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Aquaculture 189 (2000) 11–21

Aquaculture

www.elsevier.nl/locate/aqua-online

Quantification of white spot syndrome virus DNA through a competitive polymerase chain reaction

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Received 3 October 1999; received in revised form 1 March 2000; accepted 10 March 2000

Abstract

A competitive polymerase chain reaction (PCR) method for quantification of white spot syndrome virus (WSSV) genome was developed. A pair of WSSV primers, designated WSSV341F/R, was selected to amplify a 341-bp DNA fragment from the WSSV genome. For a competitive internal standard, we constructed and cloned a 289-bp DNA fragment, the result of a 52-bp deletion from the 341-bp amplicon. In a competitive PCR reaction, we co-amplified the target WSSV DNA with known concentrations of the internal standard using WSSV341F/R primers. The amplicons from WSSV DNA and from internal standard DNA differed in size and could be distinguished after gel electrophoresis. The concentration of WSSV genomes was determined from its relation to the concentration of the internal standard. The log–log plot of the ratio of the amplicons (internal standard: WSSV) on the internal standard concentration was linear. Using this competitive PCR procedure, we quantified WSSV DNA in the samples of hemolymph and tissues of the cephalothorax of individual WSSV-infected shrimp. The number of WSSV genomes in both hemolymph and tissues corresponded to the severity of infection determined by histological evaluation. In addition, the changes in number of WSSV genomes in the hemolymph during the course of the infection were determined.

Keywords: White spot syndrome virus; Competitive PCR; Penaeid shrimp

1. Introduction

White spot syndrome virus (WSSV) has caused high mortality in many species of penaeid shrimp and other aquatic crustaceans (Lightner, 1996; Lo et al., 1996a; Maeda

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0044-8486/00/$ - see front matter
PII: S0044-8486(00)00367-7

et al., 1998) throughout the world. Disease attributed to this virus was first observed in east Asia during 1992–1993 (Huang et al., 1994; Chen, 1995; Inouye et al., 1994; Nakano et al., 1994). The disease spread very rapidly, and by 1996 it had severely impacted most of the shrimp farming regions of southeastern Asia (Flegel, 1997) including Japan, Thailand, Taiwan, China, India and Korea (Inouye et al., 1994; Takahashi et al., 1994; Wongteerasupaya et al., 1995; Huang et al., 1995; Hameed et al., 1998; Park et al., 1998). Subsequently, WSSV spread to many countries of the western hemisphere. In the United States, WSSV was first recorded in Texas in 1995 (Lightner, 1996); and it was found in South Carolina in 1997 and 1998 (Lightner, 1999). Recently, WSSV was detected in Central and South America (Jory and Dixon, 1999).

The clinical signs of WSSV disease, in many penaeids, are the appearance of white spots inside the carapace and a reddish discoloration of the body. Histological analysis reveals distinct hypertrophied nuclei in the cuticular epithelial cells, connective tissue cells, and hemocytes (Lightner, 1996).

WSSV is an enveloped, rod-shaped virus, containing double-stranded DNA (Wang et al., 1995; Wongteerasupaya et al., 1995). It is large, 70–150 nm × 275–380 nm (Wang et al., 1995; Durand et al., 1996), and extremely virulent; mortalities in affected shrimp stocks can reach 100% within 3–10 days (Lightner, 1996).

The number of viral genomes in the infected animals has become one of the most important parameters used to monitor the progression of the disease. However, quantification of WSSV has been hampered by the lack of a continuous cell culture system for shrimp. The recent development of the quantitative competitive polymerase chain reaction (PCR) circumvents this obstacle and provides an attractive tool to quantify the number of WSSV genomes in individual shrimp.

The competitive PCR was developed by incorporating an internal standard into each PCR tube. This internal standard is co-amplified with a specific target sequence, both using the same pair of primers. Quantification is obtained by comparing the quantities of the amplicon of the specific target sequence with those of the amplicon from the internal standard at known concentrations (Wang et al., 1989; Gilliland et al., 1990). This method has been applied in the quantification of many important viruses, such as human immunodeficiency virus (HIV) and hepatitis B virus (HBV) (Piatak et al., 1993a; Chen et al., 1995), in monitoring viral replication and response to antiviral treatments.

In this report, we describe the development of a WSSV competitive PCR and the application of this method in analyzing the changes in the number of WSSV genomes in the hemolymph and tissues during the progression of white spot disease.

