Short communication

Modification of PCR Program for Detection of Infectious Myonecrosis Virus

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ABSTRACT—Infectious myonecrosis (IMN) causes high mortality in the white leg shrimp *Litopenaeus vannamei* and is listed by the OIE. In the current OIE manual (2016), a commercial kit is recommended to conduct one-step RT-PCR for the detection of IMNV. However, the reagent kit is no longer sold in Japan. We therefore tested different programs for RT-PCR using SuperScript[®] III One-Step RT-PCR System, which is different from the one that is recommended in the OIE manual for the diagnosis of the disease. The sensitivity of virus detection was clearly higher when the program of 3-step PCR after reverse-transcription at 55°C was adopted than the detection using the program described in the OIE manual, which is 2-step (shuttle) PCR after reverse-transcription at 60°C. The result suggests that the 3-step PCR program with SuperScript[®] III is useful for the diagnosis of IMN.

Key words: Infectious myonecrosis virus, IMNV, one-step RT-PCR, *Litopenaeus vannamei*

Infectious myonecrosis (IMN) causes high mortality (40%–70%) in the white leg shrimp (*Litopenaeus vannamei*) (Andrade *et al.*, 2007) and is specified as an OIE-listed disease (<http://www.oie.int/>). IMN is caused by IMN virus (IMNV), the genome of which consists of a single, double stranded RNA (Poulos and Lightner, 2006). As the import of aquatic animals into Japan has been increasing, the Japanese government has strengthened quarantine measures (Food Safety and Consumer Affairs Bureau, Ministry of Agriculture, Forestry and Fisheries, 2016). On July 27, 2016, new

regulations were put into effect, and IMN is now a subject of quarantine.

In the current OIE manual, a commercial kit containing *rTth* Enzyme and EZ Buffer (Applied Biosystems, #N808–0178) is recommended to conduct one-step RT-PCR for the detection of IMNV. The kit, however, is no longer sold in Japan, although the *rTth* enzyme itself is available. Therefore, we tested another commercial kit, SuperScript[®] III One-Step RT-PCR System with Platinum[®]Taq (Invitrogen, Thermo Fisher Scientific).

Materials and Methods

IMNV inoculum

Gills (about 20 mg) from frozen white leg shrimp (*n* = 1) that had clinically developed IMN in a past outbreak of the disease were homogenized in 200 µL of sterilized artificial sea water and added to additional 1.3 mL of the sterilized sea water. This was filtered through a 0.45 µm filter (Millipore) to obtain an inoculum of IMNV. These procedures were conducted in the Main Center for Brackishwater Aquaculture, Jepara, Indonesia, and the filtrate was brought to Japan.

Experimental infection by injection

White leg shrimp *L. vannamei* (average weight 3 g) were purchased from a shrimp farm and reared in our laboratory for 19 days. During this period, the shrimp grew well and appeared to be healthy. The IMNV inoculum was injected into ten white leg shrimp (100 µL/shrimp). Six animals were sampled at 3 days after the injections and the cephalothoracic muscle of about 20 mg was taken from each specimen. The gills of one of the six shrimp were also sampled to prepare sequentially diluted RNA template to compare the sensitivity of different programs. Sampled tissues were stored at –80°C. The remaining four shrimp died at 7, 8 and 11 days after inoculation. The abdomen of two shrimp, which were found immediately after death on day 11, were cloudy and slightly reddish. The muscle, (approximately 6 g) from these two dead shrimp was sampled as the source of another experimental infection. For the control, the sterilized artificial sea water was injected into five white leg shrimp (100 µL/shrimp).

Experimental infection by ingestion of virus-contaminated tissue

The muscle of the two dead shrimp sampled on day 11 was fed to intact white leg shrimp (*n* = 2). Eleven days after the ingestion, when one shrimp died and the other was moribund, the muscle (approximately 20 mg/shrimp) from these shrimp was sampled and stored at –80°C for later analyses.

Tissue sampling and RNA extraction

Each tissue sample (20 mg/sample) was

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homogenized in 200 μ L of RNAiso Plus (TaKaRa), and additional 800 μ L of RNAiso Plus was added to make a total of 1 mL. RNA extraction was performed according to the manufacturer's instruction for RNAiso Plus.

The RNA template prepared from the gills of affected shrimp was further diluted to either 10^{-2} or 10^{-4} with RNA suspension (100 ng/ μ L) from the muscle of white leg shrimp which was IMNV-negative by RT-PCR with both OIE and NRIA (National Research Institute of Aquaculture) programs described later (Table 1).

One-step RT-PCR Programs

The final reaction mixture for one-step RT-PCR using SuperScript[®] III One-Step RT-PCR System with Platinum[®] Taq (Invitrogen, Thermo Fisher Scientific) was prepared by mixing the following reagents: 2 $\times$ Reaction Mix (a buffer containing 0.4 mM of each dNTP, 3.2 mM MgSO₄), 12.5 μ L; Template RNA, 500 ng; primer F (100 μ M), 0.125 μ L; primer R (100 μ M), 0.125 μ L (final concentration of each primer was 0.5 μ M); SuperScript[®] III RT/ Platinum[®] Taq Mix, 1 μ L; nuclease free water to make the total volume 25 μ L. The primers used for RT-PCR to detect IMNV were adopted from the OIE manual; they are 4587F, 5'-CGA-CGC-TGC-TAA-CCA-TAC-AA-3' and 4914R, 5'-ACT-CGG-CTG-TTC-GAT-CAA-GT-3', which would amplify the DNA fragment of 328 bp (Poulos and Lightner, 2006).

