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Evaluation of methods for improved detection of *Cryptosporidium* spp. in mussels (*Mytilus californianus*)

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Abstract

Bivalve molluscs concentrate *Cryptosporidium* oocysts from fecal-contaminated aquatic environments and are therefore useful in monitoring water quality. A real-time TaqMan polymerase chain reaction (PCR) system was developed to allow for large scale quantitative detection of *Cryptosporidium* spp. in mussels (*Mytilus californianus*). The TaqMan sensitivity and specificity were compared to conventional PCR and direct immunofluorescent antibody (DFA) assays, with and without immunomagnetic separation (IMS), to identify the best method for parasite detection in mussel hemolymph, gill washings and digestive glands. TaqMan PCR and two conventional PCR systems all detected 1 or more oocysts spiked into 1 ml hemolymph samples. The minimum oocyst detection limit in spiked 5 ml gill wash and 1 g digestive gland samples tested by TaqMan PCR and DFA was 100 oocysts, with a 1 $\log_{10}$ improvement when samples were first processed by IMS. For tank exposed mussels, TaqMan and conventional PCR methods detected *C. parvum* in <5% of hemolymph samples. No gill washings from these same mussels tested positive by TaqMan PCR or DFA analysis even with IMS concentration. All methods detected the highest prevalence of *C. parvum*-positive samples in digestive gland tissues of exposed mussels. In conclusion, the most sensitive method for the detection of *C. parvum* in oocyst-exposed mussels was IMS concentration with DFA detection: 80% of individual and 100% of pooled digestive gland samples tested positive. TaqMan PCR was comparable to conventional PCR for detection of *C. parvum* oocysts in mussels and additionally allowed for automated testing, high throughput, and semi-quantitative results.
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1. Introduction

Sensitive and specific detection of pathogenic protozoa such as *Cryptosporidium* spp. is of critical importance to public health authorities. Many outbreaks of cryptosporidiosis have been documented worldwide, including the 1993 contamination of a Milwaukee pub-

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lic water supply with *Cryptosporidium parvum* that infected over 400,000 people and caused over 100 deaths (MacKenzie et al., 1994). Clinical disease in immunocompetent humans generally consists of self-limiting diarrhea resulting from either *Cryptosporidium hominis* or the anthropozoonotic *C. parvum* genotype 2 (Morgan-Ryan et al., 2002; Okhuysen et al., 1999). Immunocompromised humans can be chronically infected, sometimes fatally, with these as well as other *Cryptosporidium* species (Pieniazek et al., 1999). The oocyst stages of *Cryptosporidium* spp. are shed in the feces of animals and humans, which may then enter sewage treatment facilities via wastewater or persist in the environment. Depending on the type of sewage treatment, some but not all oocysts will be removed prior to environmental discharge of treated water (Bonadonna et al., 2002; Payment et al., 2001). *Cryptosporidium* parasites are endemic in many domestic and wild animal populations, with young animals often shedding over a million oocysts during initial infection, while the infective dose of *C. parvum* in humans can be as low as 10–100 oocysts (Atwill et al., 2001; Heitman et al., 2002; Okhuysen et al., 1999).

Diagnosis of acute cryptosporidiosis has historically been based on acid fast staining, fecal flotation, or direct immunofluorescent antibody (DFA) detection methods. However, for these methods the limit of detection is approximately 600–1000 oocysts/g feces, and DFA methods cannot determine *Cryptosporidium* genotype (Pereira et al., 1999; Xiao and Herd, 1993). The analytical sensitivity of oocyst detection in feces can be increased by 1–2 $\log_{10}$ units using immunomagnetic separation (IMS), which concentrates oocysts and facilitates analysis of a larger sample volume (Pereira et al., 1999). When coupled with IMS, amplification of parasite DNA using conventional polymerase chain reaction (PCR) methods provides molecular data to determine the *Cryptosporidium* genotype, with a minimum detection limit of about 100 oocysts/g feces (Deng et al., 2000; Webster et al., 1996). Unlike DFA, conventional PCR techniques do not provide quantitative data and do not allow for oocyst visualization. The real-time TaqMan PCR system described herein was designed to provide quantitative results, high throughput potential, an AmpErase UNG (uracil-*N*-glocosylase) system to prevent PCR product carry-over, and sensitive detection of all *Cryptosporidium* species. Other *Cryptosporidium* real-time PCR systems have been described (Fontaine and Guillot, 2003a; Guy et al., 2003; Higgins et al., 2001; Limor et al., 2002; MacDonald et al., 2002), but their protocols were thought to lack the analytical sensitivity and specificity to identify the variety of *Cryptosporidium* genotypes and the low levels of oocyst contamination that are expected in environmental samples (Hallier-Soullier and Guillot, 2000; Rose, 1997).

Environmental monitoring for *Cryptosporidium* spp. can be problematic, partly because of the dilution effect that occurs as oocysts are disseminated from terrestrial to aquatic ecosystems, and also because particulate matter can inhibit or interfere with *Cryptosporidium* detection methods (Feng et al., 2003; Hallier-Soullier and Guillot, 2000). Oocysts can be concentrated from large volumes of water mechanically, but expensive equipment and supplies are required. Alternatively, filter feeding invertebrates such as bivalve molluscs, which can filter over 2 L of water/hr/shellfish, can act as a natural concentration system (McMahon, 1991). These bivalves can then be collected and tested for pathogens, providing an indication of water quality (Fayer et al., 2002; Freire-Santos et al., 2000; Graczyk et al., 2003; Miller et al., 2005; Tamburrini and Pozio, 1999). Studies in North America and in Europe have shown that bivalves can act as indicators of aquatic fecal contamination with *Cryptosporidium* spp. and that molecular characterization can distinguish human from animal genotypes to assess potential fecal loading sources (Fayer et al., 1998; Gomez-Bautista et al., 2000; Gomez-Couso et al., 2004; Graczyk et al., 1999). The IMS concentration technique is well accepted as an integral part of water testing methods (Sturbaum et al., 2002; US EPA, 2001), but its potential to improve detection of *Cryptosporidium* spp. in bivalve tissues has not been critically evaluated. The goal of this study was to evaluate a real-time TaqMan PCR system and compare it with conventional PCR and DFA methods, with and without IMS, for accurate detection of *Cryptosporidium* spp. in mussel tissues using laboratory spiked bivalve samples and samples from a tank exposure experiment.

