

Quantitative assessment of viable *Cryptosporidium parvum* load in commercial oysters (*Crassostrea virginica*) in the Chesapeake Bay

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Abstract The epidemiological importance of increasing reports worldwide on *Cryptosporidium* contamination of oysters remains unknown in relation to foodborne cryptosporidiosis. Thirty market-size oysters (*Crassostrea virginica*), collected from each of 53 commercial harvesting sites in Chesapeake Bay, MD, were quantitatively tested in groups of six for *Cryptosporidium* sp. oocysts by immunofluorescent antibody (IFA). After IFA analysis, the samples were retrospectively retested for viable *Cryptosporidium parvum* oocysts by combined fluorescent in situ hybridization (FISH) and IFA. The mean cumulative numbers of *Cryptosporidium* sp. oocysts in six oysters (overall, 42.1 ± 4.1) were significantly higher than in the numbers of viable

C. parvum oocysts (overall, 28.0 ± 2.9). Of 265 oyster groups, 221 (83.4%) contained viable *C. parvum* oocysts, and overall, from 10–32% (mean, 23%) of the total viable oocysts were identified in the hemolymph as distinct from gill washings. The amount of viable *C. parvum* oocysts was not related to oyster size or to the level of fecal coliforms at the sampling site. This study demonstrated that, although oysters are frequently contaminated with oocysts, the levels of viable oocysts may be too low to cause infection in healthy individuals. FISH assay for identification can be retrospectively applied to properly stored samples.

Introduction

Cryptosporidium parvum is a waterborne parasite frequently identified in oysters intended for human consumption worldwide (Graczyk 2003). This enteric protozoan causes diarrheal disease (Graczyk et al. 1997a) and contributes significantly to the mortality of immunocompromised people and morbidity of immunocompetent individuals due to the lack of fully effective therapy (Dawson 2005). *Cryptosporidium* is dispersed in water via its minute (approximately 5 μm), environmentally robust transmissive stage, the oocyst.

Although multiple studies have identified human-infectious *Cryptosporidium* in commercial oysters worldwide (Graczyk 2003), there are no published reports of cryptosporidiosis from oyster consumption despite a long list of other infections acquired via eating oysters (OzFoodNet Working Group 2005). The epidemiological reason for this discrepancy is unclear, but it may be related to the concentration and viability of *C. parvum* oocysts in oysters consumed raw and the acquired immunity status of the consumers. A common technique for identification of

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C. parvum in oysters, immunofluorescent antibody (IFA) detection (Fayer et al. 1998), overestimates the infectious pathogen load because the antibodies cross react with species of *Cryptosporidium* that are not infectious to humans (Graczyk et al. 1996) and small unicellular algae (Clancy et al. 1994), and nonviable oocysts (Graczyk et al. 1997b). Most of the studies on *Cryptosporidium* in oysters utilize molecular identification techniques such as polymerase chain reaction (PCR), nested PCR, and/or restriction fragment length polymorphism (RFLP) (Graczyk 2003), which, although very sensitive, do not assess viability of the detected oocysts. As a consequence, the data obtained in published reports are not fully applicable to assess the risk of foodborne cryptosporidiosis, although they provide solid scientific information on contamination of oysters with human-infectious *Cryptosporidium*.

Recovery of oocysts from oyster tissue is a technologically complex process, but even more challenging are subsequent species-specific identification and viability assessment of oocysts recovered from tissue or environmental samples. The fluorescent in situ hybridization (FISH) technique meets both these challenges, that is, providing species-specific identification with simultaneous viability assessment of identified parasites (Smith et al. 2004). The FISH utilizes a fluorescently labeled oligonucleotide probe, that is, CRY-1, designed to hybridize with specific sequences of 18S rRNA of *C. parvum* (Smith et al. 2004). Because rRNA is only present in large copy numbers in viable organisms (Smith et al. 2004), FISH allows species-specific detection by providing visualization of the oocysts and facilitates enumeration of viable *C. parvum* oocysts (Graczyk et al. 2004). Most importantly, *Cryptosporidium* RNA and DNA in preserved samples is stable for years (Amar et al. 2005), which can facilitate retrospective analyses. The purposes of the present study was to determine if FISH assay for identification of viable *C. parvum* oocysts can be retrospectively applied to samples and to enumerate viable oocysts of *C. parvum* in the tissue of Eastern oysters (*Crassostrea virginica*) commercially harvested from the Maryland portion of Chesapeake Bay.