One-step RT-PCR was performed using two different programs, one of which was the program adopted in our laboratory and the other was the one recommended in the OIE manual for IMN. In addition to the programs of NRIA and OIE, two other programs were tested to clarify the steps that would possibly affect the sensitivity. One of them is the same as the NRIA program except that the temperature for reverse transcription was adjusted to 60°C as the OIE-recommended program (Table 1). In the other, 3-step PCR cycle of the NRIA program was changed to 2 steps as the OIE program while the reverse transcription was conducted at 55°C.

The RT-PCR programs used in this study are summarized in Table 1. The PCR products were electrophoresed using 2% agarose gel containing ethidium bromide to confirm the amplified product and its size.

Results and Discussion

The analyses using the diluted RNA-template revealed that both the higher temperature for the reverse transcription and the 2-step PCR program in the OIE program affect the sensitivity of the RT-PCR when SuperScript III is used (Fig. 1). For the reverse transcription, degradation of the enzyme at the higher temperature may have caused a reduction in the detection sensitivity in the RT-PCR for the OIE program, because the temperature of 60°C is the upper limit of the range recommended for SuperScript III for reverse transcription (45–60°C). For subsequent PCR programs, the total time for extension (45 s including annealing) in each cycle of the 2-step PCR might not enough compared with the total time of 75 s for annealing and extension in the 3-step PCR. In any case, the 2-step (shuttle) PCR program does not seem to be suited for RT-PCR using SuperScript III. The manufacturer recommends a general 3-step PCR program for this enzyme, whereas a 2-step PCR program is recommended by the manufacturer of the *rTth*, which is the suggested enzyme in the OIE manual.

For shrimp that clinically develop IMN, which have a large amount of IMNV, however, the virus would be detected in most cases by the RT-PCR with OIE program even with SuperScript III, since in the experimentally infected shrimp by the injections or oral administration, the virus was detected from 7 out of 8 specimens by the OIE program, although all shrimp were found to be positive with the NRIA program (Fig. 2). The sequences of the amplified DNA fragments of 288 bp that does not contain the 40 bp primer regions were all identical and exhibited 99.3% to 99.7% identity with IMNV from Indonesia (Accession No.; KJ636788.1,

Table 1. Programs for one-step RT-PCR tested in the present study. SuperScript III (Invitrogen, Thermo Fisher Scientific) was used for all programs.

Programs	NRIA ¹⁾	High RT temp. ²⁾	2 step PCR ³⁾	OIE ⁴⁾
Reverse Transcription for cDNA synthesis	55°C 30 min	60°C 30 min	55°C 30 min	60°C 30 min
Denaturation	95°C 2 min	95°C 2 min	95°C 2 min	95°C 2 min
PCR amplification	95°C 15 s	95°C 15 s	95°C 45 s	95°C 45 s
39 cycles	60°C 30 s	60°C 30 s	60°C 45 s	60°C 45 s
	68°C 45 s	68°C 45 s		
Final extension	68°C 2 min	68°C 2 min	60°C 7 min	60°C 7 min

¹⁾ The program routinely used with SuperScript III (Invitrogen, Thermo Fisher Scientific) in the Diagnosis and Training Center for Fish Diseases, National Research Institute of Aquaculture.

²⁾ The temperature for reverse transcription is modified to 60°C from the NRIA protocol.

³⁾ PCR steps are modified to 2 steps from the NRIA program.

⁴⁾ The program recommended in the OIE manual for infectious myonecrosis. This program is designed to use *rTth* Enzyme (Applied Biosystems, Thermo Fisher Scientific).

Fig. 1. Results of one-step PCR using the RNA from the gills of *Litopenaeus vannamei* that developed infectious myonecrosis by experimental infections. One-step RT-PCR was performed using 4 different programs. The details for PCR programs are summarized in Table 1. The temperatures of either 55°C or 60°C indicated on the top of the results are the temperatures for the reverse transcription for the four RT-PCR programs. 10⁻², RNA template was diluted at 1/100. 10⁻⁴, RNA template was diluted at 1/10000.

Fig. 2. Results of one-step PCR for infectious myonecrosis using the RNA from muscle of *Litopenaeus vannamei* that developed clinical disease by experimental infections. M, marker; lanes 1–6, specimens infected with injection; 7 and 8, specimens fed on IMN-infected muscle; N_N, negative control tested with NRIA program; N_O, negative control tested with OIE program. The details for PCR programs are summarized in Table 1.

KJ636784.1, KJ636783.2, KJ636782.2, KJ636787.1, KJ636785.1, EF061744.1, KF836757.1) and Brazil (Accession No.; KR815474.1, KT003689.1, KJ556923.1, AY570982.3) (data not shown), indicating that IMNV was detected specifically. In any case, the results of the present study suggest that a 3-step PCR after reverse transcription at 55°C, such as the NRIA program, should be adopted for the detection of IMNV when SuperScript III is used. The 3-step standard NRIA PCR might be applicable for practical use, since IMNV was clearly detected with this method from shrimp 3 days after the exposure to the virus, when no mortality had yet been observed. In addition, the import of the white leg shrimp should be conducted with great caution, not only because the shrimp can be a host of various important diseases, but also the disease cannot always be detected even with PCR, particularly when the level of pathogen is low.

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