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Real-time reverse transcription loop-mediated isothermal amplification for rapid detection of yellow head virus in shrimp

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A B S T R A C T

Article history:

Received 22 April 2009

Received in revised form 13 July 2009

Accepted 21 July 2009

Available online 29 July 2009

Keywords:

Real-time RT-LAMP

YHV

Penaeus monodon

Shrimp

A real-time reverse transcription loop-mediated isothermal amplification (real-time RT-LAMP) method was applied for detecting the replicase polyprotein-encoding gene of yellow head virus (YHV) in shrimp, *Penaeus monodon*. It is a novel, gene-specific assay that amplifies nucleic acid with high specificity, sensitivity and rapidity under isothermal conditions using a set of six specially designed primers that recognize eight distinct sequences of the target gene. This method works with even low copies of DNA and is based on magnesium pyrophosphate turbidity detection by an inexpensive photometer for quantitative analysis. A user-friendly protocol was developed with optimal conditions standardized at 63 °C for 60 min. With this protocol, the assay sensitivity was 10 times higher than the widely used YHV nested RT-PCR system. Cross-reactivity analysis using other shrimp virus DNA/cDNA and YHV-negative shrimp demonstrated high specificity of the assay. The real-time RT-LAMP method was performed also for an internal control gene, EF-1 α , to compare with the expressions of the YHV gene in different organs of infected shrimp, and the resulting standard curves showed high correlation coefficient values. These results suggest that this assay is applicable widely as a new quantitative detection method in the pursuit of YHV-free shrimp culture.

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1. Introduction

Yellow head virus (YHV) is a highly virulent shrimp pathogen which causes extensive mortalities in farmed penaeid shrimp. YHV was first reported as a virulent pathogen in the early 1990s causing a 100% crop loss within 3–5 days post-infection in *Penaeus 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 (Wang and Chang, 2000) and many other Asian countries (Walker et al., 2001). The causative agent is a rod-shaped, enveloped virus with positive stranded ssRNA containing four open reading frames and is classified under the genus *Okavirus*, family *Roniviridae* (Walker et al., 2005). So far, six genotypes have been identified, and only genotype 1 is known to cause typical signs of YHV infection in shrimp (OIE, 2006). Other genotypes have been found widespread in *P. monodon* shrimp populations throughout the Indo-Pacific region but

not associated with farm disease outbreaks (Wijegoonawardane et al., 2008a). Conventional diagnostic measures such as gross signs and histopathology have been used to measure the severity of YHV infection (OIE, 2006; Flegel, 2007), though typical signs are not always observed and histopathology observations are not definitive (Tang et al., 2002). Various nucleic acid and antibody based diagnostic methods have been developed to demonstrate YHV infection in shrimps (Soowannayan et al., 2003; Cowley et al., 2004; Mekata et al., 2006; Munro and Owens, 2006; Intorasoot et al., 2007; Sithigorngul et al., 2007).

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 sets of specially designed inner and outer primers. This assay is highly specific for the target sequence, because the target sequence is recognized at six independent sequences in the initial stage and at four independent sequences during the later stages of the LAMP reaction. In this study, additional two primers (Loop F and Loop B) were designed to accelerate the LAMP reaction

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where the combined primer set facilitates strand displacement. Loop primers bond with the stem-loops, excluding the prime strand displacement DNA synthesis and inner primer bound area (Mori et al., 2001; Nagamine et al., 2002). The LAMP method has been used without quantitation for diagnosis of various shrimp viruses such as white spot syndrome virus (WSSV) (Kono et al., 2004), YHV (Mekata et al., 2006), MrNV and XSU (Pillai et al., 2006), infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Sun et al., 2006) and taura syndrome virus (TSV) (Kiatpathomchai et al., 2007). These qualitative methods, however, cannot determine the copy number of the viral particles present in the sample.

Recently, real-time loop-mediated isothermal amplification (real-time LAMP) was developed to amplify nucleic acids with high specificity and sensitivity, allowing quantitative analysis of nucleic acid templates (Notomi et al., 2000). During the LAMP reaction, an insoluble by-product, magnesium pyrophosphate, is produced in proportion to the amounts of the target DNA to be amplified. Therefore, real-time quantitation can be achieved by measuring the turbidity caused by magnesium pyrophosphate 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 viral diseases of shrimp, the real-time LAMP method has been employed for white spot disease virus (Mekata et al., 2009) and IHHNV (Sudhakaran et al., 2008). In order to improve the epidemiological status of YHV, it has become even more important to have a rapid and applicable method for detection of the virus. In the present study, a comparatively less expensive quantitative real-time RT-LAMP assay was successfully applied for detection of YHV in the field and proven to have high sensitivity and specificity.

