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NOTE

Application of a multiplex PCR for the detection of protozoan pathogens of the eastern oyster *Crassostrea virginica* in field samples

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ABSTRACT: Populations of eastern oysters *Crassostrea virginica* along the east coast of North America have repeatedly experienced epizootic mass mortality due to infections by protozoan parasites, and molecular diagnostic methodologies are fast becoming more widely available for the diagnosis of protozoan diseases of oysters. In this study we applied a modified version of an existing multiplex polymerase chain reaction (PCR) for detection of the eastern oyster parasites *Haplosporidium nelsoni*, *H. costale* and *Perkinsus marinus* from field-collected samples. We incorporated primers for DNA quality control based on the large subunit ribosomal RNA (LSU rRNA) gene of *C. virginica*. The multiplex PCR (MPCR) simultaneously amplified genomic DNA of *C. virginica*, and cloned DNA of *H. nelsoni*, *P. marinus* and *H. costale*. In field trial applications, we compared the performance of the MPCR to that of the conventional diagnostic techniques of histopathological tissue examination and the Ray/Mackin fluid thioglycollate medium (RMFT) assay. A total of 530 oysters were sampled from 18 sites at 12 locations along the east coast of the United States from the Gulf of Mexico to southern New England. The modified MPCR detected 21% oysters with *H. nelsoni*, 2% oysters with *H. costale*, and 40% oysters with *P. marinus* infections. In comparison, histopathological examination detected *H. nelsoni* and *H. costale* infections in 6 and 0.8% oysters, respectively, and the RMFT assay detected *P. marinus* infection in 31% oysters. The MPCR is a more sensitive diagnostic assay for detection of *H. nelsoni*, *H. costale*, and *P. marinus*, and incorporation of an oyster quality control product limits false negative results.

KEY WORDS: Diagnostics · Eastern oyster · *Haplosporidium costale* · *Haplosporidium nelsoni* · Multiplex PCR · *Perkinsus marinus* · Quality control

INTRODUCTION

Protozoa, such as *Haplosporidium nelsoni* Haskin, Stauber and Mackin, 1966 (multinucleated sphere unknown, MSX), *Haplosporidium costale* Wood and Andrews, 1962 (seaside organism, SSO) and *Perkinsus marinus* Levine 1978 (Dermo disease), are the most common causes of disease in the eastern oyster and, since the 1950s, have accounted for serious, widespread, periodic oyster mortalities from the Gulf of Mexico to the shores of Maine (Mackin et. al. 1950, Andrews & Hewett 1957, Haskin et al. 1966, Kleinschuster & Parent 1995, Barber et al. 1997, Sunila et al. 1999).

Traditionally, *Haplosporidium nelsoni* and *H. costale* have been detected by identification of spore and plasmodial stages in histological sections of oyster tissue (Wood & Andrews 1962, Andrews & Castagna 1978).

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These methods are time-consuming and expensive, and histomorphological similarities between plasmodia of *H. nelsoni* and *H. costale* make diagnosis of mixed infections in the absence of species-defining spores difficult (Andrews & Castagna 1978, Stokes & Burreson 1995, 2001). In the case of *Perkinsus marinus*, the Ray/Mackin fluid thioglycollate (RMFT) assay is useful (Ray 1952, 1966). However, the assay fails to differentiate between the many different types of *Perkinsus* species found to infect mollusks worldwide (Robledo et al. 2000). It has been shown that 2 *Perkinsus* species may simultaneously affect the same individual host (Kotob et al. 1999, Coss et al. 2001). In addition, the RMFT assay has reportedly failed to detect infections below 1000 *P. marinus* parasites per gram wet tissue (Bushek et al. 1994).

