ORIGINAL ARTICLE

Real-time quantitative loop-mediated isothermal amplification as a simple method for detecting white spot syndrome virus

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Abstract

Aims: White spot syndrome virus (WSSV) continues to be the most pathogenic virus among the crustacean aquaculture causing mass mortality. In the present study, we established a one-step, single tube, real-time accelerated loop-mediated isothermal amplification (real-time LAMP) for quantitative detection of WSSV.

Materials and Methods: A set of six specially designed primers that recognize eight distinct sequences of the target. The whole process can be completed in 1 h under isothermal conditions at 63°C. Detection and quantification can be achieved by real-time monitoring in an inexpensive turbidimeter based on threshold time required for turbidity in the LAMP reaction. A standard curve was constructed by plotting viral titre against the threshold time (T_t) using plasmid standards with high correlation coefficient ($R^2 = 0{\cdot}988$).

Conclusions: Sensitivity analysis using 10-fold dilutions (equivalent to 35 ng μl^{-1} to 35 ag μl^{-1}) of plasmid standards revealed this method is capable of detecting upto 100 copies of template DNA. Cross-reactivity analysis with DNA/cDNA of IHHNV, TSV, YHV-infected and healthy shrimp showed this method is highly specific for quantitative detection of WSSV.

Significance and Impact of the Study: WSSV real-time LAMP assay appears to be precise, accurate and a valuable tool for the detection and quantification of WSSV in large field samples and epidemiological studies.

Introduction

During the past several decades, the shrimp industry has expanded and rapidly developed. White spot syndrome virus (WSSV) of shrimp was first discovered in northern Taiwan around 1992; and is currently the most serious viral pathogen in farm reared *Marsupenaeus japonicus* (Nakano *et al.* 1994; Takahashi *et al.* 1994) throughout the world (Chou *et al.* 1995; Lo *et al.* 1996a; Flegel 1997). The International Committee on Taxonomy of Viruses (ICTV) determined WSSV is the type species of the genus *Whispovirus*, family *Nimaviridae* (Mayo 2002). WSSV is a double-stranded DNA virus with a genome size of 290–305 kb depending on the origin of the isolate (van Hulten *et al.* 2001; Yang *et al.* 2001; Chen *et al.* 2002) with an enveloped rod-shaped nucleocapsids (Wongteerasupaya *et al.* 1995). The typical clinical signs of WSS include lethargy, reduced food intake and the appearance of white spots on the carapace (Lightner 1996). This virus causes 100% mortality within 3–10 days in all the life stages of both wild and cultured *Penaeus monodon* and *M. japonicus* and has a wide host range including penaeid shrimp, crabs, copepods and other arthropods (Chen *et al.* 2000; Syed Musthaq *et al.* 2006) are well-known.

Many diagnostic methods have been developed to detect WSSV, which include microscopic and molecular

methods (Flegel 1997) with various polymerase chain reaction (PCR) based assays (Lightner 1996; Lo *et al.* 1996b; Kasornchandra *et al.* 1998; Tapay *et al.* 1999; Durand *et al.* 2003), *in-situ* PCR (Jian *et al.* 2005), *in-situ* hybridization (ISH) (Chang *et al.* 1996; Wang *et al.* 1998b; Huang *et al.* 2000), microscopic observation (Wongteerasupaya *et al.* 1995) and many immunological methods (Huang *et al.* 1995; Nadala and Loh 2000; Dai *et al.* 2003; Okumura *et al.* 2005). Because of their high sensitivity, the genome based methods have been widely applied for detection of WSSV in shrimp health management (Maeda *et al.* 1998; Musthaq *et al.* 2006; Natividad *et al.* 2007).