2. Materials and methods

2.1. Experimental design

Analytic sensitivity and specificity of *Cryptosporidium* detection techniques were first evaluated using oocyst dilutions and DNA samples without bivalve tissues. Sensitivity of the PCR and DFA techniques were established by testing serial dilutions ranging from 0–10,000 *Cryptosporidium* oocysts. Specificity testing for PCR systems was evaluated using protozoal DNA extracted from *C. andersoni, C. baileyi, C. canis, C. felis, C. hominis, C. meleagridis, C. parvum, C. serpentis, Giardia duodenalis* (synonymous with *G.*

lamblia and *G. intestinalis*), *Neospora caninum, N. hughesi, Sarcocystis falcatula, S. neurona*, and *Toxoplasma gondii*, as well as non-protozoal DNA from marine mussels (*Mytilus californianus* and *M. galloprovincialis*), freshwater clams (*Corbicula fluminea*), sandcrabs (*Emerita analoga*) and dinoflagellates (*Gymnodinium* spp.) Specificity testing for DFA utilized oocysts from *C. andersoni, C. felis, C. parvum, C. serpentis,* and cysts from *G. duodenalis*.

Technique comparisons for *Cryptosporidium* detection in bivalve tissues were performed by spiking known oocyst numbers into mussel tissues. *Cryptosporidium* dilutions containing 0, 1, 10, 100 or 500 oocysts were added to 6 sets of hemolymph, gills, and digestive gland tissues prior to any washes or tissue processing, to allow the analytical sensitivity estimates to reflect any oocyst loss during processing and analysis. TaqMan PCR was compared to 2 conventional PCR methods for hemolymph analysis. TaqMan PCR was also compared to DFA analysis, with and without IMS concentration, for evaluation of gill washings and digestive gland tissues. These two tissues are commonly tested by DFA in other laboratories, so DFA was used as the standard for comparison with TaqMan PCR in our study.

A tank experiment was then conducted to compare *Cryptosporidium* detection techniques using mussels (*M. californianus*) exposed to a known quantity of *C. parvum* genotype 2 oocysts. An 8 h oocyst exposure was used to represent the pulse of pathogens that mussels may be exposed to during a storm event. Two tubs, each containing 70 mussels and 10 L of sea water, were inoculated with 1000 oocysts/L (mean, 142 oocysts/mussel) and left undisturbed for 8 h. Each mussel tank was kept at 14 °C with no additional water or nutrient supplementation. An airstone in each tank provided constant aeration and water mixing. A third tub with 70 mussels and 10 L sea water was maintained under the same conditions except that no oocysts were added to the water. After 8 h, 60 mussels were removed from each tank: 30 were processed individually, and the remaining 30 were processed as 6 pools of 5 mussels each. Hemolymph, gill washings, and digestive gland were processed as described below. Hemolymph was analyzed by TaqMan PCR and two conventional PCR methods. Gill washings and sieved digestive gland were tested by TaqMan PCR and DFA analysis, with and without IMS concentration.

2.2. Cryptosporidium oocysts

Wild-type oocysts of *C. parvum* genotype 2 used for sensitivity testing and the tank exposures were obtained from calves near the Veterinary Medical Teaching and Research Center, Tulare, California. Calf feces were sieved through a series of mesh strainers and then purified by sucrose flotation methods (Arrowood and Sterling, 1987). Additional *Cryptosporidium* genotypes and other protozoa for specificity testing were obtained from the Veterinary Medical Teaching Hospital, University of California, Davis, the California Animal Health and Food Safety Laboratory, and Drs. Bruce Anderson in Idaho and Andrew Thompson in Australia. Oocyst concentrations for spiking experiments were determined using the mean of 8 hemacytometer counts and confirmed by DFA enumeration. Oocyst suspensions were kept at 4 °C and used within one month of collection.

2.3. Mussels

Mussel spiking and tank exposure experiments were performed using wild surf mussels (*M. californianus*) harvested outside Bodega Bay, California. Mussels 5–8 cm long were individually dissected to obtain hemolymph, gill, and digestive gland. Hemolymph was extracted by first filing a notch in the mussel shell and then aspirating 0.5–1.5 ml of hemolymph from the adductor muscle using a sterile syringe and a 22 gauge needle. The hemolymph was transferred to a microcentrifuge tube and centrifuged for 3 min at 14,000 rpm. The supernatant was removed and the cell pellet was stored at −80 °C for DNA extraction and PCR analysis. Next, the adductor muscle was cut to open the mussel, and the gills and digestive gland (0.2–2 g each) were excised. Gills from each mussel were vortexed in 5 ml sterile PBS as described (Fayer et al., 1998). Gill tissue was removed and 200 μl of the gill wash were dried onto a slide for DFA testing. The remaining gill wash fluid was centrifuged for 10 min at $1000 \times g$ to obtain a pellet. The supernatant was discarded and a 100 μl aliquot of the pellet was frozen at −80 °C for TaqMan PCR analysis. The remaining gill wash pellet ≤ 0.5 ml was processed by IMS and the 100 μl product split for TaqMan and DFA analysis. Digestive gland samples were sieved through a 100 μm cell strainer and centrifuged for 15 min at $1000 \times g$, with the supernatant discarded. A 100 μl aliquot was frozen for TaqMan PCR, a 10 μl aliquot was dried onto a DFA slide, and a 0.5 ml aliquot was processed by IMS for TaqMan PCR and DFA analysis.

