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Development and Evaluation of Real-Time Loop-Mediated Isothermal Amplification Methods for the Rapid Detection of Penaeid Viruses

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Abstract：Shrimp viral diseases are a major impediment to commercial shrimp farming. The diseases affecting shrimp culture are white spot syndrome virus (WSSV), yellow head virus (YHV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), and Taura syndrome virus (TSV). Viral diseases are particularly difficult to control after the onset of infection. Therefore, prophylaxes to prevent or reduce the losses through vertical and horizontal transmission are most important. Various diagnostic methods have been developed to detect shrimp viral diseases, including bioassays, histopathology, polymerase chain reaction (PCR), and quantitative real-time PCR. Although the PCR-based methods are sensitive and highly specific, they require expensive equipment, costly reagents, and are time consuming. Therefore, a simple, quick and sensitive detection method is urgently needed to prevent the invasion of shrimp viral diseases into Japan from other countries. The loop-mediated isothermal amplification (LAMP) assay is a novel approach to amplify nucleic acid with high specificity, sensitivity, and rapidity under isothermal conditions, thereby obviating the need for a thermal cycler. Further, during the LAMP reaction, an insoluble byproduct, magnesium pyrophosphate, is produced in proportion to the large amounts of the target DNA amplified. Hence, real-time quantification can be achieved by measuring the turbidity of the magnesium pyrophosphate using an inexpensive photometer. This real-time LAMP method allows quantitative analysis of nucleic acid templates (real-time LAMP). In the present study, a comparatively less expensive quantitative real-time LAMP assay was successfully applied for detection of shrimp viral diseases and proven to have high sensitivity and specificity.

Key words：RT-LAMP, WSSV, YHV, IHHNV, TSV, Penaeid shrimp, Japan

Introduction

Shrimp viral diseases are a major impediment to commercial shrimp farming. The diseases affecting shrimp culture are white spot syndrome virus (WSSV), yellow head virus (YHV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), and Taura syndrome virus (TSV). WSSV was first discovered in northern Taiwan around 1992, and is currently the most serious viral pathogen on shrimp farms throughout the world (Chou *et al.,* 1995; Lo *et al.,* 1996; Flegal, 1997). The International Committee on Taxonomy of Viruses (ICTV) determined WSSV is the type species of the genus *Whispovirus*, family *Nimaviridae* (Mayo, 2002). The typical clinical signs of WSSV include lethargy, reduced food intake and the appearance of white spots on the carapace (Lightner, 1996). This virus causes 100% mortality within three to 10 days in all life stages of both wild and cultured *Penaeus monodon* and *Marsupenaeus japonicus*, and has a wide host range that includes penaeid shrimp, crabs, copepods and other arthropods (Chen *et al.,* 2000; Syed Musthaq *et al.,* 2006).

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YHV was first reported as a virulent pathogen in the early 1990s causing a 100% crop loss within three to five days post-infection in *P. monodon* in Thailand (Limsuwan, 1991; Chantanachookin *et al.,* 1993). Since then, several researchers have reported the occurrence of YHV in farmed and wild shrimp in Taiwan and many other Asian countries. The causative agent is a rod-shaped, enveloped virus with positive stranded ssRNA containing four open reading frames classified under the genus *Okavirus*, family *Ronivirida*. So far, six genotypes have been identified, and only genotype 1 is known to cause typical signs of YHV infection in shrimp. Other genotypes are widespread in *P. monodon* populations throughout the Indo-Pacific region, but have not been associated with farm disease outbreaks (Wijegoonawardane *et al.,* 2008).

IHHNV is a cosmopolitan virus infecting penaeid shrimp in the Asian-Pacific area and the Americas, but it has not been reported in Japan. The virus causes 90% mortality in *P. stylirostris* juveniles (Lightner *et al.,* 1983) and is detected in other penaeid shrimps (Flegel, 1997). The typical symptoms of the viral disease include reduction in growth and a runt deformity (Lightner *et al.,* 1992; Primavera and Quinitio, 2000). The virions of IHHNV are non-enveloped icosahedrons (22 nm in diameter) containing a single-strand linear DNA of 4.1 kb (Bonami *et al.,* 1990). Genome homology suggests IHHNV may be related to mosquito Brevidensovirus (Shike *et al.,* 2000).

Taura syndrome virus (TSV) is a causative agent of a major disease of the whiteleg shrimp, *Litopenaeus vannamei*. TSV was originally placed in the family *Picornaviridae*, but was later transferred to the *Dicistroviridae*. The complete TSV genome is a linear, positive-sense, single-stranded RNA virus of 10,205 bases. Taura syndrome was first recognized in shrimp farming in Ecuador in mid-1992. The loss was estimated to be close to US$100 million. Although the TSV originated in Ecuador, it was subsequently discovered in Taiwan in 2000.