The loop-mediated isothermal amplification (LAMP) method was developed for sensitive and specific detection of WSSV (Kono *et al.* 2004) and other shrimp viruses such as yellow head virus (YHV) (Mekata *et al.* 2006), taura syndrome virus (TSV) (Kiatpathomchai *et al.* 2007), infectious hypodermal and haematopoietic necrosis virus (IHHNV) (Sun *et al.* 2006) and the white tail disease associated viruses (*Mr*NV and XSV) (Pillai *et al.* 2006). All of the above diagnostic assays are qualitative except real-time PCR (Durand *et al.* 2003) which is more expensive and labor intensive.

Recently, real-time loop-mediated isothermal amplification (RT-LAMP) was developed to amplify nucleic acids with high specificity and sensitivity that allows quantitative analysis of infected samples (Notomi *et al.* 2000). The qualitative methods can diagnose WSSV infected shrimp; however, they do not determine the number of virus particles present to determine the relative infection rate. The LAMP reaction is performed using isothermal conditions with rapid amplification where 10^9 copies at 60–65°C in 1 h is described (Notomi *et al.* 2000). This uninterrupted single temperature method (non-cycling) allows for high efficiency amplification of specific products (Parida *et al.* 2004; Savan *et al.* 2005). During the LAMP reaction, an insoluble by-product, 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 (Mori *et al.* 2001).

The real-time LAMP assay was used for the shrimp virus, IHHNV (Sudhakaran *et al.* 2008c), and various other viruses including Japanese encephalitis virus (Toriniwa and Komiya 2006), West Nile virus (Parida *et al.* 2004), dengue virus (Parida *et al.* 2005), severe acute respiratory syndrome (SARS) (Poon *et al.* 2005) and hepatitis A virus (Yoneyama *et al.* 2007). With an increasing demand by the shrimp culture sector for an inexpensive quantification method for WSSV samples, we developed a rapid, one-step, real-time accelerated LAMP quantitative assay. This is the first report describing a field diagnostic detection method for WSSV using the real-time LAMP. This is another option to quantify WSSV where it is comparatively less expensive than using real-time PCR.

Materials and methods

Preparation of the viral inoculum

Naturally WSSV-infected adult shrimp (*M. japonicus)* with the prominent signs of disease with white spots in the carapace were collected from culture ponds from shrimp farms, Japan. They were used as the source of the virus to develop the real-time LAMP diagnostic method. Adult shrimp were transported on dry ice and stored in separate packs at −80°C in the laboratory. Frozen infected specimens were thawed and homogenized in a sterile homogenizer using a 10% (w/v) suspension in NTE buffer (0·2 mol l^{-1} NaCl, 0·02 mol l^{-1} Tris–HCl and 0·02 mol l^{-1} EDTA, pH 7·4). The homogenate was centrifuged at 4000 ***g*** for 20 min at 4°C; the supernatant was again clarified at 10 000 ***g*** for 10 min at 4°C; and then the final supernatant was filtered through a 0·45 μm membrane. The filtrate was then stored at −20°C before extraction of nucleic acids for development and standardization of the real-time LAMP method.

DNA extraction

Extraction of DNA was performed from the virus homogenates prepared above. From each sample, 200 μl of the homogenate was added to 600 μl of DNAzol reagent (Invitrogen, Carlsbad, CA, USA) and further steps were carried out according to the manufacturer's instructions. Extracted DNA samples were quantified spectrophotometrically at 260 nm and the presence of WSSV was confirmed using the conventional PCR method (Takahashi *et al.* 1996) before storing at −20°C.

Primers for WSSV real-time LAMP

Real-time LAMP primers for WSSV were designed using the published sequence of the WSSV-ORF36 gene (GenBank accession number: AF369029) with the LAMP primer designing software Primer Explorer version 4 (http://primerexplorer.jp/lamp4·0·0/index.html). A set of six primers: two outer (F3 and B3), two inner (FIP and BIP) and two loop primers (FL and BL) were designed according to the manufacturer's instructions where the combined primer set helps in strand displacement (Notomi *et al.* 2000). The oligonucleotide primers used for amplification are shown in Table 1 and Fig. 1.

