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Short communication

A new method for quantifying white spot syndrome virus: Experimental challenge dose using TaqMan real-time PCR assay

Fei Zhu[a,*], Haizhi Quan[b]

[a] College of Animal Science and Technology, Zhejiang Agriculture and Forestry University, Lin'an 311300, China
[b] Institute of Feed Science, College of Animal Sciences, Zhejiang University, Hangzhou 310058, China

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A B S T R A C T

White spot syndrome virus (WSSV) is an important pathogen in shrimp aquaculture. The susceptibility of crayfish (*Procambarus clarkii*) was assessed by means of serial dilutions of a solution containing WSSV. A TaqMan real-time PCR was used to quantify the WSSV challenge dose in *P. clarkii*. The results showed that WSSV copies could be detected at concentrations from 1.365×10^4 to 1.129×10^9 copies/μl. The viral infectivity (LD_{50}), measured as the mortality of infected crayfish, indicated 60% mortality in the 10^5 dilution group (1.524×10^5 copies/μl). TaqMan real-time PCR represents a novel standard method, based on the by quantitation of WSSV copies, for determining the appropriate concentration of WSSV for use in infection experiments.

1. Introduction

White spot syndrome virus (WSSV), which was first discovered in Taiwan in 1992, has caused mass mortalities and devastating production losses to shrimp farming over many areas (Huang et al., 1995; Wang et al., 1995; Wongteerasupaya et al., 1995; Lightner, 1996). WSSV possesses a large DNA genome of about 300 kb, and genome analysis has shown that WSSV may be the sole member of the monotypic family *Nimaviridae*, genus *Whispovirus* (Van-Hulten et al., 2001; Yang et al., 2001; Vlak et al., 2005). WSSV is known to infect many crustacean species, including crayfish (Hossain et al., 2001; Lo et al., 1996). Baumgartner et al. (2009) recently found that both farmed and wild *Procambarus clarkii* in Louisiana (USA) were natural hosts for WSSV. Crayfish, such as *Cherax quadricarinatus* have also been used as experimental hosts for WSSV (Shi et al., 2000).

A new real-time PCR technique, using TaqMan probe fluorescence, has been used recently to quantify the copy number of a particular target segment of nucleic acid, based on monitoring the increase in fluorescence. In previous studies, TaqMan real-time PCR was used to successfully quantify WSSV in various shrimp species, such as *Penaeus monodon, Litopenaeus vannamei* and *Fenneropenaeus chinensis* (Jang et al., 2009; Fouzi et al., 2010; Meng et al., 2010). In this investigation, a quantitative assay, involving a viral titration of WSSV in *P. clarkii*, was described and determine precise lethal dosages for use in challenge experiments.

2. Materials and methods

2.1. Crayfish

Crayfish (*P. clarkii*) of approximately 20 g were reared at 25 ± 1 °C. They were kept in tanks with sand-filtered and ozone-treated water and fed with commercial pellet feed at 5% of body weight per day. The appendages from individuals selected at random were subjected to PCR assays to ensure that the crayfish were WSSV-free prior to the experimental challenge.

2.2. WSSV stock

White spot syndrome virus-infected shrimp, *F. chinensis*, were collected from shrimp farms located near Ningbo, China. Ten grams of infected tissues (from the gills and the tail muscle) were homogenized in 500 mL TNE buffer (50 mM Tris–HCl, 400 mM NaCl, 5 mM EDTA, pH 7.5) containing a combination of protease inhibitors — (1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM benzamidine, and 1 mM $Na_2S_2O_5$) — and then centrifuged at 10,000 × *g* for 10 min at 4 °C. After filtration through a nylon net (400 mesh), the homogenate was centrifuged at 6000 × *g* for 25 min at 4 °C and filtrated using a Millipore filter (pore size 0.45 mm). The filtrate represented the original viral fluid used for further challenge tests.

* Corresponding author. Tel.: +86 571 63740815; fax: +86 571 63740815.
E-mail address: zhufei1998@yahoo.cn (F. Zhu).

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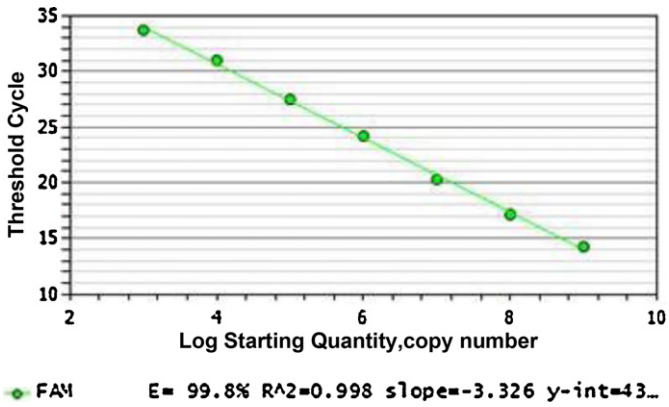

Fig. 1. The standard curve of TaqMan Real-time PCR. R: correlation coefficient; M: slope; B: intercept; Efficiency: PCR amplification efficiency.

2.3. WSSV detection by PCR and quantitative analysis by TaqMan real-time PCR

Total DNA was extracted from the gills of dead crayfish, using an animal genomic DNA mini-prep kit (Sangon, Shanghai). The primer set VP28-FW and VP28-RV (5′-CGCACAGACAATATCGAGAC-3′/5′-GTCTCAGTGCCAGAGTAGGT-3′), amplifying portion of the WSSV VP28 gene, was used to screen for WSSV-positive animals. PCR was performed with the VP28 primer set using the following protocol: 5 min at 94 °C followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. The PCR products were analyzed by electrophoresis on 1% agarose gels stained with ethidium bromide and visualized by ultraviolet transillumination.