Materials and methods

Thirty commercial-size *C. virginica* (>7.6 cm shell length) were collected by dredging (Graczyk et al. 2000) from each of 53 sites in 11 regions (Tables 1 and 2) of the Maryland portion of Chesapeake Bay. Oyster collections were carried out in July 2000 at sites approved for commercial shell fishing by the Maryland Department of the Environment (MDE). Water quality parameters such as benthic water temperature, salinity, and dissolved oxygen were measured

at each site (Table 2). The last three fecal coliform counts for each site were obtained from the MDE. Fecal coliform testing of shellfish harvesting waters is routinely carried out by the MDE with a frequency of 8–11 days.

Thirty oysters from each site were placed separately in mesh bags and transported to the laboratory in insulated coolers. Approximately 5 ml of hemolymph was collected from each oyster (Graczyk et al. 1998). Hemolymph samples of six oysters from the same site were combined into plastic 50-ml tubes to yield five groups from each sample of 30 oysters. Each of the tubes were vortexed for 2 min, centrifuged (3,000×g; 10 min), the supernatant discarded, and the pellet resuspended in 50 ml of sterile phosphate-buffered saline (PBS; pH 7.4). The oysters were shucked, and the gills were excised from each oyster and placed separately into a 15-ml plastic tube containing 5 ml of sterile PBS. The tubes were vortexed for 2 min, gills removed, and the resulting suspensions from each of six oysters from the same site were placed together into 50-ml plastic tubes. The tubes were centrifuged (3,000×g; 10 min), the supernatant was discharged, and the pellet resuspended in 50 ml of sterile PBS. The remaining meat from each set of six oysters was placed together in a plastic bag and weighed. Gill- and hemolymph-derived samples were processed separately by the cellulose acetate membrane (CAM)-filter dissolution method (Graczyk et al. 1997b). The resulting pellets were placed on three lysine-coated immunofluorescent slide wells (1.0-cm diameter). The slides were air-dried, fixed with methanol, and processed with fluorescein isothiocyanate (FITC)-conjugated fluorescent antibodies (IFA) against the cell wall antigens of *Cryptosporidium* (i.e., MERIFLUOR; Meridian Diagnostic, Inc., Cincinnati, OH) as described previously (Graczyk et al. 1996). Testing included positive and negative controls provided by the manufacturer. *Cryptosporidium* oocysts were enumerated with the aid of an Olympus BH2-RFL epifluorescent microscope, dry ×60 objective, and BP450-490 exciter filter. The slides were stored at −70°C.

Small aliquots, approximately 150 µl, of oyster hemolymph and gill washing samples from each area of Chesapeake Bay were used for PCR analysis. DNA was extracted from these aliquots using proteinase K digestion and glass bead disruption of the oocysts as described elsewhere (Pieniazek et al. 1999). The PCR amplification was performed using primers designated to amplify specific for the *Cryptosporidium* oocyst wall protein (COWP) and SSUrRNA genes following previously described protocols (Johnson et al. 1995; Pieniazek et al. 1999). Amplicons were subjected to DNA sequence analysis to confirm the species of *Cryptosporidium*. Amplified products from all PCR reactions were purified by using the Strataprep PCR purification kit (Stratagene, La Jolla, CA) according to the

Table 1 Descriptive statistics of mean cumulative number of viable *Cryptosporidium parvum* oocysts identified by combined FISH and IFA, and *Cryptosporidium* sp. oocysts identified by IFA in six commercial-size (>7.6 cm shell length) Eastern oysters (*Crassostrea virginica*)