2.4. TaqMan PCR

A real-time TaqMan PCR system was developed to detect a variety of *Cryptosporidium* species based on 18S rRNA sequences deposited in GenBank. This Taq-

Man system was intentionally designed to recognize many *Cryptosporidium* genotypes, including *C. hominis* (GenBank accession number AF093489), *C. parvum* genotype 2 (AF093490), *C. muris* (AF090496), *C. felis* (AF108862), and *C. meleagridis* (AJ493549). The TaqMan probe was adapted from Limor et al. (Limor et al., 2002), and primers were newly designed to amplify a short PCR product to maximize analytical sensitivity. The sensitivity was 50 times greater when testing complementary DNA (cDNA) transcribed from messenger RNA (mRNA) as compared to testing genomic DNA (gDNA), so cDNA was used for all further analyses. In addition, a Bivalve TaqMan PCR system to be used for quality control was designed based on a conserved region of the 18S rRNA gene targeting bivalves including clams (*Corbicula fluminea* [AF305705]) and mussels (*Geukensia demissa* [L33450], *M. californianus* [L33449], *M. edulis* [L78854], *M. galloprovincialis* [L33452], *M. trossulus* [L33453]). For each target gene, two primers and an internal, fluorescent labeled TaqMan probe (5′ end, reporter dye 6-FAM [6-carboxyfluorescein], 3′ end, quencher dye TAMRA [6-carboxytetramethylrhodamine]) were designed using Primer Express software (Applied Biosystems, Foster City, CA). The length of the PCR products was short (between 110 and 182 bp) to enable high amplification efficiencies. TaqMan primer and probe sequences are listed in Table 1.

For nucleic acid extraction, tissue samples of ≤100 mg were loaded into 96 deep well plates prefilled with 500 μl 1X ABI lysis buffer (Applied Biosystems) and stored frozen until RNA extraction. Two grinding beads (4 mm diameter, SpexCertiprep, Metuchen, NJ) and Proteinase K (Invitrogen, Carlsbad, CA) were added and samples were homogenized in a GenoGrinder2000 (SpexCertiprep) for 2 min at 1000 strokes/min. After 30 min periods at 56 °C and −20 °C, total RNA was extracted from the tissue lysates using a 6700 automated nucleic acid (ANA) workstation (Applied Biosystems) according to the manufacturer's instructions. Complementary DNA was synthesized using 100 units of SuperScript III (Invitrogen), 600 ng random hexadeoxyribonucleotide (pd(N)$_6$) primers (random hexamer primer) 10 U RNaseOut (RNase inhibitor), and 1 mM dNTPs (all Invitrogen) in a final volume of 40 μl. The reverse transcription reaction proceeded for 120 min at 50 °C. After addition of 60 μl of water, the reaction was terminated by heating for 5 min to 95 °C and cooling on ice.

Real-time TaqMan PCR reactions were conducted on 96 well plates in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Each PCR

Table 1
Nucleotide sequence of PCR primers and TaqMan probes used to detect *Cryptosporidium* or bivalve 18S rRNA

Amplification target	Primer	Primer sequence (5′-3′)	Length of PCR product	Probe	TaqMan probe sequence (5′-3′)[a]
TaqMan bivalve spp.	Clam18-412f	CGGCTACCACATCCAAGGA	110	Clam18-434p	CAGCAGGCGCGCAAATTACCCACT
	Clam18-521r	CCAATTACGGGGCCTCGAA			
TaqMan *Cryptosporidium* spp.	Crypt-193f	GGAAGGGTTGTATTTATTAGATAAAGAACCA	182	Crypt-276p	CATTCAAGTTTCTGACCTATCAGCTTTAGACGG
	Crypt-374r	CTCCCTCTCCGGAATCGAA			
PCR1 *Cryptosporidium* spp.[48]	C1F	TTCTAGAGCTAATACATGCG	1325		
	C1R	CCCTAATCTTTCGAAACAGGA			
	C2F	GGAAGGGTTGTATTTATTAGATAAAG	850		
	C2R	AAGGAGTAAGGAACAACCTCCA			
PCR2 *Cryptosporidium* spp.[32]	18sif	AGTGACAAGAAATAACAATACAGG	298		
	18sir	CCTGCTTTAAGCACTCTAATTTTC			

[a] TaqMan probe oligonucleotides were labeled with 6-FAM at the 5′ and the quencher TAMRA at the 3′.

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reaction contained 400 nM of each primer, 80 nM of the TaqMan probe and commercially available PCR mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems) containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM dNTPs, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction and 5 μl of the diluted cDNA sample in a final volume of 12 μl. Amplification conditions for the automated fluorometer were 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Cycle threshold (Ct) values less than 39.5 were considered positive based on the results of testing *C. parvum* exposed and unexposed mussels in the tank exposure experiment. For selected isolates, the PCR product was purified according to QIAGEN protocol (QIAGEN Inc., Valencia, CA), sequenced on an automated sequencer, and analyzed with Vector NTI (Informax, Frederick, MD) software for *Cryptosporidium* genotype identification.

2.5. Conventional PCR

Two conventional 18S rRNA PCR protocols, henceforth called PCR1 (Xiao et al., 1999) and PCR2 (Morgan et al., 1997), respectively, were used for comparison with the TaqMan PCR system. Primers are listed in Table 1. The PCR1 18S rRNA nested protocol first amplified a 1325 bp DNA segment and then an 850 bp DNA segment in the nested PCR step. The PCR2 18S rRNA protocol amplified a 298 bp DNA fragment. Extraction of gDNA and conventional PCR protocols were performed as described (Miller et al., 2005). For selected isolates, the PCR products were purified and the sequences analyzed as described above.

