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Detection and Quantification of Infectious Hypodermal and Hematopoietic Necrosis Virus and White Spot Virus in Shrimp Using Real-Time Quantitative PCR and SYBR Green Chemistry

ARUN K. DHAR, MICHELLE M. ROUX, AND KURT R. KLIMPEL*

Super Shrimp, Inc., National City, California, California 91950

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A rapid and highly sensitive real-time PCR detection and quantification method for infectious hypodermal and hematopoietic necrosis virus (IHHNV), a single-stranded DNA virus, and white spot virus (WSV), a double-stranded DNA (dsDNA) virus infecting penaeid shrimp (*Penaeus* sp.), was developed using the GeneAmp 5700 sequence detection system coupled with SYBR Green chemistry. The PCR mixture contains a fluorescence dye, SYBR Green, which upon binding to dsDNA exhibits fluorescence enhancement. The enhancement of fluorescence was proportional to the initial concentration of the template DNA. A linear relationship was observed between the amount of input plasmid DNA and cycle threshold (C_T) values over a range of 1 to 10^5 copies of the viral genome. To control the variation in sampling and processing among samples, the shrimp β-actin gene was amplified in parallel with the viral DNA. The C_T values of IHHNV- and WSV-infected samples were used to determine absolute viral copy numbers from the standard C_T curves of these viruses. For each virus and its β-actin control, the specificity of amplification was monitored by using the dissociation curve of the amplified product. Using genomic DNA as a template, SYBR Green PCR was found to be 100- to 2000-fold more sensitive than conventional PCR, depending on the virus, for the samples tested. The results demonstrate that SYBR Green PCR can be used as a rapid and highly sensitive detection and quantification method for shrimp viruses and that it is amenable to high-throughout assay.

Viral diseases are a major problem in the shrimp aquaculture industry worldwide. Serious viral outbreaks often cause catastrophic losses in shrimp farming around the globe (16). Infectious hypodermal and hematopoietic necrosis virus (IHHNV) and white spot virus (WSV) are the two most important DNA viruses infecting penaeid shrimp in the Western Hemisphere (16).

Natural infection by IHHNV has been reported for most shrimp (*Penaeus* sp.) species (16). In the Americas, the two most commercially important species of shrimp are *P. stylirostris* and *P. vannamei. P. stylirostris* is highly susceptible to IHHNV, and the virus causes lethal disease (up to 90% mortality) in *P. stylirostris* juveniles (16). However, the virus does not cause lethal infection in *P. vannamei*; instead, it causes reduction in growth and a variety of cuticular deformities of the rostrum, antenna, and other thoracic and abdominal areas, deformities that together are commonly known as runt deformity syndrome (12). Losses of revenue due to runt deformity syndrome range from 10 to 50% depending on the level of infection. In an effort to develop virus-resistant lines of shrimp, Super Shrimp, Inc., has developed a line of *P. stylirostris* shrimp that is resistant to IHHNV. The population originated as wild larvae from Panama, survived an IHHNV epidemic, and has undergone more than 20 generations of captive breeding. This line of shrimp shows greater than 80% survival in an IHHNV-positive grow-out system (H. Clifford, Abstr. World Aquac. Soc. Meet., abstr. 116, 1998). By histology, in situ hybridization using an IHHNV-specific probe, and PCR, Super Shrimp *P. stylirostris* postlarvae and juveniles have been shown to be resistant to IHHNV (26). The virions of IHHNV are nonenveloped icosahedrons (22 nm in diameter) and contain a single-stranded linear DNA of 4.1 kb (2). We have recently sequenced the IHHNV genome. The genome organization revealed that IHHNV belongs to the family *Parvoviridae* and is closely related to mosquito brevidensoviruses (24).

WSV is currently the most devastating viral pathogen threatening the shrimp farming industry worldwide. WSV is also known as baculoviral hypodermal and hematopoietic necrosis virus (J. Huang, X. L. Song, J. Yu, and C. H. Yang, Yellow Sea Fishery Res. Inst., abstr. 9, 1994), a rod-shaped virus of *P. japonicus* (10), penaeid acute viremia (25), systemic ectodermal and mesodermal baculovirus (31), *P. monodon* nonoccluded baculovirus (32), white spot syndrome baculovirus (18), Chinese baculovirus (19), and white spot syndrome virus (15). To avoid confusion, we used the name WSV as suggested by Cesar et al. (3). WSV was first reported in eastern Asia during 1992 to 1993 (10; Huang et al., Yellow Sea Fishery Res. Inst.). The virus rapidly spread throughout shrimp farming regions of Southeast Asia and North America in the mid-1990s (6, 11). During 1999 WSV severely impacted the shrimp industries of both Central and South America. WSV-infected shrimp become lethargic, show reduction in food consumption, lose cuticles, and often exhibit white spots under their exoskeletons, hence the name of the disease white spot syndrome. The virus has a wide host range among crustaceans, infecting about 40 different species (6). The virions are enveloped nucleocapsids,

* Corresponding author. Mailing address: Super Shrimp, Inc., 1545 Tidelands Ave., Suite J, National City, CA 91950. Phone: (619) 477-5394. Fax: (619) 477-5396. E-mail: klimpel@supershrimp.com.

TABLE 1. Details of the primers used for the amplification of IHHNV, WSV, and the β-actin gene of shrimp by conventional PCR

Virus	Primer name	Primer sequence (5′-3′)	% GC	T_m (°C)[a]	Size (bp)	Reference
IHHNV	1641F	AGAACAATGATCCGGTCACAAGTCA	44	63	1,368	24
	1148R	AACACGCCGAAGGATCAA	50	56		
	IHHN-aFor	GTACATGGTGCGTGA	53	37	329	24
	607R	AGCCTATACAATCCATGGTGACACT	44	51		
WSV	F002	GATGAGACAGCCCAAGTTGTTAAAC	44	51	306	13
	R002	CGAAATTCCATCACTGTAATTGCTTG	38	50		
β-Actin	Actin-F	CCCAGAGCAAGAGAGGTA	55	45	339	4, 7, 21
	Actin-R3	GCGTATCCTTCGTAGATGGG	55	49		

[a] T_m at 50 mM Na^+.

bacilliform, and ~275 by 120 nm in size and have a tail-like projection at one end of the particle (5, 31). The viral genome contains double-stranded DNA (dsDNA) of 305 kb (1). Based on the morphology of the virus particles and the histopathology of infected tissue, WSV was initially thought to be a member of the family *Baculoviridae* (5). However, DNA sequence data of two major structural proteins (VP 26 and VP 28) showed no homology to baculovirus structural proteins (27) and a phylogenetic study based on ribonucleotide reductase genes revealed that WSV does not share a common ancestor with baculoviruses (28). Therefore, it has been proposed that WSV may be a representative of a new virus family, *Whispoviridae* (27, 28).

Diagnosis of IHHNV and WSV was done by bioassay using indicator hosts, monitoring clinical signs, histopathology, dot blot and in situ hybridizations using a virus-specific gene probe, and PCR (15). Although conventional PCR is the most sensitive of these methods, it is unable to detect the presence of a single copy of a viral genome in the infected tissue. This ability is critical for the development of a specific-pathogen-free shrimp-breeding program, for the screening of broodstock held in quarantine facilities, and for the detection of viral pathogens in commodity shrimp imported into or exported from the United States. In addition, due to the lack of a continuous cell culture system for penaeid shrimp, quantification of viruses infecting shrimp has been severely hampered. To overcome these problems, we have developed a rapid and highly sensitive real-time quantitative PCR method using SYBR Green as a fluorescence dye (SYBR Green PCR) and the GeneAmp 5700 sequence detection system.