Bay region, oyster sites (<i>n</i>)	Number of oocysts				Cumulative wet meat weight of 6 oysters (g)
	IFA		FISH and IFA		
	Range	Mean±SD	Range	Mean±SD	
Upper Bay (8)	5–105	53.3±11.7	5–57	32.9±6.1	156.8±35.3
Chester River (4)	5–52	28.5±10.5	3–39	28.5±10.5	216.0±36.5
Eastern Bay (6)	1–75	35.7±11.4	5–57	31.2±7.9	199.1±79.4
Miles River (4)	4–49	27.0±10.1	3–29	16.5±5.7	180.2±38.2
Wye River (4)	29–125	80.0±20.5	5–106	47.8±21.2	197.4±34.9
Nanticoke River (1)	–	23	–	10	92.8±36.4
Tangier Sound (9)	4–40	24.9±5.1	2–34	13.4±3.9	167.1±27.8
Manokin River (1)	–	22	–	7	150.0±21.2
Choptank River (11)	5–80	51.3±9.1	13–66	41.4±4.9	167.1±27.8
Wicomico River (2)	16–55	35.5±19.5	5–21 13.0±8.0	–	190.8±55.2
Potomac River (3)	23–75	49.0±15.1	2–43	22.0±11.8	161.5±43.8

Thirty oysters were collected from each of 53 commercial sites located in the 11 regions (listed from North to South) of the Maryland portion of Chesapeake Bay.

FISH fluorescent in situ hybridization; IFA immunofluorescent antibody

manufacturer's instructions. Sequencing analysis was performed using cycle sequencing using the ABI BigDye version 3.0 sequencing kit (ABI, Foster City, CA). Sequencing reactions were analyzed on the PerkinsElmer ABI 3100 sequence analyzer, and the sequences were assembled using SeqMan II (DNASTAR Inc., Madison, WI).

In 2004, glass slides were removed from the freezer, thawed, coded, and used for combined FISH and direct IFA analysis (Graczyk et al. 2004). An oligonucleotide probe,

CRY-1 (5' CGG TTA TCC ATG TAA GTA AAG 3') 5' labeled with a single molecule of a fluorochrome [i.e., hexachlorinated 6-carboxyfluorescein (HEX)] was used (Graczyk et al. 2004; Smith et al. 2004). The probe hybridizes to positions between 138 and 160 on the *C. parvum* 18S rRNA (Deere et al. 1989). The probe was synthesized by the DNA Analysis Facility of the Johns Hopkins University, Baltimore, MD, in 1.0 μM scale and purified by high-performance liquid chromatography (HPLC). The slides were placed in a humidified chamber,

Table 2 Mean±standard deviation of water quality parameters at all 53 commercial Eastern oyster (*Crassostrea virginica*) harvesting sites in the 11 regions (listed from North to South) of the Maryland portion of Chesapeake Bay

Bay region, oyster sites (<i>n</i>)	Surface water			
	Temperature (°C)	Salinity (ppt)	Dissolved O ₂ (mg/l)	fecal coliforms (MPN/100 ml)
Upper Bay (8)	24.1±0.6	8.5±0.9	6.3±0.4	1.3±0.6
Chester River (4)	25.2±1.2	8.9±0.7	7.4±0.47	5.6±3.0
Eastern Bay (6)	24.6±0.5	15.8±1.7	7.3±0.83	2.1±1.6
Miles River (4)	24.8±0.2	15.8±2.2	6.0±1.4	4.6±1.4
Wye River (4)	24.8±0.2	14.7±0.4	5.5±0.46	7.7±2.2
Nanticoke River (1)	29.9	15.3	6.1	2.7
Tangier Sound (9)	25.2±1.2	12.3±2.7	6.2±0.4	1.9±0.7
Manokin River (1)	25.8	7.9	6.9	1.0
Choptank River (11)	25.2±1.2	12.3±2.7	6.2±.40	1.9±2.4
Wicomico River (2)	26.1±0.0	8.7±1.1	6.6±0.7	2.9±0.2
Potomac River (3)	25.7±0.4	8.3±0.4	6.3±0.1	3.8±0.3

Fecal coliform data courtesy of the Maryland Department of the Environment.
MPN most probable number

and all combined FISH and IFA reactions were carried out in a total volume of 200 μ l of hybridization buffer at 48°C for 1 h (Graczyk et al. 2004). FISH and IFA testing included positive and negative controls. The concentration of oligonucleotide probe was 1 mmol, and the fluorescently labeled antibodies were diluted 1:1 (Graczyk et al. 2004). After hybridization, the area of each well was examined as described above for the IFA testing, and only oocysts that showed both FISH and IFA reaction were enumerated.