Viral diseases are particularly difficult to control after the onset of infection; therefore, prophylaxes to prevent or reduce the losses through vertical and horizontal transmission are most important (Bell and Lightner, 1988; Lotz, 1997). Various diagnostic methods have been developed to detect shrimp viral diseases, including bioassays, histopathology, dot blot with *in situ* hybridization, polymerase chain reaction (PCR; Lightner and Redman, 1998), and quantitative real-time PCR (Tang and Lightner, 2001).

Although the PCR based methods are sensitive and highly specific, they require expensive equipment, costly reagents, and are time consuming. Therefore, a simple, quick and quantitative detection method is urgently needed to prevent the invasion of shrimp viral diseases into Japan from other countries. The LAMP assay is a novel approach to nucleic acid amplification that amplifies DNA with high specificity, selectivity and rapidity under isothermal conditions, thereby obviating the need for a thermal cycler.

The LAMP assay is based on the principle of autocycling strand displacement DNA synthesis (Notomi *et al.,* 2000). The reaction is performed by DNA polymerase with strand displacement activity and two or three sets of specially designed primers. The assay is highly specific for the target sequence, because that sequence is recognized at six or eight independent sequences. The LAMP method has been used without quantization for diagnosis of various shrimp viruses, such as WSSV (Kono *et al.,* 2004), YHV (Mekata *et al.,* 2006), *Mr*NV & XSV (Pillai *et al.,* 2006), IHHNV (Sun *et al.,* 2006), and TSV (Kiatpathomchai *et al.,* 2007). These qualitative methods cannot, however, determine the copy number of the viral particles present in the sample.

Real-time loop mediated isothermal amplification assay produces large amounts of the target DNA as well as an insoluble by-product, magnesium pyrophosphate, during the reaction, making it possible to perform a real-time measurement of turbidity using an inexpensive photometer (Mori *et al.,* 2001). Real-time LAMP assay has been used for many non-shrimp viruses, such as West Nile virus (Parida *et al.,* 2004), Severe Acute Respiratory Syndrome (SARS) virus (Poon *et al.,* 2005), Dengue virus (Parida *et al.,* 2005) and hepatitis A virus (Yoneyama *et al.,* 2007). In the present study, a comparatively less expensive quantitative real-time RT-LAMP assay was successfully applied for detection of shrimp viral diseases in the field and proven to have high sensitivity and specificity.

Materials and methods

Shrimp: Black tiger shrimp (*P. monodon*) and whiteleg shrimp (*L. vannamei*) with prominent signs of WSSV, YHV, IHHNV, and TSV infection, respectively were collected from shrimp farms in Songkhla, Thailand. Shrimp tissue samples were collected in separate sterile tubes and transported to the laboratory on dry ice for the real-time LAMP assay for each viral pathogen.

Nucleic acid extraction: Extraction of DNA was performed from the corresponding viral homogenates prepared from WSSV and IHHNV sources. From each sample, 200 μl of the homogenate were added to 600 μl of DNAzol reagent (Invitrogen, Carlsbad, California USA) and further steps were carried out according to the manufacturer's instructions. Total RNA was extracted from a pool of heart tissues from the infected shrimp using an RNA extraction kit (High Pure RNA Tissue Kit; Roche Diagnostics, Germany) according to the manufacturer's instructions. Extracted nucleic acid samples were quantified using Nanodrop UV Spectrophotometer ND-100 (NanoDrop Technologies, USA). Synthesis of cDNA for quantitation analysis was carried out using ReverTra Ace qPCR RT Kit (Toyobo, Japan) with 1 μg of total RNA as per the manufacturer's instructions.

Design of primers for real-time LAMP procedures: Real-time LAMP primers specific to WSSV, YHV, IHHNV and TSV were designed according to published sequences using Primer Explorer Software version 4 (https://primerexplorer.jp/lamp4.0.0/index.html, Fujitsu, Japan). The target regions of each shrimp viral pathogen include: ORF36 of WSSV, replicase polyprotein gene of YHV, non-structural protein gene of IHHNV, and coat protein gene of TSV (GenBank Accession No. AF369029, EU487200, AF218266 and AF277378, respectively). A set of six or four primers, two outer (F3 and B3), two inner primers (FIP and BIP) and two additional primers (LoopF and LoopB) were designed according to the guideline provided. The oligonucleotide primers used for the amplification are shown in Table 1.

