Development of a Reverse Transcription Loop-Mediated Isothermal Amplification Assay for Detecting Nervous Necrosis Virus in Olive Flounder *Paralichthys olivaceus*

Suebsing, Rungkarn†, Myung-Joo Oh‡, and Jeong-Ho Kim†*

†Department of Marine Bioscience, Gangnung-Wonju National University, Gangnung 210-702, Korea
‡Department of Aqualife Medicine, Chonnam National University, Yeosu 550-749, Korea

Received: January 4, 2012 / Revised: March 1, 2012 / Accepted: March 11, 2012

In this study, a reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay was developed for the rapid, sensitive, and inexpensive detection of nervous necrosis virus (NNV) in olive flounder, *Paralichthys olivaceus*, in Korea. A set of six specific primers was designed to target the RNA 2 gene encoding the coat protein of Korean NNV strains. The RT-LAMP reaction successfully detected NNV after 30 min at 65°C. When the sensitivities among RT-LAMP, RT-PCR, and nested RT-PCR were compared, the RT-LAMP was shown to be able to detect the RNA template at $2.58 \times 10^{-2}$ TCID$_{50}$/ml, whereas the RT-PCR and nested RT-PCR were only able to detect the RNA template at $2.58 \times 10^{-2}$ TCID$_{50}$/ml and $2.58$ TCID$_{50}$/ml, respectively. Thus, the sensitivity of the RT-LAMP assay was higher than those of the RT-PCR assays. In the specificity test of the RT-LAMP, 2 genotypes of NNVs (SJNNV and RGNNV) were positive; however, no other fish viruses were positive with the primers, indicating that the RT-LAMP assay is only specific to NNV. A total of 102 olive flounder were collected from hatcheries between 2009 and 2011. The occurrence of NNV in olive flounder was determined to be 53.9% (55/102) by the RT-LAMP. On the other hand, the prevalence based on the nested RT-PCR and RT-PCR results was 33.8% (34/102) and 20.6% (21/102), respectively. This result indicates that the RT-LAMP assay developed in this study is suitable for early field diagnosis of NNV with high sensitivity.

Keywords: Nervous necrosis virus, olive flounder, *Paralichthys olivaceus*, RT-LAMP, RT-PCR

Nervous necrosis virus (NNV) is a member of the family Nodaviridae, genus *Betanodavirus*. The genome is composed of two positive-sense single-stranded RNA segments, where the RNA 1 gene encodes a nonstructural protein and RNA 2 gene encodes the coat protein. Fish nodaviruses are classified into 4 different genotypes based on the partial nucleotide sequence of the coat protein gene; striped jack nervous necrosis virus (SJNNV)-type, tiger puffer nervous necrosis virus (TPNNV)-type, barfin flounder nervous necrosis virus (BFNNV)-type, and red spotted grouper nervous necrosis virus (RGNNV)-type [16].

Viral nervous necrosis (VNN) is a serious disease of larval and juvenile fish, and causes economic losses in the aquaculture industry. The disease has been reported in more than 40 fish species, with the greatest impact on sea bass (*Lates calcarifer*) [2], grouper (*Epinephelus akaara*) [4], parrotfish (*Oplegnathus fasciatus*) [28], tiger puffer (*Takifugu rubripes*) [13], and olive flounder (*Paralichthys olivaceus*) [14, 26]. In Korea, VNN was reported from seven band grouper (*Epinephelus septemfasciatus*) for the first time in 1998 [22]. The infected fish showed anorexia, dark coloration, loss of equilibrium, spiral swimming, vertebral deformity, and inflation of the swim bladder, and the mass mortality was up to 80%. Since then, several outbreaks of VNN have been reported from several cultured fish species, such as olive flounder, black rockfish (*Sebastes schlegeli*), oblong rockfish (*Sebastes oblongus*), and red drum (*Sciaenops ocellatus*), as well as wild marine fish populations in Korea [7, 12, 18, 19]. Based on phylogenetic analysis, all Korean NNVs were classified into the RGNNV type [3, 12, 19]. For example, the virus isolated from olive flounder and seven band grouper showed a homology ranging from 93% to 100% with Japanese NNV (RGNNV-type) isolated...
from flounder. In addition, NNVs isolated from red drum and oblong rockfish in Korea were clustered in the RGNNV-type with sequences similarities to the Taiwan RGNNV with sequences similarities to the Taiwan

