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Rapid and sensitive detection of Taura syndrome virus by reverse transcription loop-mediated isothermal amplification

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Abstract

Reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay is a novel method of gene amplification that amplifies nucleic acid with high specificity, sensitivity and rapidity, which can be applied for disease diagnosis in shrimp aquaculture. The method is performed under isothermal conditions with a set of four specially designed primers that recognize six distinct sequences of the target. In this study, using the RT-LAMP method, a protocol for detecting Taura syndrome virus which is a causative agent of *Penaeus vannamei* was developed. Time and temperature conditions for detection of TSV were optimized for 60 min at 63 °C. The nucleic acids of other shrimp pathogens (yellow head virus; YHV and white spot syndrome; WSSV) were not amplified by this RT-LAMP system. The detection of TSV using RT-LAMP was 10 times more sensitive than the RT-PCR but less sensitive than nested RT-PCR. However this system was more convenient, rapid, and does not require sophisticated PCR machine.

Keywords: Taura syndrome virus (TSV); *Penaeus vannamei*; Shrimp; Loop-mediated isothermal amplification; LAMP; RT-LAMP; RT-PCR

1. Introduction

Taura syndrome virus (TSV) was first discovered in Ecuador in 1992 (Jimenez, 1992). It was a serious cause of shrimp mortality for reared *Penaeus* (*Litopenaeus*) *vannamei* (*P. vannamei*) in the Americas where it spreads principally through the regional and international transfer of live postlarvae and broodstock (Brock, 1997). TSV is a small, non-enveloped icosahedral virus containing a single-stranded positive-sense RNA genome of 10,205 nucleotides (Bonami et al., 1997; Mari et al., 2002) and was classified in the family *Dicistroiridae* (Mayo, 2002, 2005). The capsid is comprised of three major proteins, i.e. 55, 40, 24 kDa designated VP1, VP2 and VP3, respectively as well as a 58 kDa minor protein designated V0 (Bonami et al., 1997; Mari et al., 2002).

Loop-mediated isothermal amplification (LAMP) assay is a novel approach that allows amplification of DNA with high specificity, sensitivity and rapidity under isothermal conditions. LAMP, originally described by Notomi et al. (2000), can amplify target nucleic acid to 10^9 copies at 60–65 °C within 1 h. The method relies on autocycling strand displacement DNA synthesis by the *Bst* DNA polymerase large fragment, a DNA polymerase with high strand displacement activity, and a set of two specially designed inner primers and two outer primers. LAMP is highly specific for the target sequence because of the recognition of the target sequence by six independent sequences in the initial stage and by four independent sequences in the later stages of the LAMP reaction. As the reaction is conducted under isothermal conditions, it can be performed with a simple and inexpensive water bath. Therefore, a thermal cycler is not required. As there is no time loss in thermal changes, the amplification efficiency of the LAMP method is extremely high (Parida et al., 2004; Savan et al., 2005).

The development of a loop-mediated isothermal amplification (LAMP) assay for detection of shrimp white spot syndrome virus (WSSV) DNA was described by Kono et al. (2004). The

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LAMP assay is also useful for RNA template detection upon the use of reverse transcriptase together with DNA polymerase (Notomi et al., 2000; Whiting and Champoux, 1998). In this paper, the RT-LAMP assay for detection of TSV RNA in *P. vannamei* is described.

2. Materials and methods

2.1. Shrimp samples

TSV infected *P. vannamei* were collected from shrimp farms in Samutsakorn province, Thailand. Shrimp haemolymph (50 μl) was collected in a syringe preloaded with 100 μl (i.e., two volumes) of 10% (w/v) sodium citrate solution (Kiatpathomchai et al., 2004).