2. Materials and methods

2.1. Shrimp

Black tiger shrimp (*P. monodon*) with prominent signs of YHV infection were collected from shrimp farms of Songkhla, Thailand.

Table 1

Primers used for real-time LAMP to detect the replicase polyprotein gene of YHV.

Primer name	Sequences 5'-3'
YHV-FIP	CATGTTCCAATTTCCGCCGTACACACTGAAAATCCTACACG
YHV-BIP	ACCAAGCACTCACCACATTCCTCCCTCATGTATAGAGTCAAGT
YHV-F3 primer	CGCTTCTCTCTGCTATC
YHV-B3 primer	AAGCATACTCTCCGATT
YHV-loop F primer	AGACCGCAGAGAATTTCCATG
YHV-loop B primer	CAAAGACATCACATTCACATTCGTC

Shrimp samples were collected in separate sterile tubes and transported to the laboratory on dry ice for the real-time LAMP assay for YHV.

2.2. RNA extraction and cDNA construction

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 RNA was then eluted in spin columns to a final volume of 100 μ l and stored at -80°C for developing the real-time LAMP diagnostic assay for YHV. Total RNA was 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 μ g of total RNA as per the manufacturer's instructions.

2.3. Design of primers for real-time RT-LAMP

Real-time RT-LAMP primers specific to YHV were designed according to the published sequence of YHV replicase polyprotein gene (GenBank accession number EU487200) using PrimerExplorer Software ver. 4 (<http://primerexplorer.jp/lamp4.0.0/index.html>, Fujitsu, Japan). A set of six primers, two outer (F3 and B3), two inner (FIP and BIP) and two loop primers (Loop F and Loop B), were designed according to the guideline provided. The oligonucleotide primers used for the amplification are shown in Table 1 and Fig. 1. Each primer has two distinct sequences corresponding to the sense

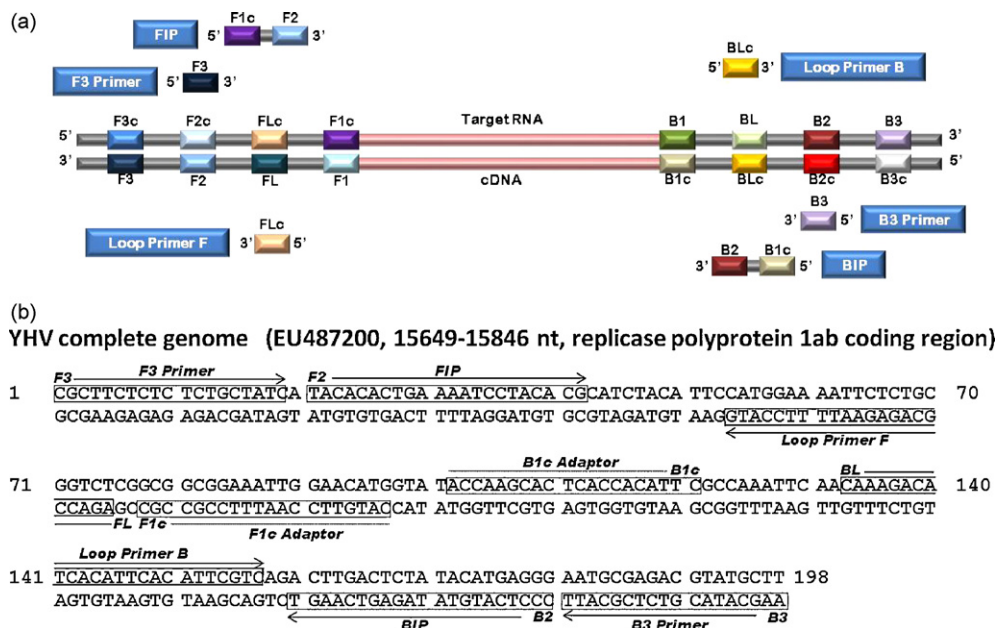


Fig. 1. (a) A diagram showing the two inner (FIP and BIP), two outer (F3 and B3) and two loop (FL and BL) primers for real-time LAMP. A part of these sequences is excerpt from the published documents of Eiken Chemical Co., Ltd. (b) The nucleotide sequence of the YHV replicase polyprotein (GenBank accession number: EU487200) used to design the inner, outer and loop primers. DNA sequences used for primer design are shown by boxes and arrows.

and antisense sequences of the target, one for priming in the first stage and the other for self-priming in later stages.

