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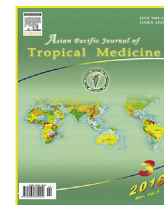

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Detection of coat protein gene of nervous necrosis virus using loop-mediated isothermal amplification

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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	

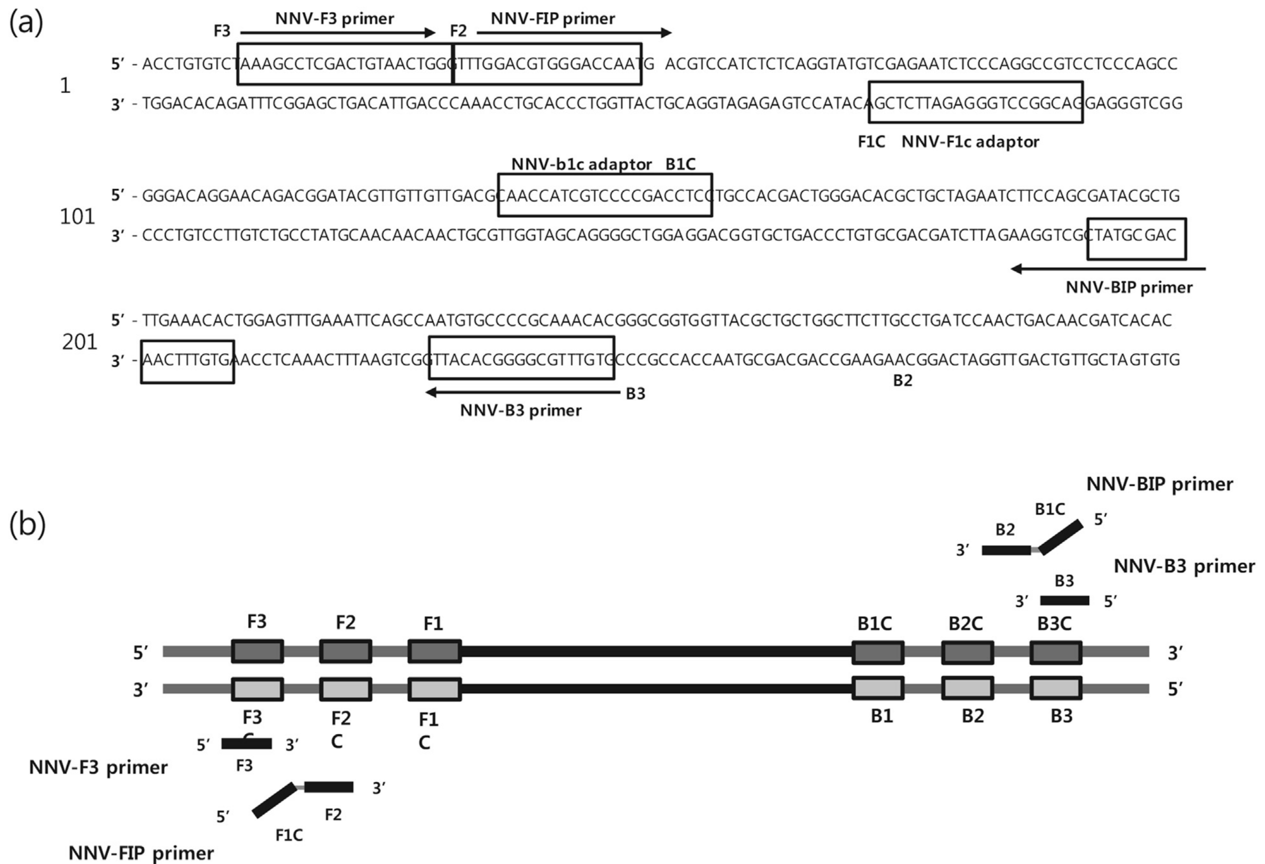

Figure 1. Nucleotide sequence of *nnv* DNA used for the inner and outer primers (a). DNA sequences used for primer design are shown by boxes and arrows.
Schematic diagram of two-outer (F3 and B3) and two-inner (FIP and BIP) primers for LAMP (b).