Polymerase chain reaction (PCR) and DNA probe methodologies have been developed and used successfully for rapid detection of *Haplosporidium nelsoni* (Stokes et al. 1995, Stokes & Burreson 2001), *H. costale* (Ko et al. 1995, Stokes & Burreson 1995, 2001), and *Perkinsus marinus* (Marsh et al. 1995, Robledo et al. 1998). PCR is ideal for specifically recognizing target DNA sequences regardless of the life history stage of the parasite or host (Stokes et al. 1995). Simultaneous detection of multiple pathogens in a single PCR reaction utilizing multiplex PCR (MPCR) technology has proven to be a successful approach for rapid, sensitive and specific identification of protozoan agents in the eastern oyster (Penna et al. 2001). In addition, with an increased reliance on PCR-based diagnostic tests for pathogenic microorganisms, internal standards are useful to obtain reliable diagnosis and to avoid false negative results (Brightwell et al. 1998). MPCR is ideally suited for incorporation of an internal standard (Sachadyn & Kur 1998); successful amplification of the internal standard confirms that the quality of DNA isolated from a sample will support amplification and that no inhibitors or technical failures were encountered.

The aim of this study was to modify an existing MPCR to include an internal standard for DNA quality control and to apply this modified MPCR in a large-scale field trial to assess its utility versus the conventional diagnostic techniques of histological examination and the RMFT assay.

MATERIALS AND METHODS

To introduce a host-specific amplicon into the MPCR protocol, primers to regions of the large subunit (LSU) ribosomal RNA (rRNA) sequence of *Crassostrea virginica* (GenBank Accession #AF137050) were designed using DNAMAN analysis software (Lynnon Corp.). In order to obtain primers of similar lengths (19 to 21 nucleotides) and melting temperatures (±1 to 2°C) to accommodate the incorporation of an internal standard, primer sets previously designed by Stokes et al. (1995), Ko et al. (1995), Marsh et al. (1995), and Penna et al. (2001) for the small subunit (SSU) rRNA gene sequences for *Haplosporidium nelsoni*, *H. costale*, and an intergenic non-transcribed sequence (NTS) between the 5S and SSU rRNA gene of *Perkinsus marinus* (GenBank Accession #U19538, U20858 and S78416, respectively) were modified or redesigned (Table 1). Primers to the SSU rRNA gene of *H. costale* were not those used by Penna et al. (2001) or Ko et al. (1995), but instead were designed from a different section of the SSU rRNA gene sequence provided by Ko et al. (1995). Putative primer sequences were aligned and checked against the SSU rRNA gene sequences of *Haplosporidium louisiana* (#U47851), *Urosporidium crescens* (#U47852), *Minchinia teredinis* (#U20320), the ciliates *Oxytricha nova* (#X03948), *Tetrahymena pyriformes* (#M98021) and *Paramecium tetraurelia* (#X03772), the dinoflagellates *Crypthecodinium cohnii* (#M64245), *Symbiodinium corculorum* (#L13717), *Prorocentrum micans* (#M14649), and *Amphidinium belauense* (#L13719). Forward and reverse primers for *C. virginica*, *H. nelsoni*, *P. marinus*, and *H. costale* were synthesized by Life Technologies. DNA was extracted using the Qiagen DNeasy tissue extraction and purification system, with several modifications to the manufacturer's animal tissue protocol. Briefly, tissue samples of gill, mantle, digestive gland and/or anus (depending on oyster size) were collected using a 4 mm diameter biopsy punch, placed in 360 ml tissue lysis buffer, heated to 95°C for 10 min, allowed to cool to 55°C, and incubated at 55°C for 24 to 36 h after the addition of 40 ml of 20 mg ml^{-1} Proteinase K. Two hun-

Table 1. Oligonucleotide primers used to amplify DNA in the multiplex PCR (MPCR)

Species	Forward primer	5' to 3' sequence	Reverse primer	5' to 3' sequence	Product size (bp)
Crassostrea virginica	H1fwd	GCTGGGAACTGTGGTGTTT	H1rev	GGCTGCCTTCACTTTCATT	805
Haplosporidium nelsoni	Msxf[a]	TGGCATTAGGTTTCAGACCT	Msxr[a]	ATGTGTTGGTGACGCTAACC	565
Perkinsus marinus	Dermo-fwd[b]	CACTTGTATTGTGAAGCACCC	Dermo-rev[c]	GGTGACATCTCCAAATGACC	305
Haplosporidium costale	sso #2fwd[d]	CTGAGAAACGGCTACCACAT	sso #2rev[d]	ACTTGGTGGTCGATAAGGCT	149