2.6. Direct immunofluorescence

Cryptosporidium oocyst DFA detection was performed on 10 μl digestive gland or 200 μl gill wash dried onto 1 of the 3 wells on a Merifluor slide (Meridian Bioscience Inc., Cincinnati, OH) and analyzed as described (Miller et al., 2005). For IMS-DFA of gill washes and digestive gland, the 50 μl IMS product containing parasites was dried onto a DFA slide well, with 2 wells per tissue sample. All slides were read by the same microscopist. *Cryptosporidium* parasites were identified as 5 μm diameter oocysts outlined in apple green, often with a midline seam. Hemolymph was not tested by DFA because preliminary tests showed that autofluorescence of mussel hemocytes interfered with the identification of oocysts.

2.7. Immunomagnetic separation

Selected gill wash and digestive gland pellets of up to 0.5 ml were concentrated and purified with IMS (Dynal Biotech, Oslo, Norway) as described (Miller et al., 2005), followed by DFA or TaqMan PCR for *Cryptosporidium* detection. During IMS concentration, the IMS bead-parasite complexes were bound to a magnetic holder while the supernatant and debris were discarded during a series of wash steps. For parasite dissociation from the beads, 2 acid washes of 50 μl 0.1 N HCl were vortexed at the beginning and end of two 10 min incubations, with the IMS product then neutralized and transferred to a slidewell or PCR tube for parasite detection. Hemolymph was not suitable for IMS because hemocytes adhered to the glass tubes.

2.8. Data analysis

Amplification efficiencies of the 18S rRNA *Cryptosporidium* and Bivalve TaqMan PCR systems were calculated using the formula: $E = 10^{1/-s} - 1$, where $E*100$ is the % efficiency and s is the slope of the standard curve. The proportion of tissues positive per technique was calculated as the number of tissues that tested positive divided by the total number of tissue samples tested for each technique. Percent recovery values for DFA testing were calculated by dividing the number of oocysts counted on the DFA slide by the number of oocysts expected per test aliquot. Expected oocysts per test aliquot were calculated by multiplying the number of oocysts spiked into a tissue by the proportion of spiked tissue analyzed. Paired t tests were used to compare the number of oocysts detected by DFA with IMS-DFA. McNemar's χ^2 test for paired data was used to compare the proportion of positive mussels detected by DFA with PCR. Statistical significance was defined as a p value <0.05.

3. Results

3.1. Oocyst spiking experiments

The TaqMan PCR detection systems were validated using DNA and *Cryptosporidium* oocyst dilutions alone. The linearity of the TaqMan standard curve dilutions of cloned *C. parvum* plasmids and cDNA extracted from oocysts is shown in Fig. 1. The amplification efficiencies were approximately 99% for both the *Cryptosporidium* TaqMan and Bivalve TaqMan systems that were designed to assess the RNA quality from extracted tissue. The regression line generated

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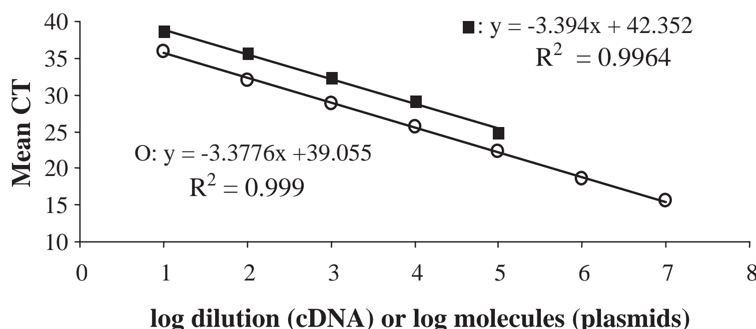

Fig. 1. TaqMan amplification of cloned *Cryptosporidium* PCR product (O) and cDNA generated on RNA extracted from 10-fold dilutions of *C. parvum* oocysts (■). Standard curves were determined in triplicate, standard deviations too small to be visible.

when 6 replicates of 10, 100, or 500 oocysts were spiked into digestive gland tissues (data not shown) was almost identical to the Fig. 1 oocysts alone cDNA regression line, suggesting that any residual mussel tissue did not inhibit the PCR reaction.

The TaqMan and conventional PCR system specificity results are shown in Fig. 2. As expected, the Bivalve TaqMan detection system detected invertebrate cDNA but not protozoal cDNA. The *Cryptosporidium* TaqMan detection system detected all *Cryptosporidium* and *Sarcocystis* species tested but not *Neospora* spp., *T. gondii*, *G. duodenalis*, dinoflagellates, or invertebrate DNA. The PCR1 system was the most specific, detecting only *Cryptosporidium* species, while the PCR2 system occasionally detected other protozoa, dinoflagellates, and invertebrate DNA in addition to *Cryptosporidium* spp. All three PCR systems were able to detect a single oocyst present in the 200 ul dilution without bivalve tissue.

The DFA technique detected 1 or more oocysts spiked onto a slide well, and was able to detect all *Cryptosporidium* genotypes tested, showing a strong apple green fluorescence outlining the *C. parvum* oocysts and more variable fluorescence with *C. andersoni, C. canis, C. felis,* and *C. serpentis*. As expected, *Giardia* cysts also showed strong fluorescence but could be distinguished by the larger oval cyst size of 10–14 μm diameter as compared to the 4–7 μm diameter size of *Cryptosporidium* oocysts.

