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Protocol

Rapid and sensitive detection of shrimp yellow head virus by loop-mediated isothermal amplification combined with a lateral flow dipstick

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A B S T R A C T

Yellow head virus (YHV) is a highly virulent pathogen that has caused severe mortality in cultivated shrimp (*Penaeus monodon* and *Penaeus vannamei*) in Thailand. There are several technologies that are applied to detect YHV for further control of the disease. RT-PCR is currently widely used in the laboratory, but it has some disadvantages related to cost, time-consuming and complexity. An alternative assay combines RT with loop-mediated isothermal amplification (LAMP) that not only provides high specificity, sensitivity and rapidity, but is also cheaper and more suitable for field applications in shrimp aquaculture than the RT-PCR. RT-LAMP is performed under isothermal conditions with a set of four to six primers designed to recognize six to eight distinct target sequences, and it has been combined with a chromatographic lateral-flow dipstick (LFD) to detect LAMP amplified product, which avoids the use of gel electrophoresis. In this study, RT-LAMP for the detection of YHV was developed by isothermal amplification at 65 °C for 45 min, followed by hybridization with an FITC-labeled DNA probe for 5 min and detected by LFD within 5 min (time required approximately 55 min, excluding RNA extraction and preparation time). The detection limit of RT-LAMP-LFD was 0.1 pg RNA extracted from shrimp infected with YHV equivalent to the nested RT-PCR, and no cross reaction was observed with other common shrimp viral pathogens. The LAMP method described in this study showed a rapid, high sensitivity and specificity and it is recommended as user-friendly for diagnosis of YHV in the field.

Contents

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1. Introduction

Yellow head virus or YHV is a highly virulent pathogen that has caused yellow head disease (YHD) and led to severe economic losses in Thailand shrimp aquaculture since 1992 (Limsuwan, 1991; Boonyaratpalin et al., 1993; Chantanachookin et al., 1993). Severe YHD is caused by YHV type-1, so far reported only from Thailand, while less virulent or non-virulent types have been reported from elsewhere in Asia (Walker et al., 2001). YHV is a member of the genus *Okavirus*, family *Roniviridae* and order *Nidovirales* with single-stranded positive-sense RNA (ssRNA) genome, rod-shaped, and enveloped viral particles (40–60 nm × 150–200 nm) containing four long open reading frames (Wongteerasupaya et al., 1995; Walker et al., 2005). Infection of YHV in shrimp may cause up to 100% crop loss within 3–5 days of the first appearance of gross signs of disease. The gross signs of YHV disease showed pale yellow coloration of the cephalothoraxes caused by the underlying yellow hepatopancreas which may be exceptionally soft when compared with the brown hepatopancreas of normal shrimp. The shrimp also exhibited erratic swimming near the surface at the pond edge (Chantanachookin et al., 1993).

Recently, various diagnostic methods such as reverse transcriptase-polymerase chain reaction (RT-PCR) (Wongteerasupaya et al., 1997; Cowley et al., 1999), nested RT-PCR (IQ2000™ YHV Detection and Preventation System), *in situ* hybridization (Tang et al., 2002), loop mediated isothermal amplification (RT-LAMP) (Mekata et al., 2006), and real-time RT-LAMP (Mekata et al., 2009) have been developed for the detection of YHV. However, these methods have some disadvantages such as time-consuming and the requirement for special instrumentation.

Loop-mediated isothermal amplification (LAMP) is a novel method that allows the amplification of DNA under isothermal conditions with high specificity, sensitivity and rapidity. This technique can amplify target nucleic acids from a few copies to 10^9 copies within 1 h based on the auto-cycling strand displacement DNA synthesis activity of *Bst* DNA polymerase large fragment (Notomi et al., 2000). LAMP is highly specific for the target sequence because six to eight independent sequences recognized the target sequences at the initial stage and four to six independent sequences amplified the target sequence at the later stage of the reaction (Mori et al., 2001). Reverse-transcription (RT) LAMP has been developed for detection of several RNA viruses (Savan et al., 2005). The LAMP products were initially detected by agarose gel electrophoresis followed by staining with carcinogenic ethidium bromide and there was the possibility that non-specific amplification products could lead to false-positive interpretation of results. To avoid these problems, authentication of LAMP products can be confirmed by restriction enzyme analysis (Notomi et al., 2000) or hybridization with specific probes (Mori et al., 2006).