[a]Modified from Stokes et al. (1995); [b]taken from Marsh et al. (1995); [c]modified from Marsh et al. (1995); [d]designed from sequence data provided by Ko et al. (1995)

dred ml of lysed and homogenized sample was frozen at –80°C, and 200 ml was further digested with 10 ml of 100 mg ml^{-1} RNAse solution and loaded onto a silica-gel membrane spin column for DNA extraction. Purified DNA was quantitated using a spectrophotometer and stored at –80°C.

MPCRs were optimized consecutively in a GeneAmp PCR System 9600 thermal cycler following protocols outlined by Henegariu et al. (1997). Amplification reactions were carried out in 50 µl volumes, each reaction mixture containing 450 to 550 ng of purified DNA, 500 mM KCl, 100 mM Tris-HCl (pH 8.3), 3 mM $MgCl_2$, 200 mM each dNTP, 1 U of AmpliTaq Gold DNA polymerase, and 48, 6, 12, and 18 pmol of each forward and reverse primer for *Crassostrea virginica*, *Haplosporidium nelsoni*, *Perkinsus marinus*, and *H. costale*, respectively. Reaction mixtures were heated to 94°C for 12 min and cycled 32 times at 94°C for 1 min, 62.5°C for 1 min, and 72°C for 2 min for each cycle, with a final extension at 72°C for 7 min. For each MPCR, a volume of 16 ml was subjected to horizontal electrophoresis in 1.5% (w/v) agarose gels. Amplification products for *C. virginica*, *H. nelsoni*, *P. marinus*, and *H. costale* were 805, 565, 305, and 149, respectively. Negative controls included a reaction mixture without template (i.e. reagent blank) and reaction mixtures with approximately 500 ng of genomic DNA from either *Salmo salar* (Atlantic salmon) or *Homarus americanus* (American lobster) that underwent simultaneous extraction and purification with the oysters sampled in this study. Positive controls were mixtures of 1 ng each of cloned SSU rDNA of *H. nelsoni*, *H. costale*, and 1 ng of cloned NTS DNA of *P. marinus* spiked into 500 ng of genomic DNA from pathogen-free *C. virginica*. Preparation of cloned SSU rDNA of *H. nelsoni* and *H. costale* followed procedures described previously by Penna et al. (2001) based on original material kindly provided by Nancy Stokes (Virginia Institute of Marine Science, Gloucester Point, VA, USA). The intergenic NTS of *P. marinus* was amplified from genomic *P. marinus* DNA (ATCC strain LICT-1) using specific *P. marinus* primers described in this article, cloned into pGEM T, and plasmid DNA was isolated using a Qiagen Plasmid Mini Kit.

In order to test the target specificity of primers in the context of the optimized MPCR, various artificial mixtures (6 combinations, 1 ng $parasite^{-1}$) of cloned DNA of *Haplosporidium nelsoni*, *H. costale*, and *Perkinsus marinus* were added to 500 ng of genomic *Crassostrea virginica* DNA and were used as template for the MPCR. In addition, primer specificity was tested in MPCRs, using cloned SSU rDNA from *H. louisiana* and *Urosporidium crescens*, genomic DNA from *Crassostrea gigas* (Japanese oyster) and *Mytilus galloprovincialis* (Mediterranean mussel), and the free-living and parasitic protozoa *Paramoeba eilhardi* and *Neoparamoeba pemaquidensis.* MPCR products were cloned as previously described, and sequenced in an automated Applied Biosystems 377 DNA sequencer. Nucleotide sequences from 3 clones of each MPCR product were aligned using DNA analysis software and then compared to the NTS sequence of *P. marinus* (#S78416) and the SSU rDNA sequences of *H. nelsoni* (#U19538), *H. costale* (#U20858), and LSU rDNA of *C. virginica* (#AF137050).