2.4. Optimization of reaction time and temperature

The real-time RT-LAMP was carried out in a total volume of 25 μ l of reaction mixture using Loopamp RNA Amplification Kit (Eiken Chemical, Japan) according to the manufacturer's instructions. Briefly, 5 μ l of target RNA was mixed with 1 μ l (40 pmol) of each YHV-FIP and -BIP, 1 μ l (5 pmol) of YHV-F3 and -B3 primers, 1 μ l (20 pmol) of YHV-loop F and B primers, 12.5 μ l of Reaction Mix (2 \times), 0.5 μ l of distilled water and 1 μ l Enzyme Mix containing *Bst* DNA polymerase and AMV reverse transcriptase. The reaction temperature (60, 63 and 65 $^{\circ}$ C) was optimized using Loopamp Real-Time Turbidimeter LA-200C (Teramecs, Japan). Real-time monitoring was performed every 3 s using spectrophotometric analysis by recording the optical density (OD) at 650 nm. Each assay was carried out three times.

2.5. Determination of sensitivity of real-time RT-LAMP assay

Ten-fold serial dilutions (10^{-1} to 10^{-7}) of RNA extracted from the heart tissues of YHV-infected shrimp were used as templates for real-time RT-LAMP under the optimized conditions. Real-time RT-LAMP reaction was performed using the Loopamp real-time turbidimeter.

2.6. Comparative analyses with the YHV nested RT-PCR system

Nested RT-PCR was carried out using a commercial kit, IQ 2000 (Farming IntelliGene Technology Corporation, Taiwan, ROC) for the detection of YHV and gill-associated virus (GAV). The first step RT-PCR was carried out in 8 μ l reaction volume containing 7 μ l of RT-PCR PreMix (reaction buffer, dNTPs, and YHV/GAV specific primers), 0.5 μ l of IQzyme DNA polymerase (2 U/ μ l), 0.5 μ l of RT Enzyme Mix and 2 μ l of RNA extracted from YHV. Ten-fold serial dilutions (10^{-1} to 10^{-7}) of RNA used in sensitivity analysis were used as the templates in nested RT-PCR reactions. The amplification regimen was 30 min at 42 $^{\circ}$ C, 2 min at 94 $^{\circ}$ C followed by 15 cycles of 94 $^{\circ}$ C for 20 s, 62 $^{\circ}$ C for 20 s, 72 $^{\circ}$ C for 30 s, and then final elongation for 30 s at 72 $^{\circ}$ C and 30 s at 20 $^{\circ}$ C. After the RT-PCR reaction was completed, 14 μ l of nested PCR PreMix and 1 μ l of IQzyme DNA polymerase were added. Nested PCR reaction setup was followed by 30 cycles of 94 $^{\circ}$ C for 20 s, 62 $^{\circ}$ C for 20 s and 72 $^{\circ}$ C for 30 s, and then final elongation for 30 s at 72 $^{\circ}$ C and 30 s at 20 $^{\circ}$ C. Nested PCR products were electrophoresed in a 2% agarose gel to visualize the detection limit of the assay system.

2.7. Specificity of real-time RT-LAMP detection

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

2.8. Quantitative real-time RT-LAMP

To quantitate the target mRNA from YHV using the real-time RT-LAMP assay, YHV cDNA fragment (988 bp) was cloned into a plasmid. The amplified PCR product was obtained using the upstream (5'-CATTCTACCGTGGTGCTCA-3') and downstream (5'-GCTCTGGCAGCAAAGGTAAC-3') primers and cloned into pGEM-T Easy Vectors (Promega, USA) according to the manufacturer's instructions. Quantitation of the constructed plasmid (pGEM-YHV) was achieved using the NanoDrop spectrophotometer, and ten-fold

Table 2
Primers used for real-time LAMP of the EF-1 α gene.

Primer name	Sequences 5'-3'
EF-1- α -FIP	GCGAACTTGACAGCAATGTGAGCAGATCCAGG-CTGGCTACT
EF-1- α -BIP	TGGTAAGGAAGCTGGAAGCCGGACATCTTTAC-GATGCAGGAGT
EF-1- α -F3 primer	ATCGTCCTTAACCCACTT
EF-1- α -B3 primer	CATGGGCTTGCTGGGAAC

serial dilutions (10^2 to 10^9) were made to evaluate the real-time RT-LAMP. 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 YHV replicase gene 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.

