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A QUANTITATIVE COMPETITIVE POLYMERASE CHAIN REACTION ASSAY FOR THE OYSTER PATHOGEN *PERKINSUS MARINUS*

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ABSTRACT: A quantitative competitive polymerase chain reaction (QCPCR) assay was developed for the oyster parasite *Perkinsus marinus.* PCR primers for the rRNA gene region of *P. marinus* amplified DNA isolated from *P. marinus* but not from *Perkinsus atlanticus, Crassostrea virginica,* or the dinoflagellates *Peridinium* sp., *Gymnodinium* sp., or *Amphidinium* sp. A mutagenic primer was used to create a competitor plasmid molecule identical to the *P. marinus* target DNA sequence except for a 13-bp deletion. Both *P. marinus* and competitor DNA amplified with equivalent efficiencies. Each of 25 oysters was processed by 5 *P. marinus* diagnostic methods—Ray's fluid thioglycollate medium (FTM) tissue assay, FTM hemolymph assay, whole oyster body burden assay, QCPCR of combined gill and mantle (gill/mantle) tissue, and QCPCR of hemolymph. The QCPCR assay enabled detection of 0.01 fg of *P. marinus* DNA in 1.0 μg of oyster tissue. QCPCR of gill/mantle tissue or hemolymph as well as the body burden assay detected infections in 24 of 25 oysters. Ray's FTM tissue assay detected only 19 infections. The FTM hemolymph assay detected only 22 infections. Regression analysis of QCPCR results and FTM results indicated that the QCPCR assays were effective in quantitating *P. marinus* infections in oyster tissues.

Perkinsus marinus has been a significant cause of mortality of the eastern oyster *Crassostrea virginica* along the east coast of the United States since the 1950s (Andrews, 1988; Burreson and Ragone Calvo, 1996). Investigators generally rely on Ray's fluid thioglycollate medium (FTM) culture of *P. marinus* cells from oyster tissue (rectum, gill, and mantle) for the diagnosis of infected oysters (Ray, 1952). An oyster hemolymph FTM assay was developed to allow monitoring of *P. marinus* infections without sacrificing the host and to improve quantitation of systemic infections (Gauthier and Fisher, 1990). Using these FTM methods, quantitation relies on accurate counting and the use of a subjective scale developed by Mackin (1962) and modified by Craig et al. (1989). Diagnosis based on FTM methods makes several assumptions. It is assumed that all life stages of *P. marinus* found in the host are retrieved and that the number of parasites remains constant during incubation in FTM. Furthermore, it is assumed that the distribution of *P. marinus* in the assayed tissues is representative of the distribution of the parasite throughout the oyster. To overcome this latter assumption, a body burden assay was developed (Bushek et al., 1994) that employs a procedure using sodium hydroxide to digest oyster tissues after incubation in FTM (Choi et al., 1989). The body burden assay is quantitative and allows enumeration of the total number of parasites in whole oyster homogenates (Bushek et al., 1994; Fisher and Oliver, 1996). FTM culture diagnostic methods are relatively simple to perform; however, insensitivity of the assays often causes very light infections to be overlooked.

To date, the polymerase chain reaction (PCR) has been used for parasite detection and semiquantitative assays for pathogen diagnosis in shellfish (Hudson and Adlard, 1994; Ko et al., 1995; Marsh et al., 1995; Stokes et al., 1995). Limitations of precise quantitation using PCR techniques stem from the lack of consistent initial exponential increase in product during the amplification reaction. Replicate samples can be subjected to variations in reaction conditions such as inhibitor concentrations, polymerase integrity, and even thermal cycler block positions. These differences can result in variation in amplification efficiencies that may obscure differences in the amounts of DNA or RNA that are being measured and thus preclude accurate quantitation (Siebert and Larrick, 1993). In competitive PCR, the competitor and target DNA are present in the same reaction tube. The target DNA and competitor DNA compete for the same DNA polymerase and deoxynucleotide triphosphates (Sur et al., 1995). In addition, the 2 DNA templates are equally affected by tube-to-tube variations in the PCR conditions including inhibitor effects (Gilliland, Perrin et al., 1990; Soong and Arnheim, 1995). Furthermore, competitive PCR overcomes the need to perform quantitation in the exponential phase because both the competitor and the target are equally affected by the changes in amplification parameters that occur as the reactions enter the plateau phase (Siebert and Larrick, 1993; Soong and Arnheim, 1995). Temperature cycling into the plateau phase, therefore, does not interfere with quantitation and even increases the sensitivity of the assay (Morrison and Gannon, 1994).

Ultimately, quantitation by competitive PCR involves a set of reactions that include a constant volume aliquot of DNA containing the target sequence and a dilution series of known concentrations of the competitor DNA. At the point where the molar amounts of the 2 products are equivalent, the amount of original target DNA present in the sample is equivalent to the amount of competitor initially added (Piatak et al., 1993). Thus, quantitation of the unknown target DNA is based on the attainment of an equivalence point at a known concentration of competitor DNA.

Internal competitive standards for quantitative PCR typically have sequences that are homologous to the target nucleic acids and amplify with the same or slightly modified primers. The target and the competitor, therefore, amplify with the same kinetics (Ferre, 1992). Altering the size of the competitor molecule relative to the target sequence has been shown to be an excellent method for competitor construction (Porcher et al., 1992; Cottrez et al., 1994; Lu et al, 1994; Repp et al, 1995; Thiery et al., 1995). This method eliminates the need to use restriction enzymes that may have variable digestion efficiencies to cut competitor molecules engineered with unique sites (Clementi et al., 1993). Using automated sequencers, a 1-bp

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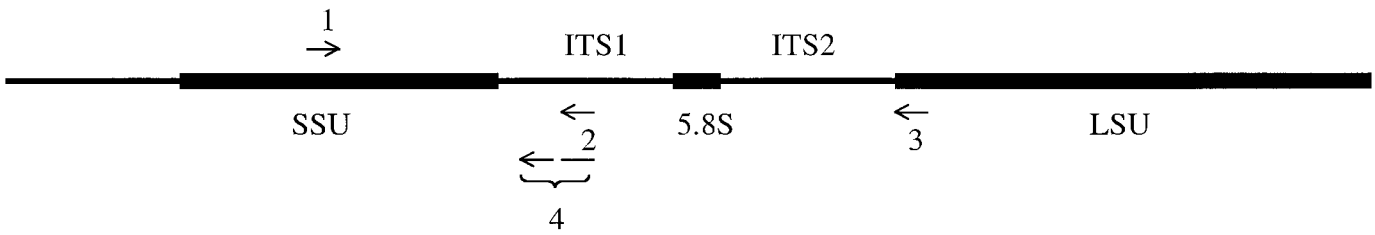

FIGURE 1. Schematic representation of the *Perkinsus marinus* rRNA locus indicating the annealing sites for the PCR primers used in the development and implementation of the QCPCR assay. Direction of primer extension is represented by the arrows. Numbered arrows indicate the primers as follows: 1 = PER-18S (5′CCTACGGGATTGGTTGGTATCAG3′), 2 = PER-ITS (5′CATCTCGCAACTCTCTAACAAAAG3′), 3 = ITS-25 (5′TATGCTTAAATTCAGCGGGT3′), and 4 = ITS-MUT (5′CATCTCGCAACTCTCTAACAAAAGagcaagagagagcGAGACCGCTG3′). Lowercase letters in the ITS-MUT sequence indicate bases present in the *P. marinus* ITS1 sequence that are missing from the primer sequence allowing for construction of the competitor molecule with a 13-bp deletion.

size difference between PCR products can be detected, allowing extremely similar competitor molecules to be utilized.

