

DEVELOPMENT AND VALIDATION OF A REAL-TIME QUANTITATIVE PCR ASSAY FOR THE DETECTION AND QUANTIFICATION OF *PERKINSUS MARINUS* IN THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*

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ABSTRACT *Perkinsus marinus* causes a devastating disease, known as Dermo, in the Eastern oyster *Crassostrea virginica*. Routine detection of the disease is traditionally accomplished by the use of the Ray/Makin assay, using Fluid Thioglycollate Medium (RFTM). A simple real-time quantitative PCR assay was developed as a diagnostic tool to detect and quantify *P. marinus*, to complement and serve as an alternate to the RFTM method. Using a dual-labeled probe approach, a sensitive assay was designed to accurately detect a range of one to several thousand *P. marinus* organisms present in oyster tissues. A simple extraction method was used to increase throughput of the assay. Cultured *P. marinus* cells were quantified prior to DNA extraction, generating a standard curve and allowing cell counts to be derived from PCR cycle threshold values. Direct comparison of the RFTM and real-time PCR methods was accomplished by using tissue samples from the same oyster for both tests. Plotting cycle threshold values against the known Mackin index value generated a standard curve with a coefficient of regression of 0.9. Our results indicate that correlations could be made between this molecular based approach and traditional methods, allowing results generated from the PCR assay to be easily translated into the understood Mackin scale.

KEY WORDS: Dermo, *Perkinsus marinus*, real-time PCR, RFTM, eastern oyster, *Crassostrea virginica*

INTRODUCTION

Over the past 50 y, populations of the Eastern oyster *Crassostrea virginica* (Gmelin 1791) have been experiencing dramatic declines along the Atlantic coast of the United States (Andrews 1988, Rothschild et al. 1994, Gauthier et al. 2006). Among the various diseases that have plagued oyster populations, the protozoan endoparasite *Perkinsus marinus* (Mackin 1951) has remained a top offender as the causative agent of Dermo disease. It has been responsible for mass mortalities experienced by the Eastern oyster (Burreson & Ragone-Calvo 1996, Ford 1996, Ray 1996), having devastating effects on the commercial shellfish industry as well as the environment (Andrews 1988, Villabla et al. 2004). The continuing decline in oyster populations has led to an increased need for population restoration and management strategies. Among these strategies is the development and improvement of sensitive, accurate diagnostic tests for the detection of *P. marinus* in the environment and for the evaluation of infection intensity in oyster populations.

The life cycle of *P. marinus* occurs exclusively in hemocytes of the eastern oyster (Chu 1996). Within these cells, the life cycle of *P. marinus* involves the development of unicellular trophonts into multicellular meronts (Chu 1996). The meronts rupture releasing individual merozoites, which develop into meronts and the cycle repeats in the tissue. Under certain conditions (fluid thioglycollate incubation), parasites enlarge to form hypnospores (Andrews 1996, Ford & Tripp 1996). The most common method for *P. marinus* detection, the Ray's Fluid Thioglycollate Medium (RFTM) assay, involves culturing oyster tissue in fluid thioglycollate medium, allowing any trophonts present to develop into hypnospores (Ray 1952). The tissue is then stained with Lugol's Iodine, which is absorbed by the thick walls of the hypnospores, giving the parasite a blue-

black appearance under a light microscope. The amount of hypnospores observed is used to estimate infection intensity. Unfortunately, this method is not specific for *Perkinsus* species and cannot discriminate between species in areas where more than one exist.

This study reports a simple DNA extraction method and the development of a dual-labeled probe based, quantitative real-time PCR assay to detect and quantify the abundance of *P. marinus* cells in oyster tissue. The effectiveness of the assay was validated by processing naturally infected oysters that expressed a range of infection intensities. The results generated from this assay were extensively compared with the RFTM assay, to correlate this molecular approach with the traditional method. For simplifying interpretations, standard curves were generated with known *P. marinus* cell densities, to allow the integration of data sets obtained from both detection methods. Furthermore, two different extraction methods were evaluated to develop an efficient technique of recovering reliable target sequences. Our results indicate that this quantitative PCR assay is a sensitive and specific alternative to the RFTM assay.