As a substitution for usual LAMP product detection by gel electrophoresis, a field-based detection system combined with a lateral flow dipstick (LFD) has recently been developed for the detection of several shrimp pathogens such as taura syndrome virus (TSV) (Kiatpathomchai et al., 2008), white spot syndrome virus (WSSV) (Jaroenram et al., 2009), infectious myonecrosis virus (IMNV) (Puthawibool et al., 2009) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Arunrut et al., 2011). In these studies, generic LFD strips (Milenia® GenLine HybriDetect, Germany) were employed to detect biotin-labeled LAMP amplified products that have been hybridized with an FITC-labeled DNA probe. This technique required 5–10 min compared to 45–60 min for gel electrophoresis. The LFD did not require special instrumentation and reduced total assay time that was suitable for the field diagnosis. Therefore, the objective of this study was to develop a rapid and sensitive visual detection method for YHV in the field by combining an RT-LAMP with LFD.

2. Materials and methods

2.1. Shrimp samples and RNA extraction

Specific pathogen-free (SPF) and *Penaeus Vannamei* infection with YHV were collected from shrimp farms in Samutsakorn Province, Thailand. Total RNA was extracted from shrimp pleopods by using TRI Reagent® (Molecular Research, USA) according to the manufacturer's instructions. The concentration and quality of RNA were measured by spectrophotometer analysis at 260 and 280 nm, and then adjusted to 100 ng/μl by RNase-free water.

2.2. Primers and probe for the RT-LAMP assay

RT-LAMP primers for YHV detection were designed according to the published sequence of YHV-PmA replicase polyprotein 1ab gene (GenBank accession no. EU977578.1) by the Primer Explorer version 3 software (Eiken Chemical, Japan). The primers and the FITC-labeled oligonucleotide probe were synthesized by Bio Basic, Canada. The details of primers are shown in Fig. 1 and Table 1.

2.3. Recombinant plasmid construction and in vitro RNA transcription

The YHV-PmA replicase polyprotein 1ab gene was amplified by RT-PCR using F3 primer (5′ ACC CTG TAA TTG GCG ATG TT 3′) and B3 primer (5′ TGC AGT TAA GAT GGT CAC AG 3′), yielding a PCR product of 186 bp. After ligation into pGEM®-T Easy plasmid Vector (System II, Promega, USA), the recombinant plasmid was transformed into 100 μl of *Escherichia coli* competent cells strain JM109, followed by blue-white colony selection using colony PCR. Positive colonies were cultured for 16 h in 2 ml of LB broth medium containing 100 μg/ml of ampicillin. The plasmid DNA was purified using the GeneJET™ Plasmid Miniprep Kits (Fermentas, USA), followed by UV spectrophotometric analysis at 260 and 280 nm. The plasmids were completely linearized by *Sal*I restriction and purified using a PCR purification kit (QIAGEN, Germany). RNA *in vitro* transcription was carried out using the RiboMAX™ Large Scale RNA Production System-SP6 and T7 kit (Promaga, USA) according to the manufacturer's instructions.

2.4. Optimization of YHV RT-LAMP conditions

To determine the optimum amplification temperature, the RT-LAMP reactions were carried out at 60, 63 and 65 °C for 1 h, followed by heat inactivation at 93 °C for 3 min to terminate the reactions. The reaction mixture contained 2 μM each of inner primers (5′biotin-labeled BIP and FIP), 0.2 μM each of outer primers (F3 and B3), 1.4 mM of dNTPs mix (Promega, USA), 0.6 M betaine (Sigma–Aldrich, USA), 6 mM $MgSO_4$, 8 U of *Bst* DNA polymerase (large fragment; New England Biolabs, USA), 1× of reaction buffer, 0.25 U of AMV (avian myeloblastosis virus) reverse transcriptase