

Quantitative PCR assay to determine prevalence and intensity of MSX (*Haplosporidium nelsoni*) in North Carolina and Rhode Island oysters *Crassostrea virginica*

Ami E. Wilbur^{1,*}, Susan E. Ford², Julie D. Gauthier³, Marta Gomez-Chiarri⁴

¹Department of Biology and Marine Biology, Center for Marine Science, University of North Carolina Wilmington, 5600 Marvin K. Moss Lane, Wilmington, North Carolina 28409, USA

²Rutgers University, Haskin Shellfish Research Laboratory, 6959 Miller Avenue, Port Norris, New Jersey 08349, USA

³Department of Biology, Loyola University, New Orleans, 6363 St. Charles Ave., New Orleans, Louisiana 70118, USA

⁴Department of Fisheries, Animal and Veterinary Science, University of Rhode Island, CBLS Room 169, Kingston, Rhode Island 02881, USA

ABSTRACT: The continuing challenges to the management of both wild and cultured eastern oyster *Crassostrea virginica* populations resulting from protozoan parasites has stimulated interest in the development of molecular assays for their detection and quantification. For *Haplosporidium nelsoni*, the causative agent of multinucleated sphere unknown (MSX) disease, diagnostic evaluations depend extensively on traditional but laborious histological approaches and more recently on rapid and sensitive (but not quantitative) end-point polymerase chain reaction (PCR) assays. Here, we describe the development and application of a quantitative PCR (qPCR) assay for *H. nelsoni* using an Applied Biosystems TaqMan[®] assay designed with minor groove binder (MGB) probes. The assay was highly sensitive, detecting as few as 20 copies of cloned target DNA. Histologically evaluated parasite density was significantly correlated with the quantification cycle (C_q), regardless of whether quantification was categorical ($r^2 = 0.696$, $p < 0.0001$) or quantitative ($r^2 = 0.797$, $p < 0.0001$). Application in field studies conducted in North Carolina, USA (7 locations), revealed widespread occurrence of the parasite with moderate to high intensities noted in some locations. In Rhode Island, USA, application of the assay on oysters from 2 locations resulted in no positives.

KEY WORDS: MSX · Oysters · qPCR · Diagnostic assay · Histology · Parasite density

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INTRODUCTION

The use of polymerase chain reaction (PCR) assays for detecting parasites of commercially important bivalves has progressed from initial development (Anderson et al. 1995, Stokes et al. 1995, Robledo et al. 1998, Penna et al. 2001, Carnegie et al. 2003) to implementation in field studies (Lyons et al. 2005, Carnegie et al. 2008, Tun et al. 2008, Ford et al. 2009a) and is beginning to be used for seed certifica-

tion (e.g. Ford et al. 2009b). The greater sensitivity of PCR, compared to histology or other non-molecular semi-quantitative assays for bivalve pathogens, has been demonstrated repeatedly in these studies. This improved sensitivity has resulted in detection of the presence of a parasite (or parasite DNA) when more traditional histological methods were unable to detect its presence (Ford et al. 2009a). PCR assays allow recognition of target DNA regardless of the life stage present; they can be used to detect cryptic life

*Email: wilbura@uncw.edu

history stages in atypical hosts, vectors, and/or environmental samples, and they provide a sensitive tool for detecting the pathogens at early stages of infection.

Traditional PCR assays analyze end-point amplification products that generally require post-amplification handling for quantification, reducing their utility for some questions. These limitations have prompted interest in real-time or quantitative PCR (qPCR) assays, which have followed a trajectory similar to end-point PCR, from initial development as a diagnostic tool (Day et al. 2000, Yarnall et al. 2000, Audemard et al. 2004, Gauthier et al. 2006, Lyons et al. 2006, De Faveri et al. 2009) to application for understanding the ecology of the parasite in the environment, and mechanisms of host–pathogen interactions (Audemard et al. 2006, Marty et al. 2006, Gast et al. 2008, Liu et al. 2009). These applications have been made possible by the fact that qPCR assays permit the absolute or relative quantification of the target. For species of parasites that can be propagated *in vitro*, a qPCR assay can be calibrated using counts of cultured parasites (Yarnall et al. 2000, Audemard et al. 2004, Gauthier et al. 2006, De Faveri et al. 2009) to convert relative to absolute abundance. The inability to propagate some important bivalve pathogens complicates the development of qPCR assays for those pathogens. In such cases, calibration can be achieved by using semi-quantitative rating systems for infection intensity (Corbeil et al. 2006, Marty et al. 2006) or serial dilutions of cloned targets (Ulrich et al. 2007a, Liu et al. 2009).

One of the parasites for which *in vitro* propagation has remained problematic is *Haplosporidium nelsoni*, the causative agent of multinucleated sphere unknown (MSX) disease in eastern oysters *Crassostrea virginica*. MSX disease was responsible for catastrophic oyster mortalities in the mid-Atlantic US states in the late 1950s and early 1960s. Widespread and recurrent mortalities persisted in the Delaware and Chesapeake Bays, and by the 1980s, *H. nelsoni* had been reported in oysters from Maine to Florida (Haskin & Andrews 1988). Outside the mid-Atlantic, however, the impact of the parasite has been more uncertain, with its presence not necessarily being associated with extreme mortalities. To the north, the lack of epizootic outbreaks was temporary, with severe mortalities being reported subsequently from Long Island Sound, USA, to Nova Scotia, Canada (Matthiessen et al. 1990, Barber et al. 1997, Sunila et al. 1999, Stephenson et al. 2003). To the south, the prevalence of histologically detected *H. nelsoni* infections has remained low since quantitative data

collections began in the mid-1980s (Lewis et al. 1992, Dougherty et al. 1993, Bobo et al. 1996). For this study, we targeted one area in the northern range (Rhode Island) and one in the southern range (North Carolina), where relatively little information exists on the distribution and prevalence of *H. nelsoni*.

