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Detection and Identification of *Oncorhynchus masou* virus (OMV) by Polymerase Chain Reaction (PCR)

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Abstract

A polymerase chain reaction (PCR) was established for detection and identification of *Oncorhynchus masou* virus (OMV). A set of primers was prepared for amplification of a target gene of DNA from OMV reference strain 00-7812, and the PCR was able to amplify the DNA of a 439 bp from four representative strains of OMV isolated from masu salmon (*Oncorhynchus masou*), coho salmon (*O. kisutch*) and rainbow trout (*O. mykiss*), and from liver, kidney, brain and nervous tissues of OMV infected fish. It was also able to amplify about 800 bp from *Herpesvirus salmonis*, the reference strain of salmonid herpesvirus type 1, isolated from rainbow trout in the USA.

Key words: Polymerase chain reaction, *Oncorhynchus masou* virus, *Herpesvirus salmonis*, Salmonid herpesvirus, Masu salmon, Coho salmon, Rainbow trout

Introduction

Salmonid herpesvirus is distributed in the USA and Japan. It was first isolated from apparently normal adult stocks of rainbow trout (*Oncorhynchus mykiss*) in the USA. This virus is pathogenic to rainbow trout fry and was named *Herpesvirus salmonis* by Wolf et al. (1978). In Japan, herpesvirus infection of salmonid fish was first reported by Sano (1976) with the isolation of Nerka Virus Towada Lake, Akita and Aomori Prefecture (NeVTA) from moribund kokanee salmon (*O. nerka*) in Towada Lake. Subsequently in 1978, a herpesvirus was isolated from ovarian fluid of apparently normal mature masu salmon (*O. masou*) cultured at the Otobe Salmon Hatchery in Hokkaido. It was named *Oncorhynchus masou* virus (OMV) from the scientific name of its host species (Kimura et al., 1981a). Since then, many herpesviruses serologically identified to be OMV have been isolated from cultured and wild salmonid populations in northern Japan (Yoshimizu et al., 1993). OMV is oncogenic and causes ulcers on the skin of salmonid species (Kimura et al., 1981b, Yoshimizu et al., 1987). Susceptible species include kokanee salmon, masu salmon, chum salmon (*O. keta*), coho salmon (*O. kisutch*) and rainbow trout (Yoshimizu et al., 1995). Since 1988, OMV has been isolated from coho salmon, and in 1991, it was found in rainbow trout. Recently OMV has become a major problem for rainbow trout culture in Honshu, Japan (Yoshimizu and Nomura, 2001).

At present, the basic OMV diagnosis is based on direct isolation of the virus in salmonid cell lines and confirmation with immunological identification by a neutralization test (Yoshimizu, 1997), which takes up to two weeks to reach a diagnosis. However, rapid diagnosis by immuno-fluorescent tests using polyclonal (Kumagai et al., 1994) and monoclonal (Hayashi et al., 1993) antibodies has been developed and is now routinely used. This method can diagnose diseased fish, but its detection limit is 10^5 tissue culture infectious dosages ($TCID_{50}$)/g tissue. The present paper describes a sensitive PCR method for OMV detection and its application for detecting OMV in tissues.

Materials and Methods

Cells and Viruses

Cell lines used for propagation and determination of virus infectivity included rainbow trout gonad (RTG-2) (Wolf and Quimby, 1962), striped snake head (SSN-1) (Frerichs et al., 1996), grouper fin (GF) (Nakajima et al., 1997) and hirame natural embryo (HINAE) (Kasai and Yoshimizu, 2001). RTG-2 cells were maintained in Eagles' Minimum Essential Medium (MEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 IU/ml penicillin G (Sigma), 100 μg/ml streptomycin (Sigma), 0.15% $NaHCO_3$ and 1.6% Tris-buffer (Sigma). HINAE, GF and SSN-1 cells were maintained in Leibovitz's 15 (L-15) media (Gibco) supplemented with 10% FBS and antibiotics.