2.9. Establishment of quantitative real-time RT-LAMP for the internal control gene for expression analysis

To confirm the quality of mRNA and the application of real-time RT-LAMP assay for expression analysis, LAMP primers were designed for an internal control gene, elongation factor 1 α (EF-1 α) according to the published sequence (GenBank accession number DQ021452). A set of four primers, two outer (F3 and B3) and two inner primers (FIP and BIP), were designed according to the guideline using PrimerExplorer ver. 4 (Table 2). EF-1 α cDNA fragment (199 bp) was cloned into a plasmid using PCR products obtained from EF-1 α -F3 and EF-1 α -B3 primers. The constructed plasmid (pGEM-EF-1 α) was serially diluted to construct a standard curve for the quantitation of templates of unknown concentrations. The standard curve of EF-1 α gene was generated for each analysis. The reaction setup was the same as the one optimized above and carried out in the Loopamp real-time turbidimeter. The data obtained from the real-time RT-LAMP analysis were subjected to one-way analysis of variance followed by an unpaired, two-tailed *t*-test. Differences were considered significant at $P < 0.01$.

2.10. Quantitative detection of YHV in different organs using real-time RT-LAMP

Total RNA was extracted from different organs such as the stomach, muscle, lymphoid tissue, intestine, brain, gills, hepatopancreas and the heart of YHV-infected, moribund shrimp using an RNA extraction kit (High Pure RNA Tissue Kit) according to the manufacturer's instructions and used as a template for quantitative real-time RT-LAMP analysis. The copy numbers of each reaction were calculated based on standard curves using the software provided along with the Loopamp real-time turbidimeter. The software was also used to compare the relative expression of YHV and EF-1 α genes.

3. Results

3.1. Optimization of real-time RT-LAMP assay conditions for YHV detection

Real-time RT-LAMP was performed using RNA as a template in order to determine the optimal temperature and reaction time. Out of the three different temperatures (60, 63 and 65 $^{\circ}$ C) the best results were obtained at 63 $^{\circ}$ C. The most rapid amplification was achieved at this temperature, requiring less than 20 min for the initiation of amplification as determined by a change in the turbidity by magnesium pyrophosphate (Fig. 2). Amplification was efficient

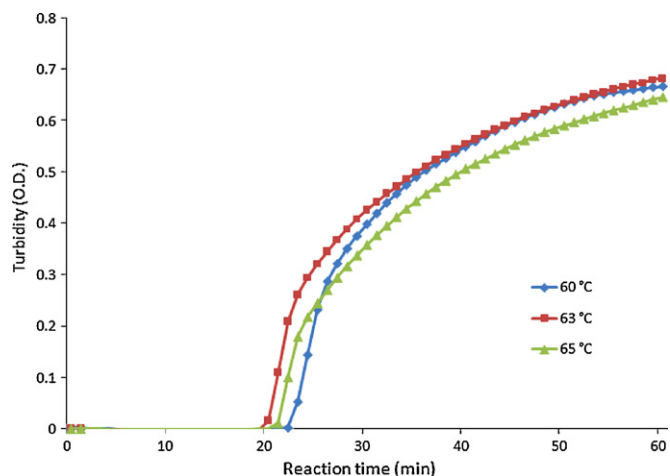


Fig. 2. Results of YHV real-time LAMP at 60, 63 and 65 °C performed for optimization of the reaction temperature.

at all temperatures tested; however, 63 °C for 60 min of reaction time was selected as optimal conditions for further experiments.

3.2. Comparison of real-time RT-LAMP with nested RT-PCR

In order to determine the detection limit of real-time RT-LAMP, various concentrations (10^{-1} to 10^{-7} dilutions) of RNA extracts from the heart tissues of YHV-infected shrimp were analyzed by nested RT-PCR. Results revealed that real-time RT-LAMP assay could detect RNA at 10^{-1} to 10^{-5} dilutions, whereas the maximum dilution of RNA detected by the nested RT-PCR system was 10^{-4} . Thus, the real-time RT-LAMP assay was one order more sensitive than the nested RT-PCR detection method (Fig. 3a and b).

3.3. Specificity of real-time RT-LAMP detection

Cross-reactivity analysis was performed to examine the specificity of real-time RT-LAMP assay. DNA/cDNA of other shrimp viral disease viruses (WSDV, IHNV and TSV) and healthy shrimp were used to determine the specificity of YHV real-time RT-LAMP assay. As shown in Fig. 4, the real-time RT-LAMP assay was highly specific to YHV without any cross-reaction with other shrimp viruses.