In North Carolina, the first reports of *Haplosporidium nelsoni* date to the early 1960s when the parasite was found in Pamlico Sound oysters (Haskin & Andrews 1988), but the epizootic mortalities associated with the pathogen just to the north, in Chesapeake Bay, apparently did not occur, or at least were not reported. In fact, the potential association of *H. nelsoni* with oyster mortalities was not investigated until the fall of 1988, when large-scale mortalities prompted the testing of oysters from 11 locations in North Carolina. At that time, only 2 sites were found to be positive by histological examination for *H. nelsoni* (Fig. 1, Crab Slough and Wysocking Bay), whereas oysters from 9 sites exhibited infections with *Perkinsus marinus* (cause of Dermo disease) to which the mortalities were attributed (NCDMF 2008). A more expansive survey of oysters in the fall of 1989, using the hemolymph assay of Bureson et al. (1988), indicated that *H. nelsoni* was more widespread in North Carolina, with oysters from 11 of 36 locations exhibiting detectable infections (Morrison et al. 1992). The sites where *H. nelsoni* was reported were restricted to the

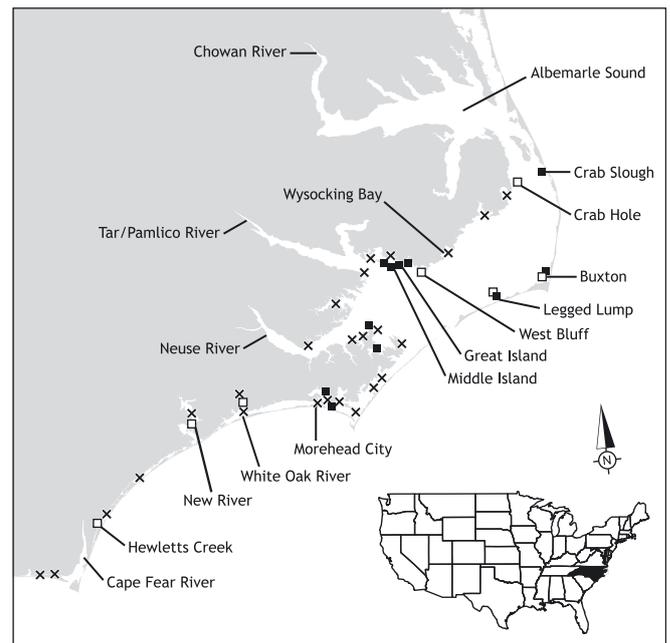


Fig. 1. Sample locations in North Carolina, USA, tested for *Haplosporidium nelsoni*. Sample sites where *H. nelsoni* was detected (■) or not detected (x) by Morrison et al. (1992). (□) Sites sampled for this study

northern waters of the state, with the southernmost occurrence being in the Newport River near Morehead City. At only 2 sites (near the mouth of the Pamlico River, see Fig. 1) were the infections deemed substantial (11 of 40 oysters infected from Great Island, 12 of 43 from Middle Ground, with infections ranging from 1 to 1000 plasmodia per 100 000 hemocytes; Morrison et al. 1992). Subsequent sampling conducted during 1990 to 1992 revealed no high-intensity infections, and severe hurricane activity in the mid-1990s, which kept salinities low, was suggested as the reason that *H. nelsoni* has not proliferated in the Pamlico River (NCDMF 2008).

Similarly, Rhode Island oysters have likely experienced some infection pressure from *Haplosporidium nelsoni* since the 1960s, when the parasite was reported in the adjacent states of Connecticut and Massachusetts (Krantz et al. 1972, Haskin & Andrews 1988), but the extent of oyster morbidity and mortality caused by this parasite is unknown. A survey in the 1980s failed to find the parasite (Cooper & Durfee 1982), but *H. nelsoni* was observed in oysters from a coastal pond in Rhode Island in another survey performed in 1991 to 1992 (Andamari et al. 1996). A state-funded annual monitoring program that began in 1998 using a combination of histology and PCR (Stokes et al. 1995) determined that the prevalence of haplosporidian parasites in Rhode Island oysters is usually low and restricted to a few locations (Alves 2007). The highest levels of infection were observed in 1998, coincident with the timing of an MSX-disease epizootic experienced by Connecticut oysters in 1997 to 1998 (Sunila et al. 1999). It is unclear, however, whether the heavy mortalities observed in wild oyster populations in Rhode Island at this time were due to MSX or to Dermo disease, which is prevalent in wild oysters.

The differences in the progression of the host-parasite relationship in epizootic areas and those where epizootics are rare or have not been reported raise questions regarding the factors that trigger or inhibit MSX disease outbreaks. The expansion of oyster restoration and aquaculture in both North Carolina and Rhode Island increases the need to understand what can cause the shift from an apparently benign relationship (present without epizootic mortalities) to a more deleterious one. It may be that the relationship remains benign as long as infective stages are scarce, the oysters are resistant, or the parasite has reduced virulence. The parasite could be scarce because of an environmental condition (i.e. recurring low salinity) or low abundance of an alternate or immediate host (Ford et al. 2009b). Evidence

contradicts the hypothesis that oysters in these regions are resistant to MSX disease, as they become heavily infected when transplanted to known epizootic waters (Ford & Haskin 1987, Ragone-Calvo et al. 2003). The inability to culture *Haplosporidium nelsoni* has restricted investigation of variation in its virulence.

We developed a qPCR assay for *Haplosporidium nelsoni* that will allow the rapid and quantitative detection of parasites in monitoring programs, as well as facilitate the study of MSX disease epizootiology. We calibrated the assay against different measures of parasite abundance: (1) cloned *H. nelsoni* DNA copy number, (2) a semi-quantitative rating system, and (3) estimates of parasite density made using counts of *H. nelsoni* in histological sections. The assay was tested in 2 different laboratories, using 2 different platforms, and samples from 7 populations of oysters in North Carolina and 2 sites in Rhode Island.