Sensitivity of the MPCR was determined by spike/recovery. Serial 10-fold dilutions of an initial mixture consisting of 1 µl (100 ng $µl^{-1}$) each of cloned DNA of *Haplosporidium nelsoni*, *H. costale*, and *Perkinsus marinus* were spiked into a constant amount (500 ng) of genomic DNA of pathogen-free *Crassostrea virginica*, and used as templates for the MPCR.

Oysters (*Crassostrea virginica*) were collected from 18 sites at 12 locations along the Atlantic Coast and Gulf of Mexico of the United States between June 2000 and August 2001 (Fig. 1). Two of the sites, Oyster Bay, Long Island, New York, and Norwalk, Connecticut, were sampled year-round. The rest of the field stations were sampled once between June 6, 2000 and December 10, 2000. In order to test and compare the results of this modified MPCR protocol to those of the RMFT assay and histopathological examination of tissue sections, individual oysters were subjected to all 3 diagnostic techniques to determine the presence of each

Fig. 1. Map of the East Coast of the United States indicating oyster collection sites. Site 1: Katama Bay, Martha's Vineyard, MA; Site 2: Barrington River, RI; Sites 3–6: Norwalk, CT; Site 7: Amagansett, Long Island, NY; Site 8: Mattituck Creek, Long Island, NY; Sites 9–12: Oyster Bay, Long Island, NY; Site 13: Port North, Delaware Bay, NJ; Site 14: Ocean City, Sinepuxent Bay, DE; Site 15: Lower Cedar Point, Potomac River, Chesapeake Bay, MD; Site 16: Cornfield Harbor, Potomac River, Chesapeake Bay, MD; Site 17: Clambank Creek, North Inlet, SC; Site 18: Grand Isle, LA

protozoan pathogen in each oyster. Upon receipt, oysters were examined grossly, measured and opened individually using aseptic technique. Tissues for the RMFT assay were sampled using a sterile 4 mm biopsy punch through the digestive gland and anus, and were cultured according to the methods of Ray (1966). *Perkinsus marinus* hypnospore counts were assigned a semi-quantitative estimate of infection based upon Mackin's scale (Mackin 1962). Tissues for DNA extraction and purification were sampled from the digestive gland, mantle and gill (approximately 25 mg wet tissue) using a sterile 4 mm biopsy punch for each individual oyster. Oysters were then shucked and fixed in 10% neutral buffered formalin for 24 h. Transverse sections through the visceral mass, mantle and gill were routinely processed for paraffin-embedding, sectioned at 4 mm, stained with hematoxylin and eosin (H&E), and examined by light microscopy.

Simultaneous amplification of the 4 target gene segments for *Crassostrea virginica*, *Perkinsus marinus*, *Haplosporidium nelsoni*, and *Haplosporidium costale* was achieved using PCR cycling parameters at a primer annealing temperature of 62.5°C.

RESULTS

Primer specificity was tested in MPCR experiments using combinations of cloned DNA of *Haplosporidium nelsoni*, *H. costale*, and *Perkinsus marinus* added to genomic *Crassostrea virginica* DNA. There was no cross-reactivity between species (Fig. 2). In addition, when a single-species target DNA sequence was added to the MPCR reaction, DNA fragments of expected molecular weight resulted. When cloned SSU rDNA of *H. nelsoni* template alone was present in the MPCR mixture, an additional product of approximately 980 bp was visualized above the targeted 565 bp amplification product. However, in the optimized MPCR protocol containing the quality control target sequence of *C. virginica* and any other combination of target DNA templates, this higher molecular weight product of *H. nelsoni* was not detectable. Direct sequence analysis of cloned PCR amplification products revealed 100% homology to sequences obtained from GenBank.