Optimization of reaction temperature: The real-time LAMP was carried out in a total volume of 25 μl of reaction mixture using Loopamp DNA and RNA Amplification Kit (Eiken Chemical, Japan) according to the manufacturer's instructions for analysis of DNA and RNA viruses. Briefly, the specified

Table 1. Primers used for the LAMP assay for diagnosis of WSSV, YHV, IHHNV and TSV

Primer name	Sequences 5′– 3′
WSSV-FIP	TCCGTCTTCAGGGAATACATATGCTCAGGGAAGAAATAGACCATG
WSSV-BIP	GGACCCAAATCGAAATATAAGGCCTATGTTGCCCAAGATCCAC
WSSV-F3	AAACACCGGATGGGCTAA
WSSV-B3	CAAGGCAATACAGAATGCG
WSSV-loopF	GTTAAGAATGATGCATCTAGTGCGA
WSSV-loopB	TGGAACAAAAGATGCTGCTCA
YHV-FIP	CATGTTCCAATTTCCGCCGCTACACACTGAAAATCCTACACG
YHV-BIP	ACCAAGCACTCACCACATTCCCCTCATGTATAGAGTCAAGT
YHV-F3	CGCTTCTCTCTCTGCTATC
YHV-B3	AAGCATACGTCTCGCATT
YHV-loopF	AGACCGCAGAGAATTTTCCATG
YHV-loopB	CAAAGACATCACATTCACATTCGTC
IHHNV-FIP	GAAAACTGGAACAGTTCTTCAGACAAATCAAGACCCTAAACCCAC
IHHNV-BIP	ACGAGGAAGACAACTCTCAAACTGTTATCCACGCAGACCTTAG
IHHNV-F3	TCTCCAAGCCTTCTCACC
IHHNV-B3	TCCCTCTCGAATTCCCAG
TSV-FIP	AGTTCATCTCAATGCCAGGAAATGAAGACATCAATTATTCGACGC
TSV-BIP	GCAGTCTGAAGCTCGAGCTATTGTTATTCACATTTCTGGGGTT
TSV-F3	TGGAATAAGATGAATGCTAAGC
TSV-B3	GACTCAGAACGGAAAGCC

amount of target nucleic acid was mixed with 1 μl (40 pmol) of each -FIP and -BIP, 1 μl (5 pmol) of -F3 and -B3 primers, 12.5 μl of Reaction Mix (2 ×), 1 μl Enzyme Mix containing *Bst* DNA polymerase and distilled water used to make up to 25 μl. AMV reverse transcriptase was used in the case of YHV and TSV. The reaction temperature (60, 63, and 65°C) was optimized using Loopamp Real Time Turbidimeter LA-200C (Teramecs, Japan). Real-time monitoring was performed every six seconds using spectrophotometric analysis by recording the optical density (OD) at 650 nm. Each assay was carried out three times.

Specificity of real-time LAMP detection: The specificity of the real-time LAMP method was evaluated using different sources of DNA/cDNA templates prepared from WSSV-, YHV-, IHHNV- and TSV-infected shrimp and healthy shrimp. Each assay was carried out in duplicate.

Quantitative real-time LAMP: To determine the quantity of unknown nucleic acid using the real-time LAMP assay, the specific target fragments of each viral disease were cloned into plasmids. The amplified PCR product was cloned into pGEM-T Easy Vectors (Promega, USA) according to the manufacturer's instructions. Quantization of the constructed plasmid (using pGEM vector) was achieved using the NanoDrop spectrophotometer, and ten-fold serial dilutions (10^1-10^9) were made to evaluate the real-time LAMP assay. The copy numbers of the plasmid DNA were calculated based on the molecular weight and Avogadro's number, and a standard curve was constructed. The standard curve of the specific virus was generated each time during the analysis of samples. The reaction setup was the same as that optimized above, and the reactions were carried out in the Loopamp real-time turbidimeter.

Results

Optimization of real-time LAMP assay conditions for WSSV, YHV, IHHNV & TSV detection: Real-time LAMP assay was performed using DNA/RNA as a template in order to determine the optimal temperature and reaction time for various shrimp viral pathogen. Out of the three different temperatures (60, 63, and 65°C), the best results were obtained at 63°C. The most rapid amplification was achieved at that temperature, requiring less than 20 minutes for the initiation of amplification as determined by a change in the turbidity by magnesium pyrophosphate (Fig. 1). Amplification was efficient at all temperatures tested; however, 63 °C for 60 minutes of reaction time was selected as optimal conditions for further experiments.