This work describes a specific and sensitive quantitative competitive PCR (QCPCR) assay for *P. marinus.* QCPCR conditions were tested and optimized with competitor and control target plasmid DNAs. A quantitative standard curve was generated using genomic DNA of unifected oysters spiked with known numbers of *P. marinus*-cultured cells. Oysters were collected from sites endemic for *P. marinus* and used for comparing the QCPCR assays of DNA from hemolymph and tissue to the more traditional FTM methods for diagnosis of *P. marinus* infections in oyster tissues.

MATERIALS AND METHODS

Perkinsus marinus-specific primers

Species-specific PCR primers, designated PER-18S and PER-ITS, were developed based on published *P. marinus* DNA sequences for the rRNA gene (Fong et al., 1993) and the adjacent internal transcribed spacer (ITS-1) region (Goggin, 1994). These primers specifically amplified a 1,210-bp fragment of DNA from within the small subunit rRNA gene to within the ITS-1 of the ribosomal DNA region. The locations of these primers within the rRNA locus and their sequences are shown in Figure 1. Specificity of these primers was tested against DNA isolated from various geographic isolates of *P. marinus, Perkinsus atlanticus* (tentative identification), and 3 dinoflagellate species as described below.

Isolation of DNA from cultured cells

Perkinsus marinus cells were maintained in culture according to the methods of Gauthier and Vasta (1993) or La Peyre et al. (1993). Cells were pelleted by centrifugation, washed twice with TE buffer (10 mM Tris pH 8.0, 1 mM EDTA), and resuspended in 0.5 ml of lysing solution (50 mM Tris-HCl pH 8.0, 100 mM EDTA, 1% sarkosyl, 0.5 mg/ml proteinase K). Samples were incubated overnight at 50 C on a rotator. After incubation, the samples were extracted with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1) 1–2 times as needed to remove cellular protein and once with chloroform–isoamyl alcohol (24:1). DNA was precipitated with 0.04 volumes of 5 M NaCl and 2 volumes of 95% EtOH, incubated overnight at −20 C. Following centrifugation, pellets were resuspended in TE buffer. Genomic DNA was digested for 2 hr at 37 C with *Xba* I. There is no recognition site for this enzyme within the 1,210-bp PCR target region.

Cultured cells tentatively identified as *P. atlanticus* were obtained from Dr. Stephen Kleinschuster (Haskin Shellfish Research Laboratory, Rutgers University, Port Norris, New Jersey) (Kleinschuster et al., 1994). DNA from various *P. marinus* isolates, including isolates from infected oysters collected in Virginia (VA-1), Connecticut (CT-1), Louisiana (LA-1), South Carolina (SC-1), and Texas (TX-1) (Bushek, 1994; Reece, Bushek, and Graves, 1997), was isolated as described above. Cultured dinoflagellates, *Amphidinium* sp., *Peridinium* sp., and *Gymnodinium* sp., were obtained from Carolina Biological Supply Company (Burlington, North Carolina). DNA was isolated from these cells using a microwave lysis preparation protocol developed by Goodwin and Lee (1993) followed by hexadecyltrimethylammonium bromide (CTAB) extractions (Ausubel et al., 1988). DNA isolation was followed by additional purification with ethidium bromide/high salt extractions (Stemmer, 1991).

PCR with PER-18S and PER-ITS

PCR reactions contained reaction buffer (Invitrogen 5× buffer C: 300 mM Tris-HCl pH 8.5, 75 mM $[NH_4]_2SO_4$, 2.5 mM $MgCl_2$) (Carlsbad, California), 12.5 pmoles of each primer, 200 μM each 2′-deoxynucleotide 5′-triphosphate (dATP, dCTP, dGTP, and dTTP), 400 ng/μl bovine serum albumin (BSA), 1 unit of Ampli*Taq* DNA polymerase (Perkin Elmer, Norwalk, Connecticut), and 1 μl DNA in a total reaction volume of 25 μl. Samples were denatured initially at 94 C for 5 min and then cycled 35 times in a DeltaCycler II thermal cycler (Ericomp, San Diego, California) at 94 C for 1 min, 59 C for 1 min, and 72 C for 3 min followed by a final extension period of 5 min at 72 C.

Construction of competitor DNA

Competitor plasmid was constructed with cloned product generated from PCR primers PER-18S and ITS-MUT. The ITS-MUT primer contained the entire sequence of the PER-ITS primer; however, the ITS-MUT primer created a gap of 13 nucleotides in the sequence to be amplified by linking to a region of sequence several bases upstream within the ITS-1 region (see Fig. 1). This modification results in the competitor having the same sequence as the PCR product generated by amplification using PER-18S and PER-ITS, except for a 13-bp deletion upstream of the 3′ primer (1,197 vs. 1,210 bp products). Amplification with the ITS-MUT and PER-18S primers was carried out as described above except that the annealing temperature was 50 C.

The PER-18S and ITS-MUT PCR product was purified with a PCR Select III spin column (5 Prime→3 Prime, Inc., Boulder, Colorado) and cloned using the TA Cloning Kit (Invitrogen, Carlsbad, California). Plasmid DNA (pITS-MUT) was isolated using a PERFECTprep™ kit (5 Prime→3 Prime, Inc.) according to the manufacturer's recommendations.

To ensure that the deletion had occurred, the pITS-MUT insert was sequenced by the Sanger dideoxy chain termination method (Sanger et al., 1977) using M13 universal forward and reverse primers labeled with IRD41 fluorescent dye (LI-COR Inc., Lincoln, Nebraska). The ThermoSequenase kit (Amersham Life Science, Piscataway, New Jersey) was used for the sequencing reactions. Reactions were visualized on a 66-cm 4% polyacrylamide gel with a LI-COR DNA 4000L automated sequencer, and the sequence was determined using the LI-COR BaseImagIR (Lincoln, Nebraska) software package. The 1,197-bp PCR product of PER-18S and ITS-MUT was homologous to the 1,210-bp PCR product from the original primer pair PER-18S and PER-ITS except for the 13-bp deletion.

The pITS-MUT DNA was quantitated using a Hoefer DyNA Quant 200 Fluorometer (Pharmacia Biotech Inc., Piscataway, New Jersey). Finally, pITS-MUT was digested with *Xba* I for 2 hr at 37 C to linearize the plasmid. Recognition sites for *Xba* I are not found within the 1,197-

bp cloned PCR product but only within the cloning vector sequence. Digested plasmid DNA was stored at 4 C until its subsequent use in all QCPCR reactions.

Construction of the control target plasmid

Control target plasmid contains the DNA sequence amplified by PER-18S and PER-ITS and was constructed with cloned product generated from PCR primers PER-18S and ITS-25 (primer D from Goggin [1994]). The locations of these primers within the rRNA region are shown in Figure 1. PCR was performed on *P. marinus* genomic DNA with the primers PER-18S and ITS-25 as described above for amplification with PER-18S and PER-ITS. The annealing temperature, however, was 55 C. The resultant PCR product was cloned into a plasmid vector via the methods described above for the pITS-MUT competitor. The plasmid DNA, pITS-WT, was isolated, quantitated, and digested with *Xba* I as described for the ITS-MUT competitor plasmid. The plasmid pITS-WT was used as the control target plasmid.

QCPCR

QCPCR was performed in a series of reaction tubes with various concentrations of competitor plasmid and constant amounts of target DNA. Both competitor and target were amplified with the PER-18S and PER-ITS primers. The primer PER-18S was labeled on its 5′ end with the fluorescent dye IRD41 (LI-COR). All DNA (genomic and plasmid) used in QCPCR reactions was digested with *Xba* I to reduce the possibility of secondary structures in the DNA interfering with the primers and polymerase accessing the DNA regions targeted for amplification. *Xba* I did not cut within the amplified region.

