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Stable Yellowhead Virus (YHV) RNA Detection by qRT-PCR during Six-Day Storage

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Abstract

Storage conditions of haemolymph samples which contain yellowhead virus (YHV) may result in a decline of YHV RNA concentration or false-negative results in the detection of YHV. We evaluated the stability of YHV RNA in haemolymph stored at different temperatures for 6 d with conventional RT-PCR and TaqMan qRT-PCR. Specific pathogen-free individuals of *Litopenaeus vannamei* were challenged with YHV92TH isolate, and haemolymph samples of 3 groups of 10 pooled moribund shrimp were aliquoted and stored at 4 and 25°C for 0, 2, 6, 12, 24, 48, 72, 96, 120, and 144 h. All samples were evaluated by conventional RT-PCR and qRT-PCR. After the optimization of experimental conditions, TaqMan qRT-PCR showed a very strong linear relationship between the log scale of the standard DNA copy number and the C_T values ($R^2 = 0.999$) over a 7-log range from 10^2 to 10^8 copy number per reaction. Even though the haemolymph was stored at either 4 or 25°C for a 6-day period, the viral load number at 4°C was not significantly different from that stored at 25°C. The only difference was between the samples stored for 144 h at either 4 or 25°C and those stored at −80°C. We conclude that shrimp haemolymph can be drawn from shrimp at farms or in the wild and stored at either 4 or 25°C for 3–5 d without a significant reduction in measured YHV RNA levels and without having to immediately freeze the samples.

Keywords: yellowhead virus, haemolymph, qRT-PCR, stability of viral RNA, storage

1. Introduction

Yellowhead virus (YHV), lethal to most commercially cultivated penaeid shrimp species (Walker, 2006), was first described as an epizootic from Thai shrimp farms (Limsuwan, 1991); subsequent outbreaks of YHV have been reported from cultivated shrimp in many locations in Asia (Walker, 2006). The YHV agent has been reported from frozen imported commodity shrimp in the United States (Nunan et al., 1998; Durand et al., 2000) and from *Litopenaeus vannamei* (according to Pérez Farfante and Kensley [1997], also referred to as *Penaeus vannamei* by others [Flegel, 2007]) and *L. stylirostris* cultured on the northwest coast of Mexico (de la Rosa-Vélez et al., 2006). As an important shrimp emerging disease, YHV has caused an estimated economic loss of $500 million since its discovery in 1991 until 2006 (Lightner, 2007 Shrimp Pathology Short Course). The YHV and 2 other related disease-causing viruses from Australia, gill-associated virus (GAV) and lymphoid organ virus (LOV), have been placed in the family Roniviridae of the order Nidovirales (Cowley and Walker, 2002; Gorbalenya et al., 2006).

Many molecular methods have been developed to diagnose YHV, such as conventional RT-PCR (Wongteerasupaya et al., 1997; Cowley et al., 2004; Kiatpathomchai et al., 2004), gene probe (Tang and Lightner,1999; Tang et al., 2002), qRT-PCR (Dhar et al., 2002), and loop-mediated isothermal amplification (LAMP) (Mekata et al., 2006). Among these, qRT-PCR has become the "gold-standard" for various research and clinical studies because of its sensitivity and specificity.

Haemolymph has been widely used as the source of material to assess acute shrimp viral diseases, especially the viral dynamics and host gene expression after viral challenge. But a common protocol was to draw the haemolymph quickly and to store it at −70°C or liquid nitrogen as soon as possible. The question remained whether the haemolymph drawn at a shrimp farm or some other sites distant from the testing laboratory and stored at either 4°C or room temperature for 3–5 d would still contain active YHV or a high enough copy number for diagnosis. The objective of this study was to determine whether haemolymph was still useful for diagnosis and quantification of YHV using conventional and qRT-PCR after being stored at different temperatures and time periods.

2. Materials and methods

2.1. Experimental challenge and sample storage

A 100 μl inoculum of 1:1000 diluted homogenate of YHV isolate 92TH was injected into the third abdominal segment of 30 specific pathogen free (SPF) shrimp (*Litopenaeus vannamei*, Kona TSV-sensitive stock, Oceanic Institute, average 15 g). These shrimps were fed once a day with commercial pelleted feed (Rangen, Buhl, Idaho) divided among 6 aerated 19-l aquariums containing 20 ppt artificial seawater, maintained in a water bath at 26.0 ± 0.5°C. When some shrimp became moribund after 3 d post-inoculation, the haemolymph from 3 random groups of 10 shrimp each was drawn into an EDTA coated 1-mL syringe and then pooled, producing 3 sample groups. Then 21 aliquots of 50 μl haemolymph were collected from each sample group. They were divided by storing 9 aliquots at 4°C, another 9 aliquots at room temperature (~25°C), and the remaining 3 aliquots at –80°C. At 2, 6, 12,

24, 48, 72, 96, 120, and 144 h of storage, 1 subsample from each aliquot of the 3 sample groups at 4 and 25°C (for a total of 54 subsamples with an additional 9 subsamples at –80°C) was randomly picked and stored at –80°C until analysis.

