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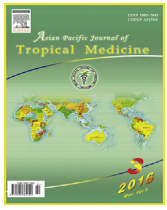

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Detection of coat protein gene of nervous necrosis virus using loop-mediated isothermal amplification

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ABSTRACT

Objective: To establish a novel and highly specific loop-mediated isothermal amplification (LAMP) assay for the identification of nervous necrosis virus (NNV) infection.
Methods: A set of synthesized primers was used to match the sequences of a specific region of the *nnv* gene from the National Center for Biotechnology Information database, not originating from NNV-infected fish, the efficiency and specificity of LAMP were measured dependent on the concentration of DNA polymerase and the reaction temperature and time. In addition, to determine species-specific LAMP primers, cross reactivity testing was applied to the reaction between NVV and other virus families including viral hemorrhagic septicemia virus and marine birnavirus.
Results: The optimized LAMP reaction carried out at 64 °C for 60 min, and above 4 U *Bst* DNA polymerase. The sensitivity of LAMP for the detection of *nnv* was thus about 10 times greater than the sensitivity of polymerase chain reaction. The LAMP assay primers were specific for the detection NNV infection in *Epinephelus septemfasciatus*.
Conclusions: The development of LAMP primers based on genetic information from a public database, not virus-infected samples, may provide a very simple and convenient method to identify viral infection in aquatic organisms.

1. Introduction

Nervous necrosis virus (NNV) belongs to the viral genus *Betanodavirus* in the family Betanodaviridae and is the causative agent of viral nervous necrosis (VNN) in marine fish [1].

The nodavirus genome consists of linear and positive-sense single-stranded RNA composed of two segments, RNA1 and RNA2. RNA1 encodes a protein that has multiple functional domains, including a transmembrane domain, an RNA-capping domain, and an RNA-dependent RNA polymerase, while RNA2 encodes a viral coat protein [2]. This virus has attracted a great deal of attention because of its economic and ecological impacts on the aquaculture industry, especially the considerable economic losses that it causes [3]. Most *betanodaviruses* are neuropathogenic, causing a degenerative effect in neurons [4]. They can infect a wide range of marine fish species, resulting in uncoordinated swimming behavior and dark bodies [2,5,6]. Several detection techniques, such as polymerase chain reaction (PCR), Giemsa staining, and immunofluorescence using a monoclonal antibody, have recently been used to detect bacterial, fungal, and viral contamination, including human and animal specimens, as well as environmental sample [7]. Among them, PCR is the most widely used molecular diagnostic technique for the effective quantification and detection of viral infection. However, it sometimes fails to amplify targets such as bacteria and viruses with low copy-number genes. In addition, it requires expensive reagents and equipment. Therefore, there is a need for a simplified method of amplification and gene product detection molecular diagnosis. Loop-mediated isothermal amplification (LAMP) was recently developed to amplify nucleic acids with high sensitivity and specificity, it can easily be performed under isothermal conditions [8,9]. This technique

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uses four to six primers that recognize six to eight distinct regions of the target DNA in conjunction with the enzyme *Bst* polymerase, which has strand displacement activity, to synthesize DNA [9]. Several studies have applied this technique to detect pathogens such as bacteria and viruses in shellfish and cultured fish [10–13]. In this study, we developed a novel and highly specific LAMP assay for the identification of NNV infection. An *nnv* gene sequence registered with the National Center for Biotechnology Information (GenBank Accession Number EU391590.1) was used to design the detection primers. To the best of our knowledge, this is the first study to detect NNV in infected fish using synthesized *nnv* sequences. It provides a new method for recognizing viral infection in marine organisms.

2. Materials and methods

2.1. Nnv gene synthesis

The capsid protein (*Cp*) gene was utilized for *nnv* detection using LAMP. CP is one of the features shared by all viruses. It is the polypeptide produced at the highest level upon viral infection and has been estimated to account for up to 45% of all virion proteins. The complete *Cp* genes from a number of vertebrate and invertebrate nodaviruses have been sequenced and substantial sequence similarity has been found among their coding regions. This gene was synthesized by Bioneer Corporation, South Korea. Finally, the *nnv* gene was cloned into the pGEMT-easy vector (Promega, Inc., WI, Madison, USA).