Table 1 Primers used for the real-time LAMP from the ORF36 gene of WSSV

Primer name	Sequences 5′–3′
WSSV-FIP	TCCGTCTTCAGGGAATACATATGCTCAGGGAAGAAATAGACCATG
WSSV-BIP	GGACCCAAATCGAAATATAAGGCCTATGTTGCCCAAGATCCAC
WSSV-F3	AAACACCGGATGGGCTAA
WSSV-B3	CAAGGCAATACAGAATGCG
WSSV-FL	GTTAAGAATGATGCATCTAGTGCGA
WSSV-BL	TGGAACAAAAGATGCTGCTCA

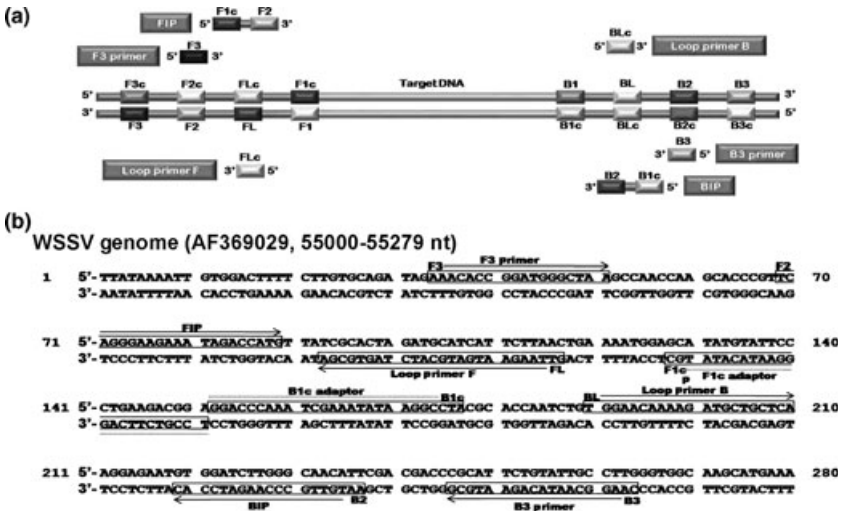

Figure 1 (a) Diagram of the two inner (FIP and BIP), two outer (F3 and B3) and two loop (FL and BL) primers for the real-time LAMP. This region description was partially from Eiken Chemical Co., Ltd published documents. (b) Nucleotide sequence of WSSV-ORF36 (GenBank accession number: AF369029) was used to design the inner, outer and loop primers. DNA sequences used for primer design are shown by boxes and arrows.

Optimization of real-time LAMP reaction conditions for WSSV detection

The real-time LAMP reaction for WSSV detection was performed in a total volume of 25 μl using a Loopamp DNA amplification kit (Eiken Chemical Co., Ltd, Japan) according to the manufacturer's instructions. Briefly, the mixture contained 40 pmol each inner primer (FIP and BIP), 5 pmol each of the outer (F3 and B3) and the loop primers (FL and BL), 12·5 μl 2 × reaction mixture (40 mmol l^{-1} Tris–HCl, 20 mmol l^{-1} KCl, 16 mmol l^{-1} $MgSO_4$, 20 mmol l^{-1} $(NH_4)_2SO_4$, 0·2% Tween-20, 1·6 mol l^{-1} Betaine and 2·8 mmol l^{-1} dNTPs), 8 U *Bst* DNA polymerase and specified amounts of target DNA (Eiken Chemical Co., Ltd). After incubating the reaction mixture for 60 min, the reaction was terminated by heating to 80°C for 2 min. The reaction temperature (60, 63 and 65°C) was optimized in a Loopamp real-time turbidimeter LA-200C (Teramecs, Kyoto, Japan). Real-time monitoring was performed every 6 s using spectrophotometric analysis recording the optical density (OD) at 650 nm.