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A total of 13 viruses were used in this study. They included *Oncorhynchus masou* virus (OMV) strain 00-7812 ; the reference strain of salmonid herpesvirus type 2 (SaHV-2), four OMV strains [coho salmon tumor virus (COTV), coho salmon tumor virus (CSTV), rainbow trout kidney virus (RKV) and rainbow trout herpesvirus (RHV)] (Yoshimizu et al., 1995), *Herpesvirus salmonis* ; the reference strain of SaHV-1, and seven other viruses from 7 different families of fish pathogenic viruses. These seven viruses were infectious hematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV) (Kimura, T. and Yoshimizu, M., 1991), hirame rhabdovirus (HIRRV) (Kimura et al., 1986), Japanese flounder lymphocystis disease virus (JF-LCDV) (Kasai and Yoshimizu, 2001), red sea bream iridovirus (RSIV) (Inouye et al., 1992), salmonid retrovirus (SRV) (Oh et al., 1995), and barfin flounder nervous necrosis virus (BF-NNV) (Watanabe et al., 1999). BFNNV, RSIV, and JF-LCDV were propagated using SSN-1, GF and HINAE cell lines, respectively, while the other viruses were propagated in RTG-2 cells.

Nucleic Acid Extraction

Nucleic acid of OMV 00-7812, COTV, CSTV, RKV, RHV, *H. salmonis*, JF-LCDV and RSIV were extracted from infected cells using InstaGene Matrix (BIO RAD Laboratories Ltd.). For DNA extraction, virus infected cells were pelleted by centrifugation at 14,800 rpm for 15 min. The pellets were washed twice with 1 ml phosphate-buffered saline (PBS) and mixed with 200 μl of InstaGene Matrix. The mixture was incubated at 56°C for 20 min in a water bath, vortexed, and then placed in a boiling water bath for 8 min. Afterwards the samples were vortexed and centrifuged at 10,000 rpm for 90 sec. The supernatant was subjected to PCR or stored at −20°C until it was assayed.

Nucleic acid of the RNA viruses IHNV, IPNV, HIRRV, BFNNV and SRV were extracted using the acid-guanidinium-thiocyanate-phenol-chloroform (AGPC) method (Yoshinaka et al., 1997). Briefly, virus infected cells were denatured with 500 μl of denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M β-mercaptoethanol). Denatured cells were vortexed, and then 50 μl of 2 M sodium acetate and 500 μl of saturated phenol and chloroform/isoamyl alcohol (49 : 1) were added and mixed after each addition. Suspensions were left on ice for 15 min before centrifugation at 14,000×g for 20 min. The supernatant was mixed with 500 μl of isopropanol and incubated for 10 min at room temperature before pelleting the nucleic acids by centrifugation at 14,000×g for 15 min. Pelleted nucleic acid was dissolved in 150 μl of denaturing solution and precipitated with an equal volume of isopropanol at −20°C for 30 min. Finally, the nucleic acid pellet was dissolved in 10 μl DEPC (diethyl-pyrocarbonate) treated distilled water, and 1 μl was used as a reverse transcription-PCR (RT-PCR) template.

Primers and PCR procedure

Two DNA oligonucleotide primers were designed using the primer selection program available at the Virtual Genome Center web site (http://alces.med.umn.edu) based on the sequence report of Bernard et al. (1994) for the major capsid protein gene of the 00-7812 strain of OMV. Ten primer sets were designed, and one of them was selected as the forward primer, F10 : 5′-GTACCGAAACTCCCGAGTC-3′ and the reverse primer, R5 : 5′-AACTTGAACTACTCCGGGG-3′ were located at nucleotides 177-195 and 598-616, respectively in the open reading frame of the major capsid protein gene. The melting temperature (*Tm*) was 55.2°C (primer F10) and 57.3°C (primer R5).

Amplification was performed in a 50 μl mixture containing 0.2 μM of each primer, 5.0 μl of 10×PCR buffer (Promega, Madison, USA), 1.5 mM $MgCl_2$, 200 μM deoxyribonucleoside triphosphate, 1.75 U of *Taq* polymerase (Promega) and 2.5 ng extracted genomic DNA as template. The reaction mixtures were incubated for 30 cycles in an automatic thermal cycler (GeneAmp PCR 9700, Applied Biosystems), with each cycle consisting of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec and extension at 72°C for 30 sec. The amplified product was analyzed for size and purity by electrophoresis (100 V, 30 min) in 2% agarose gels and stained with ethidium bromide.