Specificity of real-time LAMP detection: Cross-reactivity analysis was performed to examine the specificity of real-time LAMP assay. DNA/cDNA of other shrimp viral disease viruses (WSSV, YHV, IHHNV, and TSV) and healthy shrimp were used to determine the specificity of each viral diagnostic assay. As shown in Fig. 2, the real-time LAMP assay was highly specific to each virus without any cross-reaction with other shrimp viral pathogens.

Quantitative detection using real-time LAMP: For quantitative detection of samples of unknown concentrations, a standard curve was generated using the turbidity time (Tt) plotted against the log of the initial template using serially diluted, 10^1-10^9 copies/μl of plasmids with inserts of concern viral target DNAs (Fig. 3). High correlation coefficient values (R^2 = 0.99, R^2 = 0.99, R^2 = 0.98 and R^2 = 0.99, respectively for WSSV, YHV, IHHNV, and TSV) were obtained for each viral pathogen by using real-time LAMP assay (Fig. 4). Ten-fold dilutions were used to generate standard curves for each pathogen to run in parallel with unknown samples for quantitative and diagnostic analysis.

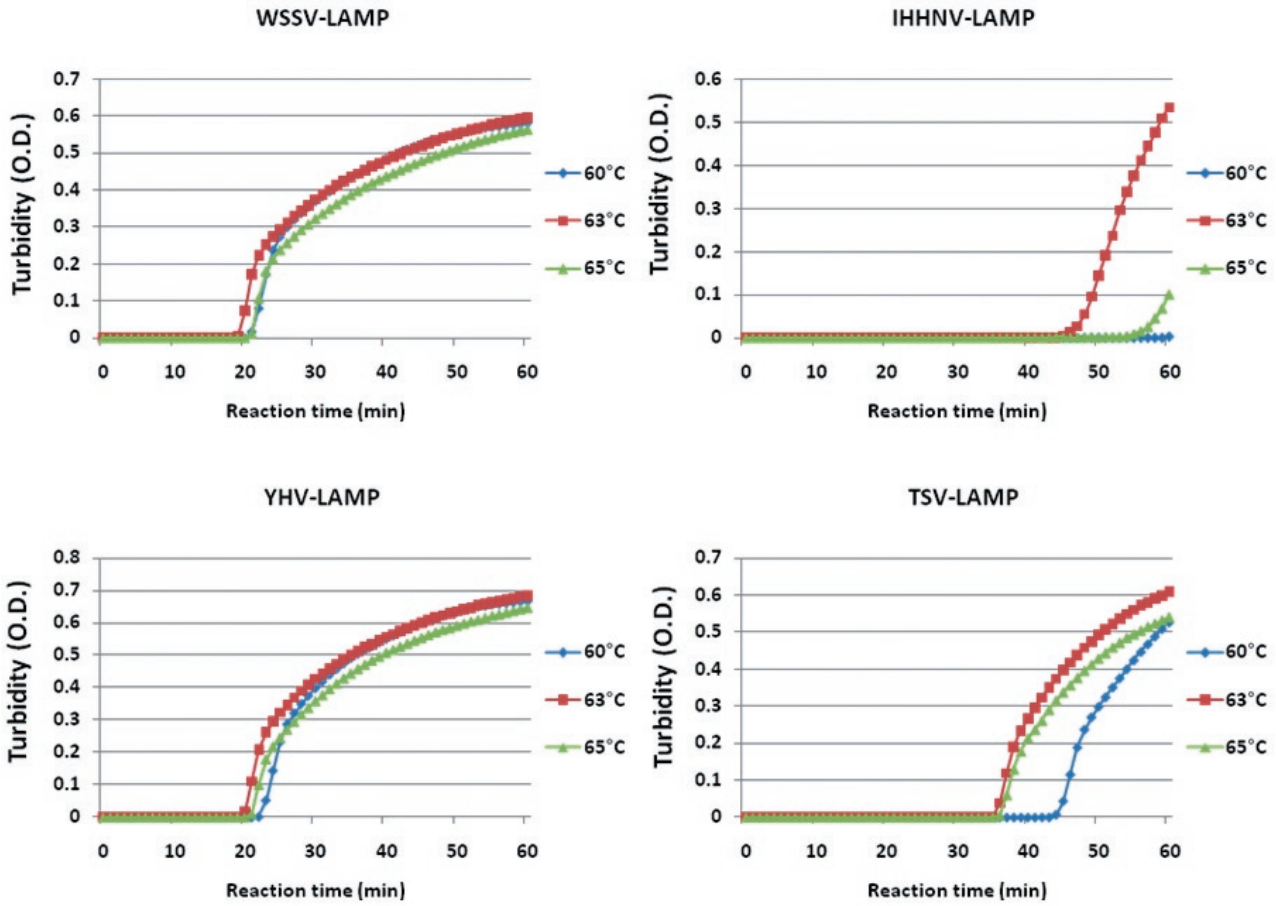

Fig. 1. Optimization of the reaction temperature for real-time LAMP assay of shrimp viral pathogens (WSSV, YHV, IHHNV and TSV) performed at 60, 63 and 65°C