2.2. Total RNA extraction

Haemolymph RNA was extracted following the protocol of the High Pure Viral Nucleic Acid Kit (Roche). Each stored 50 μl haemolymph aliquot had 150 μl autoclaved RNase-free water added; this solution was mixed with 250 μl binding buffer working solution containing poly A and proteinase K. The RNA was then eluted into 100 μl of autoclaved RNase-free water and stored at −80°C.

2.3. Conventional RT-PCR

Conventional RT-PCR was processed following the protocol of SuperScript™ III One-Step RT-PCR System with Platinum® Taq High Fidelity (Invitrogen) in which the following primers were added: primer 273F: 5′-CAA GAT CTC ACG GCA ACT CA-3′ and 273R: 5′-CCG ACG AGA GTG TTA GGA GG-3′ (Tang and Lightner, 1999). The total RNA extracted was incubated at 50°C for 30 min to synthesize the cDNA. Inactivation of the reverse transcriptase at 94°C for 2 min was followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 68°C for 30 s. A final extension step at 68°C for 5 min completed the reaction after which it was held at 4°C.

2.4. qRT-PCR

The iScript™ One-Step RT-PCR Kit for Probes (BioRad) was used to perform qRT-PCR with primer YHV141F: 5′-CGT CCC GGC AAT TGT GAT C-3′, YHV206R: 5′-CCA GTG ACG TTC GAT GCA ATA-3′ (Dhar et al., 2002), and YHV TaqMan: 5′-/FAM™/CCA TCA AAG CTC TCA ACG CCG TCA/TAMRA™-Sp-3′ (Integrated DNA Technologies, Inc.). The positive control used for the standard curve was a 72-bp synthesized oligo (Invitrogen Co.) which contained the 66-bp amplicon and an extra 3 bp on both ends based on GenBank accession no. AF148846. A gradient cycler (PTC200 DNA Engine) was used to adjust the annealing temperature to an optimal condition before performing qRT-PCR. The amplification products were separated using 2% agarose gel electrophoresis and analyzed with a Fluor-S™ MultiImager (BioRad). The qRT-PCR was optimized by using salt-free YHV positive oligo and different concentrations of primers and probe.

The qRT-PCR amplifications were undertaken in an iCycler Thermocycler (BioRad). The qRT-PCR was conducted in duplicate, with each 25-μl reaction volume containing 2 μl RNA (= 1 μl original haemolymph), 12.5 μl 2 × RT-PCR reaction mix for probe, 300 nM of primers, 100 nM probe, and 0.5 μl iScript Reverse Transcriptase Mix for One-Step RT-PCR. The thermal profile of qRT-PCR was 10 min at 50°C for cDNA synthesis, and 5 min at 95°C for iScript reverse transcriptase inactivation, with 40 cycles of 15 s at 95°C and 30 s at 56°C (data collection step) for amplification.

3. Results

3.1. RT-PCR

When RNA samples were tested for YHV by the conventional RT-PCR method, subsamples of all 3 groups tested YHV-positive during the 6-day storage at 4 and 25°C; Figure 1 shows the RT-PCR result for 1 group of subsamples during the 6-day storage at 4 and 25°C.

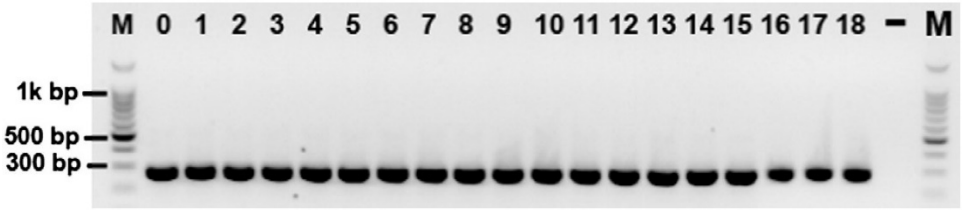

Figure 1. Conventional RT-PCR amplification of RNA from aliquoted haemolymph from *Litopenaeus vannamei* stored at different temperatures over 6 days. M, 1k bp DNA ladder (Promega, G829A); lane 0, stored at −80°C; lanes 1, 3, 5, 7, 9, 11, 13, 15, and 17 are samples stored at 25°C for 2, 6, 12, 24, 48, 72, 96, 120, and 144 h, respectively; lanes 2, 4, 6, 8, 10, 12, 14, 16, and 18 are samples stored at 4°C for 2, 6, 12, 24, 48, 72, 96, 120, and 144 h, respectively.

