Polymerase Chain Reaction (PCR) Amplification of DNA of Red Sea Bream Iridovirus (RSIV)

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The polymerase chain reaction (PCR) was used to amplify DNA of the red sea bream iridovirus (RSIV). Four oligonucleotide primer sets based on the ATPase gene, DNA polymerase gene, and a *Pst* I-restriction fragment of RSIV genomic DNA were synthesized. PCR products of the expected size were amplified with each primer set after 30 cycles using template DNA which was extracted from the supernatant of tissue-cultured GF cells infected with RSIV. These amplified products were shown to be specific for the genomic DNA of RSIV by Southern blot hybridization. In addition, PCR products were obtained from the DNAs of the spleen and blood of red sea bream, *Pagrus major*, artificially infected with RSIV. Furthermore, the PCR products were detected from the tissues of cultured marine fish naturally infected with RSIV and from the supernatant of tissue-cultured GF cells infected with RSIV isolated from cultured marine fish. However, PCR products were not obtained at 3 months post-challenge from the spleens of red sea bream infected by intraperitoneal inoculation of RSIV. None of the PCR products were obtained from the supernatant of tissue-cultured FHM cells infected with frog virus 3 or from the lymphocystis tissue of Japanese flounder, *Paralichthys olivaceus*, infected with fish lymphocystis disease virus.

Key words: red sea bream iridovirus, RSIV, red sea bream, *Pagrus major*, PCR, rapid diagnosis

An epizootic caused by a red sea bream iridovirus (RSIV) was reported to be the leading causes of high mortalities in cultured red sea bream (*Pagrus major*) in southwest Japan (Inouye *et al.*, 1992). Biological and physico-chemical properties of the virus were reported (Nakajima *et al.*, 1994) and rapid method for diagnosis of the disease using an immunofluorescence (IF) test with a monoclonal antibody was also developed (Nakajima *et al.*, 1995). However, a highly sensitive method is desirable for detecting the virus in fish at the early stage of the disease. The polymerase chain reaction (PCR) is significantly more sensitive than currently used immunological assays such as the enzyme-linked immunosorbent assay (Stoeckl *et al.* 1989, van der Ryst *et al.*, 1996, Poiesz *et al.* 1997). PCR has already been used to detect fish-pathogenic virus, epizootic haematopoietic necrosis virus (EHNV) and Bohle iridovirus which belong to the Iridoviridae (Gold *et al.*, 1995), as well as several of the fish viruses such as infectious haematopoietic necrosis virus (IHNV), channel catfish virus (CCV), infectious pancreatic necrosis virus (IPNV) and striped jack nervous necrosis virus (SJNNV) (Arakawa *et al.*, 1990, Boyle *et al.*, 1991, Lopez-Lastra *et al.*, 1994, Nishizawa *et al.*, 1994). Oshima *et al.* (1996) cloned a

small subunit of the ribonucleotide reductase (RNRS) gene of RSIV. Subsequently, PCR successfully amplified virus-specific nucleic acids with a primer set based on the RNRS gene. We also found that PCR amplification using primer sets based on genomic genes of RSIV provided a rapid, simple, and sensitive method of diagnosis.

Materials and Methods

Virus isolates and cells

The Ehime-1 strain (Nakajima *et al.*, 1995) of RSIV was used in this study. The virus was propagated in the Grunt fin (GF) cell line at 25°C in basal medium eagle (BME) (Flow Laboratories) supplemented with 10% (V/V) fetal bovine serum (Bio Whittaker), 10% (V/V) human serum (ICN Biomedicals) and 100mg/ml streptomycin and 100 IU/ml penicillin.

Virus purification

The tissue culture supernatant from GF cells cultured with RSIV was collected, pooled and centrifuged at 3,200 rpm for 5 min to precipitate cell debris. The supernatant was then successively filtered through the decreas-

ing size order of 8.0, 3.0, 0.8 and 0.45 μm membranes. The filtrate was centrifuged in a SW-40Ti rotor within a XL-90 ultracentrifuge (Beckman) at 25,000 rpm for 2h at 4°C. The resultant pellet was then soaked in a small volume of TNE buffer (0.04 M Tris, 0.15 M NaCl, 0.015 M EDTA, pH7.4) and layered on top of a continuous 10–35% (W/V) gradient of cesium chloride. The gradient was centrifuged in a SW-28 rotor at 30,000 rpm for 24h at 4°C. The virus band was harvested with a syringe and washed with TNE buffer by centrifugation. The obtained virion pellets were frozen and kept at –80°C until DNA extraction.