MATERIALS AND METHODS

Assay development

The MSX assay was developed as a TaqMan[®] assay with a minor groove binder (MGB) probe and was designed to target a relatively non-conserved region within the small subunit ribosomal RNA (SSU rRNA) gene of *Haplosporidium nelsoni* (Fong et al. 1993). TaqMan[®] assays incorporate specific forward and reverse PCR primers to amplify a short amplicon (~150 bp) and an internal probe constructed with a 5' fluorescent reporter dye, a 3' quencher, and an MGB tail to increase the stringency (Kutyavin et al. 2000). The components of this assay were designed using Primer Express software Version 2.0 (Applied Biosystems), and the target region was selected because of the substantial number of mismatches between *H. nelsoni* and other protozoan organisms known to exist within or around oysters. The primer and probe sequences for the MSX assay were as follows: forward primer: 5'-GCG CGC TAC AAT GTT GCA-3'; reverse primer 5'-CGA GAT TAC CCG GCC TTC TC-3'; and 6-carboxyfluorescein (FAM)-labeled MGB probe: 5'-CGA GTT CAA CCT TGC C-3'. TaqMan MGB assay technology is designed to detect ≥ 1 bp discrepancies within the probe region; therefore, a false positive signal is highly unlikely when mismatches of ≤ 2 bp occur. The MSX assay specificity was empirically tested against *H. costale* and other oyster protozoan pathogens known to

occur in North Carolina or Rhode Island waters (*Bonamia* spp., *Perkinsus marinus*). The assay was also tested using gametes from 12 oysters collected in 2010 (6 each from Tampa Bay, Florida, and Barataria Bay, Louisiana) to further evaluate the potential for false positives. Oysters in these areas are presumed to be free of *H. nelsoni* as determined using histology, end-point PCR, or both (Kim & Powell 2007, Ford et al. 2011). All 12 samples were evaluated in North Carolina. In Rhode Island, 6 Barataria Bay samples and 1 Tampa Bay sample were tested.

Assay calibration/validation

Haplosporidium nelsoni has not been successfully propagated *in vitro*, preventing the calibration of the qPCR assay against absolute parasite numbers. We therefore applied 3 approaches for evaluating infection in order to calibrate the assay: cloned gene target, a categorical rating of infection based on histological examination, and quantification of parasite densities from histological examination.

Cloned SSU rRNA target

Primers were designed using Primer3 (Rozen & Skaletsky 2000) to amplify a 237 bp fragment (1391 F 5'-GTG ATG CAG CAG ATG GAA GA-3'; 1627 R 5'-GTA CAA AGG GCA GGG ACG TA-3') of the *Haplosporidium nelsoni* SSU rRNA sequence (Fong et al. 1993) containing the target region. PCR (94°C for 3 min, 24 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 2 min, followed by 72°C for 5 min) was conducted using 2 µl of template (~0.5 µg ml⁻¹ DNA from North Carolina oysters heavily infected with *H. nelsoni*). The product was captured in pGEM[®]-T Easy vector (Promega) and cloned in EC100 Transformax Electrocompetent cells (Epicentre) for blue-white colony selection on Amp-100 (100 µg ml⁻¹ Ampicillin) Luria-Bertani agar plates according to the pGEM-T Easy protocol. White colonies were screened for inserts using the above primers, cells were grown overnight in Amp-100 LB (Luria-Bertani broth), and plasmid was isolated using Qiagen Mini-Prep reagents and protocol. The cloned SSU rRNA target was serially diluted, and duplicate reactions run for cloned target alone and cloned target spiked into 200 ng (routine assay concentration) of oyster DNA from an uninfected oyster. qPCR was performed as described below. A cloned SSU rRNA target was also prepared in Rhode Island using a local infected sam-

ple and similar conditions, with the exception that the PCR fragment was cloned using the TOPO TA cloning kit (Invitrogen) following the manufacturer's instructions. Concentration of the plasmid including the cloned target was determined using a Nanodrop 8000 (Thermo Scientific), and copy numbers were determined using the size of the plasmid.

Histological quantification of parasite densities

Susceptible oysters exposed to *Haplosporidium nelsoni* in enzootic waters of New Jersey and selected oysters collected by the Rhode Island disease monitoring program were selected for comparison of histological and qPCR assays. The New Jersey oysters were shucked by breaking the hinge attachment with an oyster knife, then severing the adductor muscle from the valves. The tissues were rinsed in tap water to remove bits of sand and shell. A 5 mm section across the visceral mass and gill was removed, placed posterior side down in a cassette, and fixed in Davidson's fixative. A second section of gill immediately posterior to the histological section, approximately 2 mm wide and including all 4 demibranchs, was removed and fixed in 95% ethanol (EtOH) for qPCR analysis. A second piece of tissue, from the visceral mass and also immediately posterior to the histology section, was preserved separately in 95% EtOH. Shucking knives were rinsed in tap water and then in bleach solution between oysters, and dissecting instruments were dipped in 70% EtOH and then flamed between tissues. The histological sections were embedded in paraffin, processed into tissue slides using standard methods, and stained with hematoxylin and eosin. The orientation of embedded tissue was such that the histology and qPCR tissues were as close as possible to each other. Tissues fixed for qPCR were stored at -20°C until shipped overnight on ice from the Haskin Shellfish Research Laboratory to the University of North Carolina Wilmington. Rhode Island oysters were processed similarly, with the exception that tissues for qPCR were collected prior to collection of the histological sections without considering proximity to the histological section. Samples of gills, visceral mass, and mantle from each oyster were stored in the same tube containing 95% EtOH.