METHODS

Study Site & Sampling

Hatchery raised *Crassostrea virginica* seed originating from Edgartown Great Pond (Martha's Vineyard, MA) broodstock were deployed in Edgartown Great Pond in 2005. Persistent infection by *Perkinsus marinus* has been detected in oysters at this site for 10 y during routine surveys for oyster disease, making it a likely site for the acquisition of oysters infected with Dermo (Smolowitz, personal communication).

Oysters (size: 74.7 ± 10.3 mm, 36.5 ± 9.3 g) were collected from Edgartown Great Pond in May, July, August, and September over a two-year period (2006–2007) in conjunction

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with an ongoing disease monitoring project. Fifteen specimens from each collection date were brought back to the Marine Resources Center (MBL, Woods Hole, MA) for further processing. From each individual ($n = 120$), two sets of mantle and rectum tissues (30 mg wet weight total) were sterilely dissected. One set of tissues was stored at -20°C until DNA extraction. The other set was processed immediately using the RFTM assay following the methods of Ray (1952, 1966). Estimates of infection levels were assigned to each individual based on Mackin's scale (Ray 1954). Briefly, each oyster was assigned a ranking of 0.5 (very light; 1–20 *P. marinus* cells), 1 (light; 20–100 *P. marinus* cells), 2 (light to moderate; localized infections of 25–50 *P. marinus* cells), 3 (moderate; all fields at $\times 100$ magnification show several parasites), 4 (moderate to heavy; large numbers of *P. marinus* cells), or 5 (heavy; abundant numbers of parasites).

Cultures

P. marinus cells were obtained from Marta Gomez-Chiarri (University of Rhode Island, RI) in 2005; *P. chesapeaki* and *P. olsenii* cells were obtained from the American Type Culture Collection (ATCC 50,807 and 207) in 2008. All cultures were maintained at the Marine Resources Center as described by Gauthier and Vasta (1993). Briefly, base medium containing Dulbecco Modified Ham-F12 Media (Sigma-Aldrich, St. Louis, MO) was reconstituted with 10‰ artificial seawater (Instant Ocean, Aquarium Systems, Mentor, OH), buffered with HEPES (Fisher, Pittsburgh, PA) and sodium bicarbonate (Sigma-Aldrich, St. Louis, MO). Media was supplemented with 100 U/mL Penicillin/Streptomycin (Sigma-Aldrich) and 2% heat inactivated fetal bovine serum (Sigma-Aldrich), adjusted to pH 6.5 and 0.22 μm filtered. Three milliliters of existing cultured cells was added to 60 mL of new medium, and cultures were incubated at room temperature for 14 days.

Preparation of Standards

Cultures were washed twice in sterile filtered artificial seawater (10‰) and centrifuged at $\times 400 g$. The pelleted cells were resuspended in 1 mL sterile 10‰ seawater, and three replicate hemocytometer counts were performed on the resuspended cells to determine cell density. A series of serial dilutions was prepared over five orders of magnitude, ranging from 6,000,000–600 cells per mL, and DNA was immediately extracted.

Spiked Oyster Tissue

A second dilution series for *P. marinus* was developed by spiking uninfected oyster tissue. Uninfected oysters were collected from Tisbury Great Pond (Martha's Vineyard, MA), a monitored site that has consistently tested negative for Dermo infections during disease surveys for five years before broodstock was retrieved from the pond (Smolowitz, personal communication). Thirty milligrams (wet weight) of mantle and rectum tissues was removed from five individuals. 1 mL of serially diluted *P. marinus* cells ranging from 6,000,000–600 cells per mL was added to each sample of tissue, the mixture was briefly vortexed, and DNA was immediately extracted. This was repeated with another set of five tissues.