TaqMan real-time PCR was performed using a Perfect Real Time premix (Takara, Japan) containing a high-performance Taq antibody, Takara Ex Taq HS, for hotstart real-time PCR. Primer 3 software was used to design primers and the TaqMan probe with the WSSV whole sequence (GenBank accession no. AF332093). Primers WSSV-RT1 (5′-TTGGTTTCATGCCCGAGATT-3′) and WSSV-RT2 (5′-CCTTGGTCAGCCCCTTGA-3′) produced a fragment of 154 bp after amplification. The TaqMan probe was synthesized and labeled with the fluorescent dye 5-carboxyfluorescein (FAM) (5′-FAM-TGCTGCCGTCTCCAA-TAMRA-3′). The reaction mixture consisted of a DNA aliquot of 200 nM of each primer, 100 nM of each TaqMan probe, and 1× PCR buffer containing DNA polymerase in a final reaction volume of 20 μl. PCR amplification was performed for 4 min at 50 °C, followed by 45 cycles of 45 s at 95 °C, 45 s at 52 °C and 45 s at 72 °C. Thermal cycling was performed on an iCycle IQ5 real-time PCR detection system (Bio-RAD, USA).

2.4. WSSV challenge and measurement of viral infectivity (LD_{50})

In this study, 30 crayfish per group were used for determination of the infectivity (lethal dose 50%: LD_{50}) of WSSV. To measure the infectivity of this virus-containing fluid, 10-fold serial dilutions were made from 10^1 to 10^6 and filtered, using a Millipore filter (pore size 0.45 mm). These diluted fluids were measured by TaqMan real-time PCR, after which they were administered by intramuscular (IM) injection, individually into 30 healthy crayfish, at a dose of 0.1 mL/crayfish. In the negative control group, crayfish were IM injected with a TNE buffer at the same dose. The mortality, and clinical symptoms, of the crayfish were observed daily for the following 21 days.

3. Results

3.1. Standard curve and DNA copies detection of WSSV titration

A TaqMan real-time PCR was used to quantify the WSSV copies in the serial dilutions (10^1–10^6) of the original viral fluid. The standard curve and detection limitation are shown in Fig. 1. Strong linear correlations ($R^2 > 0.998$) were obtained between the threshold cycles (Ct) and the target plasmid standard, ranging from 1×10^3 to 1×10^9 WSSV copies in PCR, with a high reaction efficiency ($E = 0.998$) and proper slope ($M = -3.326$) (Fig. 2). The amplification curves (Fig. 3) show results from seven dilutions of standard samples (Fig. 3). However, the negative control did not show any amplification for each run. The 10-fold serial dilutions of WSSV (from 10^1 to 10^6), using TaqMan real-time PCR, were then detected for the DNA copies of the genomic DNA of crayfish. This result indicates that WSSV copies were detected from 1.365×10^4

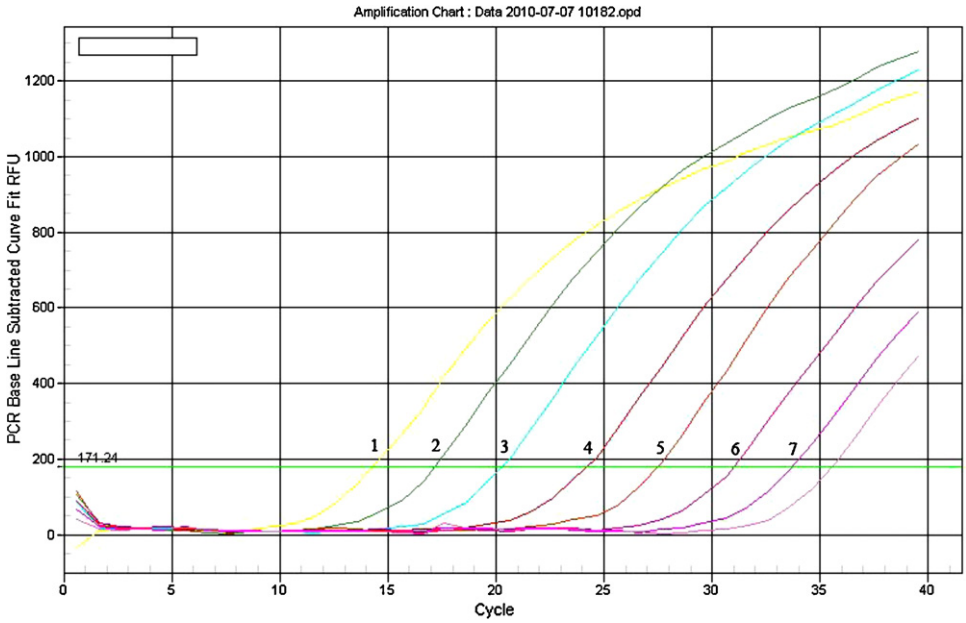

Fig. 2. Amplification curve showing 10 fold serial dilutions of the standard WSSV samples. Numbers near lines (WSSV copies/μL): (1) 1×10^9; (2) 1×10^8; (3) 1×10^7; (4) 1×10^6; (5) 1×10^5; (6) 1×10^4; (7) 1×10^3.