Sensitivity of the PCR

RTG-2 cells were infected with OMV 00-7812 at multiplicity of infection (M.O.I.) of 1.0, and then after 48 h incubation at 15°C, the cells were removed from the culture medium and a 10-fold dilution series was made using PBS. Viral DNA from each dilution (10^{-1} ~10^{-5}) was extracted using the InstaGene Matrix method. We used 1.0 μl of supernatant as the template in the PCR.

Specificity of the PCR

The specificity of the PCR was tested using four different OMV strains (COTV, CSTV, RKV and RHV) and seven other viruses from different families of fish pathogenic viruses (IHNV, IPNV, HIRRV, SRV, RSIV, JF-LCDV and BF-NNV).

Application of the PCR

Thirty sick rainbow trout fingerlings from the Nagano

Prefectural Fisheries Experimental Station, a hatchery with a known history of OMV disease, were tested using the PCR and compared with routine OMV diagnosis by cell cultivation and observation for cytopathic effect (CPE). The fish were received on ice, and portions of their brains, kidneys, livers and front, middle and rear nerves were carefully sampled for PCR and cell cultivation. Samples were homogenized in 1 : 9 (w/v) Hanks' Balanced Salt Solution supplemented with antibiotics [1,000 IU/ml penicillin G, 1 mg/ml streptomysin sulfate, 800 U/ml mycostatin (Sanko Junyaku)] and centrifuged at 10,000 rpm for 15 min. Aliquots of 100 μl and 400 μl of the supernatant were used for cell inoculation and DNA extraction for PCR, respectively. For cell inoculation, aliquots were aseptically inoculated on to RTG-2 cells and observed for OMV characteristic CPE for 10 days.

Results

PCR products

The oligonucleotide primers directed the syntheses of a 439 bp segment of DNA from OMV 00-7812 strain and about 800 bp segment of DNA from *H. salmonis*. A single band of DNA of the expected molecular weight was observed on agarose gel after electrophoresis and stained with ethidium bromide (Fig. 1).

Sensitivity of the PCR

The sensitivity of the PCR assay was examined using a 10-fold dilution series of OMV 00-7812 strain infected RTG-2 cells in PBS. The detection limit of the PCR was $10^{0.8}TCID_{50}$/ml (Fig. 2).

Specificity of the PCR

To confirm that the PCR detected different strains of OMV but not other fish pathogenic viruses, four isolates of OMV (COTV, CSTV, RKV and RHV) and seven other fish disease viruses (IHNV, IPNV, HIRRV, SRV, RSIV, JF-LCDV and BF-NNV) were used in the amplification reaction. A product of the same size as that obtained from the OMV 00-7812 strain was obtained from the other isolates of OMV (Fig. 3), and no PCR product was obtained from the seven fish disease viruses (Fig. 4).

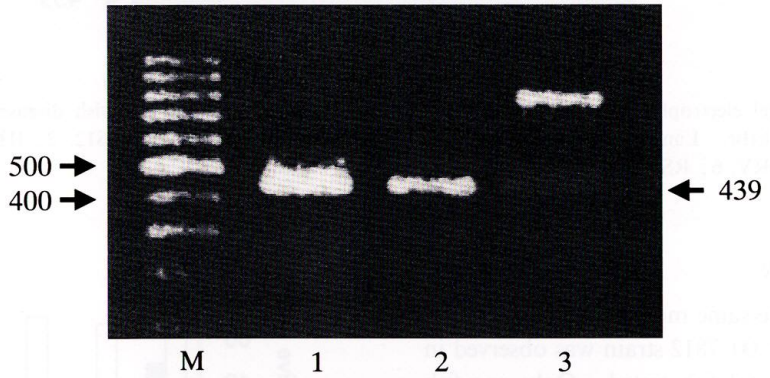

Fig. 1. Agarose gel electrophoresis of PCR amplification products from the OMV 00-7812 strain and *H. salmonis*. Gel was stained with EtBr. Lanes: M; DNA molecular weight marker, 1; purified OMV 2; cell culture OMV 3; *H. salmonis*.

