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Detection of Marine Birnavirus (MBV) from Rockfish *Sebastes schlegeli* Using Reverse Transcription and Nested PCR

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Reverse transcription (RT)-PCR and nested PCR methods (2-step PCR) were tested for their ability to detect marine birnavirus (MBV) in cultured rockfish, *Sebastes schlegeli.* One set of primers for RT-PCR was designed, based on a gene of infectious pancreatic necrosis virus (IPNV), and another set of primers for nested PCR was designed based on the VP2/NS junction region of MBV. This 2-step PCR method was specific for MBV and sensitivity was heightened when nested PCR was combined to RT-PCR. This 2-step PCR method was useful for detecting MBV not only in diseased fish, but also in asymptomatic fish. These results indicate that this 2-step PCR method is useful for detecting MBV in rockfish.

Key words: rockfish, *Sebastes schlegeli,* MBV, RT-PCR, nested PCR

The Birnaviridae are a non-enveloped, icosahedral family of viruses, having 2 segments of double-stranded RNA (12). Members of this family have various hosts, such as salmonid fish fowl, insects and, crustaceans. (3, 5, 6). Among them, infectious pancreatic necrosis virus (IPNV) has been identified as a threat to the salmonid aquaculture industry since the 1950s (6). Since 1985, birnaviruses infecting fish other than salmonids have also been reported, yellowtail *Seriola quinqueradiata* (20), flounder *Paralichthys olivaceus* (9, 10), turbot *Scophthalmus maximus* (14), and sea bream *Sparus auratus* (16). They have been discovered in bivalves (7), and in the marine environment (15). At the present time, birnaviruses infecting aquatic animals are separated into two groups : IPNV, and marine birnavirus (MBV), based on genetic differences (8).

In Korea, as marine fish culturing has intensified, economic losses due to viral diseases have become a serious problem. MBV also causes serious problems in the Japanese flounder farming industry in Korea (9, 19), and Seo *et al.* discovered MBV in cultured rockfish *Sebastes schlegeli* (18), which is a potential threat to the rockfish culture industry. MBV has been isolated from rockfish showing abnormal signs such as ascites, hemorrhages on the head, and spiral swimming (18). Rockfish is the second most popular marine culture fish in Korea, after flounder. So it is important to know how widely MBV is distributed in rockfish, as this is a necessary step for developing possible control methods. Here we investigated detection methods for MBV infection in rockfish using PCR. We employed 2-step PCR as developed by Suzuki *et al.* (21), and compared the sensitivity with simple RT-PCR.

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Materials and Methods

Virus and bacterial strains

The MBV (GC-1 strain) used in this study was first isolated in our laboratory (18). Infectious pancreatic necrosis virus (IPNV) and Infectious hematopoietic necrosis virus (IHNV) were supplied by Dr. Park (19). Avian infectious bursal disease virus (IBDV) was obtained from Dr. Mo, Department of Avian Disease, National Veterinary Research and Quarantine Service, and *Edwardsiella tarda,* and *Enterococcus seriolicida* were obtained from Dr. Park, Department of Fish Pathology at Kunsan National University. These viruses and bacterias were used for testing the specificity of primers.

Fish samples

We collected 51 samples of rockfish in the late spring and early summer of 1998 from several seawater netpen cages in Gyeongsang-Namdo Province, in southern South Korea. Among 51 fish, 29 individuals showed abnormalities such as ascites and haemorrhages on the head, with 22 individuals having no external symptoms.

Extraction of nucleic acids

Total nucleic acids of MBV were extracted from rockfish organs using the method of Suzuki *et al.* (21). Organs

from each sample were pooled and homogenized in 9 volumes of TE buffer (0.2 M Tris-HCl, pH 8.3, 0.1 M EDTA) with homogenizer. Debris was removed by centrifugation at 1,000 × g for 60 min at 4°C. The supernatant was then centrifuged at 3,000 × g for 6 hr and pelleted. 0.01 M Tris-HCl (pH 7.8), 0.05 M EDTA, 0.5% SDS and 50 g/ml proteinase K were added to the pellets, and the mixture was incubated at 55°C for 2 hr. Nucleic acids were then extracted and purified with phenol-chloroform 3 times, followed by ethanol precipitation. Extraction of bacterial nucleic acids was performed using the method of Sambrook *et al.* (17).