Statistical analysis was carried out with Statistix 7.0 (Analytical Software, St. Paul, MN). *Cryptosporidium parvum* load is presented by cumulative numbers of oocysts in hemolymph and gill washing samples of six oysters. This rationale was used because oysters are usually offered for human consumption in a raw form by the half-dozen. Variables were tested by Wilk–Shapiro/ranking plots to determine whether their distribution conformed to a normal distribution, and if so, parametric tests, were used. Results are presented as the mean \pm SD for continuous variables and as the number or percentage for categorical data. The statistical significance of association between variables was assessed by Pearson correlation coefficient. Statistical significance was considered to be $P<0.05$.

Results

Testing of positive controls processed by IFA, stored at -70°C , and then reprocessed by combination of FISH and IFA yielded similar and not statistically different enumeration values for *C. parvum* oocysts.

FISH and IFA labeling clearly differentiated viable *C. parvum* oocysts from nonviable or non-*C. parvum* oocysts (i.e., oocysts that were stained only with IFA). Oocysts labeled by FISH and IFA were predominantly intact, revealing a small gap between the oocyst wall and internal structures, and in most of them, the sporozoites were visible. In comparison, dead *C. parvum* oocysts, that is, oocyst shells, or non-*C. parvum* oocysts frequently had discernable damage to their walls. No fluorescence of other particles in the samples was observed except for weak autofluorescence of nonstructural debris.

Oocyst isolates represented *C. parvum* as determined by PCR amplification confirmed by DNA sequencing analysis.

The cumulative numbers of oocysts identified in oysters are presented in Table 1. As determined by the combined FISH and IFA technique, the mean cumulative numbers of viable *C. parvum* oocysts in six oysters varied from 2 to 106; IFA testing revealed from 4 to 125 *Cryptosporidium* sp. oocysts (Table 1). Of a total of 265 groups of six oysters, 221 (83.4%) contained viable *C. parvum* oocysts as determined by combined FISH and IFA technique, and 235 (88.7%) were *Cryptosporidium* sp.-positive by IFA. Oysters

from Wye River, Choptank River, Upper Bay, and Eastern Bay had the highest oocyst amounts, and those from Manokin River and Nanticoke River had the lowest overall levels, irrespective of the testing technique (Table 1). In general, the mean cumulative numbers of viable *C. parvum* oocysts in six oysters were significantly lower (two-sample t test; $t=1.96$, $P<0.01$) for samples processed by combination of FISH and IFA (overall, 28.0 ± 2.9) than the number of *Cryptosporidium* sp. oocysts in samples processed by IFA alone (overall, 42.1 ± 4.1) (Table 1). Testing of oysters by combined FISH and IFA techniques yielded lower numbers of viable *C. parvum* oocysts by approximately 34% compared with *Cryptosporidium* sp. oocysts enumerated by IFA; however, the numbers of oocysts enumerated by these two methods were statistically correlated (Pearson correlation; $R=0.84$, $P<0.01$). Overall, using FISH and IFA, from 10 to 32% (mean, 23%) of the total oocyst number was identified in hemolymph in six oyster pools compared with 25 to 52% (mean, 39%) by IFA.

The mean wet weight of oyster meat varied considerably among the 11 Bay subregions, from 92.8 g at Nanticoke River to 216.0 g at Chester River (Table 1). The weight of oysters was not related to the level of oocysts in their tissue within the region or a site.

The fecal coliform counts varied from 1.0 at Manokin River to 7.7 most probable number (MPN)/100 ml at the Wye River site. The highest mean fecal coliform values (Table 2) coincided with the highest *C. parvum* load only at the Wye River site (Table 1). The relation between fecal coliform counts and *C. parvum* level in oysters was not statistically significant in any of the 11 Chesapeake Bay regions.

Discussion

The present study, together with other studies on *C. parvum* in oysters for human consumption (as reviewed in Graczyk 2003), leave no doubt that such oysters can be contaminated with this pathogen for which fully effective therapy is currently unavailable (Dawson 2005). Because depuration of oocysts from oysters is ineffective (Gomez-Couso et al. 2003), and they remain viable in estuarine waters for over a year (Tamburrini and Pozio 1999), reports from the world's highest seafood-producing regions suggest implementation of a rational plan to prevent or reduce *Cryptosporidium* contamination in edible molluscan shellfish (Gomez-Couso et al. 2003, 2004). However, lack of evidence of human cryptosporidiosis linked to oysters generates a great deal of skepticism on the epidemiological importance of these reports and the necessity of reducing or eliminating the presence of waterborne *Cryptosporidium* in oyster harvesting waters.