Stained sections were screened for the presence of *Haplosporidium nelsoni*, and the infections were scored from 0 to 6 based on categories established by Ford & Haskin (1982) (Table 1). Forty-five oysters from New Jersey were selected to encompass the

span of infection intensities observed, which ranged from rare (a single plasmodium detected) to very heavy. All 45 sections contained plasmodia (the multinucleate stage of *H. nelsoni* most often found in oysters) in the gills; 12 also had infections in the visceral mass. These 57 samples were further analyzed by both histology and qPCR. An additional 15 samples in which no infections were detected histologically were also analyzed by qPCR.

Estimation of parasite density was performed as follows. For each section, plasmodia were counted using a gridded ocular placed randomly over the tissues (at least 5 over each demibranch for gill infections). In most samples, tissues were viewed with a 25× objective, and at least 20 (40 × 40 μm) grids were counted in the gill and 20 in the visceral mass. The number of grids counted increased when tissues were lightly infected to increase the chances of encountering plasmodia. Some very heavily infected tissues were examined with a 100× objective and 60 (10 × 10 μm) grids were counted. A correction was made to account for sections in the gill where a portion of the grid fell over a non-tissue space by counting the number of intersections within the grid that fell over tissue and standardizing the plasmodial count to the total possible 16 intersections. Standardized plasmodial counts were averaged and multiplied by the area of the grid and the thickness of the section (6 μm) to calculate a mean parasite per μm³ of fixed tissue. This value was then divided by 2 to correct for the fact that *Haplosporidium nelsoni* plasmodia are generally greater than 6 μm in diameter and would be expected to fall into 2 consecutive sections. The final numbers were then multiplied by 0.9

Table 1. *Haplosporidium nelsoni*. Descriptive evaluations of categories for infections assessed by tissue-section histology. N: none, no parasites found in the section; R: rare, 10 parasites in entire section; VL: very light, 11 to 100 parasites in the entire section; L: light, >100 parasites per section, but averaging fewer than 1 per 1000× field; M: moderate, averaging 1 to 5 parasites per field; H: heavy, averaging >5 parasites per field

Category	Distribution	Intensity rating
0	–	N
1	Epithelial	R
2	Epithelial	VL, L
3	Subepithelial/local	L, M ^a
4	Systemic	L
5	Systemic	M
6	Systemic	H

^aCan be locally moderate, but light overall

to correct for an estimated 10% volume shrinkage upon fixation. The result, converted to cm³ was considered an estimate of parasites g⁻¹ wet weight (wet wt), as the specific gravity of oyster tissue is approximately 1 g cm⁻³ (Andrews 1961). Three gill sections contained only 1 or 2 detected plasmodia and were assigned a parasite density of 10⁻⁴ g⁻¹ wet wt based on previous calculations suggesting that value as the minimum density that can be reliably detected by tissue section histology (Ford et al. 1999). Samples used in Rhode Island for the calibration of the assay were selected from an archive of 29 pools of gill, mantle, and digestive tissue or DNA samples from oysters collected in 2007 and 2008 from 7 subtidal locations and with known levels of *H. nelsoni* as determined by histology (Farley 1968) and end-point PCR (Stokes et al. 1995).

DNA extractions

Tissue samples analyzed in North Carolina were extracted using a PureGene^R Extraction kit (Qiagen) as described by Gauthier et al. (2006). DNA concentration (μg μl⁻¹ = absorbance at 260 nm [A_{269nm}] × 0.05 × dilution factor) was determined on a GenesysTM8 spectrophotometer, and all samples were adjusted to 100 ng μl⁻¹ prior to qPCR analysis. The 260:280 nm absorbance ratio was used to determine the purity of DNA and was between 1.8 and 2.0 for all samples assayed. Extracts tested as part of the assay application were also used in genetic analysis (16s mitochondrial gene sequencing and microsatellites) validating the effectiveness of the extraction process in yielding amplifiable DNA. DNA from the tissue samples from Rhode Island oysters was isolated using the Chelex protocol as previously described (DeFaveri et al. 2009), and subsequent analysis implementing an internal control has produced no indication of qPCR inhibition in samples extracted in this manner.

qPCR assay conditions and application

qPCR was performed using 2 different platforms: the ABI 7500 Real-Time PCR System (Applied Biosystems) in North Carolina and the Roche Light-Cycler[®] 480 real-time qPCR (Roche Applied Science) in Rhode Island. Common reagents for both platforms included the primers and the probe (see above, Applied Biosystems). For the ABI platform (North Carolina samples), reagents were added in

the following proportions to each well of 96-well optical reaction plates (ABI 4306737): 12.5 μl MasterMix (ABI 4324018), 1.25 μl primer+probe mixture (ABI 4331348: reaction concentrations were 900 nM for each primer and 250 nM for the probe), 9.25 μl double-distilled water (ddH₂O), and 2 μl of template (100 ng μl^{-1}) for a 25 μl reaction volume. Plates were sealed with MicroAmp[®] optical strip caps (ABI 4323032), and reactions were run on an ABI 7500 Real-Time PCR System using the ABI 7500 System SDS Software with the default temperature program (95°C for 10 min; 40 cycles of 95°C for 15 s followed by 60°C for 1 min) for a total run time of 1.5 h. Positive and negative controls were included in every run. Positive controls were 3 oysters that were previously determined to show a range of infection intensities and were included to assess the consistency of assay performance across runs. The negative controls were spiked with sterile water instead of DNA.

Each reaction for the Roche platform (Rhode Island samples) included 10 μl of LightCycler[®] 480 Probes Master mix (Roche), 1.2 μl of each primer (IDT DNA, 600 nM final concentration), 0.5 μl probe (final concentration of 250 nM), 5.1 μl ddH₂O, and 2 μl of DNA template for a 20 μl reaction volume. Reactions were run on a Roche LightCycler[®] 480 real-time qPCR system (95°C for 5 min; 40 cycles of 95°C for 15 s followed by 60°C for 1 min). The controls in the Rhode Island runs included 2 oysters previously determined by histology to be positive, and the negative control was sterile water. A standard curve of dilutions from cloned DNA was also included in each Rhode Island qPCR run.

