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Polymerase chain reaction (PCR) amplification of RNA of striped jack nervous necrosis virus (SJNNV)

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ABSTRACT: The polymerase chain reaction (PCR) was used to amplify a portion of the coat protein gene (RNA2) of striped jack nervous necrosis virus (SJNNV), the causative agent of viral nervous necrosis (VNN) of larval striped jack *Pseudocaranx dentex*. Based on the sequence data of SJNNV RNA2, 2 forward (F1 and F2) and 3 reverse (R1, R2 and R3) PCR primers were synthesized and the 5 potential target regions were amplified with a combination of these primers. After reverse transcription of genomic RNA extracted from SJNNV and 25 cycles of PCR amplification, products of the expected size were detected separately on agarose gels stained with ethidium bromide. Southern blot hybridization confirmed that all of the amplified products were specific to cDNA of SJNNV RNA2. Two primer sets, F1–R2 and F2–R3, produced the specified 180 bp and 430 bp products. The PCR system, using the F2–R3 primer set, was able to detect 100 fg of SJNNV RNA after 25 cycles and was also able to efficiently amplify the target region of SJNNV gene in the total nucleic acids extracted from larval striped jack affected with VNN.

KEY WORDS: Viral nervous necrosis · Nodavirus · PCR

INTRODUCTION

Striped jack nervous necrosis virus (SJNNV) is the causative agent of viral nervous necrosis (VNN) in hatchery-reared larvae of striped jack *Pseudocaranx dentex* (Mori et al. 1992, Arimoto et al. 1993). SJNNV has been identified as a member of the family Nodaviridae based on the properties of its structural proteins and nucleic acids (Mori et al. 1992). The spherical unenveloped virus, about 25 nm in diameter, contains 2 single-stranded, positive-sense, non-polyadenylated RNAs of 1.01×10^6 Da (RNA1) and 0.49×10^6 Da (RNA2). RNA1 encodes a non-structural protein of 100 kDa and RNA2 encodes a structural protein of 42 kDa of the virus. The gene of RNA2 consisting of 1.48 kb has been cloned and sequenced, and the open reading frame determined (Mori et al. unpubl.).

In VNN disease of larval striped jack, the central nervous system is affected and numerous virus particles exist in the cytoplasm of infected nerve cells. Because virus isolation using fish cell lines has not been successful, cell culture techniques cannot be applied at present for the detection of the virus. Arimoto et al. (1992) developed an enzyme-linked immunosorbent assay (ELISA) using a rabbit anti-SJNNV serum for the detection of viral antigens from diseased larvae and gonads of parental spawners. However, the sensitivity of this ELISA was not high enough to detect the virus from fish in the latent or carrier state.

Reverse transcription followed by polymerase chain reaction (PCR) is a powerful technique for amplification of RNA (Innis et al. 1990, Xu & Larzul 1991), which has been applied for the detection of infectious hematopoietic necrosis virus (Arakawa et al. 1990). This paper describes a PCR amplification technique as a rapid and sensitive method for the detection of SJNNV.

MATERIALS AND METHODS

Preparation of viral nucleic acid. The SJNNV was purified from hatchery-reared larval striped jack af-

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fected with VNN following the procedure described by Mori et al. (1992). Briefly, a pooled sample of diseased larvae (net weight: 50 g) from an outbreak at Nagasaki Prefecture, Japan, in 1992 was homogenized with chloroform - 100 mM Tris-HCl buffer (pH 7.2) containing 1 mM EDTA and 2 mM 2-mercaptoethanol. After centrifugation at 8000 × *g* for 20 min, the supernatant was centrifuged at 100 000 × *g* for 2.5 h. The resultant pellet was resuspended in 100 mM Tris-HCl containing 1 mM EDTA and the virus was purified by 10 to 40% sucrose gradient centrifugation (80 000 × *g*, 2 h) and 36% CsCl equilibrium density gradient centrifugation (210 000 × *g*, 16 h). Nucleic acids of SJNNV were extracted with phenol saturated with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) followed by a mixture of chloroform and isoamyl alcohol (24:1). The nucleic acids were precipitated by addition of 0.1 volume of 3 M ammonium acetate and 2.5 volumes of 100% ethanol, dried, and resuspended in diethyl pyrocarbonate treated water.