2. Materials and methods

2.1. WSSV isolate

The WSSV used in this study was obtained from infected *Penaeus monodon* collected in Thailand in 1995. The virus has been maintained at the University of Arizona by re-infection of SPF (specific pathogen free) *P. vannamei*. The inoculum was prepared from a homogenate of infected tissues in TN buffer (0.02 M Tris–HCl, pH 7.2,

0.4 M NaCl). The tissue homogenate was then diluted with 2% NaCl and injected into tail muscle of healthy SPF *P. vannamei* (average weight: 1 g). At various time points after injection, hemolymph was collected from individual shrimp. The heads were each sectioned longitudinally in half, one half was fixed in Davidson's AFA fixative (Lightner, 1996), and the other was stored at −70°C for later DNA extraction.

2.2. Construction of competitive internal standard for WSSV

The WSSV primers (WSSV341F/R) used in this study were chosen from sequence data of WSSV clone A68 (Dr. S. Durand, University of Arizona, Tucson, USA, unpublished data). The primers amplified a 341-bp amplicon from the WSSV genome. The sequences of primer WSSV341F is 5′-TGGCTACATCTGCATTGCTC-3′, and that of primer WSSV341R is 5′-TAGAGACGTGGCTGGAGAGG-3′. A WSSV DNA competitor was constructed that was 52-bp shorter than the 341-bp amplicon; it could be amplified with the same PCR primers. This deletion mutant was constructed using the procedures described by Celi et al. (1993). This 289-bp DNA fragment was cloned into pGem T-easy vector (Promega, Wisconsin, USA) in accordance with standard procedures. The recombinant was sequenced, which showed that it only contained one copy of the insert with the WSSV341F and WSSV341R at each end. The recombinant plasmid was purified, quantified spectrophotometrically, and serially diluted with H_2O for use as an internal standard in the competitive PCR.

2.3. DNA extraction

Total DNA was extracted, from shrimp hemolymph and tissues, with a High Pure™ PCR template preparation kit (Roche-Boehringer Mannheim, Germany).

2.4. PCR amplification

For multiplex PCR detection of WSSV DNA and shrimp 18S rRNA DNA, the PCR reaction mixture included: 10 mM Tris–HCl, pH 9.0, 50 mM KCl, 0.1% Triton ® X-100, 200 μM dNTP, 2 mM $MgCl_2$, 0.3 μM WSSV primers (WSSV341F/R), 0.45 μM 18S rRNA primers (18S441F: 5′-TTGTACGAGGATCGAGTGGA-3′; 18S441R: 5′-ATGCT-TTCGCAGTAGGTCGT-3′; primers were designed from a shrimp 18S rRNA DNA sequence available in GenBank AF186250), extracted DNA (2 μl), and 1.3 U of Taq DNA polymerase (Promega, Wisconsin, USA). Amplification was performed in a DNA thermocycler (GeneAmp 480, Perkin-Elmer, New Jersey, USA) with the following cycle parameters: initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C, 30 s, 52°C, 30 s, and 68°C, 1.5 min, and a final extension at 72°C for 7 min. This PCR reaction generated two amplicons, 341-bp from WSSV genome, and 441-bp from shrimp 18S rRNA DNA.

For WSSV competitive PCR, the reaction mixture contained: 10 mM Tris–HCl, pH 9.0, 50 mM KCl, 0.1% Triton ® X-100, 200 μM each dNTP, 1.5 mM $MgCl_2$, 0.3 μM WSSV primers (WSSV341F/R), extracted DNA (1 μl), and 0.625 U Taq DNA polymerase. Serial dilutions of the internal standard were added to the corresponding

tubes, and the PCR amplification was carried out as: initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C, 30 s, 55°C, 30 s, and 72°C, 1 min, and a final extension at 72°C for 7 min. Following this, an aliquot of the PCR products was analyzed in a 2% agarose gel electrophoresis stained with ethidium bromide and then photographed. The PCR reaction generated two DNA fragments, a 341-bp WSSV amplicon and a 289-bp internal standard amplicon. The gel photos were digitized with a scanner. We quantified the digitized images through densitometric analysis using the Whole Band Analysis program (Genomic Solution, Michigan, USA). Densitometric readings were adjusted by multiplying reading of the internal standard amplicon by size of 341-bp/size of 289-bp. The amount of WSSV DNA was calculated by determining the equivalence point in a logarithmic linear regression plot (Piatak et al., 1993b).