Construction of RSIV genomic DNA library and determination of nucleotide sequence of the viral genomic DNA

A virus genomic DNA library was constructed by the following procedure. Initially, genomic DNA of RSIV was extracted from the virions. To achieve this, pelleted RSIV virions were soaked and incubated with TE buffer (0.01 M Tris-HCl, 0.001 M EDTA, pH8.0) containing 0.5% (W/V) SDS and 1 mg/ml proteinase K at 55°C for 30 min followed by phenol-chloroform extraction and dialysis against TE buffer overnight. The extracted viral genomic DNA was digested with *Sph* I, *Pst* I, *Sac* I and digested partially with *Sau*3A I. DNA fragments digested with each enzyme were inserted into the multi-cloning site of the plasmid pGEM3zf (+), and the recombinant plasmids were used to transform to *Escherichia coli* strain XL1-Blue MRF' which is capable of cloning methylated DNA. Some fragments of recombinant plasmid DNAs were prepared by an alkaline lysis method (Sambrook *et al.*, 1989). Double-stranded template DNAs were used with the Sequitherm cycle sequencing

kit (Air Brawn). The electrophoresed fragments were sequenced in an automated DNA sequencer (Li-Cor Model 4000). The determined nucleotide sequences were analyzed using the GENETYX-MAC (SDC, software) computer program.

Artificial infection of red sea bream with RSIV

Juvenile red sea bream (mean body weight 27.3 g) were inoculated intraperitoneally with 0.1 ml of the supernatant of the GF cell culture containing $10^{4.0}\text{TCID}_{50}/\text{ml}$ of RSIV. The fish were held in a 50 l tank supplied with running sea water maintained at 25°C.

Preparation of samples for PCR

Samples of the spleens of red sea bream artificially infected with RSIV and those of uninfected fish, as well as spleens of cultured marine fish (1 sample from each cultured marine fish), which were diagnosed as naturally infected with RSIV by an IF test, were used for DNA extractions and these DNAs were used as templates for the PCR. The DNA extracted from lymphocystis tissue of Japanese flounder infected with fish lymphocystis disease virus (FLDV) was also used for template DNA. These DNAs were extracted using Puregene DNA extraction kit (Gentra System Inc.). Template DNA from blood of a moribund red sea bream was prepared using a Takara single-tube PCR kit (Takara Shuzo). Template DNAs from the supernatant of GF cell cultures infected with RSIV and the supernatant of FHM cell culture infected with FV3 were prepared using Instagene Matrix (Bio-Rad Laboratories). These preparations were performed according to supplier's protocol. DNAs extracted from the spleens of various kinds of cultured marine fish (Table 1) with disease caused by natural

Table 1. PCR samples from tissues of naturally infected cultured marine fish and cultures of the virus infected cells

Samples	Fish	Scientific name	Area
1. (Spleen)	Red sea bream	<i>Pagrus major</i>	Ehime
2. (/)	Japanese parrot fish	<i>Oplegnathus fasciatus</i>	"
3. (/)	Striped jack	<i>Caranx delicatissimus</i>	"
4. (/)	Sea bass	<i>Lateolabrax sp.</i>	"
5. (/)	Yellowtail	<i>Seriola quinqueradiata</i>	"
6. (/)	Amberjack	<i>Seriola dumerili</i>	"
7. (/)	Albacore	<i>Thunnus thynnus</i>	Kohchi
8. (Culture)	Red sea bream	<i>Pagrus major</i>	Ishikawa
9. (/)	Japanese parrot fish	<i>Oplegnathus fasciatus</i>	Ehime
10. (/)	Striped jack	<i>Caranx delicatissimus</i>	"
11. (/)	Sea bass	<i>Lateolabrax sp.</i>	"
12. (/)	Albacore	<i>Thunnus thynnus</i>	Kohchi

RSIV infection and from the supernatant of GF cells cultured with RSIV isolated from various cultured marine fish were also used as templates in this study.

PCR primers and amplification of viral genomes

A total of four oligonucleotide primer sets were synthesized for use in the PCR assays. Two primer sets, No. 1 (1-F and 1-R) and No. 2 (2-F and 2-R), were designed from the nucleotide sequence of the 959-bp *Pst* I restriction fragment (DDBJ accession number: AB006954) of RSIV genomic DNA. The primer sets

of No. 3 (3-F and 3-R) and No. 4 (4-F and 4-R) were based on nucleotide sequences of the putative DNA polymerase gene (DDBJ accession number: AB007366) and adenosine triphosphatase (ATPase) gene (DDBJ accession number: AB007367) cloned from RSIV, respectively. These primer sequences and the expected lengths of the amplified fragments are shown in Table 1. For nested PCR, the primer sets of No. 5 (1-F and 2-R) and No. 6 (2-F and 1-R) were used (Table 2).

