Molecular and Cellular Probes 24 (2010) 244–249

Contents lists available at ScienceDirect

Molecular and Cellular Probes

journal homepage: www.elsevier.com/locate/ymcpr

Rapid and sensitive detection of *Macrobrachium rosenbergii* nodavirus in giant freshwater prawns by reverse transcription loop-mediated isothermal amplification combined with a lateral flow dipstick

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ARTICLE INFO

Article history:
Received 19 April 2010
Received in revised form
30 June 2010
Accepted 14 July 2010
Available online 22 July 2010

Keywords:
Macrobrachium rosenbergii nodavirus
White tail disease
Macrobrachium rosenbergii
Giant freshwater prawn
Loop-mediated isothermal amplification
Lateral flow dipstick
LAMP
PCR,
LFD
*Mr*NV

ABSTRACT

Loop-mediated isothermal amplification (LAMP) allows rapid amplification of nucleic acids under isothermal conditions. It can be combined with a chromatographic lateral flow dipstick (LFD) for much more efficient, field-friendly detection of *Mr*NV. In this work, RT-LAMP was performed at 65 °C for 40 min, followed by 5 min for hybridization with an FITC-labeled DNA probe and 5 min for LFD resulted in visualization of DNA amplicons trapped at the LFD test line. Thus, total assay time, including 10 min for rapid RNA extraction was approximately 60 min. In addition to advantages of short assay time, confirmation of amplicon identity by hybridization and elimination of electrophoresis with carcinogenic ethidium bromide, the RT-LAMP–LFD was more sensitive than an existing RT-PCR method for detection of *Mr*NV. The RT-LAMP–LFD method gave negative test results with nucleic acid extracts from normal shrimp and from shrimp infected with other viruses including DNA viruses [PstDNV (IHHNV), PemoNPV (MBV), PmDNV (HPV), WSSV] and RNA viruses (TSV, IMNV, YHV/GAV).

1. Introduction

The giant freshwater prawn (*Macrobrachium rosenbergii*) is an economically important farmed crustacean species cultured in Caribbean countries and Asian countries including China, India, Thailand and Vietnam. Yearly production of cultured freshwater prawns increased from 7140 million tons in the year 2000 to 30,450 million tons in 2003 in India and the country ranks third in total production after China and Vietnam [1,2]. After 2001, the expansion and intensification of aquaculture together with careless transboundary movement of farmed crustacean species have all exacerbated the rapid and easy spread of previously localized viral diseases. A new disease, named “white muscle disease (WMD)” or “white tail disease (WTD)”, has been observed in freshwater prawn hatcheries and nursery ponds since 1992. It was first reported from the French West Indies [3], and thereafter from the People’s Republic of China [4], India [5,6] and Thailand [7].

The causative agent of WTD is *M. rosenbergii* nodavirus (*Mr*NV) associated with extra small virus (XSV) [4]. *Mr*NV is a small icosahedral, non-enveloped virus, 26–27 nm in diameter, that occurs in the cytoplasm of connective tissue cells in infected prawns. The viral genome is formed by two pieces of single-stranded, positive-sense RNA (RNA1 and RNA2), of about 2.9 and 1.3 kb in length, respectively. The viral capsid contains a single polypeptide of 43 kDa [1]. XSV is icosahedral in shape and 15 nm in diameter and its genome consists of a linear single-stranded RNA of 796 nucleotides, encoding a single structural protein of 17 kDa (CP-17) [1,8]. Although the respective roles of the two viruses in the pathogenesis of WTD are not yet known, it currently appears that *Mr*NV is the major cause of pathology [9]. It is hypothesized that XSV constitutes a new species of satellite virus [1,8].

This disease has caused high mortalities and huge economic losses [10]. It affected hatchery-reared larvae and post-larvae (PL) as well as early juveniles in the nursery. The clinical signs are opaque-white or milky-white muscle in abdominal segments,

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doi:10.1016/j.mcp.2010.07.003

commonly accompanied by a progressive reduction in feeding and swimming ability. Mass mortality (40–90%) can occur within a few days. Outbreaks continue to the present and are spreading gradually to different states in India. Various diagnostic methods have been developed to detect *Mr*NV/XSV including, reverse transcriptase-polymerase chain reaction techniques (RT-PCR) [5,8,11], loop-mediated isothermal amplification (LAMP) (Pillai et al., 2006), histopathology [10] and *in situ* dot blot hybridization using nucleic acid probes [12], and ELISA [11,12].

Loop-mediated isothermal amplification (LAMP) allows amplification of DNA with high specificity, sensitivity and rapidity under isothermal conditions. LAMP, described originally by Notomi [13], 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 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 carried out with a simple and inexpensive water bath so that a thermal cycler is not required. As there is no time-loss for thermal changes, amplification efficiency is extremely high [14,15]. LAMP for detection of the shrimp DNA virus white spot syndrome virus (WSSV) has been described by Kono et al., (2004) [16]. However, LAMP is also useful for RNA template detection by the use of reverse transcriptase together with DNA polymerase [13,17].