A set of host-related, environment-derived, and *Cryptosporidium*-specific factors interplay in the epidemiology of foodborne cryptosporidiosis. *Cryptosporidium parvum* isolates differ in their virulence to humans (Okhuysen et al. 1999; Messner et al. 2001). Volunteer challenge trials that utilized healthy *C. parvum*-seronegative individuals demonstrated that the median infective dose (ID₅₀) for the three main *C. parvum* isolates (i.e., TAMU, IOWA, and UCP) varied from 9 to 18, from 87 to 190, and from 1,042 to 2,980 oocysts (Okhuysen et al. 1999; Messner et al. 2001), respectively. Considering the worst case scenario, when oysters are contaminated with the most virulent *C. parvum* isolate (i.e., TAMU; ID₅₀=9 oocysts) (Okhuysen et al. 1999), 205 (77.4%) of 265 groups of six oysters in the present study potentially can cause infection in 50% of the exposed individuals. This means that when consumed by 265 people (i.e., meal per person), 265 oyster meals can potentially cause infection in approximately 100 people, assuming that all these individuals are healthy (i.e., immunocompetent) and do not have preexisting anti-*C. parvum* serum immunoglobulin G (IgG). For partially immune immunocompetent people with systemic IgG, which is indicative of previous exposure, the ID₅₀ becomes more than 20-fold higher (Chappell et al. 1999; Dann et al. 2000; Okhuysen et al. 2004). Thus, our results indicate that infections are not likely to develop in oyster consumers who were partially immune, even upon consumption of raw oysters contaminated with the most virulent isolate of *C. parvum*. In addition, as demonstrated in the present study, even the most heavily contaminated oysters did not contain more viable oocysts than the ID₅₀ value of a moderately virulent *C. parvum* isolate, that is, IOWA, ID₅₀=190 oocysts (Messner et al. 2001). Cryptosporidiosis has life-threatening consequences in people with various immune deficiencies, and it is thought that in this population, a single oocyst can cause infection (Rose et al. 2002). However, it is also believed that people with immunological impairments are usually more aware of the potential health hazards associated with consumption of raw food items, particularly molluscan shellfish, and are therefore more likely avoid this type of food (Rippey 1994).

As demonstrated in the present study, approximately 23% of the infectious *C. parvum* oocysts were found in oyster hemolymph. Therefore, it is reasonable to assume that the amount of oocysts ingested by a person eating raw oysters is actually lower than reported. Shucking of oysters severs both sides of the adductor muscle, a hemolymph-rich organ, causing hemolymph leakage.

The assessment of FISH assay as suitable for identification of *C. parvum* oocysts was initiated in 1989 (Deere et al. 1989); however, the present study demonstrated that this particular assay can be applied retrospectively. The storage of dry, methanol, fixed samples at -70°C did not affect the

quality of rRNA, as demonstrated by the similar number of *C. parvum* oocysts identified in positive controls of IFA- and FISH-processed slides. Although RNA in dry smears is practically indestructible, fixation with alcohol and freezing has been shown previously to be particularly beneficial in long-term preservation of pathogen RNA or DNA, particularly for *Cryptosporidium* (Amar et al. 2005; Vincek et al. 2005; Perlmutter et al. 2004). It is recommended to examine IFA-processed smears without oil immersion and without coverslips because otherwise, such slides are not suitable for long-term storage, and the fluorescence will deteriorate rapidly.

The combined FISH and IFA approach has some advantages over IFA or various PCR methods, with the main advantage being the assessment of viability of even a single oocysts (Vesey et al. 1998; Smith et al. 2004). Such resolution is not available, or impractical, in any other technique. For example, using mouse bioassay and highly sensitive RT-PCR for RNA detection, the lowest number of *C. parvum* oocysts that can be assessed for their viability was 10³ (Jenkins et al. 2000). The practicality of the FISH method used in the present study relies on the elimination of the permeabilization step (Smith et al. 2004) because this was done through the CAM-filter dissolution method protocol (Graczyk et al. 1997b).