Cloning of WSSV DNA

To determine the quantity of unknown WSSV DNA using the real-time LAMP assay, WSSV-ORF36 DNA was amplified using PCR with the upstream (5′-AAACACCG-GATGGGCTAA-3′) and downstream (5′-CAAGGCAATA-CAGAATGCG-3′) primers (GenBank accession number: AY369029). The amplified product was cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) according to the manufacturer's instructions. The plasmid (pGEM-ORF36) constructed from this system was used to make standard concentrations of WSSV DNA used for quantitative analysis with the real-time LAMP after measuring the DNA concentration using a Nanodrop UV spectrophotometer ND-100 (NanoDrop Technologies Inc., Wilmington, PA). Standard reactions were carried out in duplicate for accuracy.

Specificity of the real-time LAMP

The specificity of the real-time LAMP primers designed for WSSV was examined in duplicate using DNA/cDNA templates prepared from various shrimp pathogens: YHV, IHHNV and TSV and healthy shrimp.

Detection limit of IHHNV real-time LAMP

A 10-fold serial dilution of pGEM-WSSV plasmid DNA in different concentrations from 1×10^{10} to 1×10^{1} copy numbers, equivalent to 35 ng μl^{-1} to 35 ag μl^{-1} was used as template to identify the detection limit of WSSV real-time LAMP assay. The reaction conditions

used were as determined earlier and carried out in duplicate.

Results

Determination of the conditions for WSSV detection using real-time LAMP

The real-time LAMP was evaluated using WSSV-DNA as the template to confirm the optimum temperature and reaction time. For best amplification the optimum temperature was 63°C for the activation of *Bst* DNA polymerase. It required 15–25 min for initiation of amplification to cause a change in the turbidity by magnesium pyrophosphate (Fig. 2). Although we could get good amplification at every temperature, the best parameters were found to be 60 min at 63°C (Fig. 2).

Quantification of WSSV-infected samples using real-time LAMP

Ten-fold dilutions of WSSV plasmid (pGEM-ORF36) from 10^5 to 10^9 copies μl^{-1} were used as standards for the quantitative analysis. A standard curve was generated plotting turbidity time (T_t) *vs* the log of the initial template concentration. A high correlation coefficient ($R^2 = 0{\cdot}988$) was found in the reaction of the real-time LAMP assay (Fig. 3a,b).

Specificity of real-time LAMP to other shrimp pathogens

A cross-reaction analysis using other shrimp viral disease viruses and normal shrimp DNA/cDNA was performed with YHV, TSV, IHHNV and healthy shrimp to determine the specificity of the WSSV real-time LAMP method (Fig. 4). The data show the real-time LAMP method developed here is highly specific to WSSV without cross-reaction.

A 10-fold dilution (1×10^{10}–1×10^{1}) of plasmid DNA was used to analyse the sensitivity limit of WSSV real-time LAMP assay. The results showed that the assay is detectable up to 1×10^2 copies, which is equivalent to 0·35 fg μl^{-1} (Fig. 5).

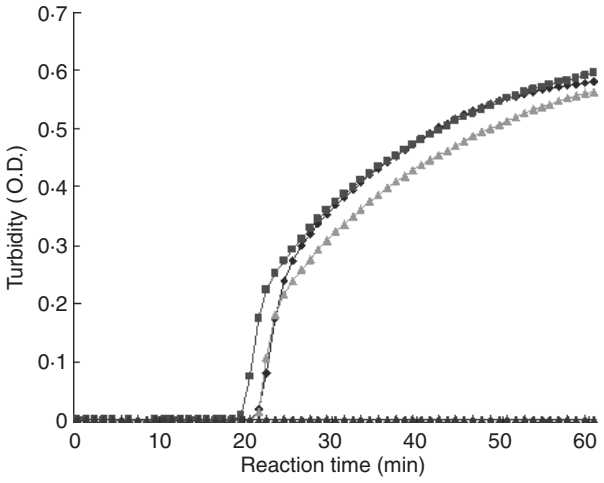

Figure 2 Optimization of the reaction temperature for WSSV real-time LAMP performed at 60, 63 and 65°C. —◆— 60°C, —■— 63°C, —▲— 65°C, —▲— negative control.