2.2. Construction of LAMP primers

The LAMP method requires a set of four specially designed primers [(B3, F3), backward inner primer (BIP), and forward inner primer (FIP)] that recognize a total of six distinct sequences (B1, B2, B3, F1, F2, and F3) in the target DNA. Primers for RSIV-6 LAMP were designed against the *nnv* gene sequences in GenBank (NCBI) using Primer Explorer V4 (http://primerexplorer.jp/e) software. Primer details are listed in Table 1. The primer sequences and their respective binding sites are shown in Figure 1.

2.3. Optimization of LAMP conditions

The LAMP reaction mixture contained 1 μL of 10× *Bst* DNA polymerase reaction buffer [20 mM Tris–HCl, 10 mM $(NH4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100 (final concentrations)], 1.6 μL of 10 mM dNTPs, forward inner primer (FIP) and backward inner primer (BIP; 1.6 μM each), outer F3 and B3 primers (0.4 μM each), and 1 μL of template DNA in a final volume of 20 μL with 0–16 U of *Bst* DNA polymerase (New England Biolabs, Ipswich, MA, USA). Extra $MgSO_4$ [final concentration (2–10) mM] was added. The LAMP reaction was performed at different temperatures (54, 56, 58, 60, 62, 64, 66, and 68 °C), and the reaction products were analyzed by gel electrophoresis using a 1% agarose gel stained with ethidium bromide (EtBr). Fluorescence was visualized by adding 1 μL of diluted SYBR Green I (Invitrogen, New York, NY, USA) and observing the sample under natural and ultraviolet light.

2.4. Comparison of the sensitivity of LAMP and PCR

In order to compare the sensitivity of the LAMP and PCR assays, the synthesized *nnv* template was serially diluted. PCR was performed in a reaction mixture with a total volume of 20 μL (primers: F, ATGGTACGCAAAGGTGAGAA, and R, GGATCCTTAGTTTCCCGA G). The program used was as: 50 °C for 30 min, 15 min initial denaturation at 95 °C, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and elongation at 72 °C for 50 s, followed by extension for 7 min at 72 °C and cooling to 4 °C. The products were electrophoresed and analyzed on a 1% agarose gel.

2.5. LAMP specificity test using internal spacer sequences

Among the four primers used for loop formation between FIP and BIP, additional spacer sequences between F2 and F1c were employed to improve the efficiency of loop formation. We tested the efficiency of an additional three inserted spacer sequences between F2 and F1c. Primers with additional spacers were prepared, and the efficiency of each spacer with two thymines (T2), four thymines (T4) and six thymines (T6) was tested. The LAMP reaction conditions were the same as described previously, (except for the additionally inserted spacers) and we observed DNA laddering on the gel.

2.6. Application to a wild-type sample and other virus families

Isolates from liver samples of uninfected and wild-type NNV infected fish [*Epinephelus septemfasciatus* (*E. septemfasciatus*),

Table 1
Oligonucleotide primers developed for detecting *nnv* using the LAMP assay.

	LAMP primer	PCR primer
nnv F3	AAAGCCTCGACTGTAACTGG	*Nnv*-F ATGGTACGCAAAGGTGAGAA
nnv B3	TGTTTGCGGGCACATTG	*Nnv*-R GGATCCTTAGTTTCCCGAG
nnv FIP(F1c–F2)	ACGGCCTGGGAGATTCTCGA-GTTTGGACGTGGGACCAA	
nnv BIP(B1–B2c)	CAACCATCGTCCCCGACCTCGT-GTTTCAACAGCGTATCGC	
nnv FIP (+T2)	ACGGCCTGGGAGATTCTCGA-TTGTTTGGACGTGGGACCAA	
nnv BIP (+T2)	CAACCATCGTCCCCGACCTCGT-TTGTTTCAACAGCGTATCGC	
nnv FIP (+T4)	ACGGCCTGGGAGATTCTCGA-TTTTGTTTGGACGTGGGACCAA	
nnv BIP (+T4)	CAACCATCGTCCCCGACCTCGT-TTTTGTTTCAACAGCGTATCGC	
nnv FIP (+T6)	ACGGCCTGGGAGATTCTCGA-TTTTTTGTTTGGACGTGGGACCAA	
nnv BIP (+T6)	CAACCATCGTCCCCGACCTCGT-TTTTTTGTTTCAACAGCGTATCGC	