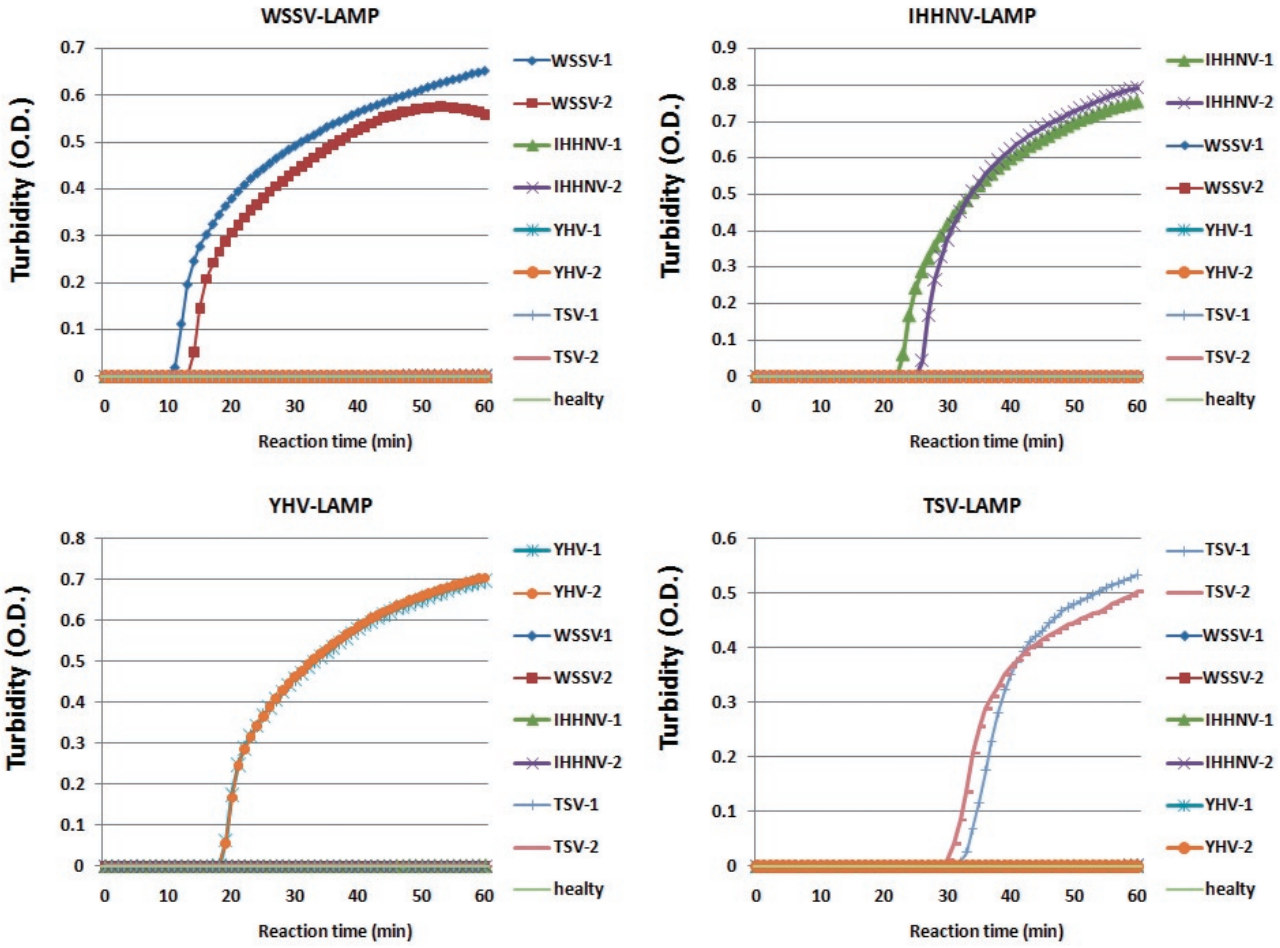

Fig. 2. Specificity of the real-time LAMP assay. Results of cross-reaction analysis with templates of individual shrimp pathogen with other major shrimp viruses (WSSV, YHV, IHHNV and TSV- DNA/cDNA, and with a healthy shrimp DNA template are shown).