3.4. Quantitative detection using real-time RT-LAMP

For quantitative detection of samples of unknown concentrations, a standard curve was generated using the turbidity time (T_t) plotted against the log of the initial template using serially diluted, 10^2 to 10^{10} copies/ μ l of pGEM-YHV. A high correlation coefficient ($r^2=0.9869$) was obtained by the real-time RT-LAMP assay (Fig. 5a and b). Ten-fold dilutions (10^4 to 10^9 copies/ μ l) of pGEM-EF-1 α were used to generate a standard curve with a high correlation coefficient ($r^2=0.9729$) and run in parallel with YHV real-time RT-LAMP for normalization during expression analysis (Fig. 6a and b). Expression analysis was carried out for the YHV replicase polyprotein gene using real-time RT-LAMP and normalized with the expression level of the internal control EF-1 α gene. The organs positive for YHV gene expression were stomach, muscle, lymphoid tissue, intestine, brain, gills, hepatopancreas and heart in moribund YHV-infected shrimp (Fig. 7a and b). Relative expression of YHV replicase polyprotein gene is very high in the heart and very low in the stomach compared to those of the other organs such as the muscle, lymphoid tissue, intestine, brain, gill and hepatopancreas (Fig. 8).

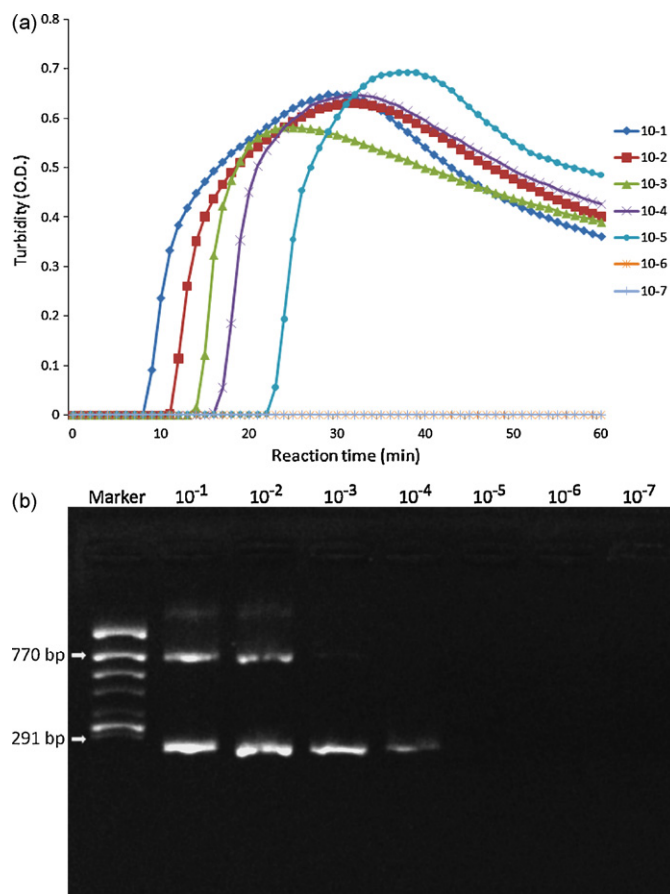


Fig. 3. Comparative analysis of YHV real-time RT-LAMP with the YHV nested RT-PCR system using same dilutions of RNA templates (10^{-1} to 10^{-7}). (a) YHV real-time RT-LAMP. (b) YHV nested RT-PCR (IQ2000 YHV/GAV detection system, Farming IntelliGene Tech.).

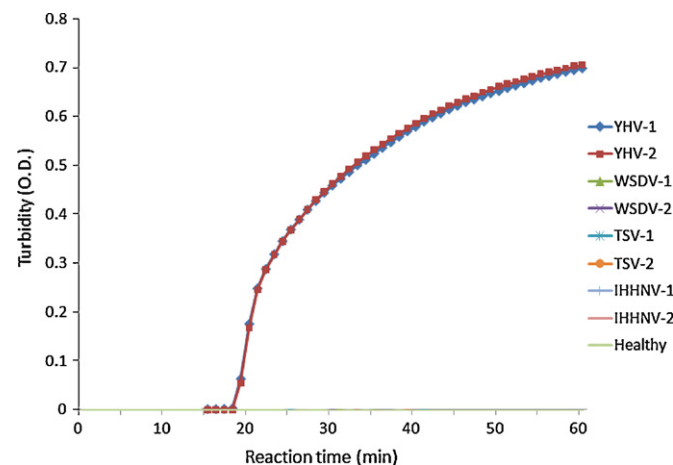


Fig. 4. Specificity of the real-time YHV RT-LAMP method. Results of cross-reaction analysis with templates of other major shrimp viruses, IHNV, WSDV and TSV-DNA/cDNA, and with a healthy shrimp DNA template are shown.