QCPCR was conducted in 2 phases. First-phase PCR used a broad range of competitor dilutions to determine the linear range of quantitation. The second phase used a narrow range of competitor dilutions within the linear range identified in the first phase for precise quantitation based on the methods of Gilliland, Perrin, and Bunn (1990). Constant amounts of target DNA were used for both phases. All PCR reactions were electrophoresed on a LI-COR automated sequencer and the equivalence point, the concentration at which the competitor and the target DNAs were equivalent, was determined by densitometric analysis of the amplification products' band intensities (see below).

The first phase PCR included 23 μl of master mix containing reaction buffer (300 mM Tris-HCl pH 8.5, 75 mM $[NH_4]_2SO_4$, 2.5 mM $MgCl_2$), 3.75 pmoles of each primer (labeled PER-18S and unlabeled PER-ITS), 200 μM each of dATP, dCTP, dGTP, and dTTP, 400 ng/μl BSA, and 1 unit of Ampli*Taq* DNA polymerase (Perkin Elmer). A constant volume (1 μl) of target DNA of unknown concentration was added to each of a series of reactions containing the master mix and 1 μl of a known dilution of the competitor (5 pg/μl, 500 fg/μl, 50 fg/μl, 5 fg/μl, 0.5 fg/μl, or 0.05 fg/μl). Temperature cycling was carried out as described above.

Samples were diluted in TE to a concentration within the linear range identified in the first phase for the QCPCR second phase reactions. The second phase, narrow range PCR, consisted of 23 μl of master mix containing reaction buffer (Expand™ High Fidelity buffer with 1.5 mM $MgCl_2$, Boehringer Mannheim Corporation, Indianapolis, Indiana), 3.75 pmoles of each primer (labeled PER-18S and unlabeled PER-ITS), 200 μM each of dATP, dCTP, dGTP, and dTTP, 400 ng/μl BSA and 2.6 units of the Expand™ High Fidelity DNA polymerase (cocktail of *Taq* DNA polymerase and *Pwo* DNA polymerase, Boehringer Mannheim). A constant volume (1 μl) of the diluted target DNA was added to each of a series of reactions containing the master mix and 1 μl of a known dilution of the competitor (1 fg/μl, 0.5 fg/μl, 0.1 fg/μl, 0.05 fg/μl, 0.01 fg/μl, or 0.005 fg/μl). Temperature cycling was carried out as described above.

Competitor and target DNA PCR products were separated and visualized by 4% denaturing polyacrylamide (Long Ranger gel solution, FMC BioProducts, Rockland, Maine) gel electrophoresis on 25-cm plates with a LI-COR 4000L automated sequencer. After PCR, reactions were diluted 1:3 in loading dye (95% formamide, 10 mM EDTA, 0.1% bromophenol blue, pH 9) and denatured at 92 C for 2 min prior to loading. The automated sequencer was programmed to run at 2,000 V, 25 mA, and 45 W at 45 C. The motor speed for the laser was set to 2, which corresponds to scan speed of 5.4 cm/sec. After focusing, the signal gain was changed to 25 and the signal offset was changed to 102 to minimize background signal. Data were collected by the LI-COR BaseImagIR Data Collection version 3.0 software.

Amplification efficiencies were initially determined for Ampli*Taq* DNA polymerase and the Expand™ High Fidelity PCR system individually using control target and competitor plasmids. *Perkinsus marinus* genomic DNA isolated from cultured cells was used as the target to determine the amplification efficiency of genomic DNA with the plasmid competitor. Reproducibility of the assay was assessed by replicate QCPCR for each enzyme system.

Oyster collection

Oysters were collected from Wreck Shoal (n = 14) and Point of Shoals (n = 11) in the James River, Virginia in October 1996, December 1996, or January 1997. Each oyster was analyzed for the presence of *P. marinus* infection by QCPCR of hemolymph and QCPCR of combined gill and mantle (gill/mantle) tissue as well as by Ray's FTM tissue method, FTM hemolymph assay, and whole oyster body burden FTM analysis.

FTM hemolymph diagnosis

Oyster shells were notched and hemolymph samples were taken using a 23-gauge needle and a 3-cc syringe. Approximately 0.6 ml of hemolymph was removed from each oyster and dispensed in equal volumes into 2 microcentrifuge tubes. One hemolymph aliquot was used for DNA isolation (described below) and the other was analyzed using the FTM method of Gauthier and Fisher (1990). Three replicates of 100 μl each were aspirated onto 0.22-μm filter paper, and the stained *P. marinus* cells were counted by light microscopy at 50× magnification. Samples containing large numbers of cells were diluted and counts were performed on the diluted samples. The entire sample volume was counted when less than 10 cells were present in 100-μl aliquots.

Standard FTM tissue diagnosis

Oysters were shucked and pieces of gill, mantle, and rectal tissues weighing approximately 0.25 g were dissected and incubated together in 10 ml FTM supplemented with penicillin/streptomycin (500 units/ml) for 7 days. After incubation, the tissues were removed, minced with a razor blade, and stained with Lugol's iodine. Samples were examined under a light microscope for *P. marinus* infections according to the methods of Ray (1952).

Body burden FTM diagnosis

After tissue pieces had been removed from the oyster for the standard FTM assay and for the QCPCR assay, the remaining oyster tissue was minced with a razor blade. Minced tissue was weighed and added to a tube containing 20 ml of FTM supplemented with penicillin/streptomycin (500 units/ml FTM). The whole tissue assay was performed based on the procedure of Fisher and Oliver (1996). Three replicates of 100 μl each were aspirated onto 0.22-μm filter paper. Stained *P. marinus* cells were counted using a light microscope at 50× magnification. When less than 20 cells were present in the replicates, the entire sample volume was counted. If more than 300 cells were present in each replicate, dilutions were made and counts were performed on the diluted aliquots.

Isolation of DNA from hemolymph

A 0.3-ml aliquot of hemolymph from each oyster was used for DNA isolation. Hemolymph samples were processed according to the methods of Stokes et al. (1995). Samples were further purified by the ethidium bromide/high salt extraction procedure described above. Before use in QCPCR, DNA was digested with *Xba* I as described above.

Isolation of DNA from gill/mantle samples and rectal samples

Genomic DNA was isolated from the samples of oyster gill/mantle tissue using a modification of the method by Hill et al. (1991). Tissue samples (0.25 g) were homogenized with disposable grinders in 0.25 ml of TE buffer in microcentrifuge tubes. The cells were pelleted by centrifugation, washed in TE buffer, and incubated in 25 μl of 5.9 M guanidine thiocyanate at 60 C for 90 min to lyse the cells. Lysates were diluted with dH_2O to achieve a final guanidine thiocyanate concentra-

tion of 0.3 M and extracted twice with equal volumes of chloroform–isoamyl alcohol (24:1). DNA was precipitated at −20 C overnight with 0.6 volume isopropanol and 0.1 volume 3 M NaOAc (pH 5.2). Following centrifugation, DNA was resuspended in TE and further purified by the ethidium bromide/high salt extraction procedure as described above (Stemmer, 1991). Finally, the nucleic acids were resuspended in 100 μl TE. Before use in QCPCR reactions, the DNA was digested with *Xba* I as described above.

Rectal samples (approximately ⅓ of the rectum) were put into microcentrifuge tubes containing 0.25 ml of TE buffer. DNA was isolated from rectal samples as for the gill/mantle samples described above.

Before use in PCR reactions, all DNA isolated from hemolymph samples and gill/mantle samples was concentrated by reprecipitation with 140 μl 7.5 M ammonium acetate, 10 μg glycogen, and 1 ml 95% ethanol. Gill/mantle samples or hemolymph samples were resuspended in one-tenth or one-half the initial volume of TE, respectively.