Table 2. Primer list

Set	Primer	Sequence	Prospected Length of amplified products	Area
1	1-F 1-R	5' CTCAAACACTCTGGCTCATC 3' 5' GCACCAACACATCTCCTATC 3'	570 bp	959 bp <i>Pst</i> I fragment
2	2-F 2-R	5' TACAACATGCTCCGCCAAGA 3' 5' GCGTTAAAGTAGTGAGGGCA 3'	564 bp	959 bp <i>pst</i> I fragment
3	3-F 3-R	5' CAAACCACAGCGCGGCAAGT 3' 5' AGTAGCGCACCATGTCCTCC 3'	563 bp	ATPase gene ORF
4	4-F 4-R	5' CGGGGGCAATGACGACTACA 3' 5' CCGCTGTGCCTTTTCTGGA 3'	568 bp	DNA polymerase gene ORF
5	1-F, 2-R		848 bp	959 bp <i>Pst</i> I fragment
6	2-F, 1-R		248 bp	959 bp <i>Pst</i> I fragment

CTGCAGTTGCCGCTCAAACACTCTGGCTCATCTATGTCATCGTAGTCGTCCATTCCGCTGCCCCATC
 primer 1-F →
 GTCAAGCAGTGTAGGCGGTGGAGTAACATTATCGGTGTCTGTTGGCAGCTCACATGAGACACCTACAC
 AAGGCTGACTGTGAGATGAGATGCGGCTGGCGTGGCATGTGACGGTCTGCACAGGGTGAGGTTTCAGC
 TTGATGACAGACAAGATGGTACCGTCATACAGCACTCCATGCTTCAGGACTTCAGTCTGTTGCG
 GCCTACATGGACCACCTCGCCATGTACAACATGCTCCGCCAAGAGGCTGTTGCTGTCGCTTGACCAAA
 primer 2-F →
 CAATCTTCACATCCGCTCTCGAGGTACCCGCGAGCTGAGGGTGGTCTGTTGTCGATTTCAGG
 TTATAGAAGGTGGTGGCGTGAGTACAGCCACAGTCAGCAACAGAAGAAGTAGCAGGGTCGCCATTGC
 TCATGTAGCTATGATTCACAGTAGTCACCTATGACATGAGGATATTCAAAATTTTATACAAGTAAA
 GATGTTCACTGTGCTTGAGATAGGAGATGTGTTGGTCTAGTGTCCGCTGACGACATACGTGTGATGT
 ← primer 1-R
 ACAACCGTGACCCGGGACAATCTGTGGCGTTAACTAATACCACAGAGGGTGTGTCACGGTTACCGCG
 GTGCCTGACAACCGGATTATGAGACATTGCGCAGGTGTCTTATAGTCGCAGCCAGATTTTCGGTTC
 ACGGCTGCGTGTGCTATTACACTGATCCACCCAGACATGTCACAAGATGGATTGTCAATTACCAGG
 ACATGGGCACGCACATTGGCTTCTGCCCTCACTACTTAAACGCAGCGCAATGCGGTGGTTAACTAC
 ← primer 2-R
 GCCGAAGAGAACTCATCTCATCCGGGGTCCCTCAACCAATGACAATCATCGTGTATGTGTCTGC
 ACTGCAG

Fig. 1. The nucleotide sequences of DNA fragments digested with *Pst* I cloned from red sea bream iridovirus (RSIV). The nucleotide sequences of the forward and reverse primers of primer set 1 are underlined and those of primer set 2 are double underlined.

PCR amplification

Reaction mixes contained 1 mM of each primer, 200 mM of deoxynucleotide triphosphate, 1.25 U of ExTaq DNA polymerase in 20 mM Mg^{2+} PCR buffer supplied with a commercially available Takara ExTaq kit (Takara Shuzo) with a small volume of template. The mixture were incubated for 30 cycles in an automatic thermal cycler (Takara thermal cycler MP) programmed for 0.5 min at 94°C, 1 min at 58°C, and 1 min at 72°C, followed by an extension period of 5 min at 72°C after the last cycle.

Nested PCR amplification

Nested PCRs were used to amplify DNA from the spleens of red sea bream at 3 months post challenge. The first PCR amplifications were performed with primer set No. 5 and then the second amplifications were performed with primer set No. 6. Reaction conditions were the same as those described for the PCR amplification.