3. Results

3.1. Selection of WSSV-specific primers and construction of internal standard

We found that primers (WSSV341F/R), selected from a portion of the WSSV genomic sequence, could be used to amplify various WSSV isolates in PCR reactions. A 341-bp amplicon was amplified from DNA extracted from each of the six WSSV isolates (Thailand, China, India, US-South Carolina, US-Texas, US-crayfish) (data not shown). This amplicon was not generated when the genomic DNA of un-infected shrimp was used as a template. Therefore, these two primers appeared to be specific and conserved in the WSSV genome, accordingly we used this pair for the development of WSSV competitive PCR analysis. Subsequently, a WSSV competitor was constructed that had WSSV341F and WSSV341R primers at each end and a 52-bp deletion, from the original WSSV genomic sequence, upstream of the WSSV341R primer. The competitor was cloned and used as an internal standard in the competitive PCR. The amplicons of WSSV DNA and internal standard DNA can be distinguished on the basis of the size.

3.2. Competitive PCR

To determine the number of WSSV genomes in infected shrimp, we conducted a preliminary estimation of the target WSSV DNA concentration with a 10-fold dilution (10^1–10^7 copies) of the internal standard in a competitive PCR. We found that the concentration of WSSV genome in hemolymph drawn from infected shrimp to be 10^5–10^6 copies/μl hemolymph (data not shown). Then, we used two-fold serial dilutions of internal standard amplified together with constant aliquots (1 μl) of hemolymph. The PCR products were then resolved on an agarose gel (Fig.1A). Apparently, both WSSV DNA and the internal standard recognized the primers (WSSV341F/R). Two bands were detected, the larger amplicon (341-bp) from WSSV genome and the smaller amplicon (289-bp) from the internal standard. In addition, WSSV amplicon concentration progressively increased as the concentration of internal standard DNA decreased, indicating that the internal standard, indeed, competed with the WSSV DNA.

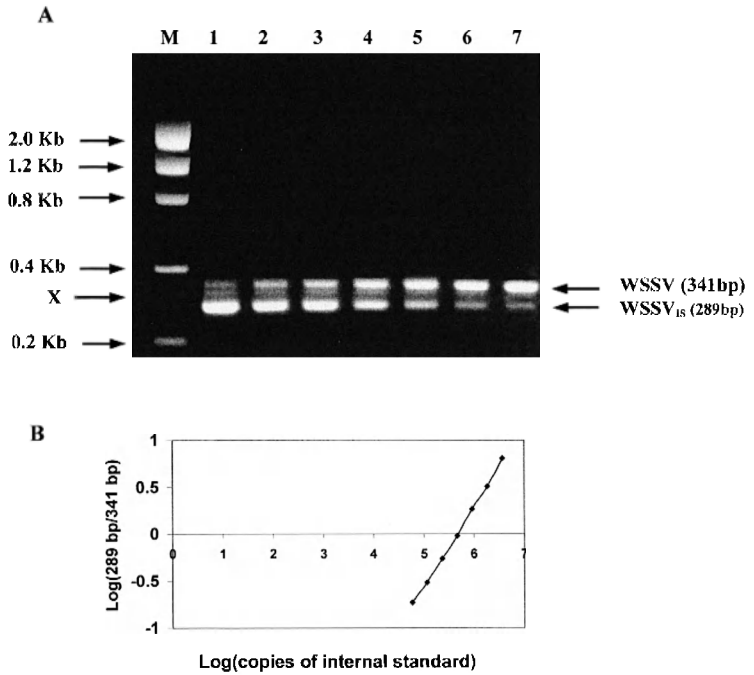

Fig. 1. Determination of WSSV DNA in hemolymph collected from WSSV-infected shrimp. (A) A two-fold serial dilution (3.6×10^6 copies in Lane 1 to 5.7×10^4 copies in Lane 7) of internal standard was amplified with WSSV341F/R primers together with aliquots (1 μl) of hemolymph in a WSSV competitive PCR as described in Materials and methods. (B) Relative intensities of the bands were densitometrically determined, and the logarithm of their ratios was plotted as a function of the logarithm of the amount of internal standard added. M: 1 Kb ladder marker. X: heteroduplex generated from WSSV and $WSSV_{IS}$.

A third band (labeled as "x" in Fig. 1A) was observed in the gel, between the WSSV amplicon and the internal standard amplicon. It is likely to be a heteroduplex. Since the internal standard amplicon was only 52-bp deletion from WSSV amplicon, its 289-bp nucleotides are identical to the WSSV amplicon, a heteroduplex could be formed. This, we presume, would incorporate a 1:1 ratio of DNA strands from the amplicons of WSSV genome and the internal standard, so that the internal standard and the WSSV amplicons were identical in concentration. This being the case, the heteroduplex formation would not adversely affect the quantification. Since equal concentrations of WSSV amplicon and internal standard amplicon would be removed, the ratio of the two amplicons would not be distorted.