SYBR Green is a minor-groove DNA binding dye with a high affinity for dsDNA and exhibits enhanced fluorescence upon binding to a dsDNA (30). The SYBR Green dye is excited at a 485-nm wavelength, and the emission is measured at a 520-nm wavelength. In the GeneAmp 5700 sequence detection system, the fluorescence of the SYBR Green dye is monitored at the end of each PCR cycle, thus allowing the detection of the product during the linear range of amplification. The increase of fluorescence above background is dependent on the initial template concentration (GeneAmp 5700 sequence detection system user manual, PE Applied Biosystems). The specificity of the amplified product is monitored by its melting curve. Since the melting curve of a product is dependent upon its GC content, length, and sequence composition, specific amplification can be distinguished from nonspecific amplification by examining the melting curve (22). The protocol described here does not need gel electrophoresis or any other post-PCR manipulation for sample analysis, which reduces the assay time and significantly eliminates PCR carryover contamination problems.

The objectives of the present study were (i) to determine the sensitivity and the specificity of SYBR Green PCR using the GeneAmp 5700 sequence detection system, (ii) to compare the sensitivity of SYBR Green PCR coupled with the GeneAmp 5700 sequence detection system to that of conventional PCR, and (iii) to determine the viral load in laboratory-challenged IHHNV- and WSV-infected shrimp (*P. stylirostris*) by SYBR Green PCR.

MATERIALS AND METHODS

Maintenance of animals and virus challenge work. Stocks of pathogen-free juvenile *P. stylirostris* (1 g) from Super Shrimp, Inc., maintained in our laboratory were used for this study. Animals were kept indoors within environmentally controlled tanks and reared on a commercially available feed formulation (MADMAC-MS Dry pellet; Aquafauna Bio-Marine, Inc., Hawthorne, Calif.). IHHNV and WSV inocula were prepared by homogenizing frozen infected tissue in 2% saline (1:10, wt/vol), centrifuging the homogenate in a tabletop centrifuge (Beckman Microfuge Lite model) at 12,000 rpm for 5 min, and filtering the supernatant through a 0.45-μm-pore-size filter. Healthy animals were injected with a viral inoculum (30 μl ≈ 10^6 copies) between the second and third tergal plates of the lateral side of the tail using a 1-ml tuberculin syringe. Control group animals were injected with a tissue homogenate from PCR-confirmed virus-negative healthy shrimp. The negative control homogenate was prepared using the same protocol.

Isolation of nucleic acids. Virus-challenged as well as mock-injected animals were sacrificed for extraction of DNA at 40 h postinjection (p.i.) for WSV and 10 days p.i. for IHHNV. The sampling time was based on the observation that, in Super Shrimp *P. stylirostris* stocks, a high viral titer is obtained at 40 h p.i. for WSV and at 10 days p.i. for IHHNV (K. R. Klimpel, unpublished data). Super Shrimp *P. stylirostris* has been shown to be resistant to IHHNV infection (26).

For the extraction of DNA, hepatopancreas tissues (50 mg) were taken from virus-injected as well as control animals. Genomic DNA was extracted using DNAZol and by following the protocol of the manufacturer (Molecular Research Center, Inc., Cincinnati, Ohio). The DNA pellets were dissolved in DNase and RNase-free distilled water. The DNA yield was quantified using a spectrophotometer (Shimadzu UV-1201), and the quality was assessed by running the samples in a 1% agarose gel using TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM sodium EDTA).

Cloning and sequencing of IHHNV and WSV DNAs. A 1,368-bp IHHNV DNA was amplified by inverse PCR using the primers 1641F and 1148R (Table 1) and by following the protocol described elsewhere (24). A 306-bp WSV DNA fragment was amplified using the primers F002 and R002 (Table 1). The reaction mixture for WSV amplification contained 100 ng of DNA, 1× PCR buffer (Sigma, St. Louis, Mo.), a 1.25 μM concentration of each forward and reverse primer, 0.2 mM deoxynucleoside triphosphate, and 0.6 U of RED *Taq* DNA polymerase (Sigma) in a 25-μl reaction volume. The temperature profile for the

TABLE 2. List of primers used for the detection of IHHNV, WSV, and the β-actin gene of shrimp by SYBR Green PCR

Virus or gene	Primer name	SYBR Green primer sequence (5′-3′)	% GC	T_m[a]	Amplicon size (bp)
IHHNV	313F	Forward: AGGAGACAACCGACGACATCA	52	49	50
	363F	Reverse: CGATTTCCATTGCTTCCATGA	42	45	
WSV	101-110F	Forward: GATAAGAGAGGTAGACACTAGTAGTGTTATTGCT	38	56	55
	101-165R	Reverse: CCACTGTGCCAGCTATTGCA	55	49	
β-Actin gene	178F	Forward: GGTCGGTATGGGTCAGAAGGA	57	51	50
	228R	Reverse: TTGCTTTGGGCCTCATCAC	52	46	

[a] T_m at 50 mM Na^+.

PCR amplification was 94°C for 2 min followed by 35 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 1 min, with an extension at 72°C for 7 min. The IHHNV and WSV amplified DNAs were cloned into a TOPO cloning vector (Invitrogen, Carlsbad, Calif.). The recombinant plasmid DNA was sequenced in an automated DNA sequencer (model ABI 373A; PE Applied Biosystems). Sequence analyses were performed using the National Center for Biotechnology Information BLAST search program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html).

PCR conditions. To compare the detection limit of conventional PCR to that of SYBR Green PCR using plasmid DNAs as well as genomic DNAs from IHHNV- and WSV-challenged *P. stylirostris* samples, 329-bp (for IHHNV) and 306-bp (for WSV) amplicons were amplified by conventional PCR. For genomic DNAs of IHHNV- and WSV-challenged animals and their corresponding healthy control animals, a 339-bp β-actin DNA was amplified in parallel. All the PCR primers were designed using Primer Express Software version 1.0 (PE Applied Biosystems) and are listed in Table 1. The primers for β-actin were designed based on the conserved sequences of the *Drosophila melanogaster*, sea urchin, and insect β-actin genes (4, 7, 21). The reaction mixture for IHHNV, WSV, and β-actin amplifications contained 1× PCR buffer (PE Applied Biosystems), 2 mM $MgCl_2$, a 0.8 μM concentration of each forward and reverse primer, 0.4 mM deoxynucleoside triphosphate, and 2.5 U of AmpliTaq Gold (PE Applied Biosystems). The amount of template DNA per reaction varied depending on the objective of the experiment (see Fig. 2 and 6). The amplifications were carried out in a thermocycler (GeneAmp 9700; PE Applied Biosystems) with the following thermal profile: 95°C for 10 min followed by 40 cycles of 95°C for 45 s, 55°C for 1 min, and 72°C for 2 min. The amplified DNAs were run in a 2% agarose gel using TAE buffer. The gel was stained with ethidium bromide and photographed.

SYBR Green PCR. The primers used for SYBR Green PCR are listed in Table 2. The primers were designed based on the sequences of 1,368- and 306-bp cloned segments of the IHHNV and WSV genomes, respectively. The primers for β-actin were designed based on a shrimp (*P. monodon*) β-actin sequence available in the GenBank database (accession number AF100987). The primers were first designed using Primer Express Software version 1.0 (PE Applied Biosystems). All primers were checked by running a virtual PCR, and the amplifications were analyzed for expected product, mispairing, and primer dimer formation using a computer program (Amplify version 1.2B; William Engles, Department of Genetics, University of Wisconsin). The best primer set was taken for the detection and quantification of IHHNV and WSV.