Diseased larvae of striped jack from the same lot as described above and normal larvae were homogenized with TE buffer containing SDS (1%) and proteinase K (1 mg ml^{-1}). After incubation at 37°C for 30 min, the total nucleic acids were extracted and precipitated by the procedure described above.

Primer design for PCR amplification. Five DNA oligonucleotide primers for PCR amplification of SJNNV gene were designed based on sequence data of the SJNNV RNA2 (Mori et al. unpubl.). These included 2 forward (sense) primers (F1 and F2) and 3 reverse (antisense) primers (R1, R2 and R3). Primer locations and sequences are shown in Fig. 1 and Table 1. Each primer consisted of 20 nucleotides and had a Tm between 63.6 and 65.4°C. These primers were synthesized using a Gene Assembler Special (Pharmacia) and established protocols. Target regions expected to be amplified by these primers were 1147 bp (T1) from F1 to R1, 875 bp (T2) from F1 to R3, 698 bp (T3) from F2 to R1, 426 bp (T4) from F2 to R3, and 175 bp (T5) from F1 to R2 (Fig. 1).

PCR amplification. For reverse transcription, viral RNA from purified SJNNV, or total nucleic acids from striped jack larvae, was pre-heated at 90°C for 5 min and incubated at 42°C for 30 min in 20 µl of PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl) containing 2.5 U of M-MLV reverse transcriptase (USB), 1.0 U of ribonuclease inhibitor (Toyobo), 0.5 µM of reverse primer, 1 mM each of 4 deoxynucleotide triphosphates (dNTP; Pharmacia), and 5 mM of $MgCl_2$. Following cDNA synthesis, the mixture was incubated at 99°C for 10 min to inactivate the reverse transcriptase and then diluted 5-fold with PCR buffer containing 0.1 µM of forward primer, 2.5 U of Tth Version 2.0 DNA polymerase (Toyobo) and 2 mM of $MgCl_2$. The mixture was incubated in an automatic thermal cycler (Astec PC-700) programmed for 1 cycle at 72°C for 10 min and 95°C for 2 min, then 25 cycles at 95°C for 40 s, 55°C for 40 s, and 72°C for 40 s, and finally held at 72°C for 5 min.