Analysis of PCR amplified products

PCR products were analyzed by electrophoresis in a 0.8% agarose-TBE buffer (0.089 M Tris-borate, 0.089 M Boric acid, 0.002 M EDTA) gels. The viral DNA, GF cell DNA and each PCR product DNA in the gel were transferred to a Hybond N⁺ nylon membrane (Amersham) and hybridized with probes of each PCR products themselves using an ECL detection system (Amersham).

Results

A 959-bp *Pst* I-digested fragment was randomly cloned from the RSIV genomic DNA library. We constructed two pairs of PCR primers (primer sets 1 and 2) to amplify 570 bp or 564 bp from the 959 bp fragment (Fig. 1). DNA fragments of expected molecular size (570 bp or 564 bp in length) were amplified from the supernatant of tissue-cultured GF cells infected with RSIV after 30 cycles of PCR amplification using primer sets 1 and 2 respectively (Fig. 2). A single DNA band of the expected molecular size (563bp or 568bp in length) was also amplified using primer sets 3 and 4 (Fig. 2). However, the same fragment was not amplified by PCR using the primer sets 1, 2, 3, or 4 with template DNA obtained from the supernatant of uninfected tissue culture (data not shown). A Southern blot analysis showed that all probes of PCR product DNAs using primer set 1, 2, 3, and 4, which were amplified from supernatant of tissue-cultured GF cells infected with RSIV, hybridized

Fig. 2. Agarose gel electrophoresis of amplification products from supernatant of tissue-cultured GF cells infected with RSIV using each primer set. Panel A is an agarose gel; Panel B, C, D, and E are autoradiographs of Southern blot hybridizations with the probes of PCR products themselves using primer set 1, 2, 3 and 4, respectively. Lane 1, λ DNA/*Hind* III molecular size marker; lane 2, RSIV genomic DNA; Lane 3, GF cell DNA; and lanes 4, 5, 6, and 7 are PCR products from supernatant of tissue-cultured GF cells infected with RSIV using primer set 1, 2, 3, and 4, respectively.

with viral genomic DNA, however, they did not hybridize with the DNA extracted from GF cells (Fig. 2).

A PCR product was not obtained from the lymphocystis tissue of Japanese flounder infected with FLDV or the supernatant of tissue-cultured FHM cells infected with FV3 (Fig. 3).

A single DNA fragment with the same molecular size was amplified not only from the spleens of red sea bream experimentally infected with RSIV (Fig. 4) but also from the blood of moribund red sea bream experimentally infected with RSIV (data not shown) using various combinations of primers. On the other hand, no PCR product was obtained from the spleens of uninfected red sea bream or from those of the fish at 3 months post-challenge (Fig. 4). Expected molecular size DNA fragment of RSIV DNA was also not detected from the spleen of red sea bream at 3 months post-challenge using the nested PCR method (data not shown).

A fragment of the appropriate size was amplified using each of the primer sets 1, 2, 3, and 4, with template DNAs obtained from the spleens of several cultured marine fish (red sea bream, Japanese parrot fish, striped jack, sea bass, yellowtail, amberjack, and bluefin tuna) (Fig. 5). All four primer sets yielded the same PCR products from the supernatant of tissue-culture d GF cells infected with virus isolated from cultured red sea bream, Japanese parrot fish, striped jack, sea bass, and bluefin tuna.

Fig. 3. Amplification of DNA of RSIV, FLDV and FV3 using PCR with four primer sets. Primer set 1 (lanes 2–4), primer set 2 (lanes 5–7), primer set 3 (lanes 8–10) and primer set 4 (lanes 11–13). Lane 1, λ DNA/*Hind* III molecular size marker; lanes 2, 5, 8 and 11, amplified DNA with the supernatant of tissue-cultured GF cells infected with RSIV; lanes 3, 6, 9 and 12, amplified DNA with the DNA extracted from lymphocystis tissue of Japanese flounder infected with FLDV; lanes 4, 7, 10, and 13, amplified DNA with the supernatant of tissue-cultured FHM cells infected with FV3.