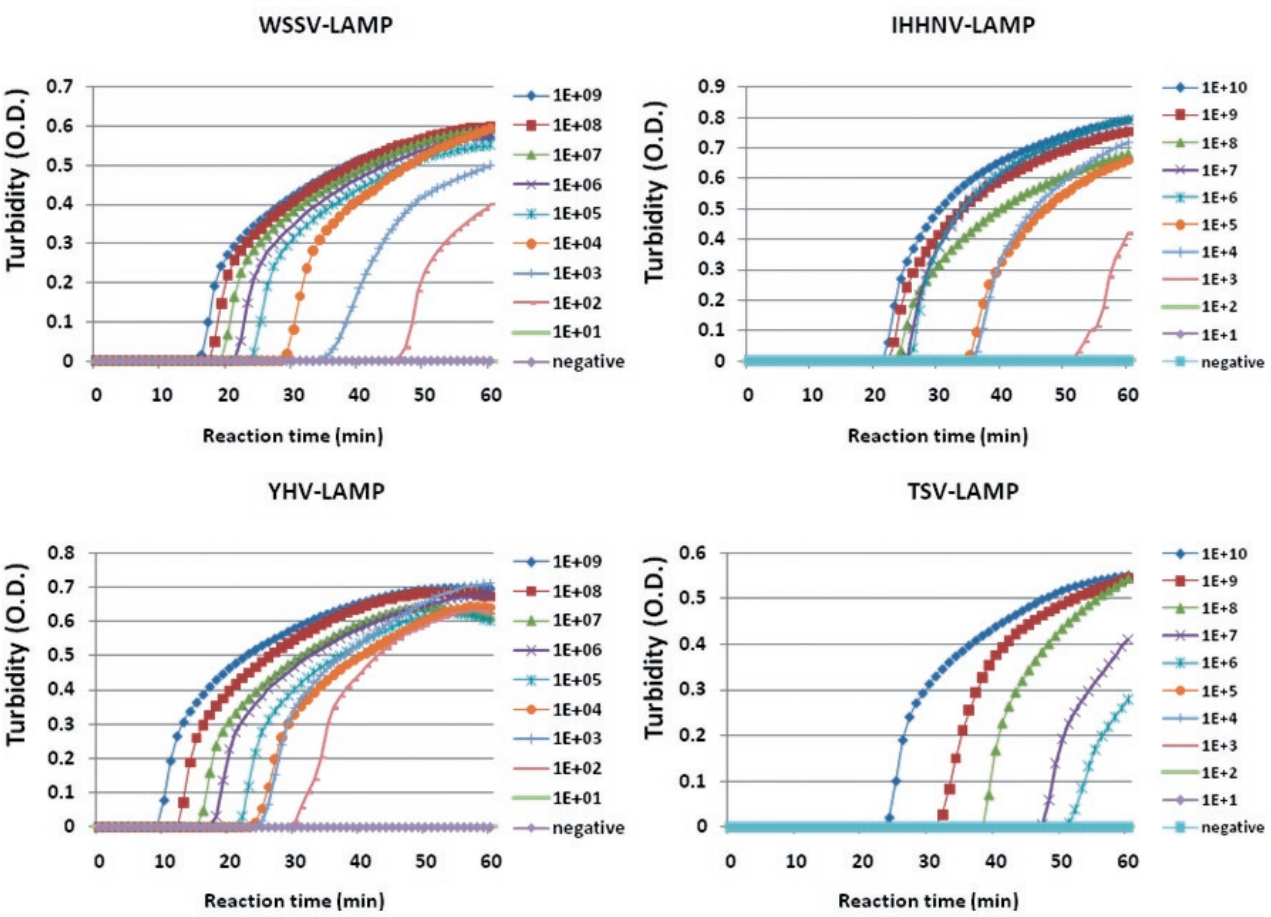

Fig. 3. Real-time amplification of shrimp viral pathogens (WSSV, YHV, IHHNV and TSV) using real-time LAMP assay. Plasmid standards corresponding to target gene of each shrimp viral pathogen at concentrations of 10^1 to 10^9 copies/μl (time is shown on the X-axis and OD at 650 nm on the Y-axis).