SYBR Green PCR amplifications were performed in a GeneAmp 9600 thermocycler coupled with a GeneAmp 5700 sequence detection system (PE Applied Biosystems). The reactions were carried out in a 96-well plate in a 25-μl reaction volume containing 7.1 μl of 2× SYBR Green Master Mix (PE Applied Biosystems), a 0.24 μM concentration of each forward and reverse primer, and 1 ng of total cellular DNA. For determining the IHHNV and WSV detection limits, the amounts of DNA used per SYBR Green PCR varied from 1.0 ng to 10 pg and from 1.0 ng to 0.1 pg, respectively. The thermal profile for all SYBR Green PCRs was 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 1 min. In each 96-well plate, a dilution series of the plasmid standard for the respective virus was run along with the unknown samples for the corresponding virus and their β-actin controls. Each sample was replicated two to three times. All reactions were repeated at least three times independently to ensure the reproducibility of the results.

Plasmid standard for quantification by SYBR Green PCR. The plasmid DNAs of 1,368-bp IHHNV and 306-bp WSV clones were linearized by *Eco*RI and *Hin*dIII (Promega, Madison, Wis.) digestions, respectively. Aliquots of the digested plasmids were run in a 1% agarose gel for checking the digestion before purification of the remaining digestion reaction mixtures with a Qiaquick gel purification kit (Qiagen, Valencia, Calif.). DNA was quantified using a spectrophotometer (Shimadzu UV-1201), and dilutions were made using sheared salmon sperm DNA (5 ng/ml) as a diluent.

Data analyses. After SYBR Green PCR amplification, data acquisition and subsequent data analyses were performed using the GeneAmp 5700 sequence detection system (version 1.3). In the SYBR Green Master Mix, there is an internal passive dye, ROX, in addition to the SYBR Green dye. The increase in the fluorescence of SYBR Green against that of ROX is measured at the end of each cycle. A sample is considered positive when the change in the fluorescence of SYBR Green relative to that of ROX (ΔR_n) exceeds an arbitrary threshold value. The threshold value is set at the midpoint of the ΔR_n and the cycle number plot. For all the amplifications described in this paper, the threshold value of the ΔR_n was considered to be 0.25. The PCR cycle at which a statistically significant increase in the ΔR_n is first detected is called the threshold cycle (C_T). Target DNA copy number and C_T values are inversely related. For example, a sample containing a high number of copies of the target DNA will cross the threshold at an earlier cycle than sample with a lower number of copies of the same target. The absolute levels of IHHNV and WSV in the experimental samples were determined by extrapolating the C_T values from the standard curves of the viruses.

For data analyses, the C_T values were exported into a Microsoft Excel Worksheet for further statistical analyses. Regression analyses of the C_T values of genomic DNA dilution series were used to determine the amplification efficiencies of IHHNV and WSV compared to those of their β-actin genes.

RESULTS

Sequence analyses of IHHNV and WSV DNAs. The nucleotide sequence of the 1,368-bp IHHNV DNA clone showed that it encompassed parts of the left and right open reading frames (ORFs) of the viral genome (GenBank accession number AF273215). In the IHHNV genome, the left ORF encodes nonstructural protein NS1 and the right ORF encodes viral capsid protein (24). The nucleotide sequence of the 306-bp WSV clone contained one additional A at nucleotide position 120 in the sequence compared to the GenBank entry (accession number WSU 89843 [13]).

Analytical sensitivity of SYBR Green PCR versus conventional PCR using plasmid DNA as the template. To determine the analytical sensitivity of SYBR Green PCR, serial dilutions of IHHNV and WSV plasmid standards were made using sheared salmon sperm DNA as the carrier. A linear relationship was observed between the input copy number of the virus template and the C_T values for the virus-specific product over 5 $\log_{10}$ dilutions, ranging from 1.88×10^5 copies ($C_T = 22.2 \pm 0.725$) to 1.18 copies ($C_T = 39.751 \pm 0.424$) for IHHNV and 2.37×10^5 copies ($C_T = 21.278 \pm 0.037$) to 1.48 copies ($C_T = 38.981 \pm 0.528$) for WSV (Fig. 1A and B; Table 3). Negative controls did not provide any amplification for either virus.