Histologically, diagnosis of NNV is based on the appearance of vacuoles in the brain and/or retina tissue under light microscopy [9]. However, fish with only a few vacuoles in the nervous tissue can be difficult to diagnose. The standard method for detection of NNV is the isolation of viruses in cell culture, followed by identification using either an indirect fluorescent antibody test (IFAT) [8, 11] or indirect enzyme-linked immunosorbent assay (ELISA) [1].

The cell line used to isolate NNV is derived from striped snakehead (*Channa striata*) (SSN-1) [6, 11]. However, the sensitivity of virus isolation is not enough to detect the virus from carrier fish or in the latent state [8, 15]. Nishizawa *et al.* [15] developed a RT-PCR for detecting SJNNV with a detection limit of 100 fg. SYBR green real-time PCR assays were also developed for NNV detection, which had a sensitivity that was 10–100 time higher than the conventional PCR [5, 10]. However, these approaches are still relatively time-consuming, requiring an expensive machine and prone to false-positive reactions due to possible contamination during procedures. Since this constraint is important for disease management, it is clearly necessary to develop a fast, sensitive, and easy diagnostic method for VNN.

Loop-mediated isothermal amplification (LAMP) is a novel method of DNA amplification, which has been used for the detection of several pathogens in aquaculture, as well as human pathogens [21]. LAMP was originally developed by Notomi *et al.* [17], and can amplify very low numbers of target sequences to millions of copies under isothermal conditions within one hour. This method depends on autocycling strand displacement DNA synthesis by the *Bst* DNA polymerase large fragment with high strand displacement activity, and a set of two specially designed inner primers and two outer primers. The LAMP is highly specific because the target sequences are detected by six independent primers in the initial stage, followed by four independent primers in the later stages of the LAMP reaction. LAMP is also applicable for RNA detection by using a reverse transcriptase (RT) together with a DNA polymerase [17]. This technique can be carried out under isothermal conditions, which can be simply achieved using a water bath or a heating block, and expensive thermal cyclers used for PCR are not required [17].

In this study, a RT-LAMP assay for detecting NNV from olive flounder in Korea was developed. A set of six LAMP-specific primers was designed, based on the coat protein sequence of Korean NNVs. The applicability of the RT-LAMP assay was evaluated by comparison with the single and nested RT-PCR assays using field samples.

**Materials and Methods**

**Sampling**

Sampling was conducted during the fall of 2009 and fall of 2011. A total of 102 olive flounder samples were collected from several hatcheries in Pohang City and Jeju Island. These samples consisted of 27 individual adults (body weight 100.1 ± 11.7 g; body length 29.9 ± 1.9 cm), 50 juveniles (body weight 51.4 ± 5.8 g; body length 18.2 ± 3.5 cm), and 25 fry (body weight 28.2 ± 10.3 g; body length 8.5 ± 1.4 cm).

**Viruses**

Striped jack nervous necrosis virus (SJNNV) and Japanese flounder nervous necrosis virus (JFNNV) were propagated in SSN (snakehead-fish)-1 cells. These viruses-infected cells were maintained in L-15 medium supplemented with 5% fetal bovine serum (FBS), 50 IU/ml penicillin, and 50 mg/ml streptomycin at 25°C. For the specificity test, infectious hematopoietic necrosis virus (IHNV) was maintained in CHSE (Chinook salmon embryo)-214 cells. Infectious pancreatic necrosis virus (IPNV), viral hemorrhagic septicemia virus (VHSV), and hirame rhabdovirus (HRV) were maintained in RTG (rainbow trout gonad)-2 cells with MEM-10 [minimum essential medium supplemented with 10% fetal bovine serum (FBS), 200 mM 1-glutamine, 50 IU/ml penicillin, and 50 mg/ml streptomycin] at 15°C. The viruses were then titrated in 96-well plates, and the tissue culture infectious dose infecting 50% of inoculated cultures per milliliter (TCID_{50}/ml) was calculated [20].