from Tong-young Bay in South Korea] were used to assess the specificity of LAMP for *nnv*, potential cross reactions with viral hemorrhagic septicemia virus (VHSV) and marine birnavirus (MABV) were examined using infected fish (*Paralichthys olivaceus*). Fish tissues that were infected with NNV and VHSV, MABV were preserved in RNA later (Life Technologies). These were then processed by grinding thoroughly in liquid nitrogen with a mortar and pestle. Twenty milligrams of tissue powder was then placed into an RNase-free, liquid nitrogen-cooled 2 mL microcentrifuge tube with 600 μL of lysis buffer. The lysate was transferred directly into a QIAamp viral RNA kit (QIAGEN GmbH, Hilden, Germany) and centrifuged at 18 000 ×*g* for 2 min. One volume (600 μL) of 70% ethanol was added to the supernatant and extraction was completed per the manufacturer's instructions. RNA was eluted in RNase-free water (70 °C) and stored at −80 °C. cDNA was constructed from total RNA isolated using a cDNA synthesis kit (Promega, Inc., WI, Madison, USA). The amplification results were observed after electrophoresis and stained to verify the specificity.

3. Results

3.1. Determination of the most appropriate conditions for rapid detection

To determine the optimal conditions for LAMP of *nnv*, LAMP was performed with different temperatures, reaction times, and $MgSO_4$ concentration, which are variables known to affect this assay [9]. As shown in Figure 2a, multiple distinct patterns of DNA ladders, as LAMP products, were observed at temperatures from 56 °C to 64 °C; they were not seen at 54 °C or below or at 66 °C or above. Notably, among the different temperatures, we observed the most distinct pattern of ladders at 64 °C. In addition, LAMP was successful with a reaction time of at least 60 min; DNA ladders did not appear or produced only very weak signals when the reaction time was less than 60 min (Figure 2b). The sensitivity of the LAMP reaction for *nnv* was analyzed using a template serially diluted from 1×10^6 copies/mL of NNV–pGEM-T Easy clone to 1/10 million (1×10 copies/mL). Notably, the distinct pattern of DNA ladders was observed at all concentrations of template, except the lowest one ($1/10^6$), which showed no DNA ladder on the gel (Figure 2c). Next, to determine the optimum quantity of LAMP polymerase for the reaction, LAMP was performed with three different amounts of *Bst* DNA polymerase (4, 8, and 16 U). All of the positive LAMP reactions produced a characteristic ladder with multiple bands on the agarose gel (Figure 2d). In addition, one of the main determinants in the LAMP reaction, $MgSO_4$, was also tested at different concentrations [(5–40) mM]. While LAMP amplification did not occur at an $MgSO_4$ concentrations of 5 mM, the characteristic DNA ladder appeared at concentrations at 10 mM and above (Figure 2e). Overall, the optimized conditions for the LAMP reaction were as follows: master mix containing FIP and BIP at 1.6 μM each, F3 and B3 at 0.4 μM each, 10 mM dNTP mix, 40 mM $MgSO_4$, and above 4 U of *Bst* DNA polymerase in a final volume of 20 μL. The reaction was carried out at 64 °C for 60 min and then terminated by increasing the temperature to 80 °C for 5 min.