RFTM Incubated Versus NonIncubated Oyster Tissue

To test the sensitivity of the assay to various life stages of *P. marinus*, two sets of tissues were removed from a subset of oysters assumed to be infected with Dermo ($n = 10$). One set of tissues was immediately extracted for DNA. The other set was first cultured in 1 mL of fluid thioglycollate medium for five days. Tissues were sterilely removed from the medium after incubation and extracted with Chelex.

DNA Extraction: Chelex

Genomic DNA was extracted using a 10% Chelex (Bio-Rad, Hercules, CA) resin solution according to Barber and Erdmann (2000). Briefly, batches of Chelex solution were prepared by sterilely adding molecular biology grade Chelex 100 resin (200–400 dry mesh, 75–150 μm wet bead) to ultra pure water, keeping solution well mixed. Thirty mg (wet weight) of oyster tissue was sterilely macerated, added to 1 mL of 10% Chelex resin solution and incubated at 95°C for 45 min. For each batch of Chelex solution prepared, 1 mL of the solution was removed without addition of oyster tissue, and processed following the protocol to ensure that the solution was not contaminated. These samples were included in the real-time PCR analysis, and served as negative controls.

DNA Extraction: Qiagen DNeasy Blood and Tissue Kit

Mantle and rectum tissue samples from 20 infected oysters (also sampled for DNA extraction using Chelex) were processed using Qiagen DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) according to manufacturer's protocol. In brief, the samples were lysed at 70°C for 10 min, washed, precipitated and eluted with 200 μL of Qiagen AE buffer. Two additional serial dilutions of *P. marinus* cells, ranging from 6,000,000–600 cells per mL were extracted using both methods. DNA extracted from the tissue kit was stored at -20°C ; DNA extracted from Chelex was stored at 4°C .

P. marinus Quantitative PCR Assay

A real-time PCR protocol was developed for quantitative detection of *P. marinus* genomic DNA targeting the ITS region. A dual-labeled probe (5'-/56-FAM/CGC AAA CTC GAC TGT GTT GTG GTG/3BHQ1/-3') and primers (F: 5'- CGC CTG TGA GTA TCT CTC GA-3', R: 5'- GTT GAA GAG AAG AAT CGC GTG AT -3')(Integrated DNA Technologies, Coralville, IA) were designed to target this region using Primer Express Software (Applied Biosystems, Foster City, CA). Additional constraints were put on the software to ensure selection of sites with maximum dissimilarity with the comparable portion of the *P. olsenii* genome. PCR reaction mixtures contained 5 μL of sample DNA in Platinum Quantitative PCR SuperMix UDG (Invitrogen, Carlsbad, CA), 200 nM each primer and 40 nM probe, with a reaction volume of 25 μL . In addition, 10 random samples were selected to perform assays where combined reagents were increased to a volume of 50 μL , according to manufacturer's protocol (Invitrogen SuperMix UDG). This was done to characterize any volume effects. Reactions were conducted in an Opticon2 Continuous Fluorescence Detection System (Bio-Rad) for 35 cycles at 95°C for 30 sec, and 60°C for 45 sec, and a final extension step of 21°C

for 10 min. Two initial 2 min holding steps at 50°C and 95°C were included to activate the polymerase before cycling. All samples, negative controls, and standards were run in duplicate.

Statistical Analysis

Linear regression analysis was performed to determine the relationship between *P. marinus* cell density and cycle threshold (C_T) values generated by the quantitative PCR assay. Linear regression analysis was also performed to compare the infection intensity determined by the quantitative PCR assay with the infection values assigned by the Mackin scale. Paired *t*-tests were performed to compare C_T values of standard curves generated from (1) neat *P. marinus* cells with spiked tissue samples, (2) RFTM cultured oyster tissue samples with non-cultured oyster tissue samples, (3) Chelex with Qiagen kit extractions, and (4) 25 μ L with 50 μ L reaction volumes. For all statistics, a *P* value of <0.05 was considered significant.