Standard curve preparation for quantitation of *P. marinus* cells in oyster tissue

Cultured *P. marinus* cells were enumerated using trypan blue exclusion staining. Equal volumes of cells and trypan blue were mixed and a 25-μl aliquot was loaded onto a hemocytometer. Cells were counted using a light microscope at 50× magnification. Duplicate counts were made and cell viability was assessed. Dilutions of the cultures were made in 0.22-μm filtered York River water (salinity 18 ppt).

Oysters that were suspected to be uninfected were obtained in June 1997 from Deep Water Shoals, James River, Virginia. At least 1 ml of hemolymph was taken from each oyster with a 23-gauge needle and divided into 0.3-ml samples. Oysters were shucked, and the gill/mantle tissue from each oyster was cut into 0.25-g samples. The hemolymph and gill/mantle tissues were frozen by immersion in liquid nitrogen and stored at −80 C.

DNA was isolated from hemolymph samples and gill/mantle samples as described above. From each oyster, a hemolymph sample and a gill/mantle sample were screened for *P. marinus* infection using the PCR assay described above. No competitor was added to these reactions, and PCR was performed using the Expand™ High Fidelity polymerase protocol as described above.

After obtaining negative results, the hemolymph samples and the gill/mantle samples were spiked with *P. marinus* cultured cells (10^2–10^6 cells) in triplicate. DNA was isolated from the spiked hemolymph samples and the gill/mantle samples, digested with *Xba* I, and subjected to QCPCR as described above. Standard curves were constructed to relate the number of cultured *P. marinus* cells to the amount of DNA determined by QCPCR analysis.

Data analysis

GeneImagIR (RFLPscan, Scanalytics, Fairfax, Virginia) was utilized to calculate the peak areas under the Gaussian curves (integrated density of the bands) fitted to both the target and competitor PCR product bands that had been separated on the 4% polyacrylamide gels as described above. This peak area is proportional to the molar amount of the DNA fragment when fragments of similar length are compared (Hahn et al., 1995).

The logarithm of the ratio of the integrated densities of the product bands (target/competitor) was plotted against the logarithm of the amount of competitor initially added to the reaction (Cross, 1995). Linear regressions were performed on the data, including all replicates (Zar, 1984), in order to assess the relationship of the variables. The regression curve equation was given in the form $f(x) = mx + b$ where m represents the slope of the line. The equivalence point was determined by solving the regression equation for the curve when the ratio of signal intensity of the target/competitor is 1($f[x] = 0$, i.e., $\log_{10}1 = 0$) (Piatak et al., 1993; Zimmermann and Mannhalter, 1996). The amount of DNA in the target sample, as determined by the equivalence point, was corrected for dilution, the volume of DNA used in the PCR, and reprecipitation recovery. In addition, a genomic correction factor was determined in order to correct for the difference in the number of amplification target sequences in equivalent amounts of plasmid and genomic DNA. Only a fraction of the total DNA (plasmid or genomic) in the PCR reaction is targeted for amplification and the relative percentage of target in the genomic DNA is less than in the plasmid. The correction factor was determined to be the average value for correcting the equivalence point for each of the genomic concentrations within the range of quantitation. For the Expand™ High Fidelity PCR system, the genomic correction factor was determined from QCPCR with 1 μl of the *P. marinus* genomic DNA at concentrations of 5 fg/μl, 1 fg/μl, and 0.5 fg/μl. For all oyster tissue samples the genomic correction factor was used to correct the estimated DNA concentration. The 95% confidence intervals were calculated for each standard curve linear regression analysis of the relationship between the cell number and the amount of *P. marinus* DNA in the oyster tissues in order to estimate the standard error of the slope of the line. Finally, the amount of DNA calculated by QCPCR was converted to cells per g for gill/mantle samples or to cells per ml for hemolymph samples using the appropriate linear regression equation.

Comparisons of the weight-standardized parasite burden obtained from the whole tissue assay or the hemolymph assay, the intensity rank obtained from Ray's FTM assay, and the QCPCR diagnoses were made in pairs. All data were log-transformed before comparison except for the intensity rank data. Regression analyses were employed to compare the assays. The significance of the relationship was determined by the *P* value of the ANOVA *F* statistic for each pair of assays.

RESULTS

PCR sensitivity and specificity

Perkinsus marinus-specific primers PER-18S and PER-ITS amplified a 1,210-bp region of DNA from the small subunit rRNA gene and first ITS region. As little as 0.01 fg of *P. marinus* DNA in a background of approximately 1.0 μg of *C. virginica* DNA was detected. Furthermore, the DNA of 2 cultured cells in a background of approximately 5 mg of oyster tissue was detected as estimated from the results of the oyster tissues spiked with cultured *P. marinus* cells. Using 1 μl of unpurified PCR product in a second amplification step did not increase the sensitivity of the assay. The overall detection limit for quantitation was 0.005 fg of *P. marinus* DNA.

Amplification with DNA isolated from cultured *Perkinsus*-1 cells yielded the expected PCR product of 1,210 bp. Furthermore, DNA isolated from geographic isolates of *P. marinus* cultured cells from Virginia, Connecticut, South Carolina, Louisiana, and Texas amplified with the PER-18S and PER-ITS primers under the conditions described for this PCR assay. No product band was detected after PCR with uninfected *C. virginica* DNA; however, DNA isolated from *C. virginica* tissue spiked with cultured *P. marinus* cells yielded the appropriate PCR product. No product band was detected after PCR with DNA isolated from the dinoflagellates *Amphidinium* sp., *Gymnodinium* sp., and *Peridinium* sp., or from cultured cells resembling *P. atlanticus.*

QCPCR

Quantitation was possible for a wide range of control target plasmid concentrations. A series of reactions with control target plasmid DNA concentrations ranging from 100 pg/μl to 10 fg/μl is depicted in Figure 2a. Each of the 5 target concentrations is represented by an individual curve that corresponds to the series of competitor dilutions. The regression curves (log signal intensity ratio [target/competitor] vs. log concentration of competitor) show slopes ranging from −1.45 to −0.805. The equivalence points for each of the series of reactions were found to be 81.010 pg, 12.113 pg, 1.212 pg, 0.140 pg, and 0.0134 pg for 100 pg, 10 pg, 1 pg, 0.1 pg, and 0.01 pg of control target plasmid DNA, respectively. These values represent the initial amount of target DNA in the reaction before amplification.