Results of the amplifications are reported as a quantification cycle (C_q ; Bustin et al. 2009), which is representative of the number of cycles required for target amplification to reach the exponential phase and thus is inversely related to the initial concentration of the target in the sample.

Oysters were collected from subtidal reefs from 4 locations ($n = 15$ each site, 60 total) in Pamlico Sound in July 2006 and from 3 intertidal locations ($n = 90$ each site, 270 total) in southeastern North Carolina in December 2006 (Fig. 1). Oysters were measured (shell height) and shucked, and a small (~15 mg) piece of mantle was dissected and frozen for later DNA extraction following the protocols described above. In Rhode Island, a total of 107 samples collected at 2 farm locations (Portsmouth and Rome Point) in 2009 were tested by qPCR. Of those, 38 samples were also tested using end-point PCR (Stokes et al. 1995).

RESULTS

Specificity of the assay

Primer and probe sequences were aligned with SSU rRNA gene sequences from 22 taxa (GenBank accession numbers: *Haplosporidium costale*, U20858; *H. edule*, DQ458793; *H. montforti*, DQ219484; *H. lusitanium*, AY449713; *H. louisiana*, U47851; unknown *Haplosporidium* spp., AY781176, AY435093, AY449712, AF492442, DQ653412, AY449715, DQ444238; *Minchinia chitonis*, AY449711; *Minchinia* sp., EF165631; *M. teredinis*, U20319; *M. tapetis*, AY449710; *Urosporidium crescens*, U47852; *Urosporidium* sp., AY449714; *Bonamia ostrea*, AF262995; *Bonamia* sp., AY542903; *B. perspora*, DQ356000; and *B. exitiosa*, DQ312295). The average number of mismatches between the assay sequences and the non-target taxa was 17, with the differences, in general, being distributed across the primers and the probe. The fewest mismatches (7) were seen between the designed assay and an undescribed halosporidian isolated from a polychaete (Siddall & Aguado 2006), but the presence of 4 differences in the probe sequence is likely to result in no false positives attributable to co-occurrence of this parasite. Empirically, the assay as performed in North Carolina produced no signal in oyster tissues infected with *Perkinsus marinus* or *Bonamia* spp. In Rhode Island, at least 3 samples with moderate *P. marinus* infections were included among samples tested using the *H. nelsoni* qPCR and were negative. One oyster sample (provided by the OIE reference laboratory at the Virginia Institute of Marine Science) and 2 from Rhode Island that were positive for *H. costale*, as determined by histology and end-point PCR (Stokes & Bureson 2001), were negative by qPCR.

The Gulf of Mexico samples were treated as biologically relevant negative controls, since these samples are from an area where *Haplosporidium nelsoni* has not been found. A recent report of the PCR detection of *H. nelsoni* in oysters from the Gulf coast (Ulrich et al. 2007b) was not supported by histological analysis, nor did a subsequent survey using both PCR and histology (Ford et al. 2011) verify the 2007 report. Further, the Gulf samples used in the present study consisted of gametes that were removed by pipette from underneath the mantle, minimizing the possibility of detecting parasites that might be adhering to external surfaces. When tested for *H. nelsoni* using qPCR, 4 of 12 samples tested in North Carolina exhibited C_q values between 38.89 and 39.93 (mean \pm SD: 39.6 \pm

0.45), and the remaining 8 did not amplify. In Rhode Island, all of the Gulf samples showed C_q values ranging between 30.18 and 34.78 (31.8 ± 1.54). Therefore, a cut-off C_q value of 38 (for the ABI platform) and 30 (for the Roche platform) was used to designate positive samples.

Assay validation/calibration

Cloned SSU rRNA target

Cloned target was serially diluted and assayed on the ABI platform alone and in the presence of 200 ng of oyster DNA from samples determined to be negative for *Haplosporidium nelsoni* by histology and qPCR. Duplicate reactions of each treatment (alone, Fig. 2; and in the presence of oyster DNA, data not shown) were assayed and the resulting C_q values regressed against log template copy number. The regressions did not differ significantly between duplicates or treatments (analysis of covariance, $p > 0.05$), and indicate that the assay was equally efficient in detecting target with or without the presence of non-target DNA. The assay was sensitive to as few as 20 copies, and highly linear over 10 orders of magnitude ($r^2 = 0.991$). Using the Roche platform and samples from Rhode Island, the assay was linear ($r^2 = 0.995$) between 5×10^3 and 5×10^8 copies (Fig. 2).

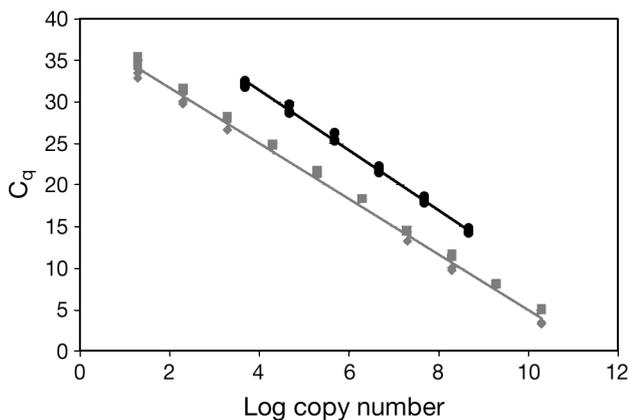


Fig. 2. *Haplosporidium nelsoni*. Regression lines for DNA copy number vs. quantification cycle (C_q). Lower line is the regression (pooled data, $y = -3.34x + 38.31$, $r^2 = 0.991$) of C_q against log template copy number for 2 replicates of 2 dilution series (\blacklozenge , \blacksquare) from 2 plasmid preparations assayed using the protocols developed in North Carolina. The upper line is the regression (pooled data, $y = -3.36x + 45.71$, $r^2 = 0.999$) for the 5 dilution series (\bullet) assayed using the protocols developed in Rhode Island