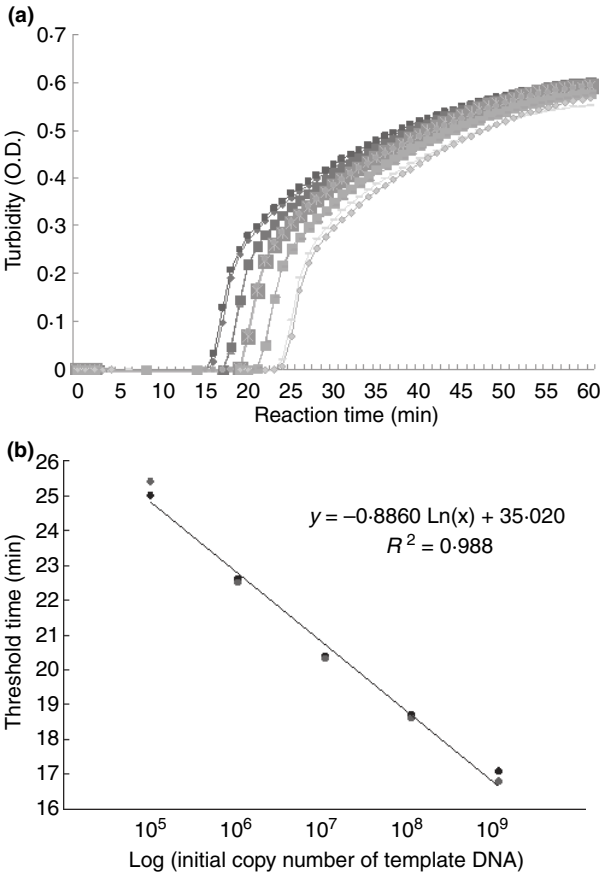

Figure 3 Real-time amplification of WSSV using the real-time LAMP standard curve. (a) WSSV DNA plasmid standards from 10^5 to 10^9 copies μl^{-1} (time is shown on the *X*-axis and optical density at 650 nm on the *Y*-axis). (b) Standard curve generated. —◆— and —■—, 1×10^5, —■— and —□—, 1×10^6, —●— and —⊠—, 1×10^7, —■— and —▲—, 1×10^8, —◆— and —■—, 1×10^9.

Discussion

Here we demonstrate a new diagnostic method for the quantitative detection of WSSV using the real-time LAMP method. Early detection of WSSV is important in the shrimp industry for effective health management and preventive measures. Previously, various nucleic acid and

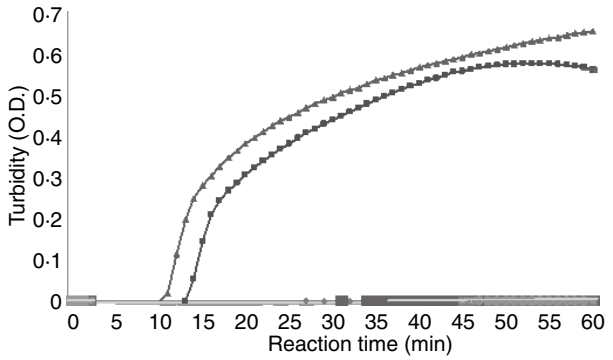

Figure 4 Specificity of the real-time WSSV LAMP method. Cross-reaction studies with other major shrimp viruses: IHHNV-, YHV- and TSV- DNA/cDNA and with a healthy shrimp DNA template. —▲— and —■—, WSSV, —◆— and —▣—, IHHNV, —⊣— and —●—, —⊣— and —●—, TSV, —⊣— and —■—, YHV, —■—, negative control.