The IHHNV and WSV plasmid standard dilutions, as used

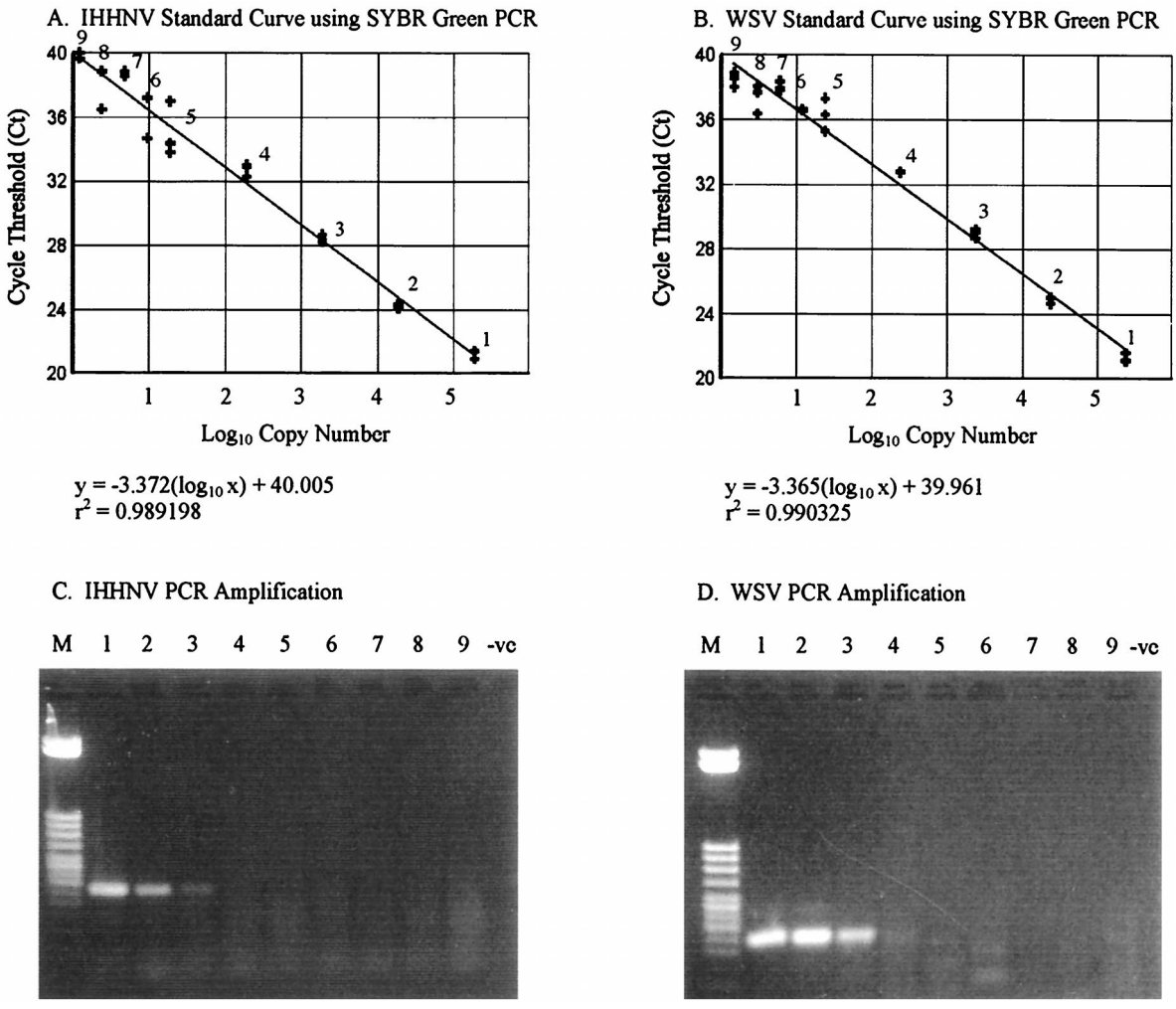

FIG. 1. Linear relationship between C_T values and the dilutions of the plasmid DNAs of IHHNV (A) and WSV (B) samples. The exact numbers of copies of the IHHNV plasmid added to each reaction mixture (corresponding to the numbers on the linear curve) were as follows: 1.88×10^5 (1), 1.88×10^4 (2), 1.88×10^3 (3), 1.88×10^2 (4), 1.88×10^1 (5), 9.4 (6), 4.7 (7), 2.35 (8), and 1.18 (9). For WSV samples, the plasmid copy numbers were 2.37×10^5 (1), 2.37×10^4 (2), 2.37×10^3 (3), 2.37×10^2 (4), 2.37×10^1 (5), 11.85 (6), 5.93 (7), 2.96 (8), and 1.48 (9). The amplifications of IHHNV and WSV DNAs from the same plasmid dilutions by conventional PCR are shown in panels C and D, respectively. The lane numbers refer to the plasmid copy numbers mentioned above. Lane M, 50-bp DNA step ladder (Sigma); lane −ve, negative control.

for SYBR Green PCR, were also tested by conventional PCR using virus-specific primers (Table 1). A 329-bp DNA was amplified for the IHHNV, and a 306-bp DNA was amplified for the WSV (Fig. 1C and D). Successful amplifications could be obtained down to 1.88×10^2 copies for IHHNV and 2.37×10^1 copies for WSV, as opposed to approximately 1 copy by SYBR Green PCR for both viruses.

Detection limits of IHHNV and WSV by conventional PCR using genomic DNA as the template. In order to determine the detection limits of IHHNV and WSV by conventional PCR, dilution series of genomic DNAs were made from laboratory-challenged *P. stylirostris* samples. For each virus, PCR amplifications were carried out using the virus-specific primers and the β-actin primers in parallel. The amplicon sizes for IHHNV, WSV, and β-actin were 329, 306, and 339 bp, respectively (Fig. 2). For IHHNV, the detection limit was 100 ng of DNA, whereas for β-actin, the detection limit for the same dilution series was 100 pg (Fig. 2A and C). For both WSV and its β-actin gene, the detection limit was 10 pg (Fig. 2B and D).

Limits of detection of IHHNV and WSV by SYBR Green PCR using genomic DNAs as templates. The limits of detection for IHHNV and WSV by SYBR Green PCR were determined by using dilution series of total genomic DNAs from laboratory-challenged *P. stylirostris* samples. The detection limits were 50 pg (average $C_T = 37.9$) (Fig. 3A) for IHHNV and 0.1 pg (average $C_T = 37.02$) (Fig. 3B) for WSV.

To compare the efficiencies of amplification of IHHNV and WSV with those of their β-actin genes, the C_T values were plotted against the dilutions of the input genomic DNAs. The slopes of the regression lines for IHHNV and its β-actin gene were −1.7307 and −1.6573, respectively (Fig. 3A). The slopes of the regression lines for WSV and its β-actin gene were −3.665 and −3.5233, respectively (Fig. 3B). Therefore, the amplification efficiencies of both the viruses and their β-actin genes were very similar.

Specificity of SYBR Green PCR. The specificities of the products amplified by SYBR Green PCR were monitored by analyzing the amplification profiles and the corresponding dis-

TABLE 3. C_T values of replicate assays of dilution series of IHHNV and WSV plasmid standards and genomic DNA of virus-infected *P. stylirostris*

Virus	No. of copies of plasmid or genomic DNA or β-actin control	C_T values[a]				Mean	SD	CV[b]
		Expt 1	Expt 2	Expt 3	Expt 4			
IHHNV	Plasmid copy no.							
	1.88×10^5	21.253	22.350	22.185	23.010	22.200	0.725	3.27
	1.88×10^4	24.220	25.700	25.315	25.115	25.088	0.627	2.49
	1.88×10^3	28.443	30.625	28.785	28.625	29.120	1.013	3.49
	1.88×10^2	32.730	32.445	32.980	NT	32.718	0.268	0.82
	1.88×10^1	35.060	35.560	35.515	35.965	35.525	0.370	1.04
	9.4	37.307	37.270	36.390	36.610	36.894	0.464	1.26
	4.7	39.130	40.000	38.635	39.620	39.346	0.593	1.51
	2.35	38.457	38.910	38.175	39.220	38.690	0.465	1.20
	1.18	39.883	40.000	40.000	39.120	39.751	0.424	1.07
	Genomic DNA sample							
	1	32.983	32.187	32.335	NT	32.502	0.424	1.31
	2	34.280	34.795	34.530	NT	34.535	0.258	0.75
	3	33.935	33.747	33.545	NT	33.742	0.195	0.58
	4	31.620	32.065	32.600	NT	32.095	0.491	1.53
	5	33.627	33.345	32.190	NT	33.054	0.761	2.30
	6	33.530	34.860	33.307	NT	33.899	0.840	2.48
	7	30.873	31.305	30.730	NT	30.969	0.299	0.97
	8	31.860	32.075	30.767	NT	31.567	0.702	2.22
	9	31.697	33.005	31.480	NT	32.061	0.825	2.57
	10	30.917	31.825	30.745	NT	31.162	0.580	1.86
	β-Actin control							
	1	23.567	23.893	24.035	NT	23.832	0.240	1.01
	2	24.755	26.030	25.580	NT	25.455	0.647	2.54
	3	24.823	25.477	25.435	NT	25.245	0.366	1.45
	4	NT	24.470	24.775	NT	24.623	0.216	0.88
	5	25.397	26.170	26.045	NT	25.871	0.415	1.60
	6	20.460	21.690	21.020	NT	21.057	0.616	2.93
	7	20.033	20.995	20.595	NT	20.541	0.483	2.35
	8	22.595	22.230	22.070	NT	22.298	0.269	1.21
	9	22.575	22.895	22.470	NT	22.647	0.221	0.98
	10	22.210	22.895	22.510	NT	22.538	0.343	1.52
WSV	Plasmid copy no.							
	2.37×10^5	21.260	21.287	21.327	21.240	21.278	0.037	0.17
	2.37×10^4	25.143	24.897	25.057	25.437	25.133	0.227	0.90
	2.37×10^3	28.860	28.987	28.455	29.373	28.919	0.378	1.31
	2.37×10^2	32.863	32.787	32.247	32.570	32.617	0.276	0.85
	2.37×10^1	35.280	36.307	35.770	35.867	35.806	0.421	1.18
	11.85	36.717	36.600	37.737	37.310	37.091	0.531	1.43
	5.93	38.057	38.053	37.837	38.270	38.054	0.177	0.47
	2.96	38.690	36.670	37.653	37.667	37.670	0.825	2.19
	1.48	39.653	38.677	38.463	39.130	38.981	0.528	1.35
	Genomic DNA sample							
	1	26.023	25.047	25.537	NT	25.536	0.399	1.56
	2	24.007	24.007	24.227	NT	24.080	0.104	0.43
	3	23.983	23.770	23.360	NT	23.704	0.259	1.09
	4	22.867	22.857	23.177	NT	22.967	0.149	0.65
	5	22.167	22.020	22.267	NT	22.151	0.101	0.46
	6	25.857	26.223	26.040	NT	26.040	0.150	0.58
	7	23.960	23.800	23.493	NT	23.751	0.113	0.48
	8	23.007	22.843	22.837	NT	22.896	0.115	0.50
	9	25.030	24.760	30.637	NT	26.809	0.191	0.71
	10	21.723	21.643	21.650	NT	21.672	0.057	0.26
	β-Actin control							
	1	23.140	23.207	23.740	NT	23.362	0.047	0.20
	2	22.983	22.113	22.303	NT	22.467	0.615	2.74
	3	21.880	22.920	23.190	NT	22.663	0.735	3.24
	4	23.130	23.227	23.323	NT	23.227	0.068	0.29
	5	21.023	21.123	21.323	NT	21.157	0.071	0.34
	6	23.710	23.773	24.030	NT	23.838	0.045	0.19
	7	18.923	19.270	19.147	NT	19.113	0.245	1.28
	8	22.403	22.360	22.197	NT	22.320	0.031	0.14
	9	24.040	24.153	27.763	NT	25.319	0.080	0.32
	10	22.290	22.223	22.240	NT	22.251	0.047	0.21

[a] For each sample, the C_T value is the average of results from two to three replicates of the SYBR Green PCR run of that day. NT, not tested.
[b] CV, coefficient of variation.