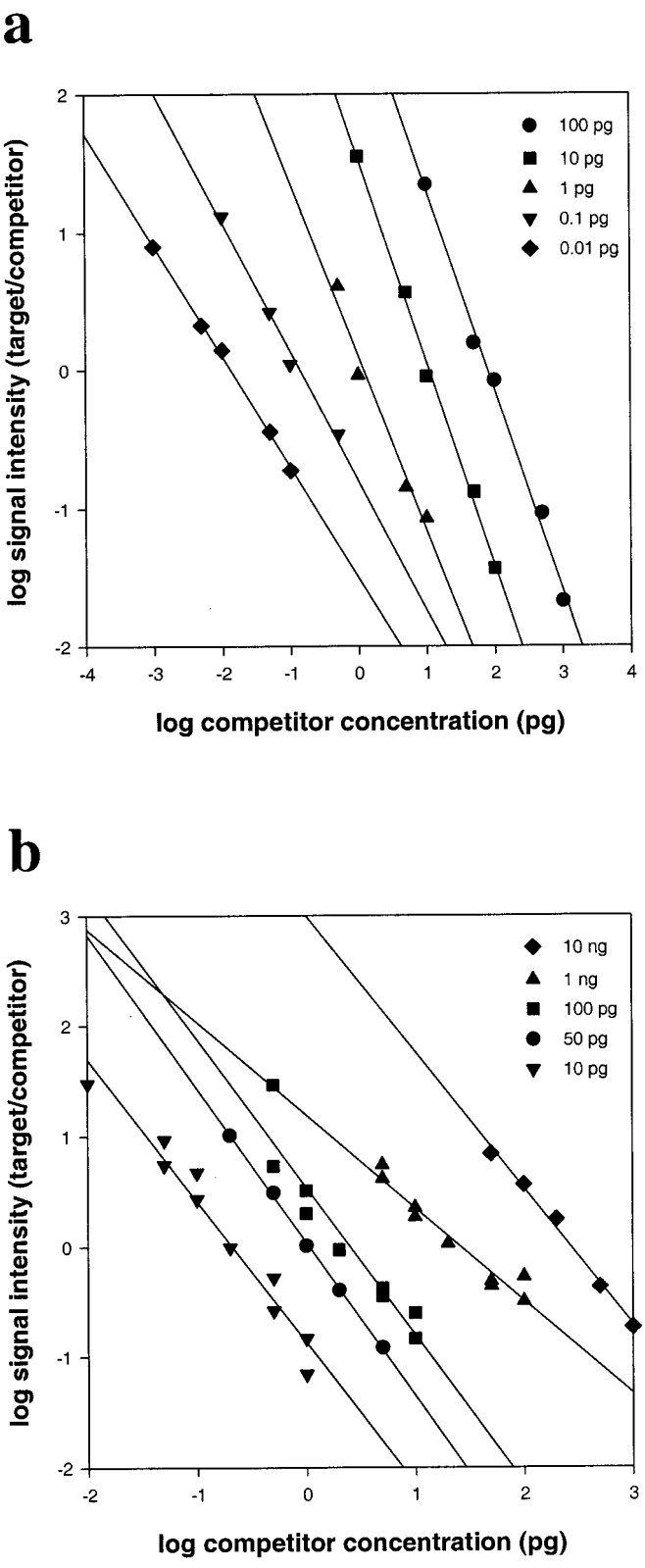

FIGURE 2. QCPCR with Ampli*Taq* DNA polymerase. Competitor plasmid DNA (pITS-MUT) was used in all QCPCR reactions. The linear regression equations are given in the form f(x) = mx + b where m is the slope of the linear curve. **a.** Control target plasmid DNA (pITS-WT): 100 pg, 10 pg, 1 pg, 0.1 pg, or 0.01 pg of the plasmid target DNA was used in each QCPCR series. The regression equations were f(x) = −1.452x + 2.772 (R^2 = 0.993), f(x) = −1.472x + 1.54 (R^2 = 0.996), f(x) = −1.262x + 0.105 (R^2 = 0.973), f(x) = −0.940x + −0.802 (R^2 = 0.990), f(x) = −0.805x + −1.506 (R^2 = 0.998), respectively. **b.** *Perkinsus marinus* genomic target DNA: 10 ng, 1 ng, 100 pg, 50 pg, or 10 pg of the genomic DNA was used in each QCPCR series. The regression equations were f(x) = −1.239x + 3.008 (R^2 = 0.991), f(x) = −0.842x + 1.193 (R^2 = 0.940), f(x) = −1.343x + 0.540 (R^2 = 0.898), f(x) = −1.393x + 0.040 (R^2 = 0.999), f(x) = −1.283x + −0.871 (R^2 = 0.957), respectively.

QCPCR with genomic target DNA ranging from 10 ng/μl to 10 pg/μl yielded slopes of the fitted curves ranging from −0.842 to −1.39, indicating the nearly equivalent amplification efficiencies of the genomic target and plasmid competitor (Fig. 2b). Digestion of the DNA with *Xba* I made the amplification efficiencies of genomic and plasmid DNA more equivalent.

The Expand™ High Fidelity enzyme mixture offered maximal sensitivity due to the presence of both *Taq* and *Pwo* DNA polymerases. *Pwo* DNA polymerase has a 3′ to 5′ exonuclease activity that allowed for increased fidelity, and overall the cocktail produced higher PCR yields. In Figure 3a, plasmid target concentrations were 100 pg/μl, 100 fg/μl, and 1 fg/μl. The corresponding equivalence points were found to be 112.720 pg, 80.168 fg, and 0.782 fg. The slopes of the log–log plots ranged from −1.21 to −1.42 and indicated that the control target and competitor plasmids were amplifying with relatively equal efficiencies. The QCPCR second phase reactions with 5 fg, 1 fg, and 0.5 fg of *P. marinus* genomic DNA and the Expand™ High Fidelity enzyme resulted in the determination of target equivalence points at 0.489 fg, 0.134 fg, and 0.119 fg, respectively (Fig. 3b). Because of the equivalent amplification efficiencies depicted, quantitation was possible for 50 pg to 0.5 fg of genomic DNA. A genomic correction factor of 7.296 ± 3.015 was calculated as described above for the Expand™ High Fidelity system based on the second phase QCPCR series. This genomic correction factor was subsequently used to correct for differences in the number of target sequences in equivalent amounts of genomic and plasmid DNA.

The assay was shown to be reproducible for each of the enzyme systems employed. Figure 4 depicts a representative plot of triplicate QCPCR with 1 pg of *P. marinus* genomic target DNA using the Expand™ High Fidelity enzyme system. From this plot, the equivalence point was found to be 110.98 ± 29.785 fg before correction and 809.7 fg after correction with the genomic correction factor.

Perkinsus marinus infection diagnosis

The first phase of QCPCR spanned 6 orders of magnitude and often produced a sigmoidal curve when the log of the signal intensity ratio (target/competitor) was plotted against the log of the competitor concentration. Because quantitation should be done on the linear region of the fitted curve, the first phase QCPCR results were only used for estimating the sample dilution for the more narrow phase (second phase) series of reactions in the linear portion of the sigmoidal plot. Figure 5a depicts a gel image of the second phase series of QCPCR reactions of a representative gill/mantle sample (Oyster 18). A plot of the log of the signal intensity ratio (target/competitor) versus the log of the concentration of the competitor for the