Histological quantification of infected oysters

qPCR results for the New Jersey oysters using the ABI platform were compared to 2 estimates of infection intensity. The first involved a categorical system in which infections were scored based on a combination of parasite abundance and infection location (Table 1). The correlation with C_q values derived from the qPCR assay was modest, though significant ($r^2 = 0.696$, $p = < 0.0001$; Fig. 3). The correlation using the parasite density as estimated from histological counts was higher and also significant ($r^2 = 0.797$, $p < 0.0001$; Fig. 4). The C_q values for the highest esti-

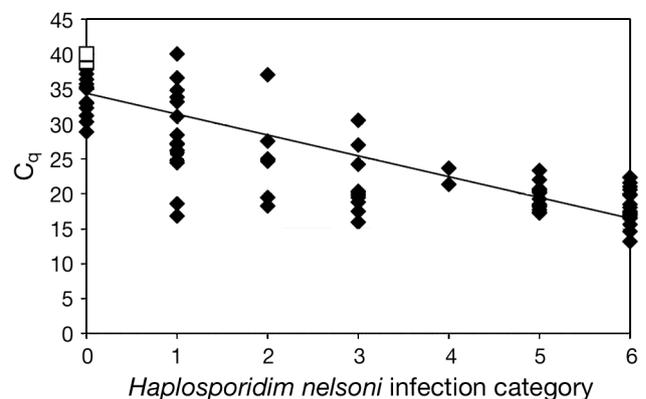


Fig. 3. *Crassostrea virginica* infected with *Haplosporidium nelsoni*. Regression of quantification cycle (C_q) values against categorical ranking of infections (see Tables 1 & 2) based on histological examination of infected Delaware Bay oysters ($y = -2.99x + 34.04$, $r^2 = 0.696$). Open squares represent samples of Gulf of Mexico oysters used as negative controls. All quantitative PCR (qPCR) results shown are from the ABI platform

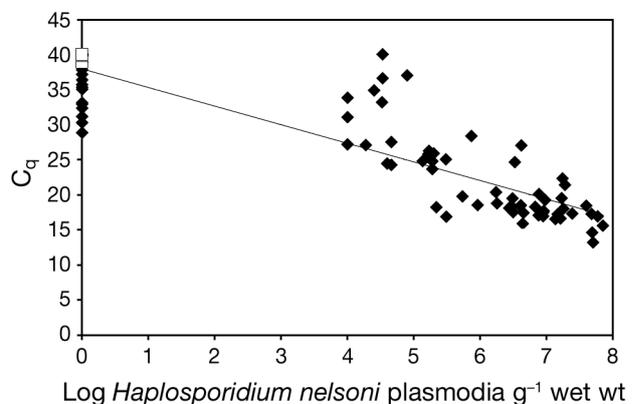


Fig. 4. *Crassostrea virginica* infected with *Haplosporidium nelsoni*. Regression of quantification cycle (C_q) values against cell counts of *H. nelsoni* determined by histological examination of infected Delaware Bay oysters ($y = -2.65x + 37.98$, $r^2 = 0.797$). Open squares represent samples of Gulf of Mexico oysters used as negative controls. All quantitative PCR (qPCR) results shown are from the ABI platform

mated densities (Category 6, density from 7.6×10^6 to 7.1×10^7 plasmodia g^{-1} wet wt, Table 2) ranged from 13.2 to 22.2. The C_q values for the lowest densities (Category 1, density from 10^4 to 9.2×10^5 plasmodia g^{-1} wet wt) ranged from 18.5 to 36.6 with an additional sample having no detectable signal ($C_q > 40$). Of the 15 oysters deemed histologically negative (no detection of plasmodia), only 3 yielded no detectable signal. The remaining 12 had C_q values ranging from 28.8 to 37.8. All but 1 had a C_q value >30 , and most exhibited values >35 .

In Rhode Island, the 29 oysters with histologically quantified levels of *Haplosporidium nelsoni* were evaluated by qPCR using the Roche platform, implementing a threshold of $C_q \leq 30$ as necessary for identifying positives. Of those 29 oysters, 24 tested either all negative (9 oysters) or all positive (15 oysters) by the 3 techniques (83% concordance; Table 3). Quantitative PCR was negative for 4 oysters that were positive by both end-point PCR and histology. All 4 of these oysters had light infections restricted to the gills as detected by histology, suggesting that the relatively small sample size used for the qPCR assay missed these light, localized infections. Neither qPCR nor end-point PCR detected the presence of parasites in any oyster that was considered negative

Table 2. *Crassostrea virginica* infected with *Haplosporidium nelsoni*. Comparison of categorical ranking of infection (see Table 1) with estimated parasite densities (g^{-1} wet wt) in infected Delaware Bay oysters

Category	No. of tissues examined	Estimated mean density	Estimated density range
1	16	6.5×10^4	$10^4 - 9.2 \times 10^5$
2	7	3.3×10^5	$4.5 \times 10^4 - 3.3 \times 10^6$
3	8	1.7×10^6	$4.6 \times 10^4 - 1.7 \times 10^7$
4	2	1.9×10^6	$1.9 \times 10^5 - 1.9 \times 10^7$
5	11	6.5×10^6	$2.7 \times 10^6 - 2.4 \times 10^7$
6	13	2.4×10^7	$7.6 \times 10^6 - 7.1 \times 10^7$

Table 3. *Crassostrea virginica* infected with *Haplosporidium nelsoni*. Comparison of histology, end-point PCR, and quantitative PCR (qPCR) in the detection of *H. nelsoni* in 29 selected Rhode Island oysters collected in 2007 and 2008. Oysters enumerated were positive (+) or negative (-) by each assay as indicated

	Concordance of assay outcomes						
	-	+	+	+	-	-	-
Histology	-	+	+	+	-	-	-
PCR	-	-	+	+	+	+	-
qPCR	-	-	-	+	+	-	+
No. of oysters	9	1	4	15	0	0	0
Percentage	31	3	14	52	0	0	0

by histology. There was a relatively good correspondence between the intensity of *H. nelsoni* as detected by histology and the numbers of *H. nelsoni* copies as detected by qPCR for the 15 oysters that were positive by both methods. Five of 15 positive oysters were classified as light infections by histology and yielded C_q values between 27 and 30 (suggestive of copy numbers between 10^4 and 10^5). Eleven were classified as moderate or heavy by histology, with 9 estimated to have copy numbers between 10^5 and 10^8 (C_q between 10 and 27) and 2 estimated to have copy number $>10^{10}$ (C_q around 8).