PCR primers did not amplify the SSU rDNA from the related haplosporidians *Haplosporidium louisiana* or *Urosporidium crescens* or the free-living and parasitic protozoa *Paramoeba eilhardi* and *Neoparamoeba pemaquidensis*, which were chosen by virtue of their accessibility and prevalence in saltwater and freshwater culture conditions. The oyster primers did amplify a single, specific, 805 bp DNA fragment from both *Crassostrea gigas* and *Mytilus galloprovincialis* genomic DNA which was predictable based on the high degree of sequence homology that exists between the rRNA genes of *C. virginica*, *C. gigas* (GenBank #AF137051), and *M. edulis* (GenBank #AF339512). The fact that DNA of *C. gigas* and *M. galloprovincialis* is also amplifiable by this MPCR does not diminish the value of the 805 bp product as a quality control target and may perhaps broaden the applicability of the MPCR to more host species.

After 1 round of MPCR amplification in spike/recovery experiments, we were able to detect cloned DNA of *Haplosporidium nelsoni*, *Perkinsus marinus*, and *H. costale* in amounts of 100 fg, 100 fg, and 10 pg, respectively (Fig. 3). Because *H. nelsoni* and *H. costale* have not been successfully cultured *in vitro*, and due to the fact that cloned DNA of *P. marinus* was used, we were not able to accurately estimate the limit of detection of the MPCR in terms of numbers of each parasite. However, MPCR was able to maintain similar levels of detection to that of single PCR amplifications for the detection of *H. nelsoni* (Stokes et al. 1995), *P. marinus* (Marsh et al. 1995, Robledo et al. 1998), and *H. costale* (Stokes & Burreson 2001).

Comparative field trial results of PCR, RMFT and histological examination for diagnosis of the 3 pathogens are presented in Table 2. Each MPCR mixture contained a standardized 450 to 500 ng of extracted DNA as template for the evaluation. The

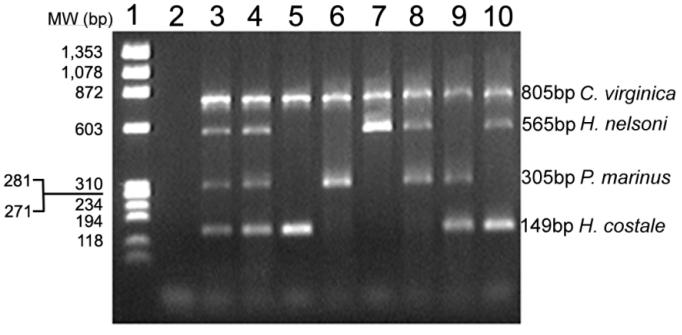

Fig. 2. Agarose gel electrophoresis of amplification products of the multiplex PCR (MPCR) demonstrating the specificity of the 4 sets of primers using various combinations of target cloned templates spiked into 500 ng of genomic *Crassostrea virginica* DNA. Lane 1: Phi X 174/ *Hae* III double-stranded DNA ladder. Lane 2: reagent blank (no template). Lanes 3–10: MPCR using *C. virginica*, *Haplosporidium nelsoni*, *H. costale*, and *Perkinsus marinus* primer sets. Lanes 3 and 4: positive control (genomic *C. virginica* spiked with 1 ng each of cloned *H. nelsoni*, *H. costale*, and *P. marinus*). Lane 5: genomic *C. virginica* and cloned *H. costale*. Lane 6: genomic *C. virginica* and cloned *P. marinus*. Lane 7: genomic *C. virginica* and cloned *H. nelsoni*. Lane 8: genomic *C. virginica* with cloned *P. marinus*, and *H. nelsoni*. Lane 9: genomic *C. virginica* with cloned *P. marinus*, and *H. costale*. Lane 10: genomic *C. virginica* withcloned *H. nelsoni*, and *H. costale*. MW: molecular weight

