

Efficiency and sensitivity determination of Shrimple[®], an immunochromatographic assay for white spot syndrome virus (WSSV), using quantitative real-time PCR

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Abstract

White spot syndrome virus (WSSV) is a prevalent and virulent pathogen affecting both wild and cultured penaeid shrimp worldwide. Molecular diagnostic tools have made detection of the virus increasingly accurate. However, these techniques are often not readily available for rapid diagnosis in the field or in shrimp production facilities. Shrimple[®], an immunochromatographic detection assay for WSSV, was designed specifically for use by shrimp producers. In this study, WSSV-infected shrimp were tested with both real-time PCR and Shrimple[®], in order to determine the range of sensitivity in which the diagnostic test kit is capable of detecting viral infection and the efficiency of the test kit when compared to the real-time PCR. *Litopenaeus vannamei* were injected with a WSSV inoculum and sampled from 1 to 32 h post injection (p.i.), prior to developing gross anatomical signs of disease. By analyzing the corresponding samples from each specimen, the Shrimple[®] test results were correlated with estimated viral copy numbers from quantitative PCR. Real-time PCR detected infections in 100% of the inoculated shrimp, while the Shrimple[®] test kits detected infection in only 34.7% of the specimens. The findings of this study indicate that the Shrimple[®] test kits fail to detect WSSV infection prior to 12 h post infection and demonstrate a significant reduction in detection efficiency during early onset of infection—failing to detect any viral infection from 1 to 8 h p.i. compared to 100% with real-time PCR. False negative results were observed for specimens containing 4–1061 viral copies/ng genomic DNA. Faint positives were observed for specimens containing 36–1784 viral copies/ng genomic DNA. Although considerably less sensitive than real-time PCR, the Shrimple[®] test kits provide a useful tool for the detection of WSSV infections prior to development of gross signs of acute disease.

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1. Introduction

White spot syndrome virus (WSSV) was identified by following the mortality events on east Asian shrimp

farms during 1992–1993 (Huang et al., 1994; Nakano et al., 1994; Zhan et al., 1998) and it has since become one of the most serious causes of disease in cultivated shrimp. Cumulative mortalities from white spot disease (WSD) can reach 100% within 5 to 7 days (Chou et al., 1995) and economic losses have been estimated at nearly US\$1 billion per year since 1994 (Lightner et al., 1998). In recent years, numerous studies have reported

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the presence of WSSV in wild crustaceans (Lo et al., 1996b; Chakraborty et al., 2002; Chapman et al., 2004) suggesting potentially significant implications for wild penaeid shrimp populations.

There are currently no treatments available for WSD, so preventative practices are needed for its control. Proposed strategies include strict biosecurity protocols and use of specific-pathogen free (SPF) shrimp stocks. Implementation depends heavily upon the availability of economically feasible, rapid assessment tools (Lightner, 1999).

Current diagnostic methods for the detection of WSSV range from clinical observations (Chou et al., 1995) to antibody-based diagnostic assays (Takahashi et al., 2003), and molecular diagnostic techniques, including real-time polymerase chain reaction (real-time PCR) (Lo et al., 1996a; Kim et al., 1998; Tang and Lightner, 2000; Durand and Lightner, 2002). Of these techniques, only real-time PCR allows simultaneous detection and quantification of WSSV infection. The cost of equipment and technical expertise necessary for real-time PCR is often beyond the means of typical commercial shrimp farms. Despite this, the virulence of WSSV makes rapid detection critical to prevent complete loss of shrimp stocks.

A commercially available immunochromatographic diagnostic test kit (Shrimple[®]) has been developed for the detection of the white spot syndrome virus from fresh samples (EnBioTec Laboratories; Tokyo, Japan). The advertised benefits of this method include diagnosis within approximately 20 min, a low cost per sample, and ease of use by untrained personnel. The aim of this investigation was to quantitatively evaluate the sensitivity range of Shrimple[®] test kits as well as to compare the efficiency of the test kit with that of the real-time PCR.