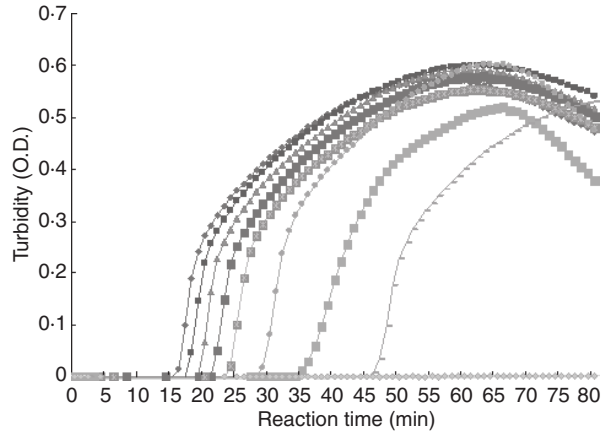

Figure 5 Sensitivity analysis of the WSSV real-time LAMP assay as monitored by the measurement of turbidity using decreasing concentrations of virus from 1×10^{10} to 1×10^{1} copy numbers of the template in a serial 10-fold dilution. —■— 1×10^{1}, —■— 1×10^{2}, —▣—, 1×10^{3}, —●—, 1×10^{4}, —⊣—, 1×10^{5}, —■—, 1×10^{6}, —▲—, 1×10^{7}, —■—, 1×10^{8}, —◆—, 1×10^{9}, —■—, negative control.

protein based assays (Sritunyalucksana *et al.* 2006) have been employed for the detection of WSSV in the shrimp culture sector. All of these assays can determine only the presence of WSSV; 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). However the cost of these experiments using real-time PCR is very high and requires technically qualified persons. The increased need for an inexpensive method to quantify WSSV 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 rapid simple detection and quantification of WSSV 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 to detect WSSV-DNA 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).

A WSSV-specific standard curve was generated using 10-fold dilutions of 10^5–10^9 copies μl^{-1} of purified WSSV plasmids and the reactions were made run in duplicate. The mean T_t 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 comparing the average T_t to the standard copy number. We obtained a high correlation coefficient ($R^2 = 0{\cdot}988$) for the unknown quantity DNA templates. Cross reactivity analysis showed the primers used were specific to WSSV and did not amplify YHV, TSV, IHHNV and healthy shrimp cDNA/DNA templates. Sensitivity analysis showed the assay was detectable up to 100 copies (0·35 fg μl^{-1}) of the template DNA, which is more sensitive than the normal LAMP and PCR based methods developed earlier. Furthermore, the gradual decrease of turbidity in the reaction was also clearly observed by naked eyes.

Various conventional diagnostic methods for WSSV has been developed and reported by several researchers worldwide with different sensitivity. Sensitivity of 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, real-time PCR has the sensitivity limit of up to 1000, 50 and 5 copies, respectively (Sritunyalucksana *et al.* 2006). Multiplex RT-PCR and 2-step nested PCR have been reported with detection limits of 5 pg μl^{-1} and 0·015 fg μl^{-1} (Khawsak *et al.* 2008; Natividad *et al.* 2008). In contrast, real-time LAMP method is found to be cost-effective quantitative assay for WSSV. As all conventional methods were developed based on a specific genome, the chances of non-specificity is found to be very less.

The real-time LAMP assay is an alternate method to conventional PCR and the LAMP assay (Sun *et al.* 2006); and in addition quantifies the WSSV DNA templates.

This gives a triplex amplification synchronization on one target gene (Cai *et al.* 2008). The real-time LAMP assay allows positive samples to become clouded (turbid) and can be viewed visually which eliminates 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 WSSV 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.

Therefore, we believe the genome specific real-time LAMP assay will be routinely used as a comprehensive WSSV detection system in most field diagnostic laboratories because of its speed, simplicity and specificity; and it is inexpensive. We are considering further studies using WSSV real-time LAMP with a fluorescence probe, SYBR-I, as the intercalation dye to increase the LAMP sensitivity to quantify very low numbers of virus.

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