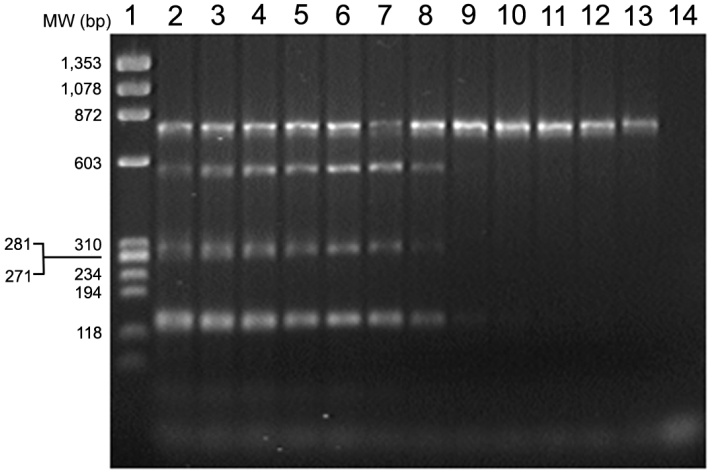

Fig. 3. Agarose gel electrophoresis of amplification products of the multiplex PCR (MPCR) demonstrating the sensitivity of the reaction. Lane 1: Phi X 174/*Hae* III double-stranded DNA ladder. Lanes 2– 13: MPCR using 10-fold serial dilutions of an artificial mixture consisting of 1 ml each of cloned NTS of *Perkinsus marinus* and cloned SSU rDNA of *Haplosporidium nelsoni* and *H. costale* spiked into 500 ng of *Crassostrea virginica* genomic DNA. Lane 2: 100 ng. Lane 3: 10 ng. Lane 4: 1 ng. Lane 5: 100 pg. Lane 6: 10 pg. Lane 7: 1 pg. Lane 8: 100 fg. Lane 9: 10 fg. Lane 10: 1 fg. Lane 11: 0.1 fg. Lane 12: 0.01 fg. Lane 13: 0.001 fg. Lane 14: 500 ng of *Homarus americanus* genomic DNA. MW: molecular wieght

Crassostrea virginica internal control was amplified in all 530/530 (100%) sample reactions, and the MPCR was negative for each of the 3 pathogens in 188/530 (35%) samples. Presence of the *C. virginica* amplifica-

Table 2. Comparative results of multiplex PCR (MPCR) assay, Ray/Mackin fluid thioglycollate (RMFT) assay and histological examination for the diagnosis of *Perkinsus marinus*, *Haplosporidium nelsoni*, and *Haplosporidium costale* (n = 530). Results expressed as number positive

Sampling site	Sampling date (m/d/yr)	Sample size (n)	MPCR			RMFT	Histology		
			P. marinus	*H. nelsoni*	*H. costale*		*P. marinus*	*H. nelsoni*	*H. costale*
1. Katama Bay, Martha's Vineyard, MA	09/22/00	30	0	0	0	0	0	0	0
2. Barrington River, RI	11/07/00	30	25	6	1	26	27	1	1
3. Norwalk, CT	10/27/00	30	12	6	0	18	6	2	2
4. Norwalk, CT	01/22/01	30	21	6	0	9	16	3	0
5. Norwalk,CT	04/26/01	30	23	11	0	4	9	0	0
6. Norwalk, CT	11/07/01	30	14	3	11	11	5	1	1
7. Amagansett, Long Island, NY	06/26/00	30	0	3	0	0	0	0	0
8. Mattituck Creek, North Fork, Long Island, NY	09/27/00	30	18	12	0	7	12	1	0
9. Oyster Bay, Long Island, NY	12/11/00	30	0	8	0	3	0	1	0
10. Oyster Bay, Long Island, NY	03/16/01	30	3	9	0	0	3	2	0
11. Oyster Bay, Long Island, NY	06/06/01	30	0	11	0	3	0	6	0
12. Oyster Bay, Long Island, NY	08/15/01	20	1	10	0	1	2	5	0
13. Portnorth, Delaware Bay, NJ	11/02/00	30	29	6	0	20	18	3	0
14. Ocean City Inlet, Sinepuxent Bay, DE	11/16/00	30	17	0	0	9	6	0	0
15. Potomac River, left channel, Chesapeake Bay, MD	11/08/00	30	3	6	0	10	2	0	0
16. Cornfield Harbor, Potomac River, MD	11/16/00	30	14	11	0	14	13	4	0
17. Clambank Creek, North Inlet, SC	12/10/00	30	21	2	0	22	11	1	0
18. Grand Isle, LA	11/29/00	30	11	0	0	6	3	0	0
	Totals	530	212	110	12	163	133	30	4
	Prevalence (%)		40	21	2.3	31	25	6	0.8

tion product in the absence of *Haplosporidium nelsoni*, *H. costale*, and *Perkinsus marinus* MPCR products illustrates that template DNA preparations were of sufficient quality to have been amplified, and that reactions were executed properly, but that the target DNA was absent, thereby validating DNA extraction quality, PCR conditions, and interpretation of these results as negative for the presence of protozoan pathogens.