2. Materials and methods

2.1. Maintenance of animals

Specific-pathogen free (SPF) Pacific white shrimp (*Litopenaeus vannamei*) were obtained from the Oceanic Institute (U.S. Marine Shrimp Farming Program; Kailua-Kona, HI) as post-larvae and reared in an indoor, environmentally-controlled, biosecure husbandry facility. Water quality parameters were monitored regularly and adjusted to maintain optimal conditions.

Experimental infections were carried out in a biosecure, environmentally-controlled challenge laboratory (27 °C, 12L:12D photoperiod) in 19 L polypropylene aquaria filled with 3 L of artificial seawater. Shrimp (5.0–7.0 g) were stocked at a density of 10 animals per aquarium and allowed to acclimate for three days prior to the injection of viral inoculum. Daily maintenance of

laboratory animals in the challenge system included a 50% water exchange prior to feeding 1 pellet of a commercial shrimp grower diet per shrimp per aquarium.

2.2. Timecourse bioassay experimental design

WSSV inoculum was prepared by homogenizing previously infected, frozen whole shrimp heads in 1% TN-buffered saline (20 mM Tris-Cl, 400 mM NaCl, pH = 7.4) (1 g infected tissue/10 mL saline), centrifuging the homogenate at 1800 g for 20 min, and filtering the supernatant through a 0.45 µm polyethersulfone (PES) filter (Prior et al., 2003). Shrimp ($n=125$) were injected with 5 µL of a 1:100 dilution of the stock viral inoculum on the lateral side between the second and third abdominal segment. The negative control group included 10 shrimp injected with 1% saline and 10 shrimp injected with an inoculum prepared from specific pathogen free (SPF) shrimp.

Shrimp were sacrificed at 1, 2, 4, 8, 12, 16, 20, 24, and 32 h post injection (p.i.) and pleopod samples were collected. Sample size varied from 10 to 30 shrimp per timepoint, based on previously determined estimates of the progression of WSSV infection. Paired pleopods were removed from segments 1 to 3. Three pleopods (one from each segment) were preserved for Shrimple[®] and real-time PCR analysis, respectively.

2.3. Shrimple[®] diagnostic test kit

All Shrimple[®] tests were performed according to the manufacturer's protocol (EnBioTec Laboratories; Tokyo, Japan). Kit components consisted of Shrimple[®] test strip, disposable eye dropper, tissue grinder, and a 1.5 mL microcentrifuge tube filled with grinding buffer. Pleopods were removed from the shrimp, placed in the microcentrifuge tube, and ground using the tissue grinder. Three drops, or approximately 75 µL, of the supernatant were applied to the test strip.

The immunochromatographic assay (Shrimple[®]) developed by EnBioTec Laboratories utilizes a sandwich immunoassay. A monoclonal rat anti-WSSV antibody-colloid gold conjugate pad is positioned next to the sample pad region on the membrane test strip. The membrane test strip is pre-coated with anti-rat IgG on the control (C) zone, where a pink band will appear if the test kit is valid and has performed properly, and monoclonal rat anti-WSSV on the test (T) zone, where a pink band will appear if the animal being tested is positive for WSSV. A test that results in pink bands both at the C-zone and at the T-zone is positive for white spot syndrome virus (EnBioTec Laboratories) (Fig. 1).

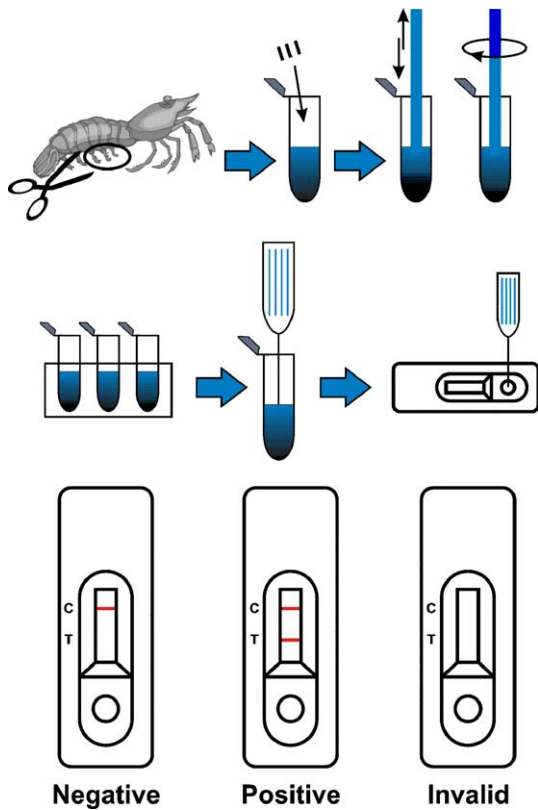


Fig. 1. Shrimple[®] diagnostic test kit procedure diagram and potential test results. Reprinted with permission from EnBioTec Laboratories Co., Ltd. Shrimple[®] Product Guide, En Bio Shrimp virus detection kit manual.