3.2. qRT-PCR

The TaqMan qRT-PCR method showed a very strong linear relationship between the log scale of the standard DNA copy number and C_T values ($R^2 = 0.999$) over a 7-log range from 10^2 to 10^8 copy numbers per reaction (Fig. 2). The PCR efficiency was as high as 95.4%.

Even though the haemolymph subsamples were stored at 4 and 25°C for a 6-day period, the viral load numbers at 4°C were not significantly different from the corresponding values at 25°C as analyzed by two-way ANOVA (Fig. 3). The only statistical difference was between the samples stored for 144 h at both 4 and 25°C and those stored at −80°C.

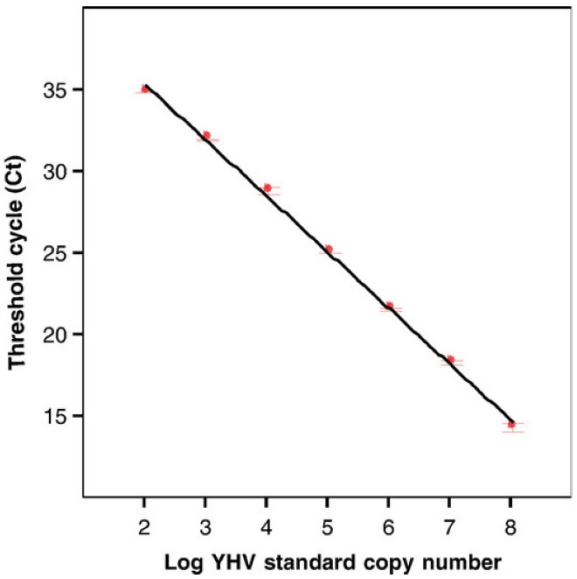

Figure 2. Standard curve generated from qRT-PCR and a 10-fold dilution series. $R^2 = 0.999$, $\hat{Y} = -3.436\ X + 42.203$.

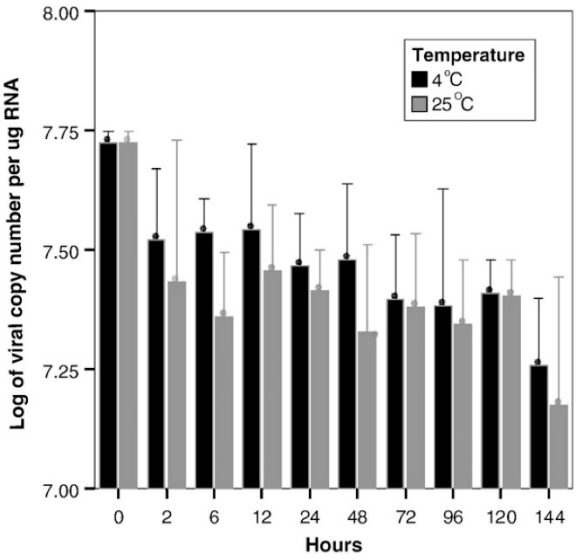

Figure 3. Quantitative analysis of log scale in *L. vannamei* haemolymph at 4 and 25°C for 6 d storage. Bars exhibit mean value, and error bars express mean ± 1 SE.

4. Discussion

The real-time PCR method has been graded as the "gold-standard" for pathogen detection, gene expression, and various studies because of its simplicity, sensitivity, and reproducibility as well as its amenability to high-output screening and ability to accurately quantify