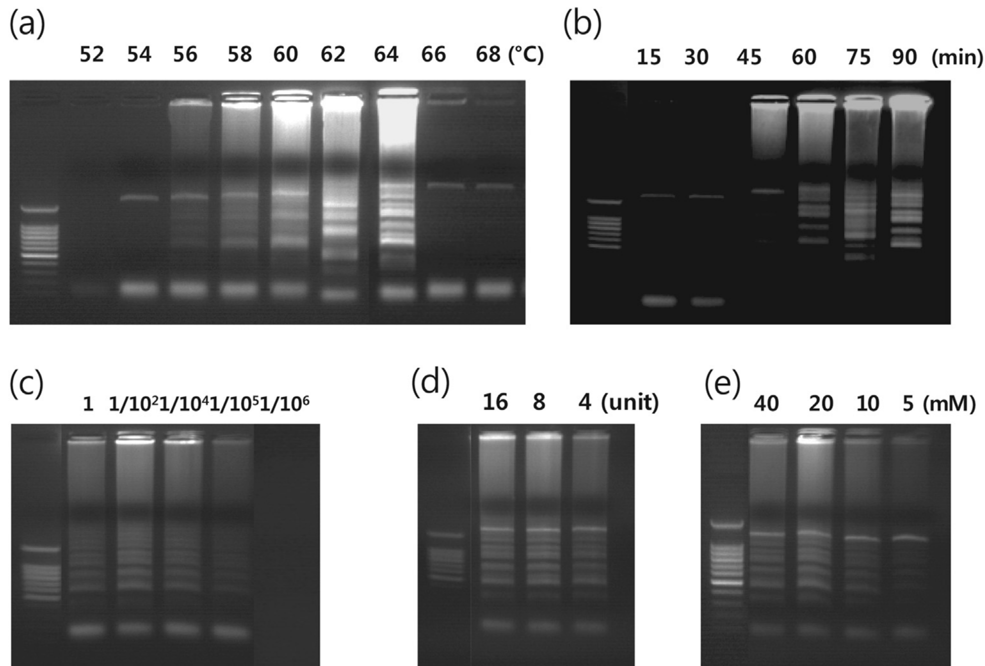

Figure 2. Optimization of the LAMP assay for detecting *nnv*. LAMP reactions at different reaction times (a), temperatures (b) and concentrations of template (c), *Bst* DNA polymerase (d), and $MgSO_4$ (e).

3.2. Comparison of sensitivity of the LAMP and PCR

We compared the detection sensitivity between LAMP and PCR. The detection limit of the LAMP reaction was tested using 10-fold serial dilutions of synthesized *nnv* and the results obtained were compared with those from PCR. The detection limit of LAMP was 10^{-5} (Figures 2c and 3a). The detection limit of the PCR was 1 to 10^{-4} (Figure 3b). The sensitivity of LAMP for the detection of *nnv* was thus about 10 times greater than the sensitivity of PCR.

3.3. LAMP specificity test using internal spacer sequences

Additional spacer sequences between F2 and F1c were used to improve the efficiency during loop formation. We tested the efficiency of an additional three inserted spacer sequences between F2 and F1c. Figure 4 shows that all spacers except T2 showed typical DNA laddering on the gel. The T2 spacers, containing the FIP (T2) and BIP (T2) primers, did not amplify the genes, showing no change of color in the reaction tubes, while primer sets containing T4 and T6 spacers led to similar results to F2 and F1c with the reaction carried out at 64 °C for 60 min.

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

We wondered whether the synthesized primers could be applied to the detection of NNV from infected fish. To test this, total viral RNA was isolated from fish, *E. septemfasciatus*, with or without NNV infection. The total cDNA was used as a template for the LAMP assay with the specific synthesized LAMP primers used in the optimized conditions described above. The change in color of positive tubes (NNV infected) from light gray to green could be observed directly by the naked eye within an incubation time of 60 min, while no color change was seen in negative tubes (NNV uninfected) using cDNA from uninfected fish (Figure 5). LAMP products from cDNA from the livers of fish (*Paralichthys olivaceus*) infected with VHSV and MABV were also detected by 2% agarose gel electrophoresis; the specific ladders of multiple bands were not visualized, but PCR bands were apparent in the *nnv* template tubes (Figure 6). These results demonstrate that the LAMP primers were specific for *nnv*.

4. Discussion

Viral infections pose a serious threat to the marine aquaculture industry and have been responsible for significant financial

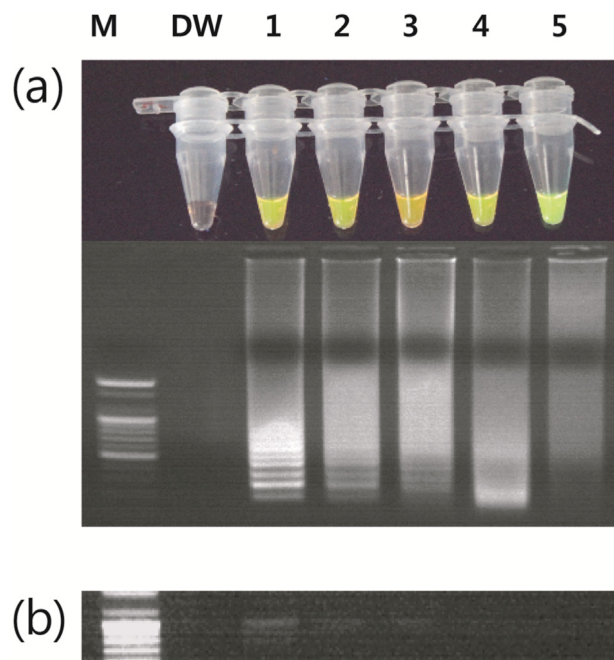

Figure 3. Comparative sensitivity of LAMP (a) and PCR (b) assays. Lane M: 100 bp DNA ladder, lanes 1–5: 10^{-1} to 10^{-5} dilutions of *nnv* template.