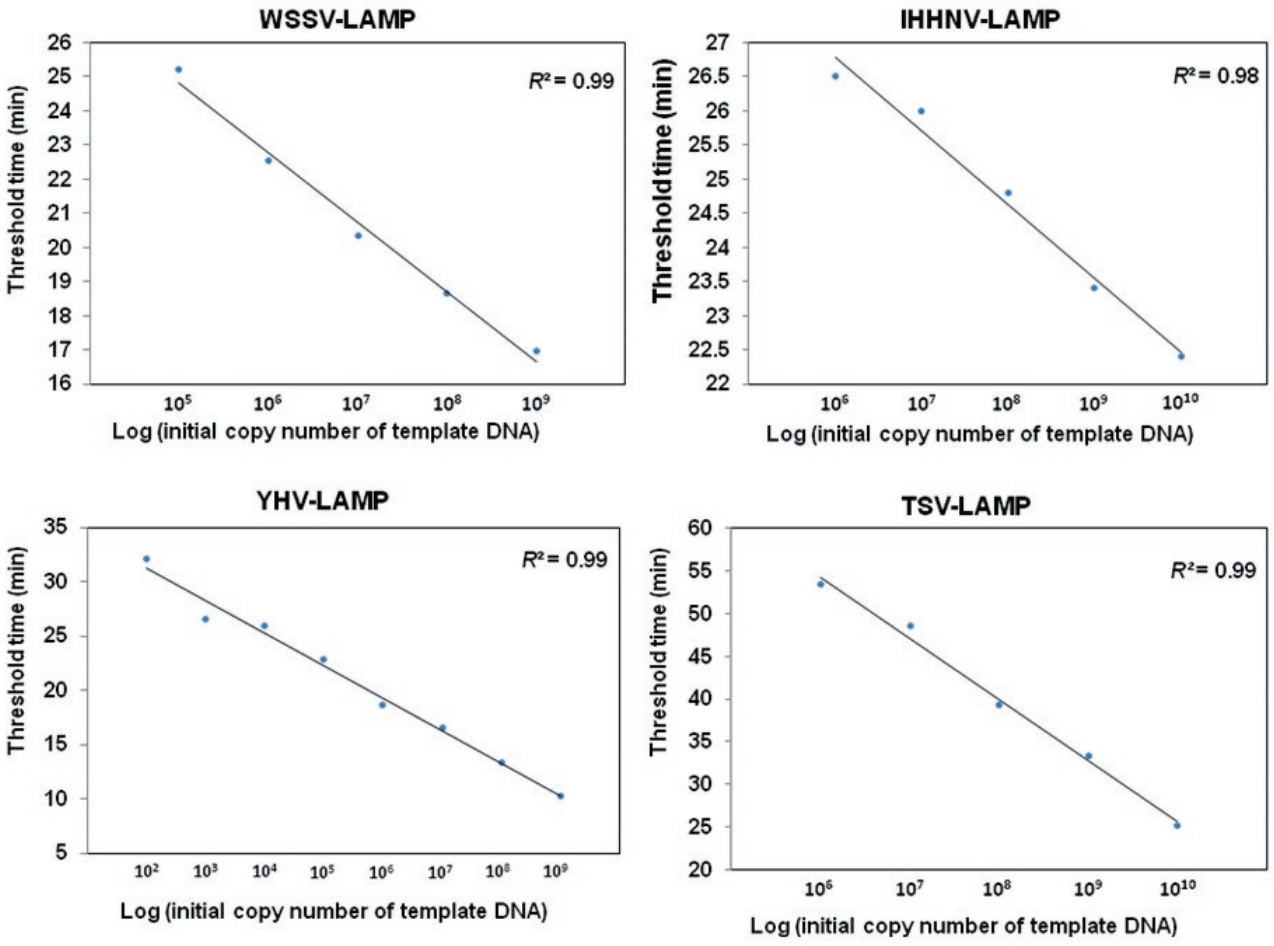

Fig. 4. Standard curves generated from plasmid standards corresponding to target gene of each shrimp viral pathogens.

Discussion

Recent outbreaks of IHHNV, YHV, and TSV virus in the Asia-Pacific area and other regions have caused severe economic loss to shrimp farmers; however, these viral disease outbreaks have not been reported in Japan. Therefore, these diseases have been designated as "a specified disease" using Japanese law, where if the disease is detected in Japan, all shrimp must be destroyed to prevent widespread dissemination. Therefore, it is essential to develop an efficient method for field surveillance for these pathogens that has high specificity and sensitivity with a short reaction time, as well as the ability to quantify the viral load. A recent outbreak of YHV in the Asia-Pacific and other regions has led to severe economic loss to shrimp farmers (Cowley and Walker, 2002) because there was no user-friendly detection assay available.

Here we demonstrate a new diagnostic method for the quantitative detection of shrimp viral pathogens (WSSV, YNV, IHHNV, and TSV) using the real-time LAMP method. Early detection of shrimp viral pathogens is important in the shrimp industry for effective health management and preventive measures. Previously, various nucleic acid and protein based assays (Sritunyalucksana *et al.*, 2006) have been employed for the detection of WSSV and other shrimp viral pathogens in the shrimp culture sector. All of these assays can determine only the presence of viral pathogen; however, they do not determine the number of virus particles present.