**RNA Extraction**

Total RNA was extracted from viruses-infected cells and the brain of olive flounders using the TRI Reagent (Molecular Research Center, USA), according to the manufacturer’s instructions. Briefly, 100 mg of tissues was homogenized in 750 μl of TRI Reagent, and incubated at room temperature for 5 min. Then, 200 μl of chloroform was added to the homogenates and mixed by vortexing. The suspension was incubated at room temperature for 10 min and centrifuged at 12,000 × g for 10 min. Then 200 μl of the upper solution was transferred into a new 1.5 ml microcentrifuge tube and precipitated by adding 200 μl of 100% isopropanol on ice for 10 min. The samples were then centrifuged at 12,000 × g for 10 min. The RNA pellet was washed with 70% (v/v) ethanol, centrifuged at 9,000 × g for 5 min, and then dried at 95°C in the incubator for 2 min or until the ethanol was evaporated. Diethylpyrocarbonate (DEPC)-treated water (Bioneer, Korea) was added to elute RNA at a final concentration of 100 ng/μl. The extracted RNA was kept at −80°C until used.

Genomic viral RNA was extracted from 500 μl of viruses-infected cells culture supernatant as described above and used to optimize the RT-LAMP conditions. After elution of the RNA in DEPC-treated water, it was stored at −80°C until used.

**Primers for the RT-LAMP Assay**

RT-LAMP primers for NNV were designed according to consensus sequences of the published coat protein sequence of fish nodaviruses of Korean strains JFNNV (DQ116037 and DQ864760), GMNNV (DQ116038), RBNNV (DQ116035), and SGNNV (DQ116036), by using LAMP primer designing software Primer Explorer version 4 (http://primerexplorer.jp/elamp4.0.0/index.html, Eiken Chemical, Japan).
The coat protein region was selected as a highly conserved region to detect most known fish nodavirus strains. The details of the primers are listed in Table 1.

### Optimization of the RT-LAMP Reaction Conditions

The RT-LAMP reaction was carried out at 60, 63, and 65°C for 10, 20, 30, 45, and 60 min. The RT-LAMP reactions were performed in a 25 µl total reaction mixture volume containing 2 µM each FIP and BIP, 0.2 µM each F3 and B3, 2 µM each LF and LB primers, 1× thermostop-supplied reaction buffer, 0.6 M betaine (Sigma-Aldrich, USA), 6 mM MgSO4 (Sigma-Aldrich, USA), 1.4 mM dNTPs mix (Solgent, Korea), 8 U of Bst DNA polymerase (large fragment; New England BioLabs, USA), and 0.25 U of AMV reverse transcriptase enzyme (New England BioLabs, USA) at 37°C for 16 h, which cleaves the RT-LAMP product at the GAGGAG site, and subsequently the RT-LAMP product was purified using the Accuprep PCR PreMix tube (Bioneer, Korea) and diluted to a final volume of 20 µl with DEPC-treated water. PCR reaction conditions were the same as described above.

### Comparison of Sensitivity and Specificity Among RT-LAMP, RT-PCR, and Nested RT-PCR Assays

To determine the sensitivity of the RT-LAMP, RT-PCR, and nested RT-PCR assays, 10-fold serial dilutions of RNA extracted from JFNV-infected SSN-1 cells were tested under the optimized conditions for RT-LAMP, RT-PCR, and nested RT-PCR as described above. The specificity was also examined using total RNAs extracted from 2 genotypes of betanodaviruses (SJNNV and RGNNV), IPNV-infected RTG-2 cells, VHSV-infected RTG-2 cells, IHNV-infected CHSE-214 cells, and HRV-infected RTG-2 cells.