3.2. Mussel tissue spiking experiments

Cryptosporidium detection techniques were next evaluated by spiking oocysts of *C. parvum* directly into mussel hemolymph, gill washings, and digestive gland, followed by tissue processing and analysis. When the TaqMan PCR assay was compared with the PCR1 and PCR2 conventional PCR assays for the

	Protozoal DNA[a,c]																	Non-protozoal DNA[b,c]				
PCR system	*Cryptosporidium andersoni* (bovine)	*Cryptosporidium baileyi* (chicken)	*Cryptosporidium canis* (canine)	*Cryptosporidium felis* (feline)	*Cryptosporidium hominis* (human)	*Cryptosporidium meleagridis* (chicken)	*Cryptosporidium parvum* (bovine)	*Cryptosporidium parvum* (river otter)	*Cryptosporidium serpentis* (snake)	*Neospora caninum* (bovine)	*Neospora hughesi* (equine)	*Sarcocystis falcatula* (equine)	*Sarcocystis neurona* (equine)	*Sarcocystic neurona* (harbor seal)	*Sarcocystis neurona* (sea otter)	*Toxoplasma gondii* (human)	*Giardia duodenalis* (bovine)	*Gymnodinium* spp. (dinoflagellate)	*Mytilus californianus* (surf mussel)	*Mytilus galloprovincialis* (bay mussel)	*Corbicula fluminea* (freshwater clam)	*Emerita analoga* (sand crab)
TaqMan Bivalve	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
TaqMan *Cryptosporidium*	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-
PCR1 *Cryptosporidium*	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
PCR2 *Cryptosporidium*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-

[a] Protozoal spp. (host from which isolate obtained).
[b] Non-protozoal spp. (common name).
[c] + = PCR positive; - = PCR negative.

Fig. 2. Specificity testing of TaqMan and conventional PCR systems with protozoal, dinoflagellate, and invertebrate DNA samples.

detection of *Cryptosporidium* DNA in spiked hemolymph samples, all three techniques were able to detect the full range of 1–500 oocysts spiked into hemolymph, with some variation in the proportion of positive samples. For the high spike doses of 100 and 500 oocysts, all three techniques detected 83%–100% of spiked hemolymph samples. When 1 or 10 oocysts were spiked into a hemolymph sample, the TaqMan and PCR1 techniques detected 33% of spiked samples while the PCR2 technique detected 50% or more of positive samples. No false positives were detected in negative control samples.

Table 2 shows the proportion of spiked gill wash and digestive gland samples that tested positive for *Cryptosporidium* by TaqMan PCR or DFA, with and without IMS. All four detection methods were able to consistently detect gill wash or digestive samples spiked with 100 or 500 oocysts. For samples spiked with 100 oocysts, 50% of gill wash and 33% of digestive gland samples tested positive by TaqMan PCR, compared to 50% of gill wash and 17% of digestive gland samples tested by DFA. The addition of immunomagnetic separation significantly improved *Cryptosporidium* detection in gill washings and digestive gland compared to TaqMan PCR or DFA detection methods alone ($p<0.01$). The oocyst detection limit in gill washings and digestive gland was improved by 1 $\log_{10}$ unit, from 100 to 10 oocysts per sample, when IMS concentration was used. For gill wash samples spiked with 10 *C. parvum* oocysts, IMS increased the proportion testing positive from 0% to 33% by TaqMan PCR and from 17% to 83% by DFA. Similarly, when 10 oocysts were spiked into digestive gland tissues, IMS increased the proportion of positive samples detected by TaqMan PCR from 0% to 17% and by DFA from 0% to 83%. Overall, the IMS-DFA technique was more sensitive than IMS-TaqMan for detection of oocysts spiked into gill wash or digestive tissues. TaqMan PCR was more successful in detecting low oocyst doses spiked into hemolymph than in gill wash or digestive gland samples. No negative control tissue samples tested positive.

3.3. Tank exposure experiment

Cryptosporidium parvum oocysts were detected in mussels that filtered oocysts during an 8 h exposure to tank inoculated sea water. There was no mussel mortality during the experiment. Thirty *C. parvum*-exposed and 15 unexposed individual mussels had gill wash and digestive glands tested by DFA and TaqMan PCR, with and without IMS concentration. Thirty hemolymph samples from exposed mussels were tested with TaqMan PCR but only 28 were tested by each conventional PCR technique due to sample volume limitations. Hemolymph from 15 unexposed individual control mussels was tested with each PCR technique. For pooled samples of 5 mussels each, 12 exposed pools and 3 unexposed pools of each tissue type were tested by each detection technique. Pooled hemolymph samples were only tested by TaqMan PCR due to sample volume limitations. All mussels from the unexposed tank tested negative in all tissues with all techniques.

The proportion of *Cryptosporidium* positive mussels in the two oocyst-exposed tanks did not differ significantly ($p=1.0$), therefore data were combined for further analysis. Table 3 shows the tissue detection method and exposure status for individual and pooled mussels tested for *Cryptosporidium*. TaqMan PCR detected *Cryptosporidium* in 3% of hemolymph samples from exposed individual mussels, compared to 0% and 4% of hemolymph that tested positive by PCR1 and PCR2 conventional PCR assays, respectively. There was no significant difference between the TaqMan and conventional PCR techniques when testing hemolymph samples ($p=1.0$). TaqMan PCR did not detect *Cryptosporidium* cDNA in any of the exposed mussel pools, and these pools could not be tested by the two conventional PCR techniques due to sample volume limitations. None of the techni-

Table 2
Proportion of spiked mussel tissues positive for *Cryptosporidium parvum* by TaqMan PCR and DFA, with and without IMS concentration[a]

Sample type	No. oocysts spiked per mussel sample	TaqMan % positive	IMS-TaqMan % positive	DFA % positive	IMS-DFA % positive
Gill wash (5 ml)	1	0	17	0	0
	10	0	33	17	83
	100	50	67	50	100
	500	100	100	67	100
Digestive gland (1 g)	1	0	0	0	0
	10	0	17	0	83
	100	33	100	17	100
	500	50	100	67	100

[a] $n=6$ replicates per spiking dose and tissue type.