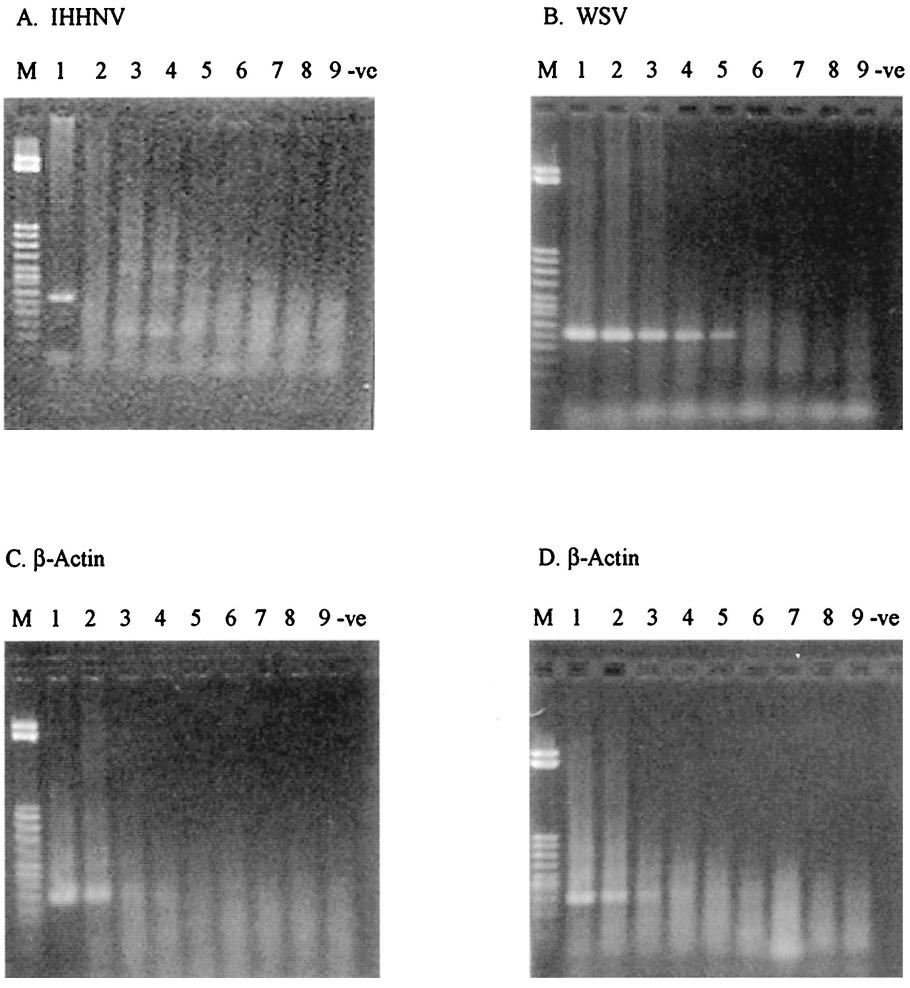

FIG. 2. (A and B) Detection limits for IHHNV (A) and WSV (B) DNAs by conventional PCR. The dilutions of genomic DNAs of IHHNV- and WSV-infected *P. stylirostris* samples were amplified using virus-specific primers. (C and D) β-Actin DNA was amplified from each sample in parallel. The sizes of the amplicons were 329, 306, and 339 bp for IHHNV, WSV, and the β-actin gene, respectively. Lanes 1 to 9 contain 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg, respectively. Lane M, 50-bp DNA step ladder (Sigma); lane −ve, negative control.

sociation curves of each amplicon. Figures 4 and 5 provide examples of amplification profiles and the corresponding dissociation curves of IHHNV, WSV, and β-actin control gene products from virus-infected and healthy shrimp. When amplification was performed using IHHNV-specific primers and DNA from an IHHNV-challenged animal, a significant increase in SYBR Green fluorescence was recorded, with a C_T value of 34.0 (Fig. 4A). When the same IHHNV-specific primers were used with the DNA from a healthy sample, the increase in SYBR Green fluorescence did not exceed the threshold limit, indicating no IHHNV-specific product (Fig. 4A). This observation was supported by the dissociation curve as shown in Fig. 4B. A dissociation curve with a single peak was obtained for the IHHNV-challenged-shrimp samples (melting temperature [T_m] = 76.2°C) but not for the healthy-shrimp samples. To rule out the possibility of variation in levels of input DNAs and the presence of an inhibitor(s) in the DNA sample from the healthy animal, β-actin amplification was performed with the DNA samples from the healthy as well as the IHHNV-challenged animals in parallel in the same 96-well plate. Both the healthy-animal and the IHHNV-challenged-animal DNA samples provided amplifications of their β-actin genes and dissociation curves with an expected T_m, 80.1°C (Fig. 4C and D). This indicates that the absence of amplification in the healthy sample by IHHNV-specific primers was not due to the presence of any inhibitors in the DNA preparation.

Similar observations were recorded for WSV amplification (Fig. 5). The WSV-challenged sample showed a virus-specific product, as exemplified by the amplification profile (C_T = 26) and the corresponding dissociation curve (T_m = 76.2°C) (Fig. 5A and B). DNA from the healthy sample did not provide any WSV-specific amplification (Fig. 5A and B). However, both the healthy-animal and the WSV-challenged-animal samples provided β-actin-specific amplification as shown in Fig. 5C. The dissociation curve (Fig. 5D) revealed that the product had a T_m of 80.3°C, as expected for the β-actin amplicon. For every SYBR Green PCR amplification described in this paper, samples were routinely analyzed by their amplification profile and the dissociation curve to ensure the specificity of the intended product.