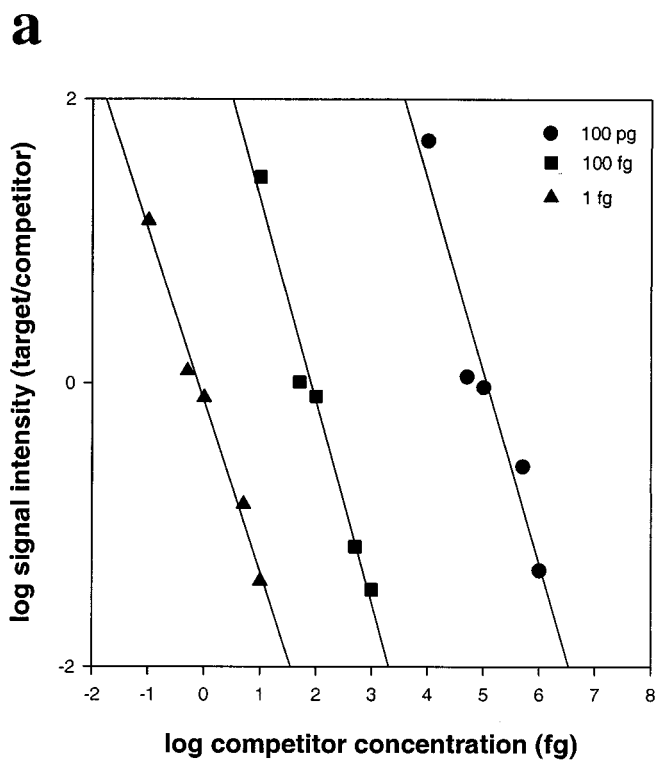

FIGURE 3. QCPCR with Expand™ High Fidelity PCR System DNA polymerase. Competitor plasmid DNA (pITS-MUT) was used in all QCPCR reactions. The linear regression equations are given in the form f(x) = mx + b where m is the slope of the linear curve. **a.** Control target plasmid DNA (pITS-WT): 100 pg, 100 fg, or 1 fg of plasmid DNA was used in each QCPCR series. The regression equations were f(x) = −1.351x + 6.824 (R^2 = 0.929), f(x) = −1.421x + 2.706 (R^2 = 0.977), f(x) = −1.208x + −0.129 (R^2 = 0.988), respectively. **b.** *Perkinsus marinus* genomic target DNA: 5 fg, 1 fg, or 0.5 fg of the genomic DNA was used in each QCPCR series. The regression equations were f(x) = −1.099x + −0.343 (R^2 = 0.989), f(x) = −1.896x + −1.654 (R^2 = 0.990), f(x) = −1.219x + −1.127 (R^2 = 0.769), respectively.

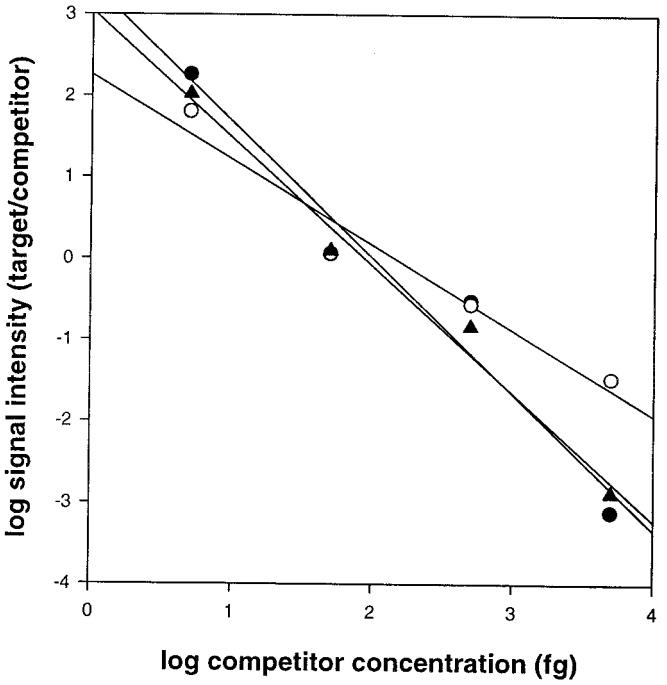

FIGURE 4. Triplicate QCPCR with 1 pg *P. marinus* genomic DNA using the Expand™ High Fidelity PCR System DNA polymerase.

same gill/mantle sample (Oyster 18) is shown in Figure 5b. The amount of target *P. marinus* DNA in the infected oyster gill/mantle (and hemolymph) samples was calculated as follows. For oyster 18 the equivalence point of 0.63 fg in the gill/mantle analysis was corrected for a 200,000-fold dilution and the genomic correction factor of 7.296 that was calculated as described above. The amount of dilution necessary for accurate quantitation varied for each sample. DNA recovery from the gill/mantle and hemolymph extraction procedures after each of three DNA precipitations was found to be 82%. Therefore, the equivalence point value was also corrected for the efficiency of recovery and the amount of tissue (0.25 g or 0.3 ml) used for the extraction to determine the quantity (fg DNA/g oyster tissue or fg DNA/ml of hemolymph) of target *P. marinus* DNA in the infected samples. Because the lysing efficiency of *P. marinus*-cultured cells by this method was found to be 99.9%, no correction for possible unlysed cells in a sample was necessary in subsequent quantitative analysis. Thus, the amount of *P. marinus* DNA in the gill/mantle sample of oyster 18 was calculated to be 6.68 ng per g of tissue.

Standard curves, generated by spiking uninfected tissues, relate the amount of DNA (fg) to the number of cultured *P. marinus* cells in gill/mantle (Fig. 6a) and in hemolymph (Fig. 6b). The regression equation f(x) = 1.216x + 0.529 from the log–log graph was used to calculate the number of *P. marinus* cells

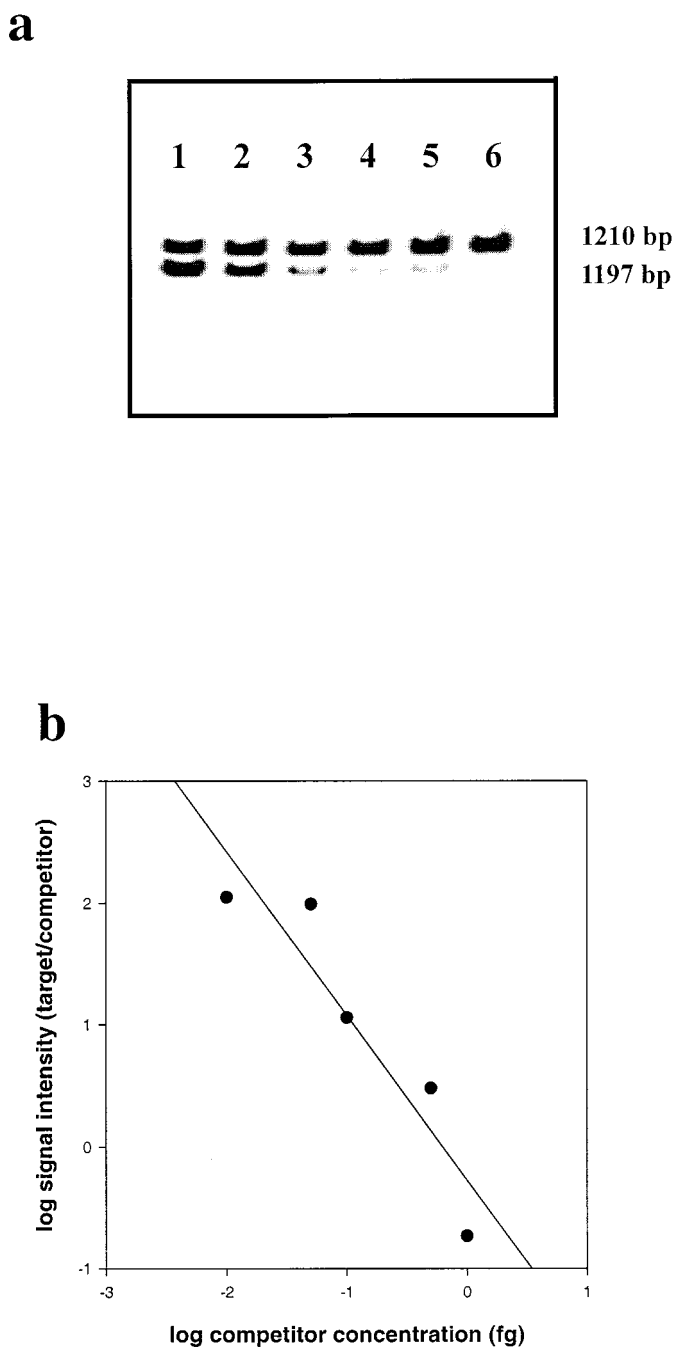

FIGURE 5. Oyster 18 gill and mantle sample. **a.** LI-COR automated sequencer polyacrylamide gel image of fluorescently labeled PCR products. The image depicts the QCPCR series of the narrow range competitor concentrations using the Expand™ High Fidelity DNA polymerase. The 1,210-bp band is the target PCR product and the 1,197-bp band is the competitor PCR product. Each lane represents a single QCPCR with a constant aliquot of genomic target DNA. One of a series of dilutions of the plasmid competitor DNA was added to each reaction: 1 fg, 0.5 fg, 0.1 fg, 0.05 fg, 0.01 fg, 0.005 fg (lanes 1–6, respectively). **b.** The log of the signal intensity ratio as determined from the integrated densities of the bands in Figure 4a plotted against the log of the amount of the competitor DNA (in fg) initially added to the reactions. The regression equation is given as $f(x) = -1.347x + -0.271$ ($R^2 = 0.863$).

