra wash step, which considerably reduced the amount of oyster tissue debris on IFA slides following elution, thereby improving slide readability and accuracy of oocyst recovery counts.

In the present study, we observed the highest recovery efficiency (i.e., 51%) from spiked samples when hemolymph was kept separate during the homogenization of the oyster meat but was then added to the pellet following diethyl ether extraction of the tissue homogenate, prior to IMS (treatment group 4). This level of recovery is within the range observed by other groups using spiked oyster samples (19). After exposure of *C. virginica* oysters to *C. parvum*-contaminated water, the greatest number of oocysts was recovered from whole-tissue homogenates that included the hemolymph (treatment group I). However, in the tissue homogenate spiking experiment, this treatment group (i.e., group 1) had the lowest recovery efficiency. Low recovery efficiency may be caused by the large amounts of tissue that result from processing a whole oyster, to which oocysts may adhere during the IMS step, interfering with their binding to the immunomagnetic beads used for isolating oocysts from the suspension. Alternatively, there may be some losses during the homogenization step due to shearing of oocysts or adherence of oocysts to the sides of the canister, making homogenization of the hemolymph undesirable. The extreme variability in oocyst recoveries observed within treatment groups of the *C. parvum*-contaminated water exposure experiment may result from variability in oyster water filtering rates. However, this makes it difficult to have confidence in comparisons among groups. For this reason, recovery efficiency using spiked samples may be a better outcome by which to choose a processing method than absolute numbers of oocysts recovered from shellfish exposed to contaminated water.

Pools of oysters are often tested for *Cryptosporidium* in order to improve the likelihood of detection and to screen large numbers of oysters quickly (5, 7, 10, 16). Processing and testing of individual oysters are far more labor-intensive and time-consuming. For testing oyster pools, the use of whole-tissue homogenates presents a processing challenge due to the large

amounts of tissue involved. In this situation, it may be beneficial to process only the gills, digestive diverticula, and hemolymph. Though dissecting oysters prior to homogenization does add another step to the processing method, the added time for oyster dissection is justified by the time that is saved by processing and testing oyster pools instead of individual oysters.

Though efficiency of recovery from spiked hemolymph samples was high, separate processing and enumeration of oocysts from hemolymph and tissue homogenates after exposure of oysters to contaminated water demonstrated that shortly after exposure, the majority of oocysts were associated with the oyster meat and not the hemolymph. However, in the samples processed 1 week after exposure to contaminated water, this ratio was very different, with nearly one-half of the total oocysts recovered coming from the hemolymph. This may result from the way that the oysters deal with oocysts taken up during filter feeding. Shortly after exposure, oocysts may be caught in the gills and other tissues of the digestive system, but with time, hemocytes scavenge these particles in an attempt to clear them from the gills and digestive tract. Since it is unlikely that the time of contamination will be known when oysters are collected from commercial harvesting sites, it is important that both oyster meat and hemolymph be processed for optimal *Cryptosporidium* oocyst detection.

It is unclear whether IFA or PCR is the best method for detecting *Cryptosporidium* oocysts in bivalve mollusks. Samples tested using both methods can produce discrepant results, indicating that using both methods on a sample increases the likelihood of detecting *Cryptosporidium* contamination (6, 11). Each method has advantages and disadvantages. IFA is a well-established method that is used in the Environmental Protection Agency method 1623 for detecting and quantifying *Cryptosporidium* in water samples (23). However, considerable amounts of debris remain even after the IMS step when oyster tissues are processed. This debris may obstruct oocysts, preventing accurate quantification. Nested PCR has great sensitivity, reported down to a single oocyst (17), but may be affected by PCR inhibitors and is difficult to use for quantification. PCR can also be used to genotype oocysts recovered from environmental samples, thereby providing information on human health risks from exposure. By dividing the IMS eluant in half, it is possible to apply both techniques to an individual sample, maximizing the likelihood of *Cryptosporidium* detection and providing information on oocyst loads as well as genotypes. In the present study, we did not observe any evidence of PCR inhibition and we were able to detect down to 10 oocysts, which was the lowest spiking level tested.

The identification of *Cryptosporidium* oocysts in oysters and other shellfish harvested for human consumption presents an important public health question on whether steps should be taken to prevent shellfish harvested from areas where contamination has been identified from reaching the retail market. To date there are no published reports of human cryptosporidiosis cases resulting from the consumption of raw oysters, despite a growing body of scientific literature on the recovery of *Cryptosporidium* oocysts from oysters collected from commercial harvesting sites (5, 7, 9, 10, 14, 16, 18, 21). The reason for this discrepancy is unclear. One explanation is that infections caused by consumption of contaminated oysters are not being diagnosed or reported. The long incubation period of the disease (i.e., 7 to 10 days) makes it difficult to associate the infection with a particular exposure (13). Healthy individuals who experience only mild, transient symptoms may not seek medical attention or may not be tested for a causative agent. Due to the increased severity of symptoms in immunocompromised individuals, *Cryptosporidium* infections in this population may be more likely to be diagnosed. However, AIDS patients on highly active antiretroviral therapy may be less susceptible to infection or may clear the infection without medical intervention, as evidenced by a decline in cryptosporidiosis prevalence among AIDS patients since the introduction of highly active antiretroviral therapy (24), resulting in fewer diagnoses among the immunocompromised. Alternatively, oocyst loads in contaminated shellfish may be too low to establish infections or the oocysts recovered may not be viable.

In the absence of other water quality indicators that correlate with *Cryptosporidium* contamination levels, assessment of shellfish safety may rely on accurate quantification of oocyst loads. To meet this public health challenge, processing methods that maximize oocyst recovery must be used. This study demonstrated that there is considerable variability in oocyst recovery among different shellfish processing methods, which has major implications for regulatory agencies charged with monitoring of molluscan shellfish safety.

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