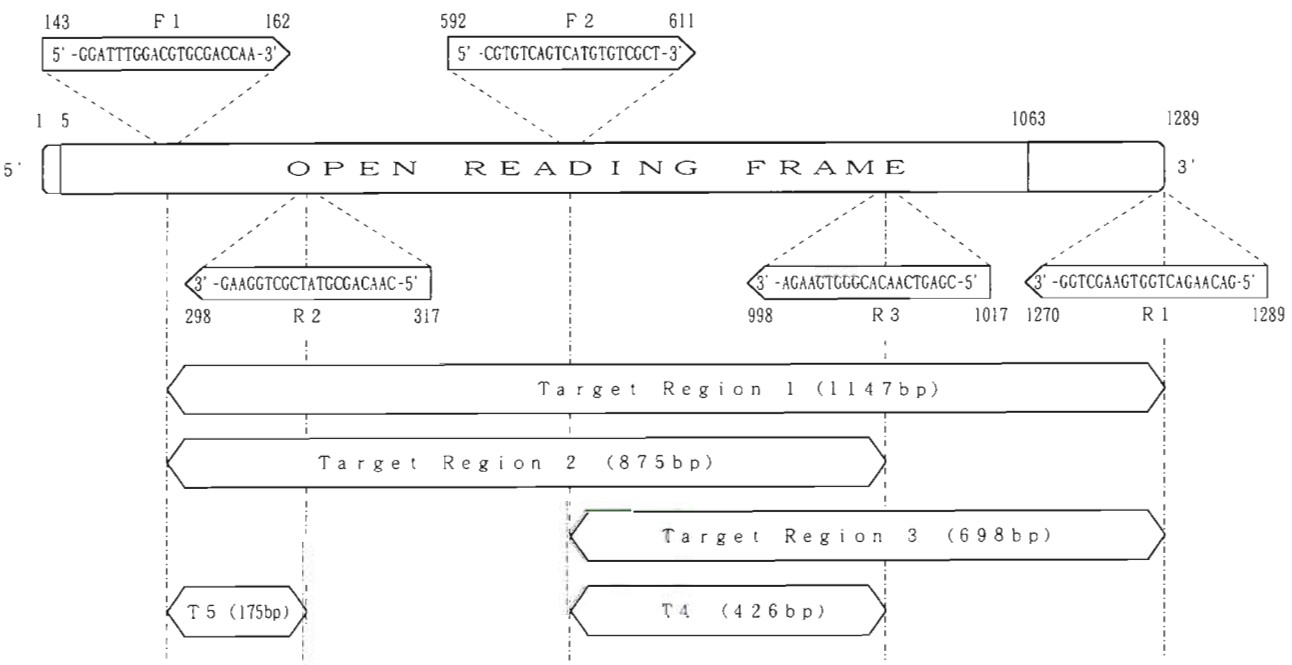

Fig. 1. Schematic illustration of the physical map of the SJNNV RNA2, 2 forward primers (F1 and F2), 3 reverse primers (R1, R2, and R3), and 5 target regions (T1, T2, T3, T4 and T5) for PCR amplification of SJNNV gene

Table 1. Sequence, location, and characterization of the oligonucleotide primers used in PCR amplification of SJNNV gene

Primer	Sequence	Location on cloned cDNA	% GC	Tm (°C)
F1	5'-GGATTTGGACGTGCGACCAA-3'	143–162	55	65.4
F2	5'-CGTGTCAGTCATGTGTCGCT-3'	592–611	55	64.5
R1	5'-GACAAGACTGGTGAAGCTGG-3'	1270–1289	55	64.8
R2	5'-CAACAGCGTATCGCTGGAAG-3'	298–317	55	63.6
R3	5'-CGAGTCAACACGGGTGAAGA-3'	998–1017	55	64.4

Analysis of PCR amplified products. Aliquots (10 µl) of amplified products were electrophoresed in a 3.0% NuSieve 3:1 agarose (Takara) - TAE buffer (40 mM Tris-acetate, pH 7.2, 1 mM EDTA) gel, then the nucleic acids were stained with ethidium bromide. For Southern hybridization, the gel was treated with 0.5 N NaOH - 0.6 M NaCl and then with 0.5 M Tris-HCl (pH 7.4) - 0.9 M NaCl. The nucleic acids in the gel were transferred to a nylon membrane (Hybond; Amersham International) by diffusion blotting in 6 × SSC (1 × SSC is 15 mM sodium citrate, 150 mM NaCl). After washing with 2 × SSC, the membrane was baked at 80 °C for 2 h to bind the DNA, then incubated at 60 °C for 6 h in prehybridization solution containing 5 × SSC, 50% (v/v) formamide, 0.5% (w/v) N-lauroylsarcosine, 0.1% (w/v) SDS, and 5% (w/v) blocking reagent (Boehringer Mannheim Yamanouchi). The cDNA against SJNNV RNA2 was labeled with digoxigenin (DIG) using a DIG labeling kit (Boehringer Mannheim Yamanouchi) and hybridization was performed at 42 °C for 12 h in prehybridization solution containing 50 ng of the DIG-labeled probe. Detection of the DIG probe, hybridized with targed DNA on the membrane, was performed as described by the manufacturer.