Assay application

In North Carolina, *Haplosporidium nelsoni* DNA was detected by the qPCR assay in all samples (ABI platform, positive threshold of $C_q < 38$). Prevalence ranged from 13% in Legged Lump to 100% in Crab Hole and in the smallest size class of oysters from the White Oak River (Table 4, Fig. 1). The vast majority of the estimated infection intensities in summer samples from Pamlico Sound were low (<2000 parasites g^{-1} wet wt). Only a single oyster from West Bluff was estimated to harbor a heavy parasite load ($>10^6$ parasites g^{-1} wet wt). Similarly, most oysters sampled from southeastern North Carolina in December exhibited low-intensity infections (<2000 parasites g^{-1} wet wt). The exception occurred in the large size class (>50 mm shell height) collected in Hewletts Creek, where 1 oyster was estimated to harbor $>10^9$ parasites g^{-1} wet wt.

The qPCR assay (Roche platform, positive threshold $C_q \leq 30$) was used to evaluate *Haplosporidium nelsoni* prevalence in 107 oysters collected in 2009 from 2 locations in Narragansett Bay, Rhode Island, including one location in East Narragansett Bay that had 8% prevalence in 2007 and 20% prevalence in 2008. None of these 1 yr old oysters (73 from East Narragansett Bay, 34 from West Narragansett Bay) was positive for *H. nelsoni* by qPCR ($C_q > 30$). A random subset of 38 oysters from these 107 was also tested and determined to be negative by end-point PCR.

DISCUSSION

We have developed a qPCR assay for the eastern oyster pathogen *Haplosporidium nelsoni* and compared

Table 4. *Crassostrea virginica* infected with *Haplosporidium nelsoni*. Quantitative PCR (qPCR) assay results for oysters collected in North Carolina. Quantification cycles (C_q) above 38 are identified as being undetectable by histology based on the results from the histologically examined New Jersey oysters (see 'Results'). Parasite densities (parasites g^{-1} wet wt) were estimated using the regression equation derived from the histological cell counts (see 'Materials and methods' and Fig. 4) and are reported for positive ($C_q < 38$) samples only. All June 2006 samples were from subtidal sites in Pamlico Sound. All December 2006 samples were from intertidal sites

Date	Site	n	Shell height (mm) (mean \pm SE)	No. positive	No. undetectable by histology	Estimated density (parasites g^{-1} wet wt)
Jun 2006	Buxton	15	83.94 \pm 6.52	3	3	14.3–1160.0
	Crab Hole	15	62.16 \pm 4.85	13	13	5.4–1333.6
	Legged Lump	15	108.61 \pm 6.22	2	2	62.9–134.4
	West Bluff	15	54.14 \pm 5.92	11	9	51.7–1769927.2
Dec 2006	White Oak	30	26.18 \pm 0.55	28	28	17.7–807.1
		30	42.47 \pm 0.97	10	10	16.6–349.5
		30	65.52 \pm 1.73	6	6	18.5–76.4
	New River	30	25.92 \pm 0.46	4	4	23.2–758.0
		30	41.28 \pm 0.93	5	5	13.3–57.0
		30	65.52 \pm 1.73	1	1	19.2
	Hewletts Creek	30	23.79 \pm 0.63	8	8	16.4–45.3
		30	40.44 \pm 0.91	12	12	13.1–223.7
		30	60.10 \pm 1.06	28	13	16.6–149420730.7

results against several measures of parasite abundance. As expected, cloned *H. nelsoni* SSU rRNA produced the typically high regression coefficient ($r^2 = 0.99$), and the assay was able to amplify target concentrations of less than 1 fg even in the presence of 200 ng of oyster DNA. The assay demonstrated specificity in that it did not detect other oyster parasites (*Perkinsus marinus*, *Bonamia* sp., and *H. costale*) known to occur on the US east coast. While the precision of the assay was not directly addressed as part of this study, subsequent investigation has shown that the inter-assay variability (same samples run multiple times) is generally 1 cycle or less. Similarly, separate extractions made from pieces of the same tissue clip differ, on average, by less than 1 cycle.

Of more practical importance for this parasite, which has not been grown in culture, are the results of calibration against both categorical ratings and quantitative estimates of parasite abundance in tissue sections. The r^2 values of 0.636 and 0.797 for C_q values versus categorical and density measures, respectively, are modest compared to that found for cloned *Haplosporidium nelsoni* target region or the 0.826 to 0.99 for calibration curves from cultured *Perkinsus marinus* (Yarnall et al. 2000, Audemard et al. 2004, Gauthier et al. 2006, De Faveri et al. 2009) and quahog parasite unknown (QPX; Lyons et al. 2006, Liu et al. 2009). Nevertheless, they are significant considering the challenges of accurate parasite quantification in histological samples and the uneven distribution of *H. nelsoni* plasmodia in the tissues, especially in early infections when parasites are

localized in the gill or sparsely scattered in the visceral mass (Ford & Haskin 1982). Gauthier et al. (2006) and De Faveri et al. (2009) also found higher values ($r^2 = 0.897$ to 0.948) comparing qPCR to Ray's fluid thioglycollate medium (RFTM) technique (Ray 1966) and the semi-quantitative rating system (Mackin 1962) for *P. marinus*-infected tissue smears. Nevertheless, the *H. nelsoni* relationships demonstrate the value of qPCR in providing a useful index to both categorical infection severity and parasite abundance in oyster tissue. Further, extrapolating the regression against parasite density provides an estimate of *H. nelsoni* numbers below (North Carolina) or similar (Rhode Island) to the threshold of reliable detection by histology (calculated to be $\sim 10^4$ g^{-1} wet wt by Ford et al. 1999).