RESULTS

Quantitative *P. marinus* Assay

Sensitivity and Specificity

The sensitivity of the quantitative PCR assay was assessed using a serial dilution of *P. marinus* cells ranging from 30,000–3 *P. marinus* cells per 5 μ L sample. A strong correlation ($R^2 \geq 0.99$, $n = 120$) was observed between *P. marinus* cell density and C_T values of the quantitative PCR assay, with detectable amplification of DNA from the most dilute sample (3 cells) (Fig. 1). To test the species specificity of the *P. marinus* assay, a real-time PCR was run using the *P. marinus* primer/probe set on the *P. chesapeakei* and *P. olseni* cultures which contained a range from 30,000–3 cells per reaction. The assay did not result in any amplification of *P. chesapeakei* or *P. olseni* DNA (Fig. 2). To evaluate the reaction volume, a quantitative PCR assay was run with 25 μ L and 50 μ L reaction volumes, using the same samples. There was no significant difference based on reaction volume ($n = 10$).

Spiked Oyster Tissue

To determine the effect of oyster tissue on the ability to detect *P. marinus* cells, the serial dilution series of *P. marinus* cells was used to spike uninfected oyster tissue. A correlation of $R^2 \geq 0.99$ ($n = 20$) was observed between *P. marinus* cell density

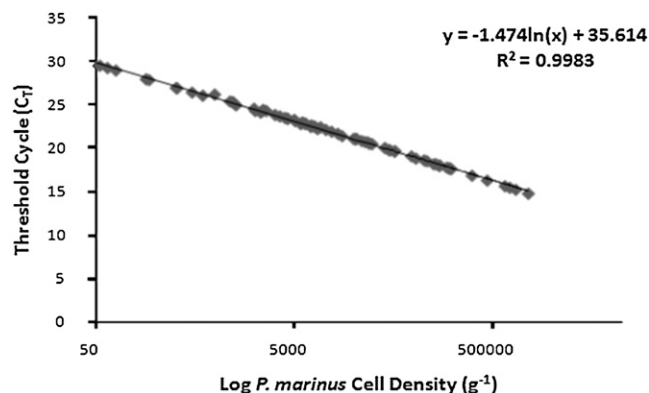


Figure 1. Standard curve representing the relationship between *P. marinus* cell density (g^{-1}) and C_T determined by real-time PCR assay.

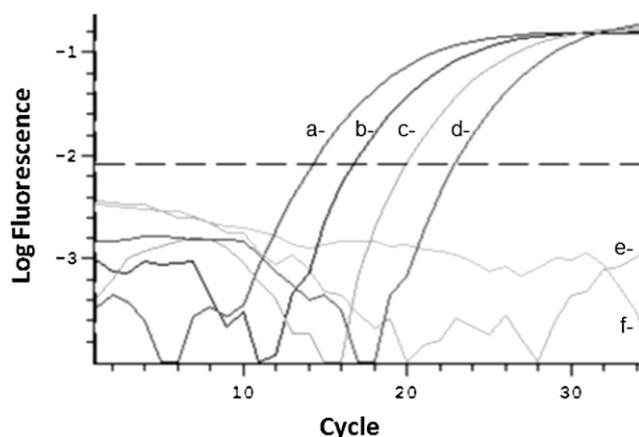


Figure 2. Amplification from a 10-fold serial dilution of *P. marinus* DNA, neat *P. chesapeakei* DNA and neat *P. olseni* DNA. a = *P. marinus* 1:10 (3,000 cells); b = *P. marinus* 1:100 (300 cells); c = *P. marinus* 1:1,000 (30 cells); d = *P. marinus* 1:10,000 (3 cells); e = *P. chesapeakei* neat (30,000 cells); f = *P. olseni* neat (30,000 cells).

in oyster tissue and C_T value. A paired *t*-test showed no significant difference between C_T values generated by the standard curves from the neat cells and spiked tissue.