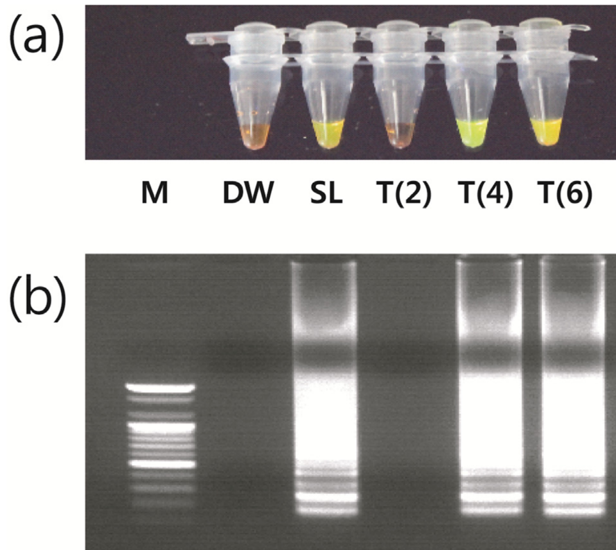

Figure 4. Comparative sensitivity of FIP and BIP primers with different spacer sequences in the LAMP assay. LAMP reactions by visual inspection with diluted SYBR Green I (a) and electrophoresis with EtBr (b). M, 100 bp DNA ladder; DW, distilled water; SL, spacer-less; T(2), TT spacer; T(4), TTTT spacer; T(6), TTTTTT spacer.

losses [14]. As such, it is important to identify various agents of fish diseases rapidly in order to prevent further disease transmission or outbreaks [15]. An example of a causative agent behind such serious fish losses is NNV, which has had a severe economic impact. The presence of this virus in fish has been reported in Asian countries including Korea, Malaysia, the Philippines, and Singapore [11,16]. Recently several detection methods have been developed to monitor and control NNV in fish culture. Among them, a real-time PCR approach has been found to be useful for NNV diagnosis [17,18]. One advantage of PCR is that it is very sensitive and small amounts of target material can be used. However, in spite of its efficiency, the sensitivity of PCR is also its major disadvantage since very small amounts of contaminating DNA can also be amplified. In addition, PCR is technically demanding and time-consuming for a definitive diagnosis. In this study, a novel method called LAMP was used for the detection of NNV in fish. The LAMP assay has numerous advantages including higher sensitivity and greater than PCR, the lack of a need for expensive equipment, and its potential application in fieldwork. The LAMP method has been shown to amplify a few copies of DNA to the level of 10^9 CFU in less than 1 h under isothermal condition [9]. Consistently with this, according to our data, an extremely low level of virus, less than 10^4 copies, was detectable by the LAMP method. However, we could not see any difference in the level of among several DNA concentrations upon serial dilutions of a purified *nnv* DNA fragment. However, another study showed a strong correlation between the number of viral DNA copies input and the corresponding turbidity at the end of the LAMP reaction [8]. Optimization of the LAMP conditions was shown to be critical for the success of this assay. For example, in the initial step, the denaturing temperature and $MgSO_4$ concentration during hybridization of the four primers to the target DNA functioned as critical effectors. Consistently with a previous study [9], our data showed that temperatures above or below certain thresholds reduced the activity of the *Bst* DNA polymerase; specifically, the denaturing temperature of primers must be (58–64) °C. These results are important, particularly when diagnosing infectious diseases for which both qualitative and quantitative diagnoses are required. Furthermore, in a previous study of LAMP tests, spacer sequences were used to improve LAMP efficiency by aiding