The mouse bioassay (Fayer et al. 1998, 2002a,b) has limited value for risk assessment of foodborne cryptosporidiosis related to consumption of oysters because the assay is based on manipulated pathogens. In this bioassay, mice are inoculated with oocysts extracted from oysters, purified, and delivered directly to their gastric region. However, under natural situations, the oocysts enter the gastrointestinal tract of a person while deeply buried in oyster tissue, which can prevent, inhibit, or delay the arrival of a set of stimuli, triggering oocyst excystation and subsequent infection. In addition, the mouse bioassay will produce false-negative results for infectivity assessment of *Cryptosporidium hominis*, which is a highly human-virulent species (Morgan-Ryan et al. 2002). For risk-assessment purposes, a bioassay that utilizes piglets is much more applicable because piglets can be fed with whole oyster meat which would mimic natural circumstances. The piglet model is commonly used for assessment of pathogenicity of *Cryptosporidium* (Enemark et al. 2003).

The present study provides useful information for formulation of risk assessment of foodborne cryptosporidiosis due to consumption of contaminated oysters in a raw form. We conclude that the lower numbers of *C. parvum* oocysts enumerated by combined FISH and IFA were due to elimination of dead oocysts and oocysts of other *Cryptosporidium* species, and elimination of small unicellular algae which are detected by IFA (Clancy et al. 1994).

We further conclude that IFA should not be used for purposes of oocyst enumeration from oysters because this assay significantly overestimates the level of *C. parvum* oocysts. However, irrespective of the identification technique, the recovery efficiency of oocysts from oyster tissue is not 100%. A previous study that utilized the tissue of molluscan shellfish from Chesapeake Bay spiked with known numbers of *C. parvum* oocysts demonstrated a 50% recovery efficiency (Graczyk et al. 1999). Thus, we conclude that the numbers of viable *C. parvum* oocysts reported in the present study were potentially underestimated.

Crassostrea virginica is a suspension feeder with a clearance rate (i.e., cleared particles in a unit time) proportionally related to their size (Newell and Langdon 1996), which means that larger oysters filter more than smaller ones, and the size of *Cryptosporidium* oocysts fall within the range of filtered particles. The present study showed that oyster size was not related to the amount of *Cryptosporidium* in their tissue, which indicates involvement of determinants other than biological factors (i.e., environmental) in recovery of waterborne oocysts by oysters.

The present study confirms previous findings that the standard water quality parameters such as fecal indicator coliform count does not correlate with the *Cryptosporidium* contamination in edible oysters (Freire-Santos et al. 2000; Gomez-Couso et al. 2003, 2004). Foodborne illnesses following consumption of raw oysters can occur even when fecal coliform testing of shellfish harvesting waters demonstrates compliance with the National Shellfish Sanitation Program criteria and the sanitation at the oyster harvesting facilities met standards set by the state authorities (Graczyk and Schwab 2000). This clearly demonstrates that monitoring of shellfish waters for fecal coliforms is not sufficient to indicate the presence of waterborne *Cryptosporidium*. We conclude that oysters harvested from natural waters and identified as suitable for human consumption in a raw form can be a potential vehicle for infectious *C. parvum* oocysts and serve as a source of human cryptosporidiosis. This epidemiological possibility should be addressed by formulation of the risk assessment model accounting for *Cryptosporidium*-specific, consumer-related, and environment-derived cofactors.