### Evaluation of the RT-LAMP Assay with Field Samples

The applicability of the RT-LAMP assay to detect NNV was assessed by comparing the detection results between RT-LAMP, RT-PCR, and nested RT-PCR using field samples. The cultured olive flounders were collected from the hatcheries (Pohang and Jeju). Brain tissues from individual fish and 5 pooled fry were used for RNA extraction as mentioned above. RT-LAMP, RT-PCR, and nested RT-PCR were then conducted under the conditions described above and the results were compared.

### Sequencing and Sequence Alignment

The PCR products were purified using a gel purification kit (Bioneer, Korea) following the manufacturer’s protocol and then 10 ng/µl of purified PCR product was directly sequenced (ABI PRISM 3730x Genetic Analyzer; Applied Biosystems, USA; available at NICEM, Seoul National University, Korea). The sequences of the

### Table 2. Oligonucleotide primers used for detecting NNV in the RT-PCR and nested RT-PCR assays.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position</th>
<th>Sequence (5'–3')</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noda-full-F</td>
<td>1–25</td>
<td>TAACTCCATACCGCTTTGCAATCAC</td>
<td>Cha et al. [3]</td>
</tr>
<tr>
<td>Noda-full-R</td>
<td>1,063–1,090</td>
<td>TCTCAGCTCATCAGCAGTACGGACT</td>
<td></td>
</tr>
<tr>
<td>Noda-partial-F</td>
<td>300–319</td>
<td>CTGGGACACGCTGCTAGAAT</td>
<td></td>
</tr>
<tr>
<td>Noda-partial-R</td>
<td>601–624</td>
<td>CGACATCTGACACCAGCAGTAG</td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td>615–634</td>
<td>CGGTGTACGTATGTCGCTG</td>
<td>Nishizawa et al. [15]</td>
</tr>
<tr>
<td>R3</td>
<td>1,022–1,041</td>
<td>CGAGTCAACACGGGTGAAGA</td>
<td></td>
</tr>
</tbody>
</table>
coat protein gene were aligned with the sequences available in the GenBank database (NCBI) according to the Clustal W using the software MEGA version 4 [25]. The nucleotide sequence was registered to the GenBank under Accession No. JN662462.

RESULTS

Optimization of RT-LAMP Reaction Conditions
RT-LAMP was conducted at 60, 63, and 65°C, to determine the optimal temperature. In this experiment, RT-LAMP products were seen as multiple bands of different sizes on 2% agarose gel at 63°C and were obviously detected at 65°C when using 100 pg RNA extracted from JFNNV-infected SSN-1 cells (Fig. 1). The RT-LAMP products were initially observed after 20 min and clearly seen after 30, 45, and 60 min (Fig. 2). Therefore, the optimal temperature and time for detecting NNV by RT-LAMP were determined to be 65°C and 30 min, respectively.

To confirm that the RT-LAMP product was amplified from the target region, the product was digested with the BseRI restriction enzyme. The enzyme was selected by using the NEBcutter V2.0 software (New England BioLabs, UK, http://tools.neb.com/NEBcutter2/index.php), based on the sequence of the expected RT-LAMP product. The enzyme digestion produced a 149 bp fragment, which was in agreement with the size predicted theoretically, and the typical ladder-like pattern of the RT-LAMP product was not observed. No bands were observed for the negative control (Fig. 3).

Comparison of Sensitivity Between RT-LAMP, RT-PCR, and Nested RT-PCR Assays
To compare sensitivity, RT-LAMP and RT-PCR were performed using a 10-fold serial dilution of RNA extracted from JFNNV-infected SSN-1 cells (2.58 × 10^7 to 2.58 × 10^3 TCID50/ml; N: negative control).
DETECTION OF NNV IN CULTURED OLIVE FLOUNDER BY RT-LAMP

10^{-3} TCID_{50}/ml). The expected size of the single RT-PCR product was 430 bp and those of nested RT-PCR products were 1,090 and 324 bp, respectively. The RT-LAMP was able to detect template at 2.58 × 10^{-2} TCID_{50}/ml (Fig. 4, Lane 7), whereas the RT-PCR was able to detect template only at 2.58 × 10^{-2} TCID_{50}/ml (Fig. 5, Lane 6) and the nested RT-PCR was able to detect template at 2.58 TCID_{50}/ml (Fig. 6, Lane 8). Thus, the sensitivity of the LAMP method was 10^4- and 100-times higher than RT-PCR and nested RT-PCR, respectively.