RT-PCR

We used 2 sets of primers described by Suzuki *et al.* (21). The set of primers (PF-PR) for RT-PCR was designed based on the IPNV gene, and another set of primers (NPF-NPR) for nested PCR was designed based on the VP2/NS junction region of MBV gene (Table 2). Primer PF and PR was added to the purified nucleic acids; the solution was then employed for denaturation by heating at 100°C for 5 min, followed by cooling on ice. To this solution, a reverse transcription mixture [50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 mM each of deoxyribonucleotide and AMV Reverse Transcriptase XL (Takara, Japan)] was added. Reverse transcription was performed in a final volume of 50 µl at 37°C for 1 hr. The RNA-DNA hybrid was denatured by heating at 100°C for 5 min and cooled on ice. The synthesized cDNA was provided for PCR amplification using Gene Cycler Thermal Cycler (Bio-RAD, U.S.A). One primer set (PF-PR) and *Ex Taq* polymerase (Takara, Japan) were used in this step. The PCR was performed for 35°C cycles: 1 min at 95°C for denaturation, 2 min at 50°C to allow annealing and 3 min at 72°C for extension. The amplified products were electrophoresed in 2% agarose gel and visualized by staining with ethidium bromide.

Nested PCR

Nested PCR amplification was performed using 3 µl of the RT-PCR product, and another primer set (NPF-NPR) (Table 2). The amplification conditions including final volume, nucleotide and salt concentration, and number of PCR cycles were the same as those of RT-PCR conditions described above. This nested PCR step was employed with samples, for which the RT-PCR step had not been effective.

Sensitivity and specificity test of 2-step PCR method

To compare the detection sensitivity of RT-PCR and nested PCR, purified viral dsRNA was serially diluted and employed as a template. The concentraion of purified dsRNA was determined by a spectrophotometer (Perkin Elmer, U.S.A). To test the specificity of the primers, we used nucleic acids from several fish pathogens other than MBV as templates.

Results

Detection of MBV from rockfish

We detected MBV genes in 26 of 29 symptomatic fish. We detected MBV from 70% of diseased fish when only RT-PCR was used, and the detection rate increased to 89% when nested PCR was combined (Table 3). We also detected MBV in 7 of 22 fish which showed no gross lesions. The detection rate was 17% when only RT-PCR was used, but increased to 32% when nested PCR was combined (Table 3). From all the PCR-positive samples, a specific 359 bp product in RT-PCR and a 168 bp product in nested PCR was detected (Fig. 1).

Sensitivity and specificity test of 2-step PCR method

We detected the specific product of RT-PCR until the viral genome was diluted to 10 pg. When combined with nested PCR, the detection limit was increased to 1 fg of

Table 1. A list of rockfish samples used in this study.

Location number	Number of rockfish	Sampling date			Average body length (cm)	Average body weight (g)
1	13	10	May	98	4.5	14.4
2	12	10	May	98	5.2	15.8
3	14	2	Jun	98	8.3	19.7
4	12	2	Jun	98	9.4	20.9

Table 2. Primers used for RT-PCR (PF-PR) and nested PCR (NPF-NPR).

Primer code	Primer sequence	Primer orientation	Product length (bp)	Map position
PF	5'-AGAGATCACTGACTTCAC AAGTGAC-3'	Sense	359	1403-1761 (Jasper strain segment A)
PR	5'-TGTGCACCACAGGAAAGA TGACTC-3'	Antisense		
NPF	5'-CAACACTCTTCCCCATG-3'	Sense	168	74-241 (Y-6 strain VP2/NS junction part)
NPR	5'-AGAACCTCCCAGTGTCT-3'	Antisense		

a. Described by Suzuki *et al.*, 1997

Table 3. Detection of birnavirus genome by 2-step PCRs and virus isolation in cell culture.