2.4. DNA isolation and quantification

L. vannamei total genomic DNA and associated viral DNA were extracted from 3 pleopods per animal using Sprint Prep kit (Agencourt Bioscience; Beverly, MA) in accordance with the manufacturer's protocol. To ensure all DNA isolations were successful and yielded intact gDNA, subsamples from the isolations were electrophoresed on a 0.8% agarose gel with ethidium bromide, and quantified on an ultraviolet spectrophotometer with a 96-well plate reader (SpectraMax Plus 384; Molecular Devices, Sunnyvale, CA) at 260/280 nm. The average concentration of isolated DNA per sample was 3.93 ± 2.13 ng/ μ L.

2.5. Real-time PCR

2.5.1. Real-time PCR amplification

Thermal cycling was performed on the Applied Biosystems 7500 Sequence Detection System with preliminary data analysis by the 7500 Sequence Detection

Software v. 1.2.2. (Applied Biosystems; Foster City, CA). Quantitative real-time PCR was performed using the Qiagen QuantiTect Probe PCR Kit (Qiagen, Inc.; Valencia, CA) according to the recommended procedure. Briefly, 2 μ L samples (5–10 ng gDNA) from each DNA isolation were added to a PCR mixture containing 0.3 μ M of each primer and 0.15 μ M TaqMan probe into a final reaction volume of 25 μ L. The amplification program consisted of 10 m at 95 $^{\circ}$ C to activate the hot-start AmpliTaq Gold polymerase, followed by 40 cycles of 15 s denaturation and 60 s annealing/extension at 60 $^{\circ}$ C. Plasmid DNA samples specific to the WSSV template of known concentration were included as absolute standards. The instrument determined fluorescence at the end of each annealing/extension cycle. Background fluorescence was monitored using ROX dye as part of the reaction mixture, and all samples were normalized to the background level.

2.5.2. Real-time PCR primers and probes

Sequences for PCR primers and TaqMan probe used for the detection of WSSV were obtained from a previously published study (Durand and Lightner, 2002). The primers (WSS1011F and WSS1079R) generated a 69 bp amplicon and were selected from a region of WSSV genomic sequence in GenBank U50923. The TaqMan probe was dual-labeled with fluorescent dyes, 5'-5-carboxyfluorescein (FAM) and 3'-N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA).

2.6. Analysis of data

Following real-time PCR amplification, a baseline and threshold were defined using the Applied Biosystems 7500 Sequence Detection Software (ABI 7500 SDS v. 1.2.2), resulting in a fractional cycle number (C_T value) assigned to each individual sample. A set of standard dilutions (from 1×10^7 to 1×10^{-1} viral copies/ μ L) was created from a WSSV plasmid prep sample of 4.0×10^7 viral copies/ μ L and run simultaneously with the samples from the timecourse experiment. Regression of the log of viral copy number and C_T value was used as a standard curve for determining viral load. Viral copy number was normalized per ng genomic DNA.