Specificity of RT-LAMP Assays

The cross amplification of RNA from other viruses pathogenic to fish (i.e., IHNV, IPNV, VHSV, HRV), as well as SJNNV and JFNNV were employed to confirm the specificity of the RT-LAMP assay. Both fish nodavirus genotypes were positive, but neither of the other viruses was positive with the primers, indicating that the RT-LAMP assay is specific to at least 2 genotypes of NNV (Fig. 7, Lanes 1 and 2).

Evaluation of the RT-LAMP Assay with Field Samples

The prevalence of NNV in olive flounder is summarized in Table 3. Among 102 samples, 55 (53.9%) samples were found to be NNV-positive by RT-LAMP, whereas the prevalence was 33.3% (34/102) and 20.6% (21/102) by the nested RT-PCR and RT-PCR, respectively. All the RT-PCR and the nested RT-PCR positive samples were RT-LAMP positive in each year.

Sequence and Phylogenetic Analysis

Ten PCR-positive samples (5 samples from Pohang and 5 samples from Jeju) were randomly selected for sequencing and a phylogenetic tree was constructed. All 10 sequences showed a high degree of 100% homology, and one sequence (JFNNV-PH) was selected for further phylogenetic analysis in this study. The nucleotide sequence of the coat protein...
had a nucleotide identity of 98% to 100% with the previously reported Korean NNV isolates from olive flounder (JFNNV; DQ116037), grey mullet (GMNNV; DQ116038), rock bream (RBNNV; DQ116035), and seven band grouper (SGNNV; DQ116036). The phylogenetic tree revealed that the JFNNV-PH isolate in this study fell within the RGNNV-type (data not shown).

**DISCUSSION**

RT-PCR has been widely used as a diagnostic tool for detecting RNA viruses, but this assay has some disadvantages, such as high cost per sample and need for additional steps, since each sample has to undergo the reverse transcription (RT) step, whereas the RT-LAMP assay is a potentially simple and rapid gene amplification technique for early detection of pathogens [21]. The assay can be accomplished within one hour under isothermal conditions by using a simple heating block or a water bath. We successfully developed a RT-LAMP assay to detect IHNV [23] and IPNV [24], and the sensitivity was higher than the conventional PCR assay. In this study, the RT-LAMP assay was developed to detect NNV from cultured olive flounder.

A RT-LAMP assay for detecting NNV was successfully developed with the reaction being complete within 30 min at 65°C. The detection limit of the RT-LAMP assay for detecting NNV was determined by amplification of 10-fold serial dilutions of RNA extracted from JFNNV-infected SSN-1 cells (2.58 × 10^7 to 2.58 × 10^3 TCID_{50}/ml). The detection limit of the RT-LAMP assay was 100- and 10^4-folds higher than the nested RT-PCR and the RT-PCR assays, respectively. In addition, the accuracy of the RT-LAMP reaction was confirmed by digestion with the BseRI restriction enzyme to ensure that the RT-LAMP products had the corresponding sequence of the coat protein gene of NNV. The BseRI restriction enzyme was found to cleave the RT-LAMP product at the recognition site and produced a 149 bp fragment. As a result, the typical ladder-like structure of the RT-LAMP pattern consisting of alternatively inverted repeats of the target sequences on the same strand disappeared after digestion.