Isolation place	Numbers of fish tested PCR (R or N)[a]			% of samples 2-step PCR-positive	Number of CPE/samples 2-step PCR-positive
G-1	AS[b]	5	+ (R 0/5, N 2/5)[d]	40	0/2
	S[c]	8	+ (R 5/8, N 3/3)	100	2/8
G-2	AS	7	+ (R 0/7, N 2/7)	29	0/2
	S	5	+ (R 2/5, N 2/3)	80	1/4
G-3	AS	6	+ (R 1/6, N 1/5)	33	0/2
	S	8	+ (R 7/8, N 0/1)	88	2/7
G-4	AS	4	+ (R 0/4, N 1/4)	25	0/1
	S	8	+ (R 7/8, N 0/1)	88	2/7

[a]R: RT-PCR; N: Nested PCR, [b]AS: asymptomatic, [c]S: symptomatic, [d]positive numbers of total tested numbers

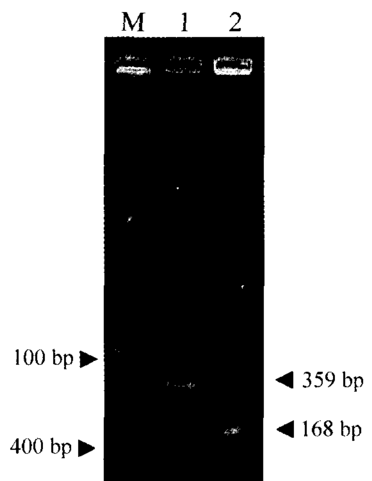

Fig. 1. PCR products in RT-PCR and nested PCR. Arrowheads on the right side indicate the location of amplified products. Lanes: M, molecular marker; 1, product of RT-PCR; 2, product of nested PCR.

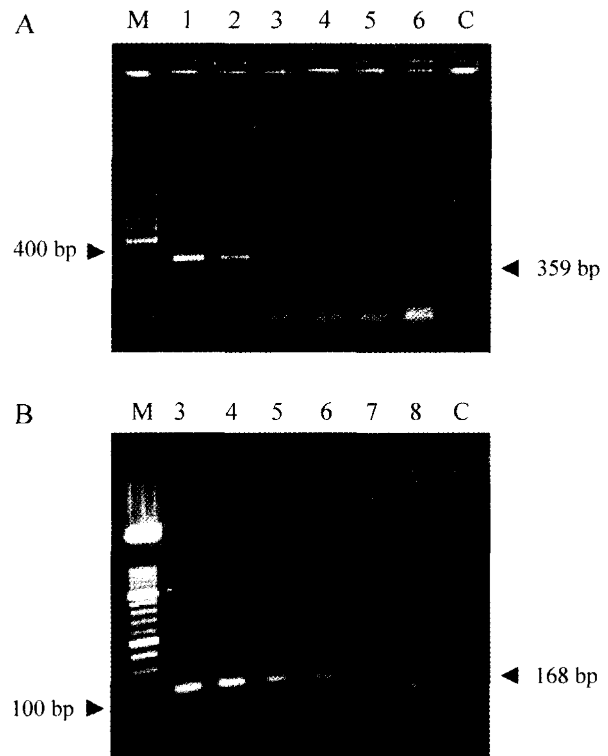

Fig. 2. Detection limits of RT-PCR and nested PCR. (A) RT-PCR, (B) nested PCR. Molecular marker (M) and CHSE-214 cell (1 ng) as control (C) were also run. Lane 1, 1 ng of template; 2, 100 pg; 3, 10 pg; 4, 1 pg; 5, 100 fg; 6, 10 fg; 7, 1 fg; 8, 100 ag.

the viral genome (Fig. 2).