RFTM Incubated versus Nonincubated Oyster Tissue

To determine the ability of the real-time PCR assay to detect DNA from various life stages of *P. marinus*, one set of oyster tissues was first incubated in fluid thioglycollate medium for five days to allow any existing trophonts to develop into hypno-spores. Another set of tissues removed from the same oysters were processed immediately, without incubation, such that DNA was extracted from any existing trophonts. The results of a paired *t*-test were only slightly significant ($P = 0.06$, $n = 10$), with C_T values from the incubated samples slightly higher.

DNA Extraction Method

The effectiveness of Chelex as an extraction method compared with the Qiagen kit was evaluated by comparing the amplification generated from both extraction methods, using serial dilutions of *P. marinus* cells, and naturally infected oysters. No differences were observed ($n = 20$) (Fig. 3).

Comparison of the Quantitative PCR and RFTM Assays for *P. marinus*

To evaluate the effectiveness of the quantitative PCR assay for detecting and quantifying *P. marinus* levels in oyster tissue, the C_T values were compared with the values assigned using the Mackin scale. A range of 14–20 oysters were used for each Mackin ranking (0.5–4); only one oyster was used for a ranking of five. The quantitative PCR yielded results that corresponded to *P. marinus* prevalence levels similar to those determined by the RFTM assay (Fig. 4). Linear regression analysis revealed a strong correlation ($R^2 \geq 0.94$) between both assays (Fig. 5). The relationship between the traditional RFTM assay and the real-time PCR assay was determined by plotting the assigned Mackin ranking against the *P. marinus* cell density, which was determined by analysis of the standard curve. A strong correlation ($R^2 \geq 0.98$) was found between infection intensity assigned by each assay (Fig. 6).

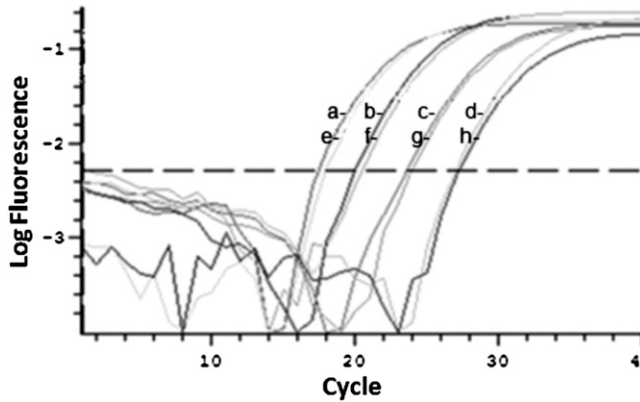


Figure 3. Amplification of a 10-fold serial dilution of *P. marinus* DNA extracted from Chelex resin and Qiagen tissue kit. a = Chelex 1:10; b = Chelex 1:100; c = Chelex 1:1000; d = Chelex 1:10000; e = Qiagen kit 1:10; f = Qiagen kit 1:100; g = Qiagen kit 1:1000; h = Qiagen kit 1:10000.

A quantitative PCR assay was run on oysters ($n = 40$) that were diagnosed as uninfected by the RFTM assay. The PCR assay detected extremely low levels of parasites (1–10 *P. marinus* cells) in 12 out of the 40 oysters, and low levels (10–50 *P. marinus* cells) in another five.