Discussion

A *Pst* I-digested fragment of 959 bp in length was cloned from RSIV genomic DNA and sequenced. No significantly homologous sequences were found in EMBL and GenBank. We designed two primer sets (primer sets 1 and 2) based on this nucleotide sequence for amplification of RSIV using the PCR method. We also constructed two primer sets (nos. 3 and 4) that were, respectively, the ORF of ATPase gene and the ORF of the DNA polymerase gene cloned from RSIV genomic DNA (Table 2). The primer sets 1, 2 and 4 could am-

Fig. 4. Detection of RSIV genomic DNA from red sea bream artificially infected with RSIV by PCR amplification using each primer sets. Panels A, B, C, and D show agarose gels after electrophoresis of amplification products using primer sets 1, 2, 3, and 4, respectively. Lane 1, λ DNA/*Hind* III molecular size markers; lane 2, amplified DNA from the supernatant of tissue-cultured GF cells infected with RSIV (positive control); lanes 3, 4, and 5, amplified DNA from the spleens of non-infected red sea bream (negative control); lanes 6, 7, and 8, amplified DNA from the spleens of dead artificially infected red sea bream; and lanes 9, 10, 11, 12, and 13, amplified DNA from the spleens of red sea bream at 3 months post infection.

Fig. 5. Detection of RSIV naturally infected with RSIV. Panels A, B, C, and D show agarose gels after electrophoresis of amplification products using primer sets 1, 2, 3, and 4, respectively. Lane 1-9, amplified DNA from the spleens. Lane 1, red sea bream artificially infected with RSIV (positive control); lane 2, non-infected red sea bream (negative control); lanes 3-9, cultured fishes naturally infected with RSIV. Fish culture locations are shown in parentheses. Lane 3, red sea bream (Ehime); lane 4, Japanese parrot fish (Ehime); lane 5, striped jack (Ehime); lane 6, sea bass (Ehime); lane 7, yellowtail (Ehime); lane 8, amberjack (Ehime); lane 9, albacore (Kochi). Lane 10, λ DNA/*Hind* III molecular size markers. Lanes 11-17, amplified DNA from the supernatant of tissue-cultured GF cells infected with RSIV strains from different sources. Lane 11, RSIV strain Ehime-1 (positive control); lane 12, no infection (negative control). Lane 13-17, viruses isolated from different cultured fishes. Lane 13, red sea bream (Ishikawa); lane 14, Japanese parrot fish (Ehime); lane 15, striped jack (Ehime); lane 16, sea bass (Ehime); lane 17, albacore (Kochi).

plify a fragment of the expected size from the supernatant of tissue-cultured GF cells infected with RSIV, and from spleens of cultured marine fish infected with RSIV. Primer set 3 amplified a common fragment of the same molecular size from marine fish described above and, in addition, yielded one or two fragments of a lower molecular size from red sea bream, striped jack, sea bass, yellow tail, and amberjack. However, the primer sets did not amplify the same molecular size fragment from the supernatant of tissue-cultured cells infected with FV3 and lymphocystis tissue of Japanese flounder infected with FLDV, which belong to Iridoviridae (Fig. 3).

Recently, Mao *et al.* (1997) demonstrated that nine iridoviruses from fish, reptile, and amphibians were more similar to FV3 of the genus *Ranavirus*. A common primer based on the conserved major capsid protein of iridoviruses was developed by Mao *et al.* (1997) for amplification of the virus-specific nucleic acid from iridoviruses infecting fish, reptiles, and amphibians. Ohshima *et al.* (1996) cloned a part of RNRS gene of RSIV and also succeeded in RSIV detection from naturally infected red sea bream by PCR using the primers based on this gene sequence. We also developed the four primer sets and could detect the RSIV by PCR us-

ing these primers not only from artificially and naturally infected red sea bream, but also from other RSIV-infected cultured marine fish including Japanese parrot fish, striped jack, sea bass, yellowtail, amberjack, and bluefin tuna. From these results we believe that our designated primer sets amplified only a specific fragment from the genomic DNA of RSIV. A PCR product was amplified from the spleen of several marine fish and also from blood of artificially infected red sea bream. It is easy to collect blood samples from live fish. Previously, we diagnosed the disease by IF test. However, it is sometime difficult to detect RSIV specific antigen from spleen samples at the early stage of the disease by IF test. So a highly sensitive method is desirable for detecting the virus even from such IF-negative samples. With the PCR method, RSIV was detected from those samples (data not shown). Consequently, our designated primer sets may provide a rapid and simple diagnostic tool for detecting RSIV from the blood of cultured marine fish.

RSIV infections have been observed widely in 20 cultured marine fish (Matsuoka *et al.*, 1996) in Japan. Indeed, iridovirus infections cause devastating epizootics in fish around the world. Further work is needed to determine whether the primer sets developed here amplify only RSIV DNA.

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