The coat protein gene of NNV was used as the target gene in the RT-LAMP assay because it is relatively conserved and one of the most important genes for the genetic relationship analysis among fish nodaviruses [16]. The RT-LAMP was previously developed for detecting NNV and the primers used were designed on the basis of the coat protein gene encoding a region of the RGNNV-type in China, but the specificity with other NNV genotypes was not determined in their study [27]. In this study, RT-LAMP primers for detecting NNV were designed based on consensus sequences of the coat protein gene of Korean NNV strains. This assay was able to detect at least two available genotypes, SJNNV and RGNNV. In addition, the RT-LAMP primer sequences were screened using BLAST (National Center for Biotechnology Information; NCBI), and the primers could detect all genotypes of NNVs (TPNNV, SJNNV, BFNNV, and RGNNV). Thus, the NNV-specific primers developed in the present study can be useful for detecting other genotypes of NNV and preventing the potential introduction of those strains in Korea. The lack of cross-reactivity with the RNA extracted from IHNV, IPNV, VHSV, and HRV also confirmed the specificity of the RT-LAMP assay developed in this study.

The applicability of RT-LAMP was also compared with those of the nested RT-PCR and RT-PCR using field samples. The detection rate was increased when using the RT-LAMP assay owing to its high sensitivity. The RT-LAMP assay is considered superior to RT-PCR for detecting NNV because it is a simple technique that can be performed in most situations where rapid diagnosis is required; that is, in field conditions and at quarantine inspection stations. Only a water bath or a heating block is needed for amplification since the method requires isothermal conditions.

Phylogenetically, the fish nodaviruses are divided into 5 genotypes (TPNNV-type, SJNNV-type, BFNNV-type, RGNNV-type, and TNV-type), and all Korean fish nodaviruses have been grouped within the RGNNV-type [3, 7, 18]. The NNV isolated from olive flounder (JFNNV-PH) also

<table>
<thead>
<tr>
<th>Year</th>
<th>Type of samples</th>
<th>Number of samples</th>
<th>RT-PCR</th>
<th>Nested RT-PCR</th>
<th>RT-LAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>2009</td>
<td>Juvenile</td>
<td>50</td>
<td>8 (16.0%)</td>
<td>18 (36.0%)</td>
<td>28 (56.0%)</td>
</tr>
<tr>
<td>2010</td>
<td>Fry</td>
<td>10</td>
<td>5 (50.0%)</td>
<td>6 (60.0%)</td>
<td>6 (60.0%)</td>
</tr>
<tr>
<td>2011</td>
<td>Adult</td>
<td>27</td>
<td>8 (29.6%)</td>
<td>10 (37.0%)</td>
<td>21 (77.8%)</td>
</tr>
<tr>
<td></td>
<td>Fry</td>
<td>15</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>102</td>
<td>21 (20.6%)</td>
<td>34 (33.8%)</td>
<td>55 (53.9%)</td>
</tr>
</tbody>
</table>

*Five fry were randomly selected and pooled as one sample.
ND: Not detected.

Table 3. Comparison of the prevalence of NNV in olive flounder determined by the RT-PCR, nested RT-PCR, and RT-LAMP assays.
shared the same branch with previous Korean isolates under the RGNNV-type. It was suggested that the members of the RGNNV-type may be responsible for the nodavirus outbreaks in Korea [3, 18]. Although the RGNNV-type is the most widespread genotype around the world [3], it is not clear when or where the fish nodaviruses were introduced into Korea. Since the first detection of fish nodavirus was reported in seven band grouper along the southern coast of Korea [22], fish nodaviruses have caused widespread outbreaks among various fish species [12]. Phylogenetic analysis showed that fish nodaviruses seemed to be easily adapted to other fish species [3]. Thus, the spread of NNV in Korean waters is thought to be due to the frequent transfer of fish between fish farms [3].

Viral infection poses a serious threat to the aquaculture industry and is responsible for significant economic losses. Thus, it is important to rapidly identify and confirm the causative agents of fish disease to prevent further disease transmission and outbreaks. The RT-LAMP assay described in this study is thought to be a highly sensitive, specific, rapid, and reliable diagnostic method for detection of NNV. This technique holds great promise for use in assessing the viral status of fish populations and can be used under field conditions for the diagnosis or surveillance of NNV.

Acknowledgments

This work was partly supported by a grant from the National Research Foundation of Korea (2009-0087136). The authors thank G. U. Kim and S. S. Joe (Gangwon Fisheries Resources Center) for their help in collecting fish samples.

REFERENCES