infection levels (Mackay et al., 2002; Espy et al., 2006; Rajendran et al., 2006,Watzinger et al., 2006; Belák, 2007). Consequently, several qRT-PCR (or qPCR) protocols have been developed for viral RNA/DNA detection, including those by either TaqMan probe or SYBR Green chemistry for infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Dhar et al., 2001; Tang and Lightner, 2001), WSSV (Dhar et al., 2001; Durand and Lightner, 2002), Taura syndrome virus (TSV) (Dhar et al., 2002; Mouillesseaux et al., 2003; Tang et al., 2004), YHV (Dhar et al., 2002), GAV (de la Vega et al., 2004), Mourilyan virus (Rajendran et al., 2006), and infectious myonecrosis virus (IMNV) (Andrade et al., 2007). However, the viral copy number in different tissues or different host individuals may vary significantly and cannot provide reasonably comparable data. Many factors may affect results significantly, such as the time point when the sample was collected, tissue type, viral dose administered, administration methods (per os or muscularly), RNA extraction method, and other aspects of qRT-PCR. Theoretically, TaqMan probe has more advantages over SYBR Green chemistry because its high specificity and stability can reduce the possibility of false-positive results produced by SYBR Green. More simplified qRT-PCR kits for probes obtained from different companies allows qRT-PCR to be used easily as a routine standard method for pathogen detection in shrimps. Even though most of the cited authors claimed that qPCR can detect as few as 10 copies of a viral molecule, one should be very cautious when interpreting data because the linear quantification may not be reliable at that level due to inevitable inconsistencies in the distribution of specific target molecules in the aliquot added to the reaction. At the same time, pipetting procedures and the experimental environment can also increase inconsistencies. When samples have about 10 copies of the viral molecule, a more reliable detection can be achieved by running a gel together with the negative control sample and determining whether an inconsistency results from a nonspecific amplification or a primer dimer.

Our conventional RT-PCR results agreed well with those of qRT-PCR because large amounts of virus occurred in haemolymph, e.g., 5.0×10^7 copies/μl at 0 h and 1.5×10^7 copies/μl at 144 h storage. When viral loading in the haemolymph is relatively low, conventional RT-PCR may not detect the viral copies that would be detectable by qRT-PCR (e.g., Chen et al., 2007).

Suboptimal storage temperatures may affect YHV RNA stability and influence viral load measurements. From our experiment, the YHV copy number of aliquoted samples was not statistically significantly different when samples were stored at 4 or 25°C for a 5-day period, even though a statistically significant difference existed between the samples stored for 6 d (144 h) at both 4 and 25°C and those immediately stored at –80°C after being collected and aliquoted. This result for YHV may be consistent with that of hepatitis C virus (HCV) RNA, even though both viruses belong to different orders. Some reports have claimed that HCV RNA in serum is stable for at least 3–4 d at 4°C or room temperature (Krajden et al., 1999b; de Moreau de Gerbehaye et al., 2002). Also, the concentration of HCV RNA remained stable in serum specimens subjected to 3 to 8 freeze-thaw cycles (Krajden et al., 1999a). Grant et al. (2000) reported that infected whole blood anticoagulated with EDTA or CPDA-1/EDTA could be stored for 5 d at ≤ 25°C without any significant loss in the plasma HCV RNA level. Moreover, Kiatpathomchai et al. (2004) used Isocode (R)

filter paper to store dried haemolymph for YHV semi-nested PCR for up to 6 months at room temperature.

Different studies using different blood collection tubes and different processing times are not really comparable. The general profile of both HCV and YHV virions may be more stable than previously thought. The reason could be because the stable virion can protect viral RNA from being degraded by chemical factors in haemolymph, as the host haemocyte and RNA can break down and degrade more easily than the RNA in an active virion. Unfortunately, no data are available about how long YHV particles retain infectivity in the environment. When in water, isolated TSV is infective for over a month in 25 ppt salinity, but the DNA virus WSSV becomes inactive in less than 1 h (unpublished). One experiment showed that the avian influenza virus (H6 N2) having a 1×10^6 mean tissue-culture infective dose can persist at 17°C in 0 ppt at pH 8.2 for 100 d and at 28°C in 20 ppt at pH 8.2 for 9 d (Stallknecht et al., 1990). Consequently, environmental factors influence the persistence of viral infectivity.

The practical aspects of our experiment show that haemolymph (1) can serve as an important diagnostic medium; it can be drawn at a shrimp farm and kept at either 4 or 25°C and then delivered to a reference laboratory for diagnosis or viral quantification and (2) does not have to be stored in RNA stabilization buffers (e.g., RNAlater™ or PrepProtect™ Stabilization Buffer) if it is used specifically for viral PCR or viral quantification as long as the viral RNA can be extracted from it within 5 d before being analyzed or stored at –80°C until analysis can be performed.

For economic and biological reasons, many Asian shrimp farms are switching from *P. monodon*, which is highly susceptible to YHV to *L. vannamei* as their animal for culture (Wyban, 2007). However, because the naïve *L. vannamei* is also susceptible to YHV (Lu et al., 1994; Lu et al., 1995; Lightner, 1996), shrimp farmers, managers, and researchers should be very cautious to avoid reemergence of the pandemic YHV disease in Asian shrimp farms. As a valuable tool, qRT-PCR can be used as a routine protocol for surveillance and diagnosis of YHV in shrimp farms and hatcheries.

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