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Table 3
Proportion of individual and pooled mussels (*Mytilus californianus*) testing positive for *Cryptosporidium parvum* after an 8 h tank exposure to 1000 oocysts/L (142 oocysts/mussel)[a]

Mussel tissue	Detection method	Exposed individuals % positive ($n=30$)[b]	Unexposed individuals % positive ($n=15$)	Exposed pools % positive ($n=12$)[c]	Unexposed pools % positive ($n=3$)[c]
Hemolymph (1 ml/mussel)	TaqMan	3	0	0	0
	PCR1	0	0	ND	ND
	PCR2	4	0	ND	ND
Gill Wash (5 ml/mussel)	IMS-TaqMan	0	0	0	0
	DFA	0	0	0	0
	IMS-DFA	0	0	0	0
Digestive Gland (1 g/mussel)	IMS-TaqMan	7	0	0	0
	DFA	23	0	33	0
	IMS-DFA	80	0	100	0

[a] Exposed mussel tanks were inoculated *C. parvum* oocysts, unexposed mussels tanks were not inoculated.
[b] $n=28$ for PCR1 and PCR2 hemolymph testing.
[c] ND = not determined due to sample volume limitations.

ques detected *C. parvum* in individual or pooled gill wash samples. Immunomagnetic separation with TaqMan PCR detected *Cryptosporidium* in 7% of individual exposed digestive glands, compared to 23% detected by DFA and 80% by IMS-DFA. Because IMS-TaqMan PCR only detected *Cryptosporidium* in 7% of individual exposed mussel digestive glands and in none of the pooled mussel digestive glands, mussel samples without IMS concentration were not tested by TaqMan PCR. Pooled digestive gland tissues tested by DFA and IMS-DFA resulted in 33% and 100% positive, respectively. Overall, the most sensitive method for detection of *Cryptosporidium* in tank-exposed mussels was IMS concentration of digestive gland followed by DFA detection.

Immunomagnetic separation with TaqMan PCR was not significantly different from DFA alone ($p=0.18$), but detected significantly fewer positive individual mussel digestive glands than IMS concentration combined with DFA detection ($p<0.01$). The mean Ct value per positive digestive gland was 32 (range, 29–36 Ct), while the mean number of oocysts detected per mussel was 1.6 (range, 1–3 oocysts) by DFA and 19 (range, 1–150 oocysts) by IMS-DFA. IMS-TaqMan PCR was not significantly different from DFA for pooled exposed mussel digestive glands ($p=0.13$) and IMS-TaqMan PCR detected significantly fewer positive samples than IMS-DFA ($p<0.01$). The mean number of oocysts per positive digestive gland pool was 1.8 (range, 1–3 oocysts) by DFA and 9.8 (range, 2–24 oocysts) by IMS-DFA. When a duplicate set of pooled digestive gland samples stored 6 months at −20 °C was tested, 0% and 83% tested positive by DFA and IMS-DFA, respectively. Many of the oocysts visualized in digestive gland that had been frozen were deformed or ruptured but still had a strong green fluorescence, suggesting that freezing does not destroy the antigens that bind antibodies in the IMS and DFA procedures, but that it does cause oocyst rupture that could make morphologic identification difficult.

Once preliminary spiking experiments established test performance parameters, quantitative detection techniques such as TaqMan PCR and DFA analysis provided data that could be used to estimate the parasite concentration per sample. In the case of TaqMan PCR, the Ct value of an "unknown" sample could be correlated to the Ct values of the oocyst standard curve as seen in Fig. 1. In the case of DFA analysis, the number of oocysts in an unknown sample could be estimated by adjusting the data based on percent recovery estimates. Table 4 shows the mean recovery efficiency of *Cryptosporidium* oocysts spiked into 6 sets of gill wash and digestive tissues at doses ranging from 0–500 oocysts. A single oocyst spiked into gill washings or digestive gland was not detected by DFA, but when 10–500 oocysts were spiked into gill washings, the mean DFA percent recovery ranged from 17% to 42% per 10 ul test aliquot and 1% to 2% per total tissue spike. The use of IMS in combination with DFA on gill washes produced mean percent recoveries ranging from 40% to 44%, which reflected the individual test aliquot as well as the total tissue spike because all test aliquots were less than the recommended volume limit of 0.5 ml pellet for IMS concentration. Thus, for gill wash tissues, IMS-DFA data could be adjusted to estimate the true number of oocysts in an unknown sample by assuming that approximately 43% of oocysts were counted during IMS-DFA analysis. For digestive gland tissues spiked with 10–500 oocysts, mean DFA percent recoveries ranged from 0% to 15% per 10 ul test and from 0% to 0.2% per whole tissue spike. The use of IMS on

Table 4
Recovery efficiency of *Cryptosporidium parvum* oocysts from spiked mussel tissues by DFA and IMS-DFA detection[a]

Sample type	No. oocysts spiked per mussel sample	DFA mean % recovery per test[b] (% range)	DFA mean % recovery per mussel[c] (% range)	IMS-DFA mean % recovery per test and mussel[d] (% range)
Gill wash (5 ml)	1	0	0	0
	10	42 (0–250)	2 (0–10)	40 (0–100)
	100	17 (0–50)	1 (0–2)	44 (10–89)
	500	37 (0–90)	2 (0–4)	44 (17–75)
Digestive gland (1 g)	1	0	0	0
	10	0	0	58 (0–150)
	100	10 (0–57)	0.2 (0–1)	58 (43–83)
	500	15 (0–43)	0.2 (0–0.4)	66 (29–86)

[a] $n=6$ replicates per dose and tissue type.
[b] % recovery per test = No. oocysts counted on a slide/No. oocysts expected in a test aliquot.
[c] % recovery per mussel = No. oocysts counted on a slide/No. oocysts spiked into the mussel sample.
[d] % recovery per test and mussel are the same because the whole sample could be analyzed in one IMS test aliquot.

digestive gland produced mean DFA percent recoveries that ranged from 58–66%, therefore raw IMS-DFA data could be adjusted to estimate the actual number of oocysts present in the sample by assuming that approximately 61% of actual oocysts were visualized during IMS-DFA analysis.