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References

- Amar CFL, East CL, Grant KA, Gray J, Itriza-Gomara M, Maclure EA, McLauchlin J (2005) Detection of viral, bacterial, and parasitological RNA or DNA of nine intestinal pathogens in fecal samples archived as part of the English infectious intestinal disease study: assessment of the stability of target nucleic acid. *Diagn Mol Pathol* 14:90–96
- Chappell CL, Okhuysen PC, Sterling CR, Wang C, Jakubowski W, Dupont HL (1999) Infectivity of *Cryptosporidium parvum* in healthy adults with pre-existing anti *C. parvum* serum immunoglobulin G. *Am J Trop Med Hyg* 60:157–164
- Clancy JL, Gollnitz WD, Tabib Z (1994) Commercial labs: how accurate are they? *J Am Water Works Assoc* 86:89–97
- Dann SM, Okhuysen PC, Salameh BM, DuPont HL, Chappell CL (2000) Fecal antibodies to *Cryptosporidium parvum* in healthy volunteers. *Infect Immun* 68:5068–5074
- Dawson D (2005) Foodborne protozoan parasites. *Int J Food Microbiol* 103:207–227
- Deere D, Vesey G, Ashbolt N, Davies KA, Williams KL, Veal D (1989) Evaluation of fluorochromes for flow cytometric detection of *Cryptosporidium parvum* oocysts labeled by fluorescent in situ hybridization. *Lett Appl Microbiol* 27:352–356
- Enemark HL, Bille-Hansen V, Lind P, Heegaard PM, Vigre H, Ahrens P, Thamsborg SM (2003) Pathogenicity of *Cryptosporidium parvum*—evaluation of an animal infection model. *Vet Parasitol* 113:35–57
- Fayer R, Graczyk TK, Lewis EJ, Trout JM, Farley CA (1998) Survival of infectious *Cryptosporidium parvum* oocysts in seawater and Eastern oysters (*Crassostrea virginica*) in the Chesapeake Bay. *Appl Environ Microbiol* 64:1070–1074
- Fayer R, Trout JM, Lewis EJ, Santin M, Zhou L, Lal AA, Xiao L (2002a) Contamination of Atlantic coast commercial shellfish with *Cryptosporidium*. *Parasitol Res* 89:141–145
- Fayer R, Trout JM, Lewis EJ, Xiao L, Lal AA, Jenkins MC, Graczyk TK (2002b) Temporal variability of *Cryptosporidium* in the Chesapeake Bay. *Parasitol Res* 88:998–1003
- Freire-Santos F, Oteiza-Lopez AM, Vergara-Castiblanco CA, Ares-Mazas E, Alvarez-Suraex E, Garcia-Martin O (2000) Detection of *Cryptosporidium* oocysts in bivalve molluscs destined for human consumption. *J Parasitol* 86:853–854
- Gomez-Couso H, Freire-Santos F, Martinez-Urataza J, Garcia-Martin O, Ares-Mazas ME (2003) Contamination of bivalve molluscs by *Cryptosporidium* oocysts: the need for new quality control standards. *Int J Food Microbiol* 87:97–105
- Gomez-Couso H, Freire-Santos F, Amar CF, Grant KA, Williamson K, Ares-Mazas ME, McLauchlin J (2004) Detection of *Cryptosporidium* and *Giardia* in molluscan shellfish by multiplexed nested-PCR. *Int J Food Microbiol* 91:279–288
- Graczyk TK (2003) Human waterborne parasites in molluscan shellfish. *J Parasitol* 89:557–561
- Graczyk TK, Schwab KJ (2000) Food borne infections vectored by molluscan shellfish. *Curr Gastroenterol Rep* 2:305–309
- Graczyk TK, Cranfield MR, Fayer R (1996) Evaluation of commercial enzyme immunoassay (EIA) and immunofluorescent antibody (IFA) tests kits for detection of *Cryptosporidium* oocysts other than *Cryptosporidium parvum*. *Am J Trop Med Hyg* 54:274–279
- Graczyk TK, Fayer R, Cranfield MR (1997a) Zoonotic potential of *Cryptosporidium parvum*: implications for waterborne cryptosporidiosis. *Parasitol Today* 13:348–351
- Graczyk TK, Cranfield MR, Fayer R (1997b) Recovery of waterborne oocysts of *Cryptosporidium parvum* from water samples by the membrane-filter dissolution method. *Parasitol Res* 83:121–125