Reproducibility of the SYBR Green PCR assay. The reproducibility of SYBR Green PCR was assessed by running the

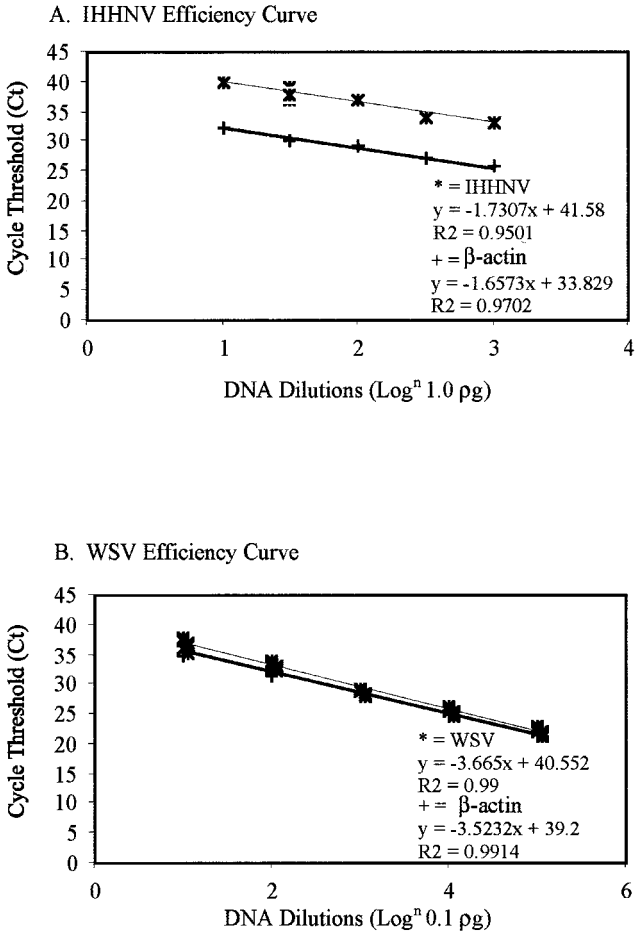

FIG. 3. Detection limits of IHHNV (A) and WSV (B) DNAs by SYBR Green PCR. The β-actin gene was amplified in parallel for each dilution of the IHHNV and WSV samples. There were three to four replicates for each dilution of DNA, and the C_T value of each dilution was used to plot the graph.

samples independently on different days. For each PCR run, samples were replicated two to three times in each 96-well plate. The C_T values of the standard plasmid dilutions as well as the genomic DNAs for both the IHHNV- and WSV-challenged-animal samples were highly reproducible, with a coefficient of variation less than 3.5% (Table 3).

Specificities of the IHHNV and WSV primers used for conventional PCR. IHHNV and WSV were detected in laboratory-challenged *P. stylirostris* using 100 ng of DNA with virus-specific primers (Table 1) and by conventional PCR. A 329-bp DNA was amplified for IHHNV, and a 306-bp DNA was amplified for WSV (Fig. 6). A 339-bp β-actin DNA was amplified in parallel for all samples. Although in the healthy animals neither virus could be detected using the corresponding virus-specific primers, β-actin DNA could be amplified successfully from the healthy-animal DNA samples. This result shows the specificities of the primers used for IHHNV and WSV amplifications.

Determining the viral load in laboratory-challenged IHHNV- and WSV-infected *P. stylirostris* by SYBR Green PCR. To measure the IHHNV and WSV loads in laboratory-challenged *P. stylirostris*, the C_T value of each sample was extrapolated into the standard curve of the corresponding virus. Among the IHHNV-challenged-animals, the viral load varied between 48 and 339 copies/ng of DNA (C_T values varied from 34.54 to 30.97), and among the WSV-challenged-animals, the viral load varied between 8,091 and 272,270 copies/ng of DNA (C_T values varied from 26.81 to 21.67). This suggests that there was considerable variation in the virus level within each group.

DISCUSSION

The management of viral diseases in shrimp aquaculture has been a challenge due to a lack of highly sensitive detection methods. Until very recently, viral detection in shrimp aquaculture was primarily based on biological and histological methods, including in situ hybridization using virus-specific gene probes (15). The detection limits for these methods are low compared to that for PCR, and the process is time-consuming. These challenges have been overcome to some extent over the past few years with the development of screening methods based on conventional PCR technology (15). Both one-step and two-step PCRs have been used with limited success for WSV screening of broodstock animals in quarantine hatchery facilities and of the postlarvae used in stocking ponds (9, 29). Traditional PCR sometimes produces false-negative findings, resulting in a putatively negative animal that served as a reservoir for the virus (9, 29).

Use of a virus-resistant line is an important preventive measure for the management of viral diseases in shrimp. A major obstacle in the development of lines resistant to shrimp viruses is the lack of a quantification method for the virus. With no established cell lines for shrimp, quantification of viruses has been very difficult. Assessment of the severity of infection in resistant versus susceptible lines relies on the observation of clinical signs, percent mortality at the end of an experiment, any detectable changes revealed by histology, or detection of the virus by PCR (15). Such methods do not provide a continuous scale of measurement for viral load and are unlikely to reflect the viral quantity accurately. In this paper, we described a detection and quantification method for two shrimp viruses addressing the concerns discussed above.

A linear relationship between input DNA and C_T values over a range from approximately 10^5 copies down to the equivalent of a single copy of the viral genome was detected by SYBR Green PCR (Fig. 1). Detection of viruses over such a wide dynamic range is very useful for measuring viral loads in animals with various levels of infection. The detection limits of conventional PCR were 23.7 and 188 copies of the WSV and IHHNV genomes, respectively, using plasmid DNAs as the templates. Lo and Kou (17) reported that 20 copies of the target WSV plasmid DNA could be detected by two-step PCR. Thus, the detection limit of conventional PCR was found to be well below (approximately 20 to 200 times below) that of SYBR Green PCR, which has been shown to routinely detect a single copy of either virus.

When dilution series of IHHNV- and WSV-infected genomic DNAs were used for conventional PCR, successful amplification could be obtained with 100 ng of DNA for IHHNV and 10 pg for WSV (Fig. 2). Such a variation in the detection limits between two viruses may be due to the differences in the pathogenesis of the viruses. In addition, Super Shrimp *P. sty-*