Intensities of the bands from the 289-bp internal standard amplicon and the 341-bp WSSV amplicon were densitometrically quantified by computer imaging and their ratios were expressed as arbitrary units. A 10-base logarithm of this relative intensity (289-bp/341-bp) was plotted against the 10-base logarithm of the amount of internal standard added. The plot of this log–log relation was linear between 5.7×10^4 and 3.6×10^6 copies of internal standard as shown in Fig. 1B. This plot was used to determine the equivalence point (i.e. the point where the logarithm of the ratio of 289 bp amplicon to

341 bp amplicon is equal to 0). The amount of WSSV DNA was equal to the amount of internal standard in the initial sample, which in this case was 4.57×10^5 copies of WSSV genomes in 1 μl of hemolymph. Samples of WSSV DNA were diluted or concentrated as required such that the DNA concentrations fell within the range of the relationship shown in Fig. 1B.

3.3. Relationship between the number of WSSV genomes and severity of white spot disease

To determine if there was a correlation between severity of infection and the number of WSSV genomes in infected shrimp, samples of the hemolymph were drawn for WSSV quantification at 24 and 35 h post-injection. For each shrimp, the head was removed; one half was used to determine the number of WSSV genomes in the tissues, and the other was fixed and examined histologically to determine the severity of the WSSV infection.

To determine if shrimp were infected with WSSV, a standard PCR assay for WSSV using WSSV341F/R primers was conducted. We detected a 341-bp amplicon using total DNA extracted from both hemolymph and tissues collected at both 24 and 35 h post-injection (data not shown). We quantified the WSSV genomes with a competitive PCR and found that the number of WSSV genomes was higher in tissues than in the hemolymph at both 24 and 35 h post-injection. At 24 h post-injection, the number of WSSV genome in the tissue was about twice that in the hemolymph, as indicated in Table 1. At 35 h post-injection, the WSSV in the tissue increased to 49 times that in the hemolymph. Comparing the rate of increase in WSSV genome between tissues and hemolymph, the WSSV in the hemolymph increased six times from 24 to 35 h post-injection, while the WSSV in the tissues increased 140 times from 24 to 35 h post-injection. The histological analysis of WSSV-infected shrimp in both 24 and 35 h post-injected shrimp displayed characteristic changes associated with the disease. Hypertrophic nuclei were found in cells of the cuticular epidermis and gills as well as in the connective tissues of the lymphoid organ, antennal gland, haematopoietic organ, and nervous system. The severity of infection of shrimp at 24 h post-injection was between G2 and G3 (Lightner, 1996). The shrimp at 35 h post-injection showed more severe infection and was graded as G4.

Table 1
Number of WSSV genomes and severity of WSSV infection in the *P. vannamei*. The shrimp were injected with a tissue homogenate containing 2.9×10^4 WSSV genomes/μl. At both 24 and 35 h after injection, hemolymph samples were drawn. Half of the head tissue was fixed in the Davidson's fixative for histological diagnosis, the other half was used for total DNA extraction. The number of WSSV genomes was determined by competitive PCR, the severity of WSSV infection was determined by histological analysis. Severity grades are: G1: mild, G2: mild to moderate, G3: moderate to severe, and G4: severe

	WSSV/μg hemolymph[a]	WSSV/μg tissue	Severity
24-h PI	6.4×10^2	1.4×10^3	G2–G3
35-h PI	3.9×10^3	2.0×10^5	G4

[a]As estimated that 1 μl of hemolymph weighs 1.04 mg.

3.4. The quantities of WSSV genome in the hemolymph during the infection

To determine the number of WSSV genomes in the infected shrimp during the progression of disease, a time-course experiment was conducted. SPF shrimp were injected with tissue homogenate containing 1.15×10^5 WSSV genome per μl, 30 μl of inoculum was injected to each shrimp. Assuming a shrimp hemolymph volume of 300 μl, the resulting WSSV concentration should be approximately 1.15×10^4 genomes per μl. At various time points (9, 14, 19, 22, 25, 28, 32, 36, and 52 h post-injection), five shrimp were collected for WSSV competitive PCR. We found that the infected shrimp appeared not to take any feed at the 22 h post-injection time, and became moribund at 35–60 h following injection. The total DNA was extracted from both hemolymph and tissues in the cephalothorax of each shrimp. Our preliminary quantification with competitive PCR showed that the number of WSSV genomes varied significantly among individual shrimp, therefore, all five DNA samples were combined for quantification at each time point.