Amplified gene products were observed only in the samples from rockfish. No bands were detected in nucleic acids from other fish pathogens and CHSE-214 cells as control (Fig. 3).

Discussion

Lopez-Lastra *et al.* (11) and Blake *et al.* (1) reported that the PCR method is useful for detecting IPNV genes. The RT-PCR method was highly sensitive and specific compared to other detection methods. However, they also mentioned that the reverse transcription step before amplification may reduce the efficiency of amplification. Recently Suzuki *et al.* (21) developed a new detection method for MBV, employing a combination of RT-PCR and nested PCR, to increase the sensitivity of detection.

We detected MBV in 89% of symptomatic fish. The remaining diseased fish, from which we could not detect MBV, might have other viral diseases such as iridovirus infection, which shows similar clinical signs to birnavirus infection. This seems likely because Oh *et al.* (14) reported that they detected iridovirus in both of 2 rockfish that they sampled in Korea. So fish samples that we tested might have both birnavirus and iridovirus, although there has been no report on double-infection of these two viruses to date. More detailed studies should be conducted to clarify if these 2 viruses infect rockfish concurrently.

We could detect MBV genes from 32% of tested fish without gross disease symptoms. Similar results were obtained by Yamamoto (22) and Bootland *et al.* (2). They mentioned that birnaviruses can be infected chronically

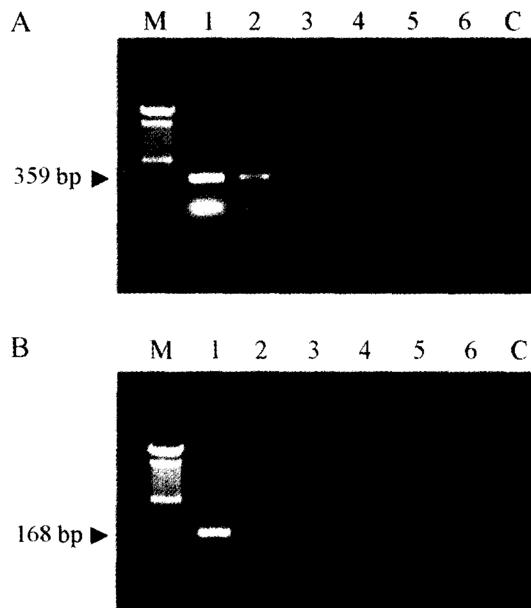

Fig. 3. Specificity of RT-PCR and nested PCR. (A) RT-PCR, (B) nested PCR. Lanes: M, molecular marker (100 bp ladder); 1, MBV (GC-1); 2, IPNV (VR-299); 3, IHNV; 4, IBDV; 5, *Edwardsiella tarda*; 6, *Enterococcus seriolicida*; C, CHSE-214 cells as control.

and existed long term in survivors after acute infection. So possibly these asymptomatic fish in our study with no gross external signs may have MBV. However, it is unclear if these asymptomatic fish in our study had recovered from acute infection or were in the early stage of infection.

We found that 2-step PCR is 1000 times more sensitive than RT-PCR (Fig. 2). This result agrees with those of Lopez-Lastra *et al.* (11) and Suzuki *et al.* (21). Furthermore, we could increase the detection rate of MBV from asymptomatic rockfish samples by using 2-step PCR. This result indicates that the 2-step PCR method is sensitive enough to detect MBV genes even though it exists in very small quantities.

We selected 3 viral pathogens and 2 bacterial pathogens for the specificity test. The result showed that the amplified PCR product was observed only in MBV from rockfish. From this result, it is suggested that primer sets used in this study can detect MBV specifically, from rockfish.

In conclusion, by using a 2-step PCR detection method, we could detect MBV in rockfish with success, although this detection seems to have been affected by the likely presence of iridovirus. This method can be used to investigate the prevalence of MBV in rockfish and to detect the virus in early infection.

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