Two other published studies have compared qPCR data with results from histological sections of oysters, both for parasites in the genus *Bonamia* (Corbeil et al. 2006, Marty et al. 2006). They compared C_q values with categorical rating systems: essentially none, light, moderate, and heavy. The C_q values for samples with no parasites detected in histological section could be differentiated statistically from those with light or moderate infections, and light or moderate infections could be distinguished from heavy infections, but within the intermediate categories of light or moderate (Corbeil et al. 2006), and minimal to mild (Marty et al. 2006) C_q values were statistically indistinguishable. As in these studies, we also found considerable overlap of C_q values for the *Haplosporidium nelsoni* infection categories, suggesting that

even in systemic infections, parasites may not be evenly distributed and/or that estimates of infection intensity are difficult to determine by visual examination of histological sections. Further, the categorical rating system for *H. nelsoni* involves infection location as well as parasite abundance. Thus, the move from Categories ≤ 3 to Categories ≥ 4 represents an important change in the severity of infection: the dispersion of parasites from the epithelium (local) into the circulation (systemic) and not always an increase in density. The increasing scatter of C_q values with decreasing parasite density (Figs. 3 & 4) is likely due to the difficulty of obtaining reliable estimates of rare or very light infections from histological sections in which parasites are patchily distributed as well as the probability that the small tissue samples analyzed by qPCR will contain yet a different number of parasites.

Our results extend the previously documented distribution limits in North Carolina (Morrison et al. 1992) into southeastern coastal waters of the state. The presence of *Haplosporidium nelsoni* along the southeastern North Carolina coast is not surprising in that the parasite has been noted in histological/cytological assays of oysters farther to the south (Lewis et al. 1992, Dougherty et al. 1993, Bobo et al. 1996). Bobo et al. (1996) reported the presence of *H. nelsoni* at 10 of 21 stations in South Carolina with prevalence as high as 42%. Lewis et al. (1992) reported *H. nelsoni* at 4 stations in Georgia with prevalence of 2 to 6%. Using qPCR methodology, we found evidence of *H. nelsoni* at all 7 sites sampled, with an average prevalence of 54% (range 13–100%). Estimated intensities, however, were relatively low with oysters at only 2 sites having infections heavy enough to be histologically detectable (i.e. $>10^4$ parasites g^{-1} wet wt): 17% (15/90) at Hewletts Creek and 13% (2/15) at West Bluff. Whether the prevalence and evidence of some proliferation of the parasite in North Carolina is indicative of an emerging disease problem is unknown, as the trigger for epizootics is also not known.

The widespread occurrence of *Haplosporidium nelsoni* and the high prevalence in some North Carolina sites, as determined by qPCR, suggests that it may not be a scarcity of parasites that prevents the development of epizootics. Unfavorable environmental circumstances, like low salinity (Andrews 1983), have been suggested (NCDMF 2008) and may indeed be contributing to the low infection estimates in samples from the Pamlico, where salinities, particularly in the northern reaches, are often low. Low salinity, however, is less likely to explain the lack of

development at high salinity sites in southeastern North Carolina. It is interesting to note the difference in estimated intensity between the high salinity sites in the White Oak River and in Hewletts Creek. C_q values for oysters from the White Oak River were never below the values associated with rare and light infections for oysters from Delaware Bay, but in Hewletts Creek, 12% (15/90) exhibited C_q values consistent with the heavily infected category (Fig. 3). While all of the oysters testing positive by qPCR were not examined by histology, 2 of the heavier infection estimates from Hewletts Creek (HC305 $C_q = 16.8$, HC312 $C_q = 17.1$) were histologically examined and infection with *H. nelsoni* was confirmed (R. Carnegie pers. comm.). Whether these higher estimated intensities caused mortalities to occur in Hewletts Creek in 2006/2007 is unknown.

The application of the assay was less informative in Rhode Island, where *Haplosporidium nelsoni* was not detected in the 2009 field samples. Although the assay was less sensitive as it was applied in Rhode Island (the C_q differed by 8 for the same cloned copy concentrations, and the standard curve flattens out between C_q s of 30 and 35), the results from the qPCR agreed with those obtained by endpoint PCR. The difference in sensitivity between the ABI (North Carolina) and Roche (Rhode Island) platforms may be attributable to the fact that reaction conditions were not thoroughly optimized for the Roche platform, since the assay developed in North Carolina for the ABI platform was used in Rhode Island with few modifications.

These results indicate that qPCR is a reliable and efficient alternative to traditional histological approaches for the detection of *Haplosporidium nelsoni* in oysters and for estimating infection intensity. The greater sensitivity usually achieved with qPCR provides the opportunity to investigate the early stages of infection before they are reliably detected by histology, and the efficiency of the assays provides results in a much quicker time frame than histology, allowing for high-throughput processing of samples. As with all PCR-based detection assays, the potential for false positives (either through contamination of the sample or the presence of non-infective parasite or parasite DNA) and false negatives (missed target) prevents the unequivocal diagnosis of a particular sample when infections are less than can be confirmed by other methods. However, such limitations plague all PCR approaches and to some extent even histological approaches (absence of parasites can only be confirmed for the small amount of tissue inspected). The assay described here has the poten-

tial to greatly expand our understanding of this important parasite and provides a powerful management tool for simultaneously monitoring prevalence and intensity in natural populations.

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