DISCUSSION

The results of 530 oysters tested in this study indicate that MPCR diagnosis of *Haplosporidium nelsoni*, *H. costale*, and *Perkinsus marinus* is a reliable alternative diagnostic test to histopathological examination and the RMFT assay. The main advantage of the MPCR is its simultaneous, rapid detection of 3 pathogens, along with an internal DNA quality control standard, using a single test. DNA quality control testing could have been accommodated by a separate PCR; however, inclusion of a host genetic target into a single MPCR reduced PCR set-up and run times, as well as reagent consumption, in a large-scale application such as ours. In this study, total time from receiving oysters to detection of amplified DNA by agarose gel electrophoresis was 36 to 48 h. Traditional pathogen diagnosis by RMFT assay or histopathological examination ranged from 5 to 7 d. *H. nelsoni*, *H. costale*, and *P. marinus* were detected by all 3 diagnostic techniques, but the MPCR detected a greater prevalence of infection by each parasite, regardless of mixed infections and infection density, when compared to other traditional tests. For the detection of *H. nelsoni* and *H. costale*, the specificity of the primers alleviates the problems associated with histomorphological differentiation of plasmodia in the absence of species-defining spores.

Our results indicate that the MPCR is able to detect oysters infected with *Perkinsus marinus* and *Haplosporidium nelsoni* throughout the respective geographic range of each parasite. The absence and low prevalence of *H. costale* from endemic and historically non-endemic regions prevented us from properly assessing the MPCR's ability to detect *H. costale*-infected oysters. Greater numbers of sampling sites from endemic coastal regions Virginia, Maryland, Delaware, and New Jersey are required to adequately compare these 2 diagnostic techniques. However, as with any diagnostic test which does not utilize the entire host organism for the detection of infectious agents, low infection densities, localized infections, and tissue-sampling techniques may account for false negative interpretation of results (Stokes et al. 1995). In addition, the 11 of 12 (2%) *H. costale* infections detected by MPCR at Site 6 (Norwalk, CT, November 7, 2001) may have resulted from laboratory error, which would enhance detection results. However, attempts to prevent laboratory contamination included use of an aseptic technique to obtain tissue samples and PCR extraction and amplification of negative controls. Moreover, a second round of DNA extraction, purification and amplification (including extraction controls) was carried out on –80°C stored samples to verify our results. Similarly, small numbers of histological or RMFT-positive/MPCR-negative samples were identified from the 530 oysters sampled. Some discrepancies between RMFT and MPCR results are likely due to the lack of specificity of the RMFT assay, which can yield false positive results because of its inability to discriminate between *Perkinsus* spp. (Robledo et al. 1998) or the presence of *Perkinsus* spp. other than *P. marinus* (Kotob 1999, McLaughlin et al. 2000, Coss et at. 2001). All oysters positive for RMFT and negative for MPCR were light infections (0.5) as determined by the Mackin scale of *Perkinsus* infection (Mackin 1962).

The wide range of geographical and environmental field-collected samples to which this MPCR was applied demonstrates its utility for *Perkinsus marinus* and *Haplosporidium nelsoni* in broad-range, large-scale surveillance programs where rapid and accurate detection of these protozoan parasites may be necessary. The usefulness of this modified MPCR will need to be explored in future applications related to oyster health and disease management, e.g. screening oysters prior to importation or transplantation, seed certification, identifying point sources of infection, and maybe elucidating unidentified life cycle stages in alternative hosts.

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