RESULTS AND DISCUSSION

Five targeted regions, T1 to T5, of SJNNV RNA2 were amplified by PCR using primer sets F1–R1, F1–R3, F2–R1, F2–R3 and F1–R2, respectively (Fig. 1). The amplified products were analyzed by agarose gel electrophoresis (Fig. 2A). The products of targets T5 and T4 were approximately 180 bp and 430 bp, respectively. A major band of about 700 bp and 2 minor bands of about 630 bp and 120 bp were detected by amplification of the T3 target region using primer set F2–R1. A major band of about 880 bp and a minor one of about 710 bp were observed by amplification of the T2 target region. The amplification of the T1 target region yielded 2 major DNA segments (about 1150 and 1080 bp) and 3 minor segments (about 880, 570 and

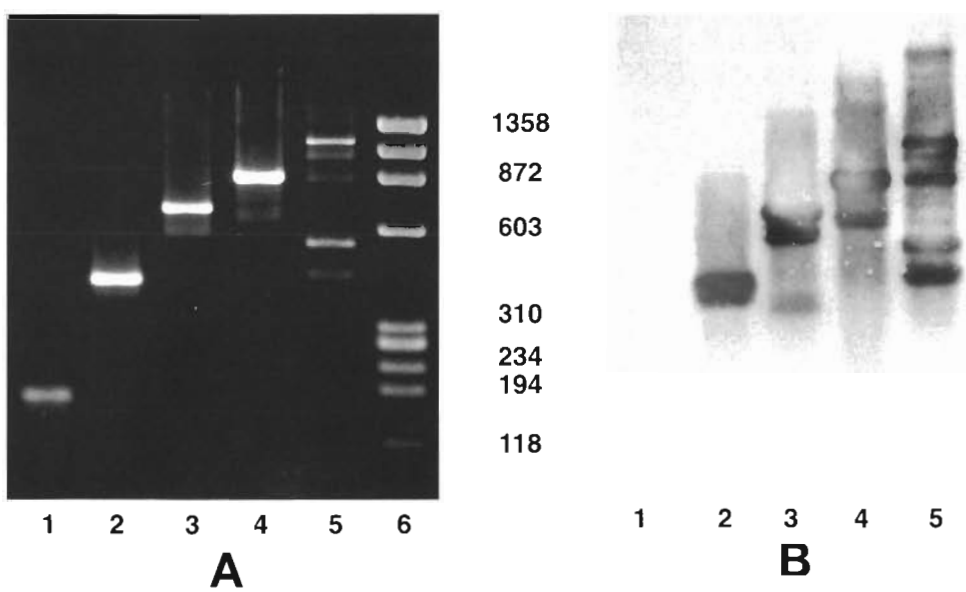

Fig. 2. Agarose gel electrophoresis of the products by PCR amplification of 5 target regions of SJNNV gene. (A) Agarose gel stained with ethidium bromide, (B) Southern blot hybridization with DIG-labeled cDNA to SJNNV RNA2. Lanes: (1) T5, (2) T4, (3) T3, (4) T2, (5) T1, (6) DNA ladder

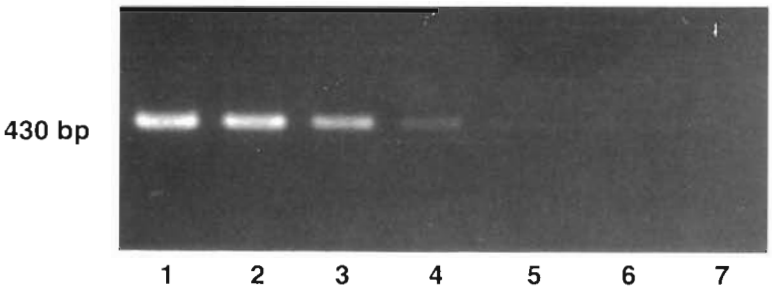

Fig. 3. Detection limit of SJNNV gene by PCR amplification with 25-cycle incubation. Amplified products in agarose gel were stained with ethidium bromide. Lanes: (1) 100 pg of SJNNV RNA, (2) 10 pg, (3) 1 pg, (4) 100 fg, (5) 10 fg, (6) 1 fg, (7) 0 g

430 bp). All of these amplified products hybridized with the DIG-labeled cDNA probe against SJNNV RNA2 (Fig. 2B). However, the F1–R1, F1–R3, and F2–R1 primer sets and their corresponding target regions, T1, T2 and T3, were not considered useful for PCR amplification since products other than those of the predicted molecular weights were obtained. These nonspecific background products might be produced by partially renatured secondary structure(s) of SJNNV RNA2 at the temperature for the reverse transcriptase reaction. The amplification of these nonspecific products could be eliminated by changing the combination of the primers used, suggesting that the secondary structure may have existed in the larger target regions of the original primer sets. Although nonspecific products were not observed in the PCR amplification of T5 and T4, the product from T5 was less stained than that from T4. From these results, T4 was considered to be the most suitable target region tested for PCR amplification of the SJNNV coat protein gene.