3. Results

3.1. Progression and detection of WSSV infection

From one hour following the injection of viral inoculum through to the end of the experiment (32 h), 100% (122 of 122) of the specimens tested positive for WSSV

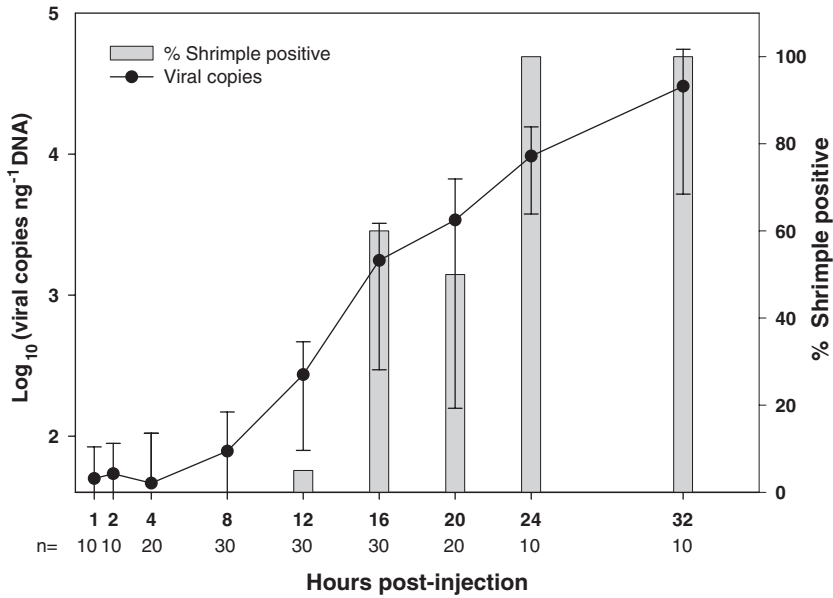


Fig. 2. Quantification of WSSV copy number, measured by real-time PCR, at various timepoints over the course of the bioassay compared to the percentage of positive Shrimple® test results for WSSV. All specimens were positive for WSSV as determined by real-time PCR. No Shrimple® positive test results were obtained from specimens prior to 12 h p.i., but all specimens were positive by 24 h p.i. Sample sizes are indicated below the x-axis.

by real-time PCR, despite displaying no gross signs of the disease. Negative control specimens (20 of 20) tested negative for WSSV with both real-time PCR and Shrimple® test kits. From 1 to 8 h p.i. none of the specimens tested positive with Shrimple®, at 12 h p.i. 5.26% (1 of 30) tested positive, increasing over time to 100%

positive by 24 h p.i. (Fig. 2). At 8 h p.i., 1 of 19 Shrimple® tests resulted in an ambiguous, faint band in the T-zone. This faint band was qualitatively different than the negative controls but was not consistent with a true positive test as it did not result in a discernable colored band. By 12 h p.i., these faint bands comprised the majority of the

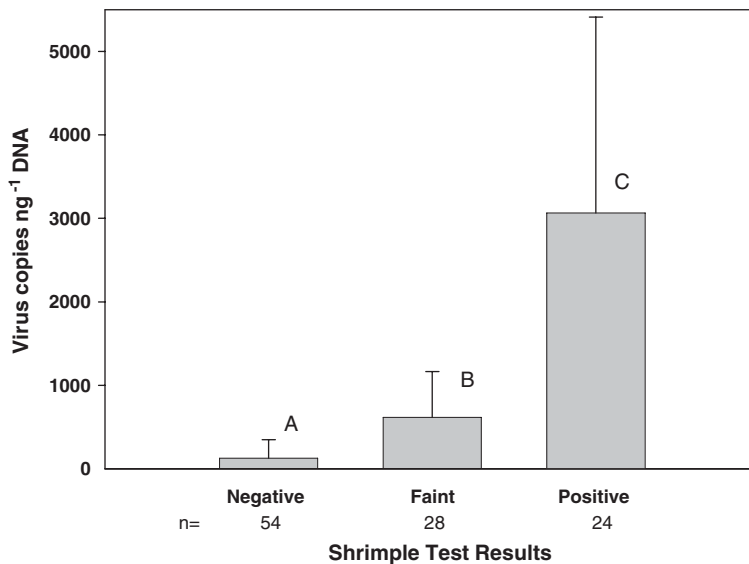


Fig. 3. Average viral copies/ng genomic DNA for specimens with negative, faint, and positive Shrimple® test results (1–24 h post injection). Test results reported as faint were characterized by an apparent, light-grey colored band not present with negative test results. However, the band was not as distinct as that for positive test results. Statistical analysis showed a significant difference between A and B, B and C, and A and C ($P < 0.001$).

test results (15 of 19); by 16 h p.i., 60% of the Shrimple® tests were unambiguously positive (18 of 30).