2.2. RNA extraction

Total RNA was extracted from 90 μl of haemolymph-citrate mixture using 750 μl of TRI Reagent® (Molecular Research Center Inc., USA). After incubation for 5 min with vigorous mixing, 200 μl of chloroform was added with vigorous mixing and the tube was incubated for 10 min before centrifugation at 12,000 × *g* for 10 min. The aqueous phase was transferred to a fresh tube followed by precipitating with 500 μl of 100% isopropanol for 10 min on ice and centrifuged at 12,000 × *g* for 10 min. The pellet was washed with 70% (v/v) ethanol, air dried and dissolved in 50 μl of RNase-free water (Kiatpathomchai et al., 2004). RT-PCR amplification was carried out using 2 μl of this RNA solution as template.

2.3. Primers for RT-LAMP

RT-LAMP primers for TSV were designed according to the published sequence of TSV structural gene (Gen-Bank accession number: AF277674; Mari et al., 2002) using Primer Explorer version 3 (http://primerexplorer.jp/lamp3.0.0/index.html). The details of the primers are given in Table 1.

2.4. Optimization of LAMP condition

The RT-LAMP reactions were carried out at 60, 63 and 65 °C for 1 h, followed by heat inactivation at 90 °C for 2 min to terminate the reaction. The reaction mixture contained 2 μM each of inner primers TSV-FIP and TSV-BIP, 0.2 μM each of outer primers TSV-F3 and TSV-B3, 1.4 mM of dNTP mix (Promega, Madison, WI, USA), 0.6 M betaine (Sigma–Aldrich, St. Louis, MO, USA), 6 mM $MgSO_4$, 8 U of *Bst* DNA polymerase (large fragment; New England Biolabs Inc., Beverly, MA, USA), 1× of the supplied buffer, 0.125 U of AMV Reverse transcriptase (Promega) and the specified amount of template RNA in a final volume of 25 μl. Different amounts of RNA template were used. Uninfected samples and reaction mix without template were included as the negative controls. To determine the optimum time for amplification, the lamp reaction was carried out at 63 °C for 30, 45 and 60 min.

2.5. Sensitivity of RT-LAMP

Ten-fold serial dilutions (10^{-1} to 10^{-7} diluted) of RNA extracted from TSV-infected shrimp was used as template for RT-LAMP following optimized conditions. The products were analyzed by 2% agarose gel electrophoresis.

2.6. RT-PCR for TSV detection

Ten-fold serial dilutions (10^{-1} to 10^{-6} diluted) of RNA extracted from TSV-infected shrimp was then amplified by RT-PCR from OIE manual of diagnostic tests for aquatic animals 2006. The primers amplify a 231 bp sequence of the TSV genome (Nunan et al., 1998). The RT-PCR products were detected by 1.5% agarose gel electrophoresis following ethidium bromide staining and visualizing on a UV transluminator.

2.7. Nested RT-PCR for TSV detection

Ten-fold serial dilutions (10^{-3} to 10^{-7} diluted) of RNA extracted from TSV-infected shrimp was then amplified by RT-PCR using IQ2000™ TSV Detection and Prevention System (Farming IntelliGene Technology Corporation) according to the manufacturer's protocol. The nested PCR products were detected by 2% agarose gel electrophoresis following ethidium bromide staining and visualizing on a UV transluminator.

2.8. Specificity of RT-LAMP detection

The specificity of RT-LAMP primers was examined using 200 ng of total RNA/DNA extracted from YHV-infected shrimp, WSSV-infected shrimp, and healthy shrimp as the template.

3. Results

3.1. Optimization of reaction temperature for TSV detection

The RT-LAMP was carried out using RNA as template in order to determine the optimal temperature and reaction time.

Table 1
Primers used for RT-LAMP of structural gene of TSV

Primer name	Genome position	Sequences 5′-3′
TSV-F3	9117-9141	CAATTGAAATTCTGAGATTAGAGTC
TSV-B3	9345-9327	GGTACATATCGAGCCACTC
TSV-FIP	9207-9184/TTTT/9144-9163	CTAGCTTCAGTGACCACGGTATAGTTTTATTTTGAGTCCAAAGCTCCA
TSV-BIP	9259-9278/TTTT/9320-9303	GCGAACCCATGCGGGTATAGTTTTCAATGCGACCAATGACTG