4. Discussion

In the present study, a novel system for rapid and simple detection of YHV RNA using real-time RT-LAMP diagnostic assay is described. Although there is a strong demand for development of a rapid and simple detection for all genotypes of YHV, the real-time RT-LAMP assay has been targeted to the most dominant genotype,

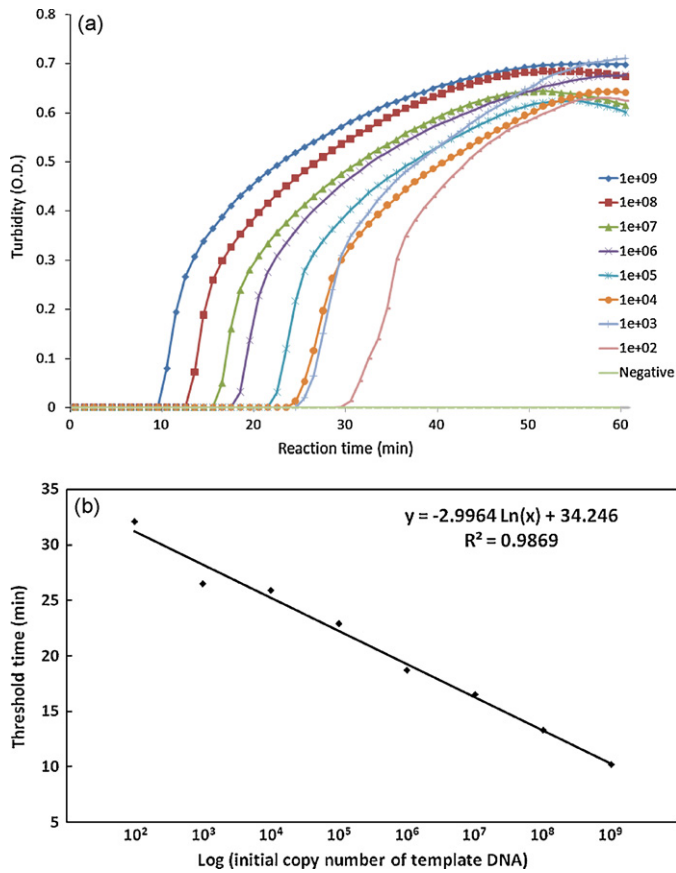


Fig. 5. Real-time amplification of the YHV replicase polyprotein gene using the real-time RT-LAMP assay and construction of a standard curve. (a) YHV DNA plasmid standards at concentrations of 10^2 to 10^9 copies/ μ l (time is shown on the X-axis and OD at 650 nm on the Y-axis). (b) A standard curve generated.

which affects the shrimp culture worldwide (Wijegoonawardane et al., 2008a). This report demonstrates a one-step, single tube, real-time accelerated RT-LAMP assay for rapid detection of YHV. Real-time RT-PCR assays have been developed for laboratory diagnosis of shrimp viruses (Poulos et al., 2008; Xie et al., 2008; Dhar et al., 2009); however, these techniques have the intrinsic disadvantage of requiring either a high-precision instrument for the amplification or a complex method for the detection of amplified products. The sensitive real-time RT-LAMP assay has been successfully applied to detect many human pathogenic RNA viruses, resulting in rapid and simple diagnostic measures (Parida et al., 2004; Imai et al., 2006; Shirato et al., 2007).

A set of six primers, consisting of pairs of outer, inner and loop primers, was used to amplify a 196-bp sequence of the YHV replicase polyprotein gene. The loop primers were used to accelerate the reaction to reduce the detection time (Yoneyama et al., 2007). The optimal condition for YHV real-time RT-LAMP assay was 63 °C with a reaction time of 60 min. Sensitivity and specificity analyses confirmed the efficiency of primer regions selected for YHV detection by real-time RT-LAMP. The developed method is specific to YHV and does not show any cross-reaction with DNA/cDNA of other shrimp disease viruses or normal shrimp DNA. Although the RT-LAMP assay for YHV (Mekata et al., 2006) has been found to be less sensitive than the nested RT-PCR detection method (IQ2000), real-time RT-LAMP is highly sensitive compared to those methods demonstrated earlier. In the present study, the YHV-positive RNA template was detectable up to a 10^{-5} dilution by real-time RT-LAMP; this sensitivity is 10 times higher than the result by nested RT-PCR. Higher