The detection limit of SJNNV RNA by PCR amplification was examined using 10-fold serially diluted samples of RNA ranging from 100 pg to 1 fg. The F2–R3 primer set was used with 25 cycles of amplification. A single species of DNA product (about 430 bp) was detected from the samples containing more than 100 fg of SJNNV RNA, but not from samples with 10 fg or less (Fig. 3). By increasing the number of PCR incubation cycles to 35, it was possible to detect a smaller quantity of SJNNV RNA, down to a level of approximately 0.1 fg.

Total nucleic acids from diseased and normal larvae of striped jack were subjected to amplification of the T4 region by PCR. The amplification using nucleic acids from diseased larvae yielded a large amount of 430 bp DNA product corresponding to that obtained from SJNNV RNA. No product was detected from nucleic acid samples from normal larvae (Fig. 4A). The amplified product from infected larvae hybridized with the cDNA of SJNNV RNA2 (Fig. 4B), confirming that the specified viral target was amplified in the reaction. In addition to being useful for detection of SJNNV from diseased larvae, the target sequence of the SJNNV gene was amplified by PCR from nucleic acids of infected larval striped jack from which SJNNV antigens were not detected by ELISA (data not shown). This demonstrates the PCR amplification of SJNNV nucleic acid to be a specific and sensitive method for the diagnosis of VNN.

Diseases of larval or juvenile marine fish with viral etiology similar to VNN in striped jack were also reported in Japanese parrotfish *Oplegnathus fasciatus* (Yoshikoshi & Inoue 1990), barramundi *Lates calcarifer* (Glazebrook et al. 1990, Renault et al. 1991, Munday et al. 1992), turbot *Scophthalmus maximus* (Bloch et al. 1991), sea-bass *Dicentrarchus labrax* (Breuil et al. 1991) and redspotted grouper *Epinephelus akaara* (Mori et al. 1991) in Japan and other countries. However, the causative viruses have been neither isolated nor purified from these diseased fish and no comparative studies of these agents have been done. Products identical to that obtained from SJNNV were amplified from diseased Japanese parrotfish and redspotted grouper by PCR using the primer set designed to amplify the T4 region of SJNNV RNA2, and they were hybridyzed with the cDNA against SJNNV RNA2 (data not shown). This amplification method will be useful for comparative studies of VNN disease agents.

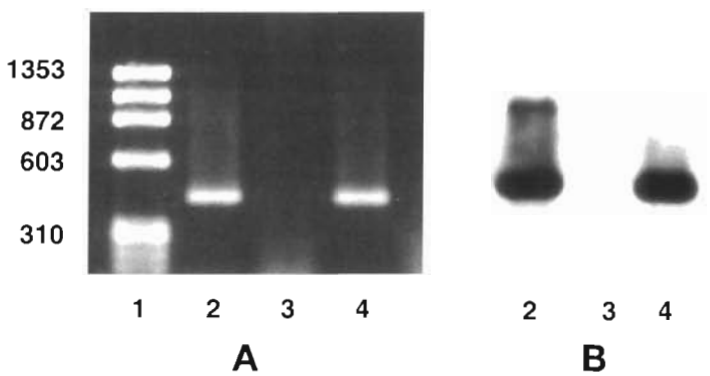

Fig. 4. Detection of SJNNV gene from larval striped jack *Pseudocaranx dentex* by PCR amplification. (A) Agarose gel stained with ethidium bromide, (B) Southern blot hybridization with DIG-labeled cDNA to SJNNV RNA2. Lanes: (1) DNA ladder, (2) amplified product from SJNNV gene, (3) amplified product from total nucleic acids extracted from normal striped jack, (4) amplified product from total nucleic acids extracted from diseased striped jack

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