DISCUSSION

There exist several methods of diagnosing Dermo, including traditional PCR (Robledo et al. 1998, Coss et al. 2001, Pecher et al. 2008), real-time PCR (Audemard et al. 2006, Gauthier et al. 2006, Ulrich et al. 2007), and the RFTM assay (Ray 1952, 1966). Although all these methods are acceptable, the latter has remained the most widely used because of its development in the early 1950s. Whereas it remains the primary diagnostic tool, the RFTM assay has several disadvantages. This technique relies on the identification of *Perkinsus* through gross morphology and thus cannot discriminate between species (Robledo et al. 1998), because hypnospores from various species may appear the same under the microscope. Stained debris and precipitated iodine can also be mistaken for *Perkinsus* hypnospores, further

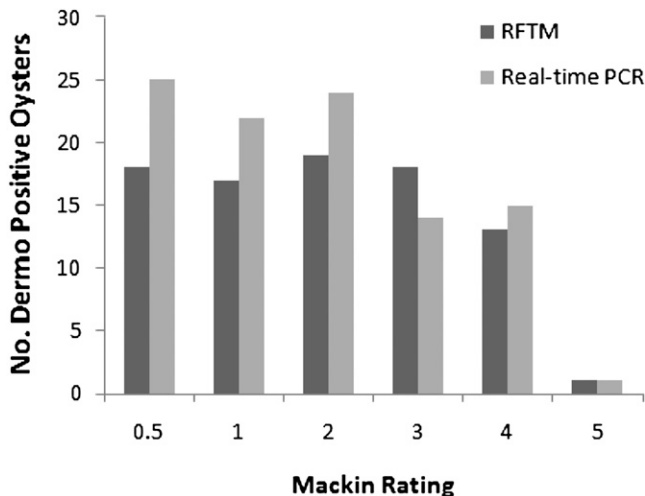


Figure 4. Comparative analysis of *P. marinus* prevalence in oyster tissue as determined by the RFTM and real-time PCR assays.

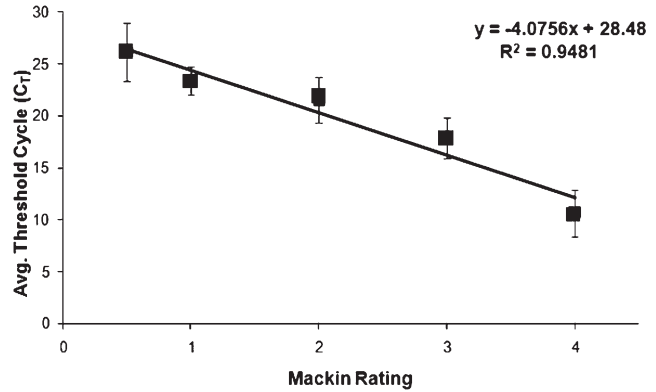


Figure 5. Relationship between *P. marinus* infection intensity determined by the Mackin rating system of the RFTM assay, and the C_T from the real-time PCR assay.

affecting the accuracy of Dermo diagnosis. Beyond this lack of specificity, the RFTM assay has limited sensitivity, with the threshold for detection requiring more than 1,000 *P. marinus* cells per gram of oyster tissue for reliable detection (Bushek et al. 1994, Ulrich et al. 2007). Samples containing fewer than this amount are more likely to be diagnosed as false negatives, reporting no occurrence of *P. marinus* where it may actually exist. Furthermore, this assay is time consuming, requiring a 5 to 7-day incubation period before the laborious task of reading out the slides.

In addition to these shortcomings, diagnosing Dermo infections using the RFTM assay is highly subjective because several scales for scoring infection exist (Ray 1954, Howard et al. 2004, Audemard et al. 2008). Because the criteria defining each intensity level vary between scales, these differences result in discrepancies of diagnoses depending on which scale is being used. Additionally, when standardizing a method of detection of a disease agent it is necessary for samples to be calibrated against accepted standards and analyzed by more than one laboratory, in an effort to synchronize results and eliminate any biases that may occur from individual interpretation (Smolowitz, personal communication, OIE 2002). This procedure is