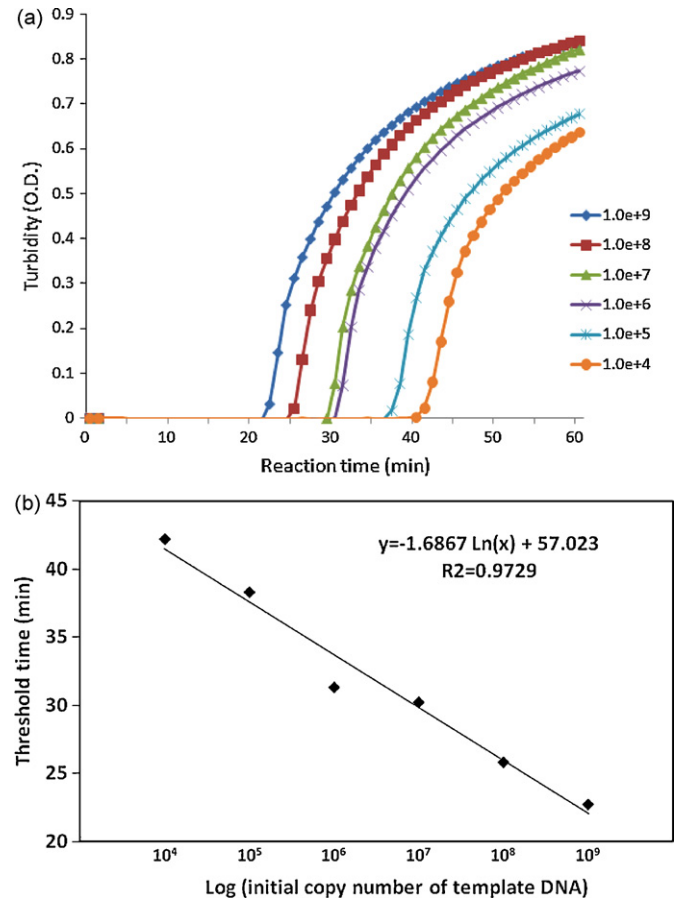


Fig. 6. Real-time amplification of the EF-1 α gene using the real-time RT-LAMP assay and construction of a standard curve. (a) EF-1 α DNA plasmid standards at concentrations of 10^4 to 10^9 copies/ μ l (time is shown on the X-axis and OD at 650 nm on the Y-axis). (b) A standard curve generated.

sensitivity of the real-time LAMP assay was accelerated by employing the loop primers (Nagamine et al., 2002).

The most beneficial property of the YHV real-time RT-LAMP assay is its rapidity; detection can be completed within a short duration of 20 min, whereas conventional PCR methods require 2–3 h (Wijegoonawardane et al., 2008b). Proportionally to the number of amplicons generated, the real-time RT-LAMP reaction yields a large amount of by-products, pyrophosphate ion, leading to the accumulation of white magnesium pyrophosphate precipitates in the reaction mixture. Hence, increase in turbidity of the reaction mixture correlates with the amount of DNA synthesized, and the turbidity is visible to the naked eye and measured by a real-time turbidimeter (Mori et al., 2001). Moreover, it is more cost effective when compared to real-time PCR, which usually requires expensive equipment as well as an expensive fluorescence probe to ensure the specificity of the reaction (Mori et al., 2004).

A standard curve was constructed using ten-fold plasmid dilutions of the YHV and EF-1 α genes with reference to mean T_t values generated using the specific software provided with the Loopamp real-time turbidimeter. Standard curve equations were calculated using regression analysis comparing the average T_t to the standard copy number. High correlation coefficient values were obtained in YHV and internal control gene, EF-1 α ($r^2 = 0.9869$ and 0.9729 , respectively). These results indicate that quantitative mRNA expression analysis of the YHV replicase polyprotein gene in different organs from YHV-infected shrimp can be reliably used even when the expression of the target gene is low. A higher expres-