Analysis of LAMP products (amplicons) is carried out usually by agarose gel electrophoresis, followed by ethidium bromide staining. As a result, non-specific amplification products may cause false positive results. To help overcome this problem, the identity of LAMP products can be confirmed by restriction endonuclease digestion [13] and hybridization with specific probes [18]. In order to further simplify and speed up the total time for the LAMP-based assay, amplicon detection by a chromatographic, lateral flow dipstick (LFD) rather electrophoresis was successfully applied for Taura syndrome virus (TSV) [19]. In that process, generic LFD strips (Milenia® GenLine HybriDetect) were used to detect biotin labeled amplicons hybridized with an FITC-labeled DNA probe linked, in turn, to a gold-labeled anti-FITC antibody. The total assay interval was approximately 60 min, including RNA preparation time, and the sensitivity was greater than that of RT-PCR detection currently used for TSV. The purpose of this study was to develop a similar RT-LAMP–LFD method for detection of *Mr*NV.

2. Materials and methods

2.1. Shrimp samples

Giant freshwater prawn (*M. rosenbergii*) adults and post-larva were obtained from shrimp farms in Ratchaburi and Kanchanaburi Provinces in Thailand and from Myanmar in August 2006. They were positive for *Mr*NV using a previously described RT-PCR detection method [20]. Normal whiteleg shrimp were obtained from Samutsakhon province of Thailand.

2.2. RNA template preparation

Shrimp muscle tissue from the 6th abdominal segment was homogenized in TRIzol™ reagent (Invitrogen®) and RNA was extracted following the manufacturer's instructions. RNA concentration and quality were measured by spectrophotometric analysis at 260 and 280 nm.

Additional method for a simple and rapid RNA extraction was carried out according to published reports [21,22]. Briefly, the muscles of *Mr*NV-infected *M. rosenbergii* samples were ground in 100 μl of 2 M guanidine thiocyanate (GuSCN) solution (2 M GuSCN). After incubation at room temperature for 5 min, the supernatant solution was used for subsequent steps without a centrifugation step [22].

2.3. Primers for RT-LAMP

RT-LAMP primers for *Mr*NV were designed according to the published sequence of *Mr*NV segment RNA-2 of the *Mr*NV genome (GenBank accession no. AY222840) [8,10] using Primer Explorer version 3 (http://primerexplorer.jp/lamp3.0.0/index.html). The details of the primers are given in Fig. 1 and Table 1. The normal primers and biotin-labeled BIP primer were synthesized by Bio Basic Inc., Canada.

2.4. Optimization of temperature for RT-LAMP

To determine the optimum temperature for amplification, the RT-LAMP reactions were carried out at 63 and 65 °C for 1 h, followed by analysis of the LAMP products by gel electrophoresis. The reaction mixture contained 2 μM each of inner primers *Mr*NV-FIP and *Mr*NV-BIP, 0.2 μM each of outer primers *Mr*NV-F3 and *Mr*NV-B3, 1.4 mM of dNTP mix (Promega, Madison, WI, USA), 0.4 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, 0.25 U of AMV Reverse transcriptase (Promega) and 2 or 20 ng of RNA extracted from *Mr*NV infected shrimp in a final volume of 25 μl. To increase sensitivity and decrease time, 1 μM of each of the loop primers (LF and LB) were added to the LAMP reaction mixture and the reaction time was changed to be 30, 40 or 60 min to determine the standard assay time. LAMP products were analyzed by gel electrophoresis.

2.5. Lateral flow dipstick (LFD) assay

A DNA probe was designed from the *Mr*NV sequence between F1c and B1c regions (Fig. 1 and Table 1). The DNA probe labeled with FITC at 5′end was synthesized by Bio Basic Inc., Canada. Twenty picomole of the FITC-labeled probe were added to the RT-LAMP products as recommended in previous reports [19,23]. After hybridization at 65 °C for 5 min, 8 μl of the hybridized product were added to 150 μl of the assay buffer in a new tube. Finally, the LFD strip was dipped into the mixture and left for 5 min.

2.6. Sensitivity of RT-LAMP by gel electrophoresis (RT-LAMP–AGE) and LFD

To test sensitivity of the detection, 10-fold serial dilutions (10^{-1} to 10^{-7}) of 100 ng μl^{-1} of total RNA extracted from *Mr*NV-infected shrimp were used as the template (2 μl) for biotin labeling RT-LAMP according to the optimized conditions. The products were analyzed by 2% agarose gel electrophoresis and by LFD as described above.

2.7. Sensitivity of RT-PCR for MrNV detection by gel electrophoresis

Ten-fold serial dilutions of 100 ng μl^{-1} of total RNA extracted from *Mr*NV-infected shrimp were used as the template (2 μl) in RT-PCR reactions for detection of *Mr*NV [20]. The amplified products were analyzed by electrophoresis in 1% agarose gels stained with ethidium bromide and visualized on a UV transilluminator.