Quantification of WSSV can be achieved by real-time PCR assays using TaqMan (Sritunyalucksana *et al.,* 2006) and SYBR chemistry (Khadijah *et al.,* 2003, Yuan *et al.,* 2007).

Various conventional diagnostic methods for WSSV has been developed and reported by several researchers worldwide with different sensitivity. Sensitivity of the normal qualitative LAMP assay of WSSV was almost equal to the real-time LAMP assay, whereas it lacks quantitative measurement (Kono *et al.,* 2004). Different PCR-based assays such as one step PCR, Nested PCR, and real-time PCR have the sensitivity limit of up to 1,000 copies, 50 copies and five copies, respectively (Sritunyalucksana *et al.,* 2006). In contrast, the real-time LAMP method is found to be a cost-effective quantitative assay for shrimp viral disease diagnosis. As all conventional methods were developed based on a specific genome, the chance of non-specificity is found to be much less. Real-time PCR assays have been developed for laboratory diagnosis of shrimp viruses; however, these techniques have the intrinsic disadvantage of requiring both high-precision instruments, high costs for the amplification, and a complex method for the detection of amplified products and technically qualified persons.

The increased need for an inexpensive method to quantify shrimp viral pathogens led us to develop the real-time LAMP method. Also the cost of the Loopamp turbidimeter is low, whereas the real-time PCR assays require fluorogenic primers and probes using an expensive fluorometer (Parida *et al.,* 2004). The sensitive real-time LAMP assay has been successfully applied to detect many human pathogenic RNA viruses, resulting in rapid and simple diagnostic measures. The rapid simple detection and quantification of shrimp viral pathogens using real-time LAMP takes less time when compared to other PCR and real-time PCR methods. The optimum conditions for the real-time LAMP reaction were 63°C for 60 min. Higher temperatures can support the rigorous binding of primer and target template in the LAMP reaction than at lower temperatures (Teng *et al.,* 2007), leading to amplicons consisting of concatemer hairpin repeats (Cai *et al.,* 2008). Using the optimized conditions to perform the assay in a short period of time (<60 min), the turbidity caused by the magnesium pyrophosphate can be visualized without an instrument (Sun *et al.,* 2006). This assay will provide a practical tool in the field for quantitative detection of viral infection in cultured shrimp, even at the early stages.

Each virus-specific standard curve was generated using 10-fold dilutions of 10^1-10^9 copies/μl of purified plasmids, and the reactions were run in duplicate. The mean Tt for the plasmid standards was generated using the specific software provided with the Loopamp real-time turbidimeter. Standard curve equations were calculated using regression analysis, which compared the average Tt to the standard copy number. We obtained a high correlation coefficient (great than or equal to R^2 = 0.988 for each shrimp viral pathogens) for the unknown quantity DNA templates. Cross reactivity analysis showed the primers used were specific to each viral pathogen and did not amplify for other shrimp viral pathogens and healthy shrimp cDNA/DNA templates. Sensitivity analysis showed the assay for each shrimp viral pathogen is detectable up to 100 copies of the template DNA, which is more sensitive than the earlier-developed LAMP and PCR based methods. Furthermore, the gradual decrease of turbidity in the reaction was also clearly observed by the naked eyes.

The real-time LAMP assay is an alternate method to conventional PCR and the LAMP assay (Sun *et al.,* 2006), and in addition, it quantifies the shrimp viral nucleic acid templates. This gives a triplex amplification synchronization on one target gene (Mori *et al.,* 2004). The real-time LAMP assay allows positive samples to become clouded (turbid) and can be viewed visually, eliminating electrophoresis for further confirmation (Mori *et al.,* 2004). The sensitivity of the loop primer was demonstrated in a previous report (Nagamine *et al.,* 2002). The real-time quantitative LAMP assay can be used for gene expression analysis, as the reaction is performed under isothermal conditions and a relatively low temperature where the reverse transcriptase can efficiently work. Thus, this assay has the potential to simplify quantitative gene expression analysis.

We believe the genome specific real-time LAMP assay will be routinely used as a comprehensive

shrimp viral detection system in most field diagnostic laboratories because of its speed, simplicity, specificity and lower cost. We are considering further studies using real-time LAMP with the fluorescence probe, SYBR-I, as the intercalation dye to increase the LAMP sensitivity to quantify very low numbers of virus. The real-time LAMP assay would be a promising technology for shrimp viral pathogen detection, which contributes to better shrimp health management and disease surveillance in shrimp hatcheries and culture ponds for prevention of disease outbreak.

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