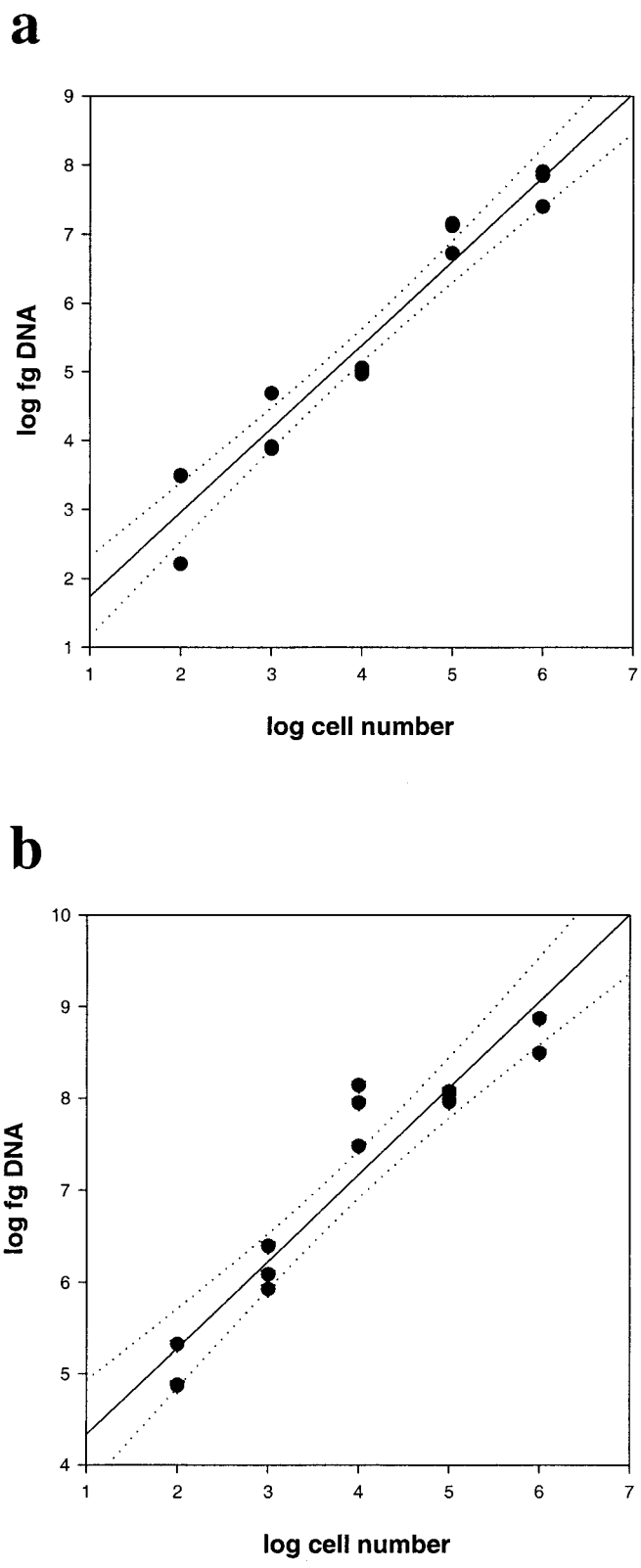

FIGURE 6. Standard curves depict the relationship between the log of the amount of DNA in fg determined by QCPCR analysis and the log of the number of *P. marinus* cells per sample. Three replicates for each cell number sample (10^3–10^6) were analyzed by QCPCR. The regression curve is shown as a solid line and the 95% confidence intervals are shown with dotted lines. **a.** Gill and mantle standard curve $f(x) = 1.216x + 0.529$ ($R^2 = 0.942$) **b.** Hemolymph standard curve $f(x) = 0.903x + 3.294$ ($R^2 = 0.823$).

TABLE I. *Perkinsus marinus* infection diagnosis by Ray's FTM tissue assay (Mackin scale rank), body burden assay, QCPCR with gill/mantle tissue, FTM hemolymph assay, and QCPCR with hemolymph.

Oyster no.	FTM tissue assay (Mackin scale rank)	Body burden (no. cells/g)	QCPCR gill/mantle (no. cells/g)	FTM hemolymph (no. cells/ml)	QCPCR hemolymph (no. cells/ml)
1	1	3,332	3,646	27	3
2	5	11,379,338	5,244,055	3,689	1,155
3	0.5	76	550	0	1
4	0.5	3,397	36	13	1
5	0.5	2,040	328	7	1
6	4	595,104	27,133	4,622	281
12	1	102,237	2,418	7	28
14	1	5,437	701	30	1
16	5	1,235,772	71,474	62,833	1,414
17	1	427,083	43,663	7,200	500
18	5	4,217,826	150,418	17,778	3,285
19	3	663,727	157,256	2,200	1,089
21	5	38,725,490	2,541,119	5,866,667	25,759
22	0	44,538	378	40	1
23	2	274,296	11,074	7,222	72
26	4	258,763	17,795	84,444	15,710
31	1	3,219	505	37	32
34	0.5	120,622	188	27	19
36	1	0	*	0	0
38	0.5	185	10	7	1
43	0	640	57	7	1
44	0	1,087	184	13	1
46	0	4,167	8	0	1
48	0	18	0	3	1
49	0	14	*	40	*

* Denotes a positive result by QCPCR; however, the sample was below the level of quantitation (<0.005 fg).

per g of gill/mantle tissue. Thus, 150,418 *P. marinus* cells per g of oyster tissue were calculated for the gill/mantle sample from Oyster 18. The 95% confidence intervals were determined in order to estimate the range of numbers of cells that could be calculated for the corresponding amount of DNA (6.68 μg for Oyster 18). For the hemolymph samples, the regression equation $f(x) = 0.903x + 3.294$ from the log–log graph was used to calculate the number of *P. marinus* cells per ml of hemolymph.

TABLE II. Comparison by regression of all *P. marinus* diagnostic assays.*

	Mackin scale	Body burden	QCPCR gill/mantle	FTM hemolymph
Mackin scale	—			
Body burden	$R^2 = 0.713$	—		
QCPCR gill/mantle	$R^2 = 0.716$	$R^2 = 0.757$	—	
FTM hemolymph	$R^2 = 0.637$	$R^2 = 0.675$	$R^2 = 0.736$	—
QCPCR hemolymph	$R^2 = 0.569$	$R^2 = 0.756$	$R^2 = 0.757$	$R^2 = 0.869$

* All data were log transformed before regression analysis except for Ray's FTM tissue assay data that were analyzed by Mackin scale rank. R^2 values are given for each comparison. All regressions were accepted as significant ($P < 0.0001$).

For rectal tissue samples, PCR inhibitors prevented consistent amplification in even the heaviest infections. PCR product bands were not detected from rectal tissues after the first amplification in 9 of 25 oysters, which were subsequently found to be positive for *P. marinus* by the 3 FTM assays.