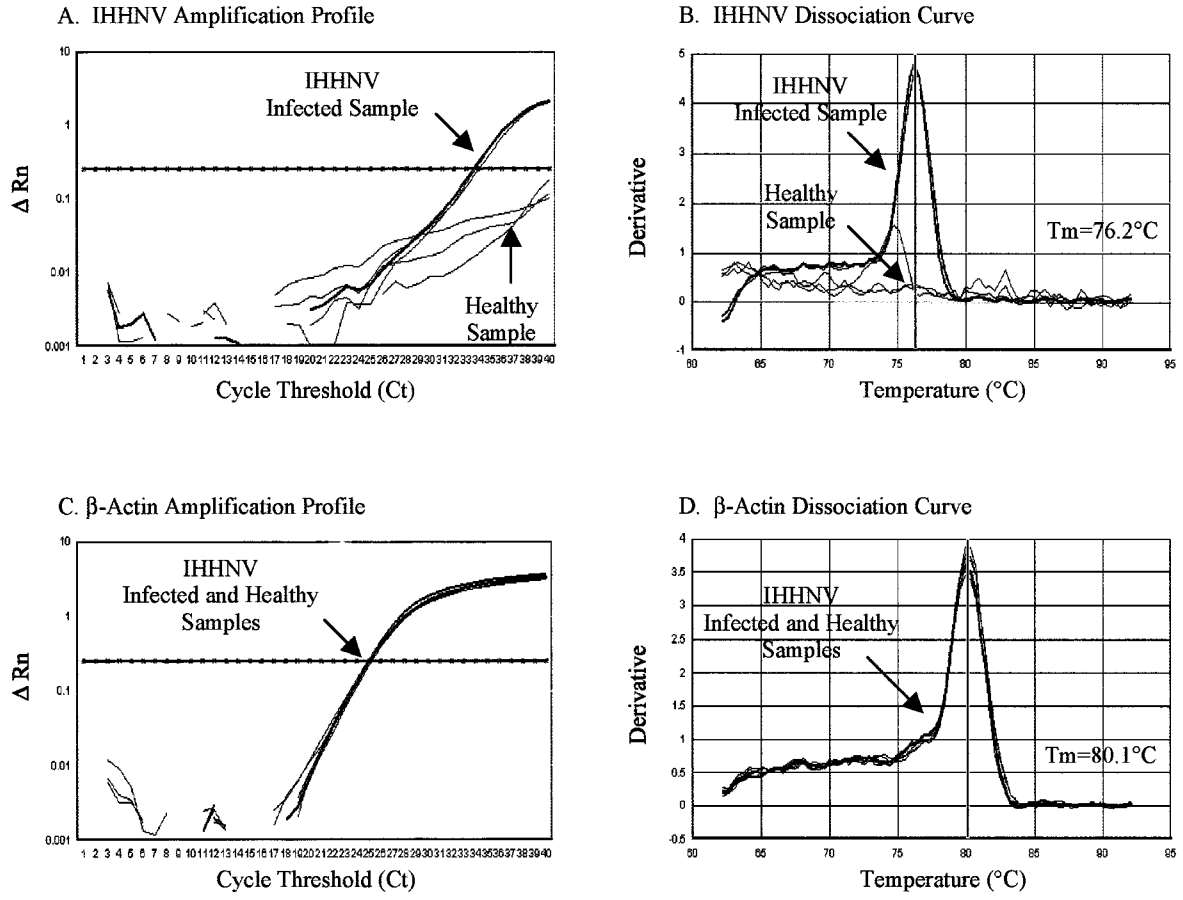

FIG. 4. Amplification profiles (A and C) and dissociation curves (B and D) of IHHNV-challenged-and healthy-*P. stylirostris* samples. Genomic DNAs of IHHNV-challenged- and healthy-*P. stylirostris* samples were amplified using IHHNV-specific (A) and β-actin-specific (C) primers by SYBR Green PCR. The dissociation curves for IHHNV and β-actin products are shown in panels B and D, respectively. The T_m values of IHHNV and β-actin amplicons are indicated alongside their corresponding dissociation curves.

lirostris stocks are resistant to IHHNV (26) but not to WSV infection. We observed a difference in the limits of detection of β-actin in IHHNV- and WSV-infected samples by regular PCR. For IHHNV samples, the detection limit for β-actin was 100 pg, whereas for WSV samples, the detection limit for β-actin was 10 pg. It is unknown at this time if the lower limit of detection of the β-actin gene in the IHHNV samples was due to some PCR inhibitors or because IHHNV infection may reduce the actin level in tissue.

The detection limit of SYBR Green PCR using genomic DNA was found to be 50 pg for IHHNV and 0.1 pg for WSV (Fig. 3). Compared to conventional PCR, these values indicate that SYBR Green PCR was 100-fold (10 pg versus 0.1 pg for WSV) to 2,000-fold (50 pg versus 100 ng for IHHNV) more sensitive. With plasmid DNA as the template, SYBR Green PCR was found to be approximately 20-fold (for WSV) to 200-fold (for IHHNV) more sensitive than conventional PCR. Therefore, there is a 5-fold (for IHHNV) to 10-fold (WSV) difference between results with plasmid and genomic DNA templates in a comparison of the sensitivities of conventional and SYBR Green PCRs. This may be due to the nature of the template (plasmid versus genomic DNA) used for the PCR. Spiking the healthy tissue homogenate with a known amount of IHHNV or WSV plasmid DNA and then extracting DNA to SYBR Green PCR may help to determine the cause of this difference.

The high sensitivity of SYBR Green PCR was found to be accompanied by its high specificity as well. Since SYBR Green dye can bind indiscriminately to dsDNA, identification of an intended product was achieved by monitoring its dissociation curve (Fig. 4 and 5). A single dissociation peak with a T_m within a variation of 2°C of the expected temperature suggests an amplification of a specific product (22). This eliminates the need to check the PCR product by gel electrophoresis, thereby significantly reducing the assay time and allowing the screening of a large number of samples in a relatively short time. When IHHNV- and WSV-infected samples along with control samples from healthy animals were tested by SYBR Green PCR using virus-specific primers, a dissociation curve with a single peak at the expected temperature was obtained only with the DNAs isolated from the virus-infected animals and not with the DNAs from healthy control animals (Fig. 4 and 5). When shrimp β-actin primers were used, DNAs from both virus-infected and healthy animals provided dissociation curves with a single peak at expected temperatures (Fig. 4 and 5). This result demonstrated the integrity of the DNA used for the amplification as well as the specificities of the virus-specific primers used for SYBR Green PCR. In recent years, a real-

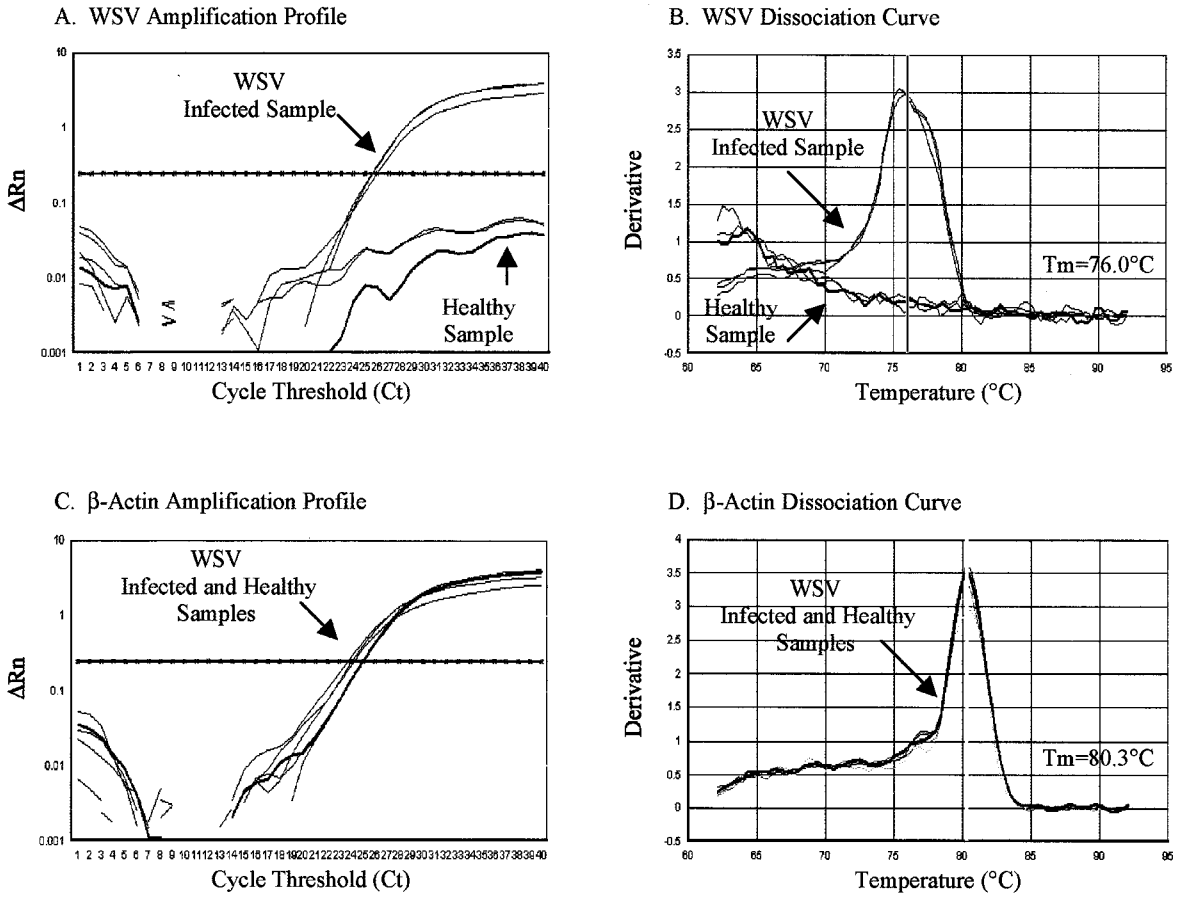

FIG. 5. Amplification profiles (A and C) and corresponding dissociation curves (B and D) for WSV and the β-actin gene amplified using genomic DNAs of WSV-challenged- and healthy-*P. stylirostris* samples. The T_m values of WSV and β-actin amplicons are indicated next to the dissociation curve of the corresponding gene product.

time PCR based on TaqMan chemistry has been used for the detection of both plant and animal viruses (14, 20, 23). The high specificity of TaqMan PCR is achieved by a target-specific dually labeled fluorogenic probe that is cleaved during polymerase extension by 5′-exonuclease activity (8). Unlike TaqMan PCR, SYBR Green PCR does not need any additional probe since the specificity of the product is monitored by analyzing the dissociation curve of the amplicon. However, in TaqMan PCR multiple targets can be amplified simultaneously using different fluorogenic probes, which is not possible with SYBR Green PCR.