- Graczyk TK, Farley CA, Fayer R, Lewis EJ, Trout JM (1998) Detection of *Cryptosporidium* oocysts and *Giardia* cysts in the tissue of Eastern oysters (*Crassostrea virginica*) carrying principal oyster infectious diseases. *J Parasitol* 84:1039–1042
- Graczyk TK, Fayer R, Lewis EJ, Trout JM, Farley CA (1999) *Cryptosporidium* oocysts in Bent mussels (*Ischadium recurvum*) in the Chesapeake Bay. *Parasitol Res* 85:518–521
- Graczyk TK, Fayer R, Jenkins MC, Trout JM, Higgins J, Lewis EJ, Farley CA (2000) Susceptibility of the Chesapeake Bay to environmental contamination with *Cryptosporidium parvum*. *Environ Res* 82:106–112
- Graczyk TK, Conn DB, Lucy F, Minchin D, Tamang L, Moura LNS, DaSilva AJ (2004) Human waterborne parasites in zebra mussels (*Dreissena polymorpha*) from the Shannon River drainage, Ireland. *Parasitol Res* 93:389–391
- Jenkins MC, Trout J, Abrahamsen MS, Higgins J, Fayer R (2000) Estimating viability of *Cryptosporidium parvum* oocysts using reverse transcriptase–polymerase chain reaction (RT–PCR) directed at mRNA encoding amyloglucosidase. *J Microbiol Methods* 34:97–106
- Johnson DW, Pieniazek NJ, Griffin DW, Misener L, Rose JB (1995) Development of a PCR protocol for sensitive detection of *Cryptosporidium* oocysts in water samples. *Appl Environ Microbiol* 61:3849–3855
- Messner MJ, Chappell CL, Okhuysen PC (2001) Risk assessment for *Cryptosporidium*: a hierarchical Bayesian analysis of human dose–response data. *Water Res* 35:3934–3940
- Morgan-Ryan UM, Fall A, Ward LA, Hijjawi N, Sulaiman N, Fayer R, Thompson RC, Olson M, Lal AA, Xiao L (2002) *Cryptosporidium hominis* n. sp. (Apicomplexa: Cryptosporidiidae) from *Homo sapiens*. *J Eukaryot Microbiol* 49:433–440
- Newell RIE, Langdon CJ (1996) Mechanism and physiology of larval and adult feeding. In: Kennedy VS, Newell RIE, Eble AF (eds) *The eastern oyster*. Maryland Sea Grant College, College Park, MD, pp 185–223
- Okhuysen PC, Chappell CL, Crabb JH, Sterling CR, Dupont HL (1999) Virulence of three distinct *Cryptosporidium parvum* isolates for healthy adults. *J Infect Dis* 180:1275–1281
- Okhuysen PC, Rogers GA, Crisanti A, Spano F, Huang DB, Chappell CL, Tzipori S (2004) Antibody response of healthy adults to recombinant thrombospondin-related adhesive protein of *Cryptosporidium* 1 after experimental exposure to *Cryptosporidium* oocysts. *Clin Diagn Lab Immunol* 11:235–238
- OzFoodNet Working Group (2005) Reported foodborne illness and gastroenteritis in Australia: annual report of the OzfoodNet network, 2004. *Commun Dis Intell* 29:165–192
- Perlmutter MA, Best CJM, Gillespie JW, Gathright Y, Gonzalez S, Velasco A, Marston-Linehan W, Emmert-Buck MR, Chuaqui RF (2004) Comparison of snap freezing versus ethanol fixation for gene expression profiling of tissue specimens. *J Mol Diagn* 6:371–377
- Pieniazek NJ, Bornay-Llinares FJ, Slemenda SB, DaSilva AJ, Moura INS, Arrowood AJ, Ditrich O, Addiss DG (1999) HIV-infected patients harbor four distinct genotypes of *Cryptosporidium parvum*: implications for diagnosis, epidemiology, and prevention. *Emerg Infect Dis* 5:444–449
- Rippey SR (1994) Infectious diseases associated with molluscan shellfish consumption. *Clin Microbiol Rev* 7:419
- Rose JB, Huffman DE, Gennaccaro A (2002) Risk and control of waterborne cryptosporidiosis. *FEMS Microbiol Rev* 2:113–123
- Smith JJ, Gunasekera TS, Barardi CRM, Veal D, Vesey G (2004) Determination of *Cryptosporidium parvum* oocyst viability by fluorescence in situ hybridization using a ribosomal RNA-directed probe. *J Appl Microbiol* 96:409–417
- Tamburrini A, Pozio E (1999) Long-term survival of *Cryptosporidium parvum* oocysts in seawater and in experimentally infected mussels (*Mytilus galloprovincialis*). *Int J Parasitol* 29:711–715
- Vesey G, Ashbolt N, Fricker EJ, Deere D, William KL, Veal DA, Dorsch M (1998) The use of a ribosomal RNA targeted oligonucleotide probe for fluorescent labeling of viable *Cryptosporidium parvum* oocysts. *J Appl Microbiol* 85:429–440
- Vincek V, Mehdi N, Block N, Welsh CF, Mehrdad N, Morales AR (2005) Methodology for preservation of high molecular-weight RNA in paraffin-embedded tissue-application for laser-capture microdissection. *Diagn Mol Pathol* 14:127–133