3.2. Shrimple® test kit sensitivity

Samples that tested negative with Shrimple® (43 of 122), but positive with real-time PCR, yielded 4 to 1061 viral copies/ng genomic DNA with an average of 126.16 ± 220.80 viral copies/ng genomic DNA (Fig. 3). Shrimple® test results classified as faint were determined by real-time PCR to contain 36 to 1784 viral copies/ng genomic DNA, or an average of 613.65 ± 551.42 . Infection levels higher than this gave a strong positive result for WSSV. A one-way ANOVA test indicated that the three groups (Shrimple® negative, faint, and positive) were significantly different than one another ($P < 0.001$).

4. Discussion

Accurate diagnostics are a necessary tool for early detection of pathogens and mitigation of economic losses associated with a disease outbreak. EnBioTec Laboratories designed the Shrimple® diagnostic test kit to detect white spot syndrome virus in an aquaculture-reared shrimp. The primary concern with such a tool is the potential for erroneous results; false negative results allow the virus the opportunity to manifest into devastating mass mortalities due to white spot disease, whereas false positive results may lead to shrimp farmers performing unnecessary rapid harvests resulting in tremendous economic losses.

A PCR-based monitoring strategy for detecting WSSV infection in earthen growout ponds has been proposed as a management practice that would allow for the detection of viral infection while at a level low enough to permit survival to harvest via a reduction in stress triggering conditions (Lo et al., 1998). The proposed management strategy relies on using nested PCR as a tool to screen for viral infections and grade infections into one of four categories—very severe infection (level 1), severe infection (level 2), light infection (level 3), and very light infection (level 4). Shrimp at infection levels 3 and 4 are able to survive from larval stages to harvest; however, infection levels 1 and 2 result in mortality within a few days. A successful management plan would detect viral infections prior to the transition stage from level 3 to level 2, thereby providing ample opportunity to reduce any potential stressors in a shrimp farm, allowing the shrimp to grow to full size prior to harvest. The proposed management protocol suggests monitoring for WSSV by a single-step and nested PCR at weekly intervals during cultivation. If the shrimp are determined positive by single-step PCR an emergency harvest is suggested.

Monitoring a shrimp population through a PCR-based approach would significantly increase the operation cost of any shrimp farm (Lo et al., 1998). The present study was designed to evaluate the sensitivity of a simpler, less expensive immunochromatographic detection kit as a potential alternative to more complex and costly PCR assays.

During the early onset of infection (from 1 to 8 h p.i.), Shrimple® test kits failed to detect WSSV infections, whereas 100% of the same specimens were determined positive for WSSV with real-time PCR. Throughout the entire study 34.7% of the specimens tested were determined positive for WSSV using the Shrimple® test kits compared to 100% with real-time PCR. Real-time PCR is more sensitive, and will detect WSSV infection earlier, than the diagnostic test kits; however, Shrimple® provides confirmation of viral presence prior to gross anatomical signs of infection and prior to mortality, which is early enough to achieve the goals of a basic shrimp aquaculture monitoring program.

Despite the variance in detection efficiencies of real-time PCR and Shrimple® test kits during early stages of infection, Shrimple® test kits provide valuable results to users. The results are rapidly obtained, even in geographically remote locations, unlike those obtained from real-time PCR where much more time is required in preparation, setup, and shipment of specimens to a diagnostic laboratory.

The present study demonstrates that Shrimple® test kits are sensitive enough to detect a relatively low-level infection, prior to gross anatomical evidence of disease. The level at which the Shrimple® test kit is capable of detecting viral infection varies. While a faint Shrimple® band was observed in test kits for specimens measured as low as 36 viral copies/ng genomic DNA, the lowest true chromatographic positive test result was observed at 356 viral copies/ng genomic DNA. Disparity in the sensitivity of the test kit exists in that some specimens that tested negative with Shrimple® were determined to have infection levels as high as 1098 viral copies/ng genomic DNA. However, in a field or aquaculture situation, the Shrimple® test kit can provide relatively reliable and sensitive detection capabilities for shrimp farmers, providing opportunities for limiting the extent of viral spread on farms infected with white spot syndrome virus.

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