4. Discussion

In the present study, a real-time TaqMan PCR system was validated and compared to conventional PCR and DFA methodologies, with and without IMS concentration, for sensitive and specific detection of *Cryptosporidium* spp. in bivalve tissues. TaqMan PCR and the two conventional PCR systems were all able to detect 1 or more oocysts spiked into 1 ml hemolymph samples, but detected *C. parvum* in $<5\%$ of tank exposed mussel hemolymph samples. In the gill wash and digestive gland spiking experiments, IMS concentration in combination with TaqMan PCR or DFA detection improved the minimum detection limit by 1 $\log_{10}$ unit, from 100 to 10 oocysts per spiked mussel sample. Despite the success of gill wash methods in detecting *C. parvum* in the tissue spiking experiments, none of the tank exposed gill wash samples tested positive for *C. parvum* by any method. However, IMS concentration in combination with DFA testing detected *Cryptosporidium* in 80% of exposed individual and 100% of exposed pooled mussel digestive glands, suggesting that more oocysts are retained in the digestive gland than in the gills or hemolymph after the mussels have filtered oocysts from the surrounding water. Both TaqMan PCR and DFA methods provided semi-quantitative results that could be used to estimate the true number of oocysts present in a bivalve sample.

TaqMan PCR has several advantages over traditional *Cryptosporidium* detection methods. TaqMan PCR is an automated, rapid, high throughput system that allows investigators to submit fresh or frozen samples to a TaqMan service in batches for pathogen detection, reducing the need for investigators to invest in expensive equipment and expertise in their own laboratories. The Bivalve TaqMan PCR system that is run concurrently with the *Cryptosporidium* TaqMan system is useful in assessing tissue extraction efficiency and can be used as a quality control measure for invertebrate studies. Additionally, the *Cryptosporidium* TaqMan PCR system detects cDNA transcribed from total RNA, a nucleic acid that is more rapidly degraded than genomic DNA after cell death. This suggests that TaqMan results are more likely to correlate with oocyst viability than assays that target genomic DNA, which is more stable than RNA (Fontaine and Guillot, 2003b). A primary goal for developing a TaqMan PCR system was to maximize analytical sensitivity. For that reason we targeted the 18S rRNA at the RNA level, which should have a higher copy number per oocyst than targeting the 18S rRNA gene (Deere et al., 1998). TaqMan assays that target low copy number genes may correlate even better with oocyst viability than 18S rRNA systems (Widmer et al., 1999). Finally, the TaqMan PCR system provides semi-quantitative results and detects many *Cryptosporidium* spp. The TaqMan and 2 conventional PCR systems detected all *Cryptosporidium* spp. evaluated in this study, but in the case of the PCR2 system, dinoflagellates and invertebrate DNA were also amplified. Other studies (Sturbaum et al., 2002) have reported similar cross reactions with dinoflagellates when testing for *Cryptosporidium*, suggesting that false positive *Cryptosporidium* results are of concern in field studies if DNA sequence analysis is not

used in conjunction with PCR. The *Cryptosporidium* TaqMan system was more specific than the PCR2 system but also detected *Sarcocystis* species. Both TaqMan and conventional PCR techniques can be used in combination with DNA sequence analysis, the gold standard for obtaining detailed molecular data that can be deposited in GenBank for use in other molecular epidemiology studies.

Before this study, TaqMan PCR systems had not been applied to bivalve studies but had been used to test water and fecal samples (Fontaine and Guillot, 2003a; Guy et al., 2003; Higgins et al., 2001). Guy et al. (2003) developed a quantitative multiplex real-time PCR system to detect *C. parvum* and *Giardia* spp. that was of comparable accuracy to immunofluorescent microscopy for testing water and sewage samples. Higgins et al. (2001) developed a TaqMan PCR system that was able to quantitate *C. parvum* oocysts in 0.2 g calf diarrhea samples, but was not able to detect *C. parvum* in 0.2 g adult cattle manure samples, possibly due to low oocyst numbers and the presence of substances inhibitory to PCR. Fontaine and Guillot (2003a) combined their TaqMan PCR system with IMS to detect 8 or more *C. parvum* oocysts in 100 L of tap water and 5 L of river water. Our TaqMan PCR system was developed for the analysis of bivalve samples; however, in conjunction with IMS concentration, it may also have valuable applications for fecal and water samples containing a variety of *Cryptosporidium* spp.

The *Cryptosporidium* DFA detection technique has been used to test fecal and water samples for over a decade, and more recently it has been applied to bivalve tissues (Fayer et al., 1998; Graczyk et al., 1998, 1999; Madore et al., 1987; Tamburrini and Pozio, 1999; Xiao and Herd, 1993). A benefit of using the DFA technique is that oocysts can be identified visually using a combination of immunofluorescence, size, and morphology to distinguish them from other organisms. Other benefits of DFA are that it provides quantitative data and can be used on formalin preserved samples, unlike conventional PCR. The limitations of the DFA technique are that it requires microscopy expertise and does not provide genotype data unless combined with PCR methods. Other studies have used DFA for testing hemolymph samples (Graczyk et al., 1998; Tamburrini and Pozio, 1999). However, in our preliminary studies the autofluorescence and small size of the hemocytes often obscured *Cryptosporidium* oocysts and interfered with accurate oocyst visualization. Therefore this detection method was not included in the present comparative study. Before the availability of IMS, the small volume of sample analyzed was also a significant limitation in detecting low numbers of *Cryptosporidium* by DFA. For example, 100 oocysts spiked into a mussel digestive gland (mean wt, 0.6 g; range, 0.2–2 g) were detected in a 10 μl DFA aliquot 17% of the time. In contrast, 100% of the same samples were positive when IMS concentration was performed before DFA detection.