Recently, a competitive quantitative PCR has been described for the quantification of WSV (26). The sensitivity of the competitive quantitative PCR was found to be 1.5 times less than that of SYBR Green PCR (see discussion in reference 26). In addition, competitive quantitative PCR is much more time-consuming, more labor-intensive, and less robust than SYBR Green PCR, although SYBR Green PCR is more expensive than competitive quantitative PCR.

The IHHNV and WSV viral loads among the samples tested varied considerably. The low levels of IHHNV DNA (48 to 339 copies/ng of shrimp DNA) suggests that the virus replicates at a very low level in Super Shrimp stocks of *P. stylirostris*. A similar finding was recently reported by Tang et al. (26). However, Super Shrimp stocks of *P. stylirostris* are highly susceptible to WSV, as indicated by the high viral loads (8,091 to 272,270 copies/ng of DNA) in the samples tested. The variation in the viral load may be due to the difference in individual host responses to WSV infection. Although a high viral load was detected in the WSV-infected animals, none of the animals showed any clinical sign of WSV infection. This finding suggests that SYBR Green PCR will be useful not only to detect a single copy of the viral genome but also to detect subclinical infection.

The high sensitivity of any detection method such as SYBR Green PCR is potentially susceptible to carryover contamination. Routine laboratory hygiene such as separation of a PCR laboratory from nucleic acid extraction and other processing facilities improved PCR reagent handling (aliquoting reagents), and the use of aerosol barrier pipette tips enabled us to eliminate any major contamination problem confounding our results. Since the protocol described here does not need any post-PCR steps such as gel electrophoresis, it significantly reduces the PCR carryover contamination problem. The total time needed to complete an SYBR Green PCR run from the time a 96-well plate is put into a thermocycler is 2 h 15 min. After the amplification, the data analyses take approximately 15 min. Thus, as little as 2.5 h is needed to complete an SYBR Green PCR run and subsequent data analyses. In a 96-well plate, for each sample, with two replicates for the virus and the

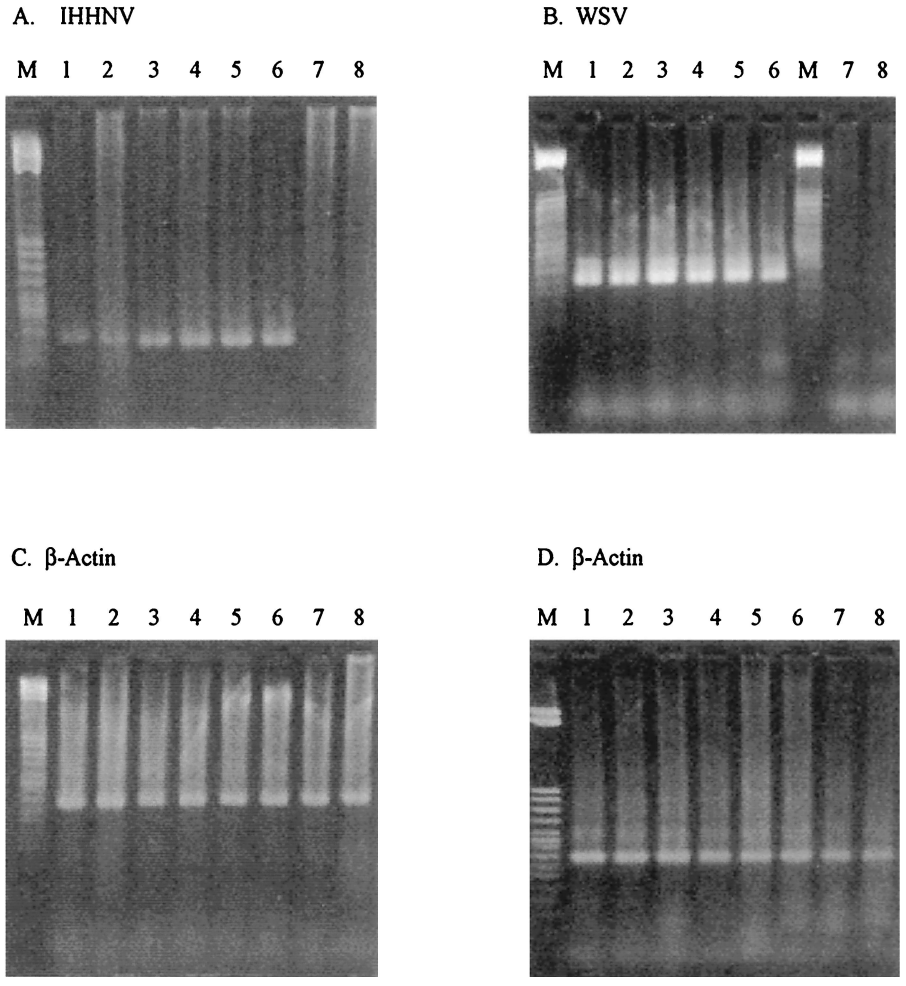

FIG. 6. PCR amplification of IHHNV (A)- and WSV (B)-specific DNA in laboratory-challenged-*P. stylirostris* samples. (C and D) β-Actin amplifications for the samples used for panels A and B. The sizes of the amplified DNAs of IHHNV, WSV, and the β-actin gene were 329, 306, and 339 bp-, respectively. In each panel, lanes 1 to 6 contain DNAs from virus-challenged samples and lanes 7 and 8 contain DNAs from healthy samples. Lane M, 50-bp DNA step ladder (Sigma).

corresponding β-actin control and two positive and two negative controls for the virus and β-actin reaction mixtures, 22 samples could be accommodated in a plate (22 × 2 [virus detection] + 22 × 2 [β-actin detection] + 2 [virus-positive control] + 2 [β-actin-positive control] + 2 [virus-negative control] + 2 [β-actin-negative control] = 96). This SYBR Green PCR saves considerable time over conventional PCR, making it a suitable method for high-throughput screening.

The rapid expansion of shrimp aquaculture industries over the last few decades has coincided with the emergence of new viral pathogens which were previously unknown to shrimp farming. In addition, there have been considerable movements of live and frozen shrimp from one country to another, increasing the risk of spread of viral diseases into the importing country. One of the important ways of preventing a viral epidemic in shrimp is the use of virus-free broodstock and postlarvae for commercial farming. In recent years, conventional two-step PCR has been used to screen broodstock and postlarvae before stocking of the ponds (9). Even two-step PCR often provides false-negative results because the virus level in the sample is beyond the limits of sensitivity of conventional PCR (9, 17). This has created a growing and an urgent need to develop a better detection method that has higher sensitivity than that of conventional PCR and that at the same time is rapid and reliable. The results described in this paper clearly established the applicability of SYBR Green PCR for the detection and quantification of shrimp viruses because of its exquisite sensitivity and high-throughput potential, which are beyond the limits of conventional PCR.

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