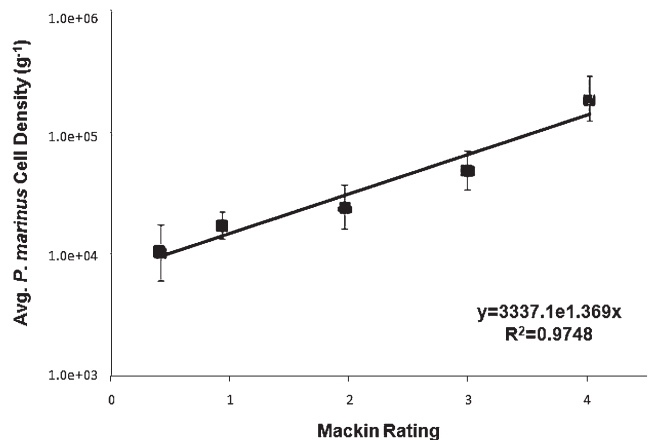


Figure 6. Relationship between *P. marinus* infection intensity determined by the Mackin rating system of the RFTM assay, and the cell density from the real-time PCR standard curve.

not practiced when assigning levels of Dermo infection in oysters, thus it is difficult to confidently coordinate diagnoses of Dermo between laboratories. The application of a real-time quantitative PCR assay provides the potential for reconciling this issue, by creating and applying a universal scale to assigning Dermo intensity in oyster tissue.

To our knowledge, this is the first study that describes a simple DNA extraction method for *P. marinus* coupled with a quantitative detection assay, which has been extensively validated alongside the conventional RFTM method.

Sensitivity and Specificity

The sensitivity of this assay was tested by creating standard curves, with and without oyster tissue, containing a range from 600–6,000,000 *P. marinus* cells per gram of oyster tissue. Detectable amplification was consistent between PCR runs, allowing cell densities to be confidently calculated from the equation generated by linear regression analysis. By plotting these values against the corresponding C_T values, a relationship between C_T values and *P. marinus* density can be established (Fig. 1). The ability to detect as few as three *P. marinus* cells in a tissue simply demonstrates that the real-time PCR assay is potentially 10 times more sensitive than the traditional RFTM assay, which cannot consistently detect less than 1,000 *P. marinus* cells in a 1 g tissue sample (Bushek et al. 1994, Ulrich et al. 2007). The extreme sensitivity of the PCR assay allows the detection and enumeration of *P. marinus* cells that would remain unaccounted for by the RFTM assay, which will ultimately reduce the occurrence of false negatives in the diagnosis of Dermo infections of oyster tissue.

When using any assay to evaluate the presence and severity of a pathogen, it is critical to confidently detect only the particular species responsible for the infection. This is particularly important in the assessment of Dermo, which is caused by *P. marinus*, because potentially more than one species of *Perkinsus* can exist in the same water and in the same oyster. Additionally, all species closely resemble one another morphologically (Robledo et al. 1998). This study addressed this issue by designing a primer/probe set to target a sequence unique to *P. marinus* in the ITS region between 5.8S and 28S ribosomal DNA regions. The specificity was validated by performing the assay on DNA extracted from *P. olseni*, the species most closely related to *P. marinus* (Roberts, personal communication), and *P. chesapeakei*, the species in closest geographic proximity. No detectable amplification of *P. olseni* or *P. chesapeakei* DNA was produced (Fig. 3).

The use of PCR assays for the detection of parasites in tissues involves the generation of standard curves, often accomplished by adding a serial dilution of known parasite numbers to uninfected tissue (“spiking”). This process increases the amount of time needed to perform a PCR assay, as it requires collecting additional tissue samples and performing a preliminary assay to verify that the sample is negative for parasite presence. This study analyzed the traditional method of “spiked” standard curve generation alongside a standard curve of parasite cells without tissue, to determine if spiking is in fact necessary. The standard curves that were produced showed insignificant variations between the two methods, demonstrating that the step of spiking oyster tissue with parasite cells can be eliminated.