Immunomagnetic separation has rarely been used in wild bivalve studies. An example is the Gomez-Bautista et al. (2000) study that used immunofluorescent antibody testing in combination with IMS to increase assay sensitivity when testing wild mussel and cockle homogenates collected along the coast of Spain. The authors detected *C. parvum* genotype 2 oocysts in bivalves collected near the mouth of rivers draining watersheds with high ruminant stocking densities but not in bivalves collected from river mouths draining more pristine areas or from sites away from river mouths. Although the study did not assess the percent recovery and sensitivity limits of their assay, the technique of testing pooled bivalve tissues with IMS holds promise for cost-efficient environmental monitoring. Immunomagnetic separation has also been used to test shellfish for pathogenic *Vibrio* spp. and hepatitis A viruses (Lopez-Sabater et al., 1997; Vuddhakul et al., 2000). The advantage of IMS is that a larger tissue volume can be analyzed (0.5 ml) as compared to 100 mg for PCR. Additionally, IMS decreases the amount of particulate matter and inhibitors in a sample. Although IMS increases the cost per test (up to $50 per test), it decreases the number of tests and time required to process a given sample volume.

The mussel tank exposure experiment was designed to simulate an 8 h pulse exposure to *Cryptosporidium*-contaminated nearshore waters. The exposure dose of 1000 oocysts/L is within the range of surface water contamination levels reported near agricultural runoff (Rose, 1997). Therefore, the results of this experiment may reflect the oocyst load to which wild shellfish can be exposed when located near fecal contaminated runoff. During an 8 h exposure to 14 °C water that originally contained 1000 oocysts/L, 10 L of salt water, and 70 mussels, each mussel was exposed to a mean of 142 oocysts. Based on the mean of 15 oocysts detected per mussel digestive gland (range from 1–150 oocysts) by IMS-DFA and adjusting the data based on the 61% recovery in spiked digestive gland tissues, up to 246 oocysts may have been present in a digestive gland, and at least 18% of the initial *Cryptosporidium* dose was likely to be retained in mussel tissues after 8 h. Graczyk et al. (2003) conducted a tank exposure experiment in which 100 *C. parvum* oocysts per day were inoculated for 31

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days into tanks containing freshwater mussels and clams. The authors found that from 7% to 32% of oocysts could be recovered in homogenized bivalve tissues, with increasing numbers of oocysts accumulated in bivalve tissues as the oocyst concentration in the water increased over time. The results of the Graczyk study complement the findings of our 8h tank exposure study by demonstrating that bivalves can accumulate oocysts after repeated daily exposures to low oocyst doses.

The most sensitive *Cryptosporidium* detection technique in our tank exposure experiment was testing digestive gland samples by DFA after IMS concentration. The majority of oocysts were detected in digestive gland samples, with 80% of exposed individual mussels and 100% of mussel pools testing positive by IMS-DFA, compared to <5% of hemolymph samples positive by PCR and 0% of gill washings positive by either DFA, IMS-DFA, or IMS-TaqMan PCR. There was no significant difference in IMS-DFA percent recovery when spiked individual mussel digestive glands (0.2–2 g before sieving) were compared to spiked pools of 5 mussel digestive glands (sieved pellet ≤ 0.5 ml) ($p = 0.33$, data not shown), suggesting that testing pooled samples could decrease cost without significant loss of sensitivity. The tissue spiking experiments had shown that low numbers of oocysts could be detected in hemolymph, gill wash, and digestive gland samples, so the differences in detection success between the tissue spiking experiments and the tank exposure experiment are likely due to the varied tissue retention and low numbers of oocysts present in the exposed mussels after they filtered the oocysts during the tank experiment. Other tank exposure studies have yielded varied results, with some studies finding that digestive gland was the best tissue for oocyst detection (Tamburrini and Pozio, 1999) while other studies concluded that gills were more sensitive for detecting *Cryptosporidium* contaminated bivalves (Fayer et al., 1997). Differences between studies could be attributable to experimental variables such as exposure time, dose, or detection techniques, or biologic variables such as bivalve species, filtration rate, or water characteristics.

In addition to determining the most sensitive *Cryptosporidium* detection method in mussels, the tank exposure experiment was used to define the TaqMan Ct cutoff for *Cryptosporidium*-positive bivalve samples. A TaqMan Ct value of <39.5 was defined as positive after comparing the TaqMan Ct values from *Cryptosporidium* exposed and unexposed mussels in the tank exposure experiment, to adjust for any background TaqMan signal. The lower the Ct value, the lower the number of TaqMan cycles required to produce detectable target DNA, with each cycle increasing the target DNA exponentially. Based on the relationship between TaqMan Ct values and standard *Cryptosporidium* dilutions, the concentration of oocysts in the test sample can be estimated. By comparing the range of Ct values (29–36) detected in the 2 positive IMS-TaqMan digestive glands in the tank experiment to the oocyst standard curve, over 100 oocysts were most likely present in those samples, which is similar to the IMS-DFA results for which 2 samples contained over 100 oocysts. This semi-quantitative capability may be useful for future studies that aim to measure changes in oocyst numbers based on environmental variables or *Cryptosporidium* treatment and inactivation regimens.

In conclusion, this study showed that TaqMan PCR was similar in analytical sensitivity and specificity to conventional PCR methods, with the additional benefits of rapid turn around time, high throughput capacity, and semi-quantitative results. TaqMan and DFA sensitivity were similar, with a 1 $\log_{10}$ unit improvement in minimum detection limits when used in combination with IMS concentration. The choice of whether to use IMS concentration before *Cryptosporidium* detection may depend on the desired minimum oocyst detection limit and the affordability of the increased IMS costs. Overall, the most sensitive *Cryptosporidium* detection technique was IMS concentration of mussel digestive gland followed by DFA detection. Both TaqMan and DFA detection methods provided semi-quantitative results but only the TaqMan system can be expected to identify the variety of *Cryptosporidium* genotypes present in environmental samples. This study provided the critical laboratory evaluations needed to select appropriate *Cryptosporidium* detection and characterization methods for future studies.

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