To determine the quality of total DNA extracted from shrimp, we employed a multiplex PCR detection for both WSSV and shrimp 18S rRNA DNA. As seen in Fig. 2A and B, two amplicons were generated, the larger amplicon (441-bp) was from shrimp 18S rRNA DNA, and the smaller amplicon (341-bp) was from WSSV genome. The presence of the WSSV genome in hemolymph was detected at 9 h post-injection, increased rapidly by 14 h post-injection, with only a small increase from 14 to 52 h

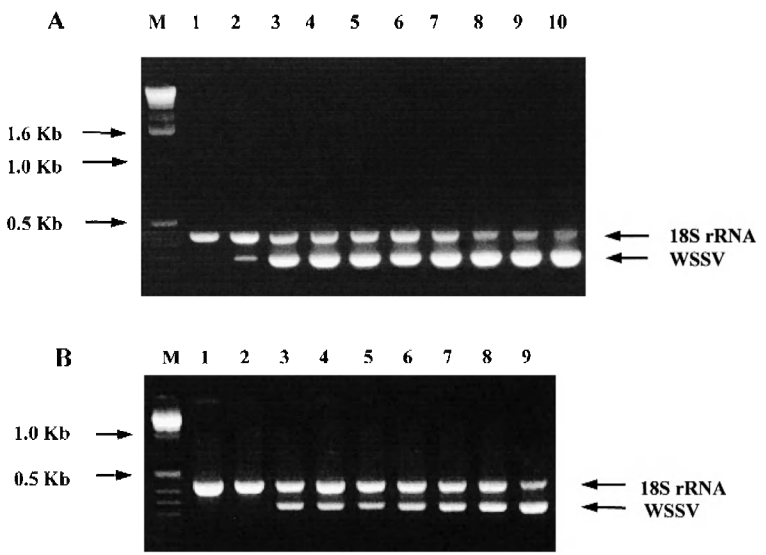

Fig. 2. Multiplex PCR detections of WSSV DNA and shrimp 18s rRNA DNA at various times after injection with WSSV. Aliquots of PCR product were analyzed on a 2% agarose gel. Lane M: 1 Kb ladder marker; Lane 1: un-injected shrimp; Lane 2: 9 h after injection; Lane 3: 14 h after injection; Lane 4: 19 h after injection; Lane 5: 22 h after injection; Lane 6: 25 h after injection; Lane 7: 28 h after injection; Lane 8: 32 h after injection; Lane 9: 36 h after injection; Lane 10: 52 h after injection. (A) Total DNA extracted from WSSV-infected shrimp hemolymph. (B) Total DNA extracted form WSSV-infected shrimp tissues.

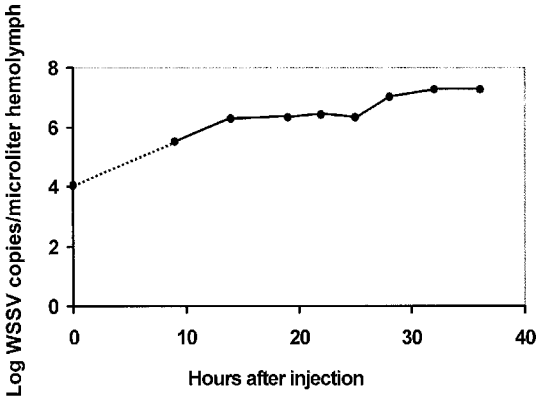

Fig. 3. The number of WSSV genomes in the hemolymph during the infection. The quantities of WSSV genomes were determined by competitive PCR. Dotted portion is approximated from number of WSSV genomes injected.

post-injection (Fig. 2A). On the contrary, the level of shrimp 18S rRNA DNA remained steady until 28 h post-injection then decreased as the infection progressed (Fig. 2A). In the tissues, changes in 18S rRNA DNA were not significant during the first 32 h post-injection. WSSV DNA was not detected until 14 h post-injection, after which it gradually increased (Fig. 2B). The number of WSSV genomes in hemolymph was quantified over the course of the infection (Fig. 3); the hemolymph contained 10^5 copies of WSSV genomes per μl early in the infection, and over 10^7 copies after 28 h post-injection. The rate of increase in the number of viral genome was greatest during the first 14 h of the infection.