The presence of PCR inhibitors has long been a recognized issue in the use of PCR assays for the detection of parasites within samples (Audemard et al. 2004, Audemard et al. 2006, Lyons et al. 2006). Organic and inorganic materials may be present in the sample, compromising the efficiency of DNA polymerases and ultimately producing a false negative. By producing standard curves that are similar with the presence or absence of oyster tissue, this study suggests that PCR inhibitors are not present in oyster tissues, and should not be a concern for this particular assay.

Within oyster tissue, *Perkinsus* can exist in several different life stages. One concern with the use of PCR based assays is the assumption that the target DNA sequences are present in all life stages (Burreson 2008), and that all life stages can be detected by the assay. To determine if this assumption is correct, sets of tissues from the same oyster were processed using the real-time PCR assay both with incubation in fluid thioglycollate medium followed by iodine stain for evaluation, and without incubation or staining. The variations within samples were slightly significant ($P = 0.06$), with the incubated samples yielding slightly lower parasite numbers. However, this can be attributed to the uneven distribution of the parasite within an individual oyster, or the binding of iodine to DNA, which makes the DNA inaccessible to PCR amplification (Marin et al. 2001). Despite the variations in parasite numbers, this assay still demonstrated the ability to accurately detect *P. marinus* DNA from both trophonts and hyphospores.

Sample Extraction

Many published studies have used quantitative PCR technology to detect infection in oysters, most of which use methods of DNA extraction including separation and purification through extraction kits (Audemard et al. 2004, Lyons et al. 2006, Gauthier et al. 2006, Ulrich et al. 2007). Whereas these kits provide high-quality DNA, they are often tedious and time consuming. The multistep protocols involve numerous transfers of products, which increases the opportunity for cross-contamination between samples, introduction of external contaminants, and pipetting errors. These multiple transfer steps and incubation periods also restrict the amount of samples that can be extracted at once. Furthermore, an overnight lysis step has been added (Audemard et al. 2004) to the procedure before following the manufacturer’s protocol, increasing the amount of time required to process each sample set. Despite the purity of the DNA produced, this method is not ideal for high throughput facilities.

This study applied an extraction method that would allow for rapid, efficient recovery of DNA without compromising its integrity. Using a Chelex resin solution, large numbers of samples were processed in a fraction of the time required for the kit. To determine the effectiveness of this extraction method for recovering the target sequence of *P. marinus* DNA, dilution series of cultured *P. marinus* cells were extracted using Chelex® resin and Qiagen Tissue Kits. The generated standard curves showed insignificant variations (Fig. 3). Sets of tissues from an additional 20 oysters were also extracted using both methods, to determine if the presence of oyster tissue affected the recovery of *P. marinus* DNA. No significant variations were observed, indicating that Chelex is a faster alternative for DNA extraction that yields DNA of a quality comparable to that of tissue kits.

Assay Validation

The development of a new assay for the detection and quantification of parasites is a sensitive issue, requiring extensive validation against the traditional methods. To directly compare the real-time PCR assay with the traditional RFTM method, tissue samples of equal size from the same oyster were processed using both tests. An obvious correlation was observed between the parasite load determined by the two diagnostic methods (Fig. 4). To demonstrate the high degree of reproducibility of the PCR assay and thus validate the test, a range of infection intensities was represented by several individual oysters. Linear regression analysis was performed on the results of the two tests (Fig. 5),

allowing the C_T values generated from the real-time PCR assay to be translated into the more familiar values associated with the traditional Mackin scale. Further, *P. marinus* cell density values were determined from C_T values and plotted against the Mackin scale (Fig. 6), allowing a precise number of cells to be associated with a Mackin value. The ability to assign each individual oyster an actual number of *P. marinus* cells provides the opportunity to fine tune the Mackin scale to include more specific categories of infection intensity. The results of this study demonstrate that the real-time PCR assay is a fast, highly reproducible alternative to the traditional RFTM method of evaluating Dermo infections in oysters.

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