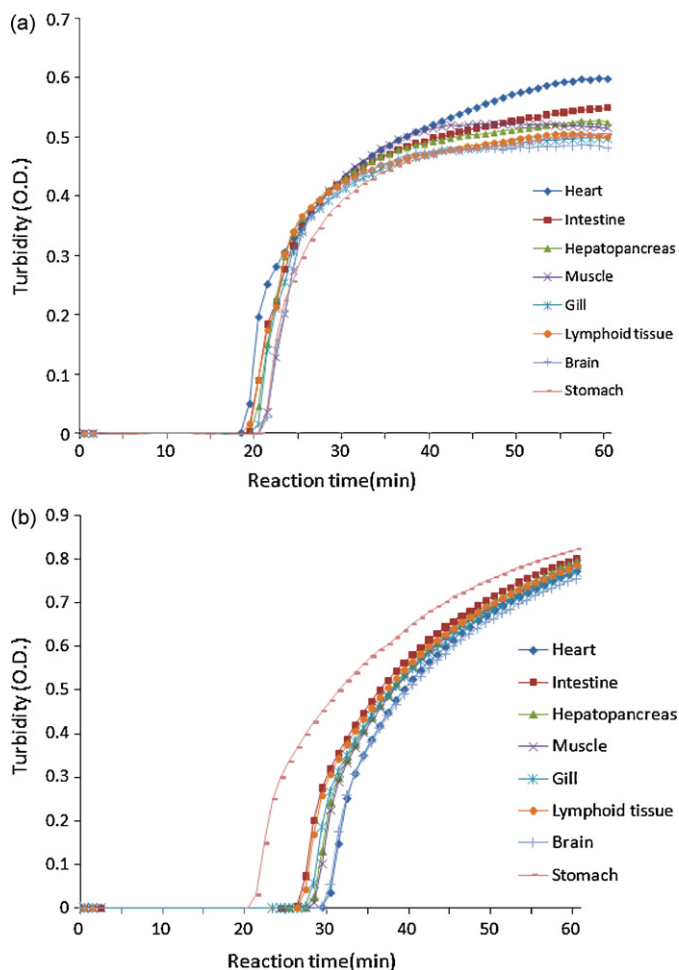


Fig. 7. Quantitative detection and expression analysis of the YHV replicase polyprotein gene and the internal control EF-1 α gene for different organs of YHV-infected shrimp, *P. monodon*. (a) Amplification of the EF-1 α gene. (b) Amplification of the YHV replicase polyprotein gene.

sion level of the YHV gene in the heart suggests the importance of selection of the organ for detecting YHV in shrimp.

This is the first quantitative real-time RT-LAMP assay described for YHV detection in shrimp with the highest sensitivity and specificity achieved within a short duration of reaction time. This assay will provide a practical tool in the field for quantitative detection of YHV infection in cultured shrimp, even at the early stages. A recent outbreak of YHV in the Asia-Pacific and other regions has led to severe economic loss to shrimp farmers (Covley and Walker, 2002) because there was no user-friendly detection assay available. The YHV real-time RT-LAMP assay would be a promising technology for YHV detection, which contributes to better shrimp health management and disease surveillance in shrimp hatcheries and culture ponds for prevention of disease outbreak.

Acknowledgements

T. Mekata and R. Sudhakaran are recipients of the Research Fellowships for Young Scientists and Postdoctoral Fellowships for Foreign Researchers of the Japan Society of Promotion of Science (JSPS). This study was supported, in part, by research grants from the JSPS and the Development Program for New Bio-industry Initiatives of Japan.

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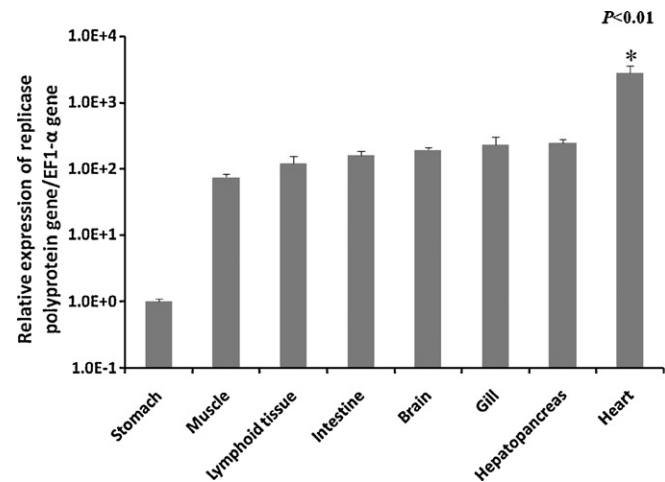


Fig. 8. Relative expression of YHV replicase polyprotein gene normalized with the expression of the internal control gene, EF-1 α , in different organs of YHV-infected shrimp, *P. monodon*. The data obtained from the real-time RT-LAMP analysis were subjected to one-way analysis of variance followed by an unpaired, two-tailed *t*-test. *Indicates a significant difference ($P < 0.01$).

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