Table I lists the *P. marinus* infection diagnosis results for all 5 procedures that were performed on each oyster. For the QCPCR results, any number greater than 0 but less than 1 was assigned a value of 1 cell. Ray's FTM tissue assay diagnosed only 19 infections, whereas the body burden whole oyster tissue assay diagnosed infections in 24 of the 25 oysters. The FTM hemolymph assay detected 22 infections. QCPCR with both the gill/mantle tissue DNA and the hemolymph DNA each detected 24 infections. Oysters that were diagnosed with negative or rare infections by Ray's FTM tissue assay were often not quantifiable by the QCPCR assay using the Ampli*Taq* DNA polymerase; however, the Expand™ High Fidelity system offered greater sensitivity and allowed molecular quantitation in 6 of 9 previously undetected infections in the hemolymph and tissue samples.

Table II lists the R^2 values obtained from the regression analysis comparing the 5 quantitative diagnostic assays for *P. marinus*. Values that appeared in Table I as positive by QCPCR but below the level of quantitation were not included in the regression analysis. All pairs analyzed resulted in highly significant correlations as determined by the ANOVA *P* value ($P < 0.0001$). The relatively high R^2 values (range 0.569–0.869) in-

dicated a good fit of the data with the regression curve and that much of the variance of the dependent variable could be accounted for by the independent variable.

DISCUSSION

This QCPCR assay imparted specificity, objectivity, and extreme sensitivity to the diagnosis of *P. marinus* infection in oyster tissues. The primers amplified DNA from various *P. marinus* geographic isolates that are thought to represent the genetic variation among available isolate cultures (Reece, Bushek, and Graves, 1997). Specificity at the species level is implied because neither DNA from the presumptive *P. atlanticus* cells nor from the closely related dinoflagellate species (Fong et al., 1993; Goggin and Barker, 1993; Goggin, 1994; Reece, Siddall et al., 1997) amplified. Ray's FTM method (Ray, 1952) has not demonstrated species-level specificity. Furthermore, employing specific molecular diagnostics allows for detection of all life stages of the parasite in oysters. Because this assay determines parasite number mathematically from the integrated densities of the PCR product bands, the subjectivity of counting stained cells, and assigning infection intensities inherent in FTM methods is eliminated.

In a semiquantitative PCR assay, Marsh et al. (1995) estimated concentrations of *P. marinus* DNA by end-point dilution with a detection limit of 100 fg by Southern blot and 10 fg by dot blot analysis. QCPCR was found to be more sensitive with a detection limit of 0.01 fg *P. marinus* DNA in 1.0 μg oyster tissue. In addition, this QCPCR assay eliminates the need for radioisotope by using fluorescently labeled primers for product detection by sensitive lasers of the automated sequencer. Standard curve results indicated that as few as 2 cells in a background of 5 mg oyster tissue were detected. This level of sensitivity minimizes the chance for false-negative diagnoses of light infections that are common to Ray's FTM tissue assay and the FTM hemolymph assay (Bushek et al., 1994).

The sensitivity of this assay was maximized by the use of the Expand™ High Fidelity system. Previous investigations by Schwieger and Tebbe (1997) demonstrated that higher PCR product yields were obtained from low copy number DNA templates with the use of the Expand™ High Fidelity system (*Taq* and *Pwo* DNA polymerases) than with *Taq* DNA polymerase alone.

Despite the extreme sensitivity and objectivity this assay offers, several disadvantages must be overcome. Because of the cost of the enzyme, the Expand™ High Fidelity enzyme was used only in the second phase reactions for actual quantitation. Small pipeting errors or degradation of the competitor resulting in nondetectable changes in concentration over time in storage may have contributed to the deviations from the slope of -1 in the plots used to calculate equivalence points. According to Cross (1995), the slope of the log of the signal intensity ratio (target/competitor) versus the log of the competitor concentration should be equal to -1 when the amplification efficiencies are equivalent; however, slight deviations from this value will not affect the determination of the equivalence point as long as a series of reactions that spans the equivalence point is performed. For these QCPCR data, quantitation would still be accurate because the series of reactions spanned this point. For future use of this assay, quantitation should be done over a broader linear range (50 fg to 0.005 fg) that should be sufficient to monitor infection levels. Error due to pipeting and minimal competitor degradation would then be minimized. Another possible disadvantage stems from the lack of documentation of potential differences, especially genome size and gene copy number, between wild-type *P. marinus* cells detected in oysters and the cultured isolates used for the generation of the standard curve.

Currently, the body burden assay can be considered the gold standard for *P. marinus* diagnostics. Because this procedure samples the entire oyster, the quantitative results are considered the most sensitive and most accurate of the FTM methods (Bushek et al., 1994). QCPCR only sampled 0.25 g of the gill/mantle tissue. In patchy, localized infections, the probability of sampling infected tissue might be low with such a small sample size; however, results from theses studies indicate that the QCPCR of gill/mantle and the body burden assay were equally sensitive, detecting infections in 24 of 25 oysters. The small tissue sample required for the QCPCR assay provides an advantage over the body burden assay when other tissue samples are needed for correlations between biochemical or histological data and disease status. Bushek et al. (1994) demonstrated that combining rectal and mantle tissue for diagnosis by FTM methods decreased the chances for false negatives. For the QCPCR assay, PCR amplification with DNA preparations from rectal tissue was inhibited, despite extra purification steps and the addition of BSA to the reaction. BSA has been shown to overcome the inhibition of *Taq* DNA polymerase by substances commonly found in environmental samples (Kreader, 1996); however, it was not effective on rectal tissue DNA samples.

Overall, the QCPCR hemolymph assay was more sensitive than the FTM hemolymph assay, which is thought to be more accurate for detecting low level infections in oysters than Ray's FTM tissue assay (Gauthier and Fisher, 1990). The FTM hemolymph assay detected infections in 22 oysters, whereas the QCPCR hemolymph assay detected infections in 24 oysters. Because the body burden assay has proven to be most effective in diagnosing infections, a highly significant correlation ($P < 0.0001$) between the QCPCR hemolymph assay and the body burden assay lends support to the applicability of this QCPCR hemolymph assay for disease monitoring. The advantages of a sensitive hemolymph assay include nondestructive sampling and the ability to sample repeatedly. Furthermore, because of the open circulatory system of oysters, the hemolymph assay reflects systemic infections as opposed to localized infections or presence of *P. marinus* cells that have not actually infected the oyster.

In addition to oyster samples, this sensitive QCPCR assay may be applied to quantitation of *P. marinus* in environmental samples. A QCPCR assay has previously been developed to detect *Pseudomonas* cells in seawater (Leser, 1995). Monitoring *P. marinus* abundance in water samples is important because infective stages of the parasite are waterborne and transmission has been reported from infected oysters to uninfected oysters via the water column (Ray and Mackin, 1954). A means for monitoring *P. marinus* specifically in the water column has not been previously documented. The common FTM methods are not easily applied to water samples and our attempts to enlarge *P. marinus* cells from the water column in FTM were unsuccessful.

In conclusion, QCPCR permitted successful quantitation of *P. marinus* DNA in oyster tissues. The disadvantages of this assay are the expense of the DNA polymerase, the relatively labor-intensive process of performing 2 series of reactions on each sample, and the equipment as well as the molecular biology expertise required to perform the assay. In addition, PCR inhibitors in oyster rectal tissue and in environmental samples (Leser, 1995) present a challenge to the preparation of amplifiable DNA. The advantages of this QCPCR assay include its exceptional sensitivity using only small tissue or hemolymph samples, its species-level specificity, and its ability to quantify infections. The QCPCR hemolymph assay provides a nondestructive, repeatable method for quantifying pathogen infection in the oyster. In addition, the potential for analyzing water samples for *P. marinus* cells is exciting in the face of necessary transmission dynamics studies and general monitoring of the water column.

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