4. Discussion

PCR has become the preferred method for measuring the exact number of DNA molecules, because it exceeds the sensitivity limits of classical methods such as Southern blot or dot blot hybridization. Here, we developed a competitive PCR assay for quantification of the WSSV genome. This method is based on competitive co-amplification of a target WSSV genome together with known concentrations of an internal standard in a one-tube reaction. The development of this assay was comprised of: (1) selection of a pair of conserved, specific WSSV primers, (2) generation of an internal standard for competing with WSSV DNA, and (3) establishment of a linear relation between the log of the standard/target ratio (internal standard amplicon/WSSV amplicon) and the log of copies of the internal standard (Fig. 1).

We applied this procedure in determining the number of WSSV genomes in infected shrimp. Our data indicated that there was a positive correlation between high viral load and severity of disease as determined by histological evaluation (Table 1). However, the grades of severity may not directly reflect the quantities of virus in the infected tissues. The number of WSSV genomes increased over 100-fold between severity levels G2–G3

and G4. While histological grading is not highly quantitative and may vary between diagnostic technicians, the competitive PCR is sensitive, quantifiable, and free from subjective bias.

Using competitive PCR, we also showed that the quantities of WSSV genomes in hemolymph were generally much lower than that in tissues, specifically, 49 times less at 35 h after injection. This is likely to be due to the higher cell mass in the tissues relative to the hemolymph. Therefore, for generation of large quantities of WSSV, the use of infected tissues as source may be preferable to hemolymph.

Time-course studies of WSSV were carried out to determine the temporal pattern of viral growth. The competitive PCR was used to quantify the WSSV genomes in hemolymph, and showed that the quantities of WSSV varied in the shrimp within each of time points, indicating that individual shrimp within a population responded differently to WSSV infection. The shrimp used in these studies were all small juveniles (average weight, 1 g). Whether or not the phenomenon of variation of WSSV quantities between individual shrimp can be extrapolated to mature shrimp remains to be seen. The WSSV appeared to accumulate at a higher rate in the early stages of infection, it took 1.9 h for the number of WSSV genomes to double during the first 14 h, and then doubling time increased to 6.9 h from 14 to 36 h post-injection.

A multiplex PCR detections of WSSV and shrimp 18S rRNA DNAs were carried out during time-course studies. We intended to use shrimp 18S rRNA DNA as a quality control for WSSV DNA extraction (Lo et al., 1996b). The data showed that the quantities of 18S rRNA DNA extracted from either hemolymph or tissues decreased during the later stages of infection. This is more evident for the hemolymph DNA, but not so obvious for the tissue DNA (Fig. 2A and B). This may be because the cephalothorax contains cell types that are not infected by WSSV, so the 18S rRNA DNA content would not be affected. On the other hand, hemocytes are the only cells in the hemolymph, and are one of the primary target cell types for WSSV infection. The use of shrimp 18s rRNA DNA as a quality and quantity control for assessing the preparation of WSSV DNA may be limited to early stages of WSSV infection. In the later stages, shrimp 18S rRNA DNA is degraded as a result of the disintegration of infected cells, and a low quantity of 18S rRNA DNA is not necessarily indicative of a bad preparation of WSSV DNA.

Through collaboration with Dr. Klimpel (Super Shrimp, California, USA), we also quantified four WSSV DNA samples with real-time PCR and SYBR-Green detection using a GeneAmp 5700 (PE Applied Biosystems, California, USA). The data indicated that the copy number generated from real-time PCR was about 1.5 times that of the competitive PCR we developed. This may be due either to a smaller WSSV amplicon that were amplified with a real-time PCR or to the higher detection sensitivity with a charge-coupled device (CCD) camera. The quantification with real-time PCR is attractive because of its rapid turnaround, however, this instrument is quite expensive.

In conclusion, we have developed a competitive PCR for quantification of WSSV DNA. This provides a sensitive tool for quantification of viral genomes. Applications include the assessment of disease progression of infected shrimp, determination of shrimp that are resistant to WSSV infection, and studies focused on the development of treatments for white spot disease.

Acknowledgements

This work was supported by the Gulf Coast Research Laboratory Consortium Marine Shrimp Farming Program, CSRS, USDA, under Grant no. 88-38808-3320, and a special grant from National Fishery Institute, Washington, DC. We thank Rita Redman for the preparation and processing of the histological sections, Qiong Wang for providing purified WSSV isolates, Jian-Ling Zhou for assistance in plasmid preparation and PCRs, and Carlos Pantoja for histological analysis.

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