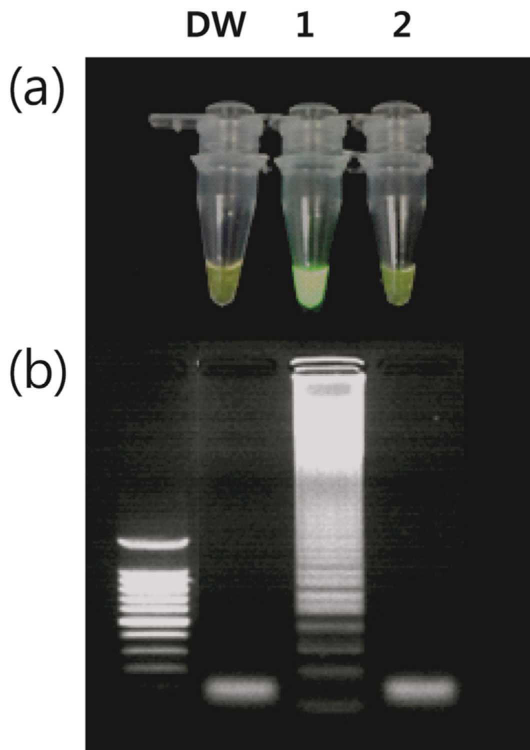

Figure 5. Detection of the NNV infection in wild fish. LAMP reactions by visual inspection with diluted SYBR Green I (a) and electrophoresis with EtBr (b). DW, distilled water; 1, NNV-infected fish; 2, no infected fish.

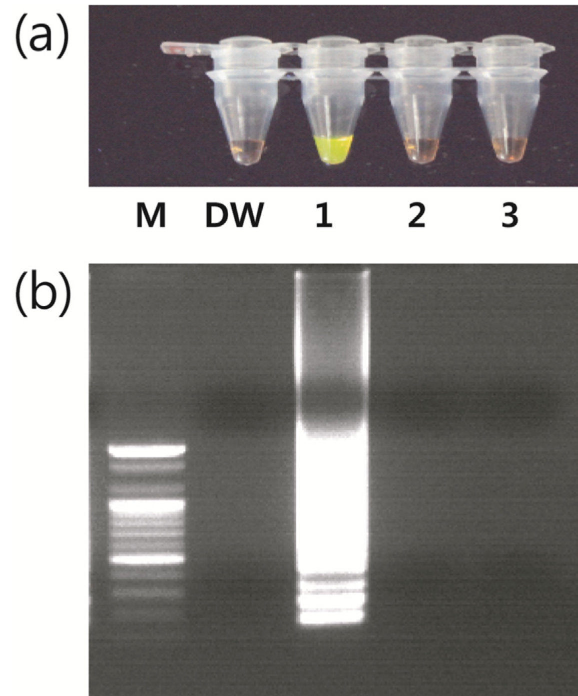

Figure 6. Cross-reactivity between NNV and other virus families. LAMP reactions by visual inspection with diluted SYBR Green I (a) and electrophoresis with EtBr (b). M, 100 bp DNA ladder; 1, NNV-infected fish; 2, VHSV-infected fish; 3, MABV-infected fish.

in loop formation with the FIP and BIP primers. In most cases, four T-bases in the primers are used as a spacer sequence [9,19,20]. In this study, we investigated the effects of NNV LAMP spacer sequences as well as the amplification efficiency associated with inserting T-bases into the spacer sequences. The best *nnv* amplification result was obtained when four bases were inserted as spacer sequences. The best *nnv* amplification result was obtained when four bases were inserted as spacer sequences. In addition, no amplification was detected when two bases were used as the spacer sequence, while the LAMP amplification efficiency with primer without spacer inserted was better than for primers with six spacers inserted. These results indicated that a two-base spacer might inhibit loop formation during the self-priming process of LAMP amplification.

This study demonstrated that synthesized primers can be used for the detection of NNV in marine organisms. NNV is believed to have a wide geographical range [21], and NNV strains isolated in Korea have been shown to share high similarity to nodavirus isolates from China [5] and Singapore [22]. This raises the possibility that the LAMP assay developed in this work may be useful for the detection and diagnosis of NNV infections in various countries with similar nodaviruses. Furthermore, we demonstrated that the synthesized primers could be efficiently applied to detect pathogenic NNV in *E. septemfasciatus* with viral species specificity. Together, these data indicate that our approach to the development of LAMP primers based on genetic information available in a public database provides a simple method for detecting viral infections in marine organisms.

LAMP is a very useful detection method due to its simple, isothermal reaction conditions and inexpensive equipment. It has been widely used to detect bacterial, viral, fungal, and parasitic pathogens in organisms including animals, plants, and humans. In addition, in aquaculture LAMP has been developed to detect a variety of pathogens such as bacteria and viruses. So far, viral genomes from infected samples have usually been used for the development of detection primers. In contrast, in this study, using synthesized nucleotides for an *nnv* gene, we established optimal LAMP conditions for the detection of NNV infection in *E. septemfasciatus*. Furthermore, we observed that the synthesized sequences could be efficiently used to determine the presence of NNV. Collectively, these findings indicate that the design of LAMP primers based on genetic information from a public database may provide a simple method for detecting viral infections in marine organisms.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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