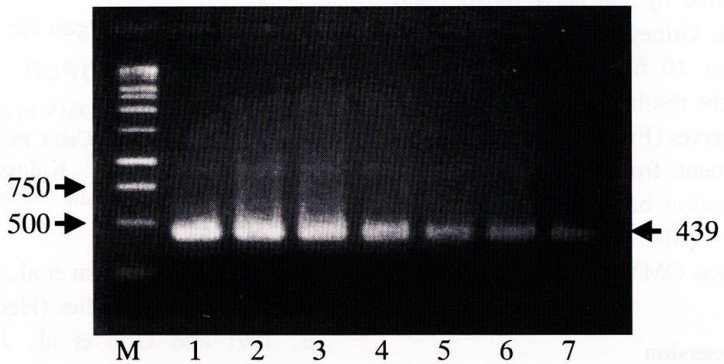

Fig. 2. Agarose gel electrophoresis of PCR amplification products from diluted virus suspension of OMV 00-7812. Gel was stained with EtBr. Lanes: M; DNA molecular weight marker, 1; OMV 00-7812 strain, 2; $10^{5.8}$ $TCID_{50}$/ml, 3; $10^{4.8}TCID_{50}$/ml, 4; $10^{3.8}TCID_{50}$/ml, 5; $10^{2.8}TCID_{50}$/ml, 6; $10^{1.8}TCID_{50}$/ml, 7; $10^{0.8}TCID_{50}$/ml.

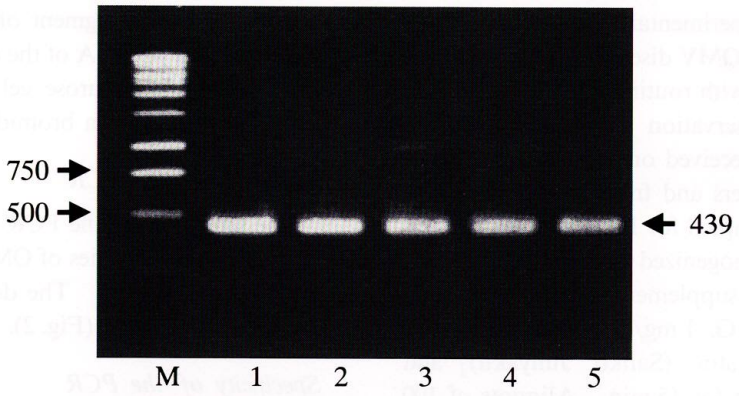

Fig. 3. Agarose gel electrophoresis of PCR amplification products from different OMV strains. Gel was stained with EtBr. Lanes: M; DNA molecular weight marker, 1; OMV 00-7812 strain 2; OMV COTV strain 3; OMV CSTV strain 4; OMV RKV strain 5; OMV RHV strain.

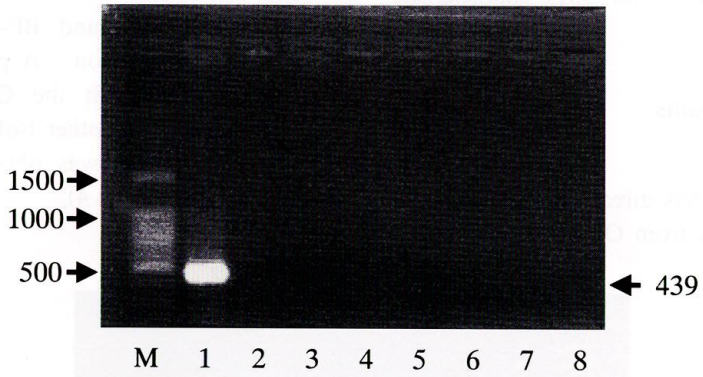

Fig. 4. Agarose gel electrophoresis of PCR amplification products from seven other fish disease viruses. Gel was stained with EtBr. Lanes: M; DNA molecular weight marker, 1; OMV 00-7812, 2; IHNV, 3; IPNV, 4; HIRRV, 5; SRV, 6; RSIV, 7; JF-LCDV, 8; BF-NNV.

Application of the PCR

A PCR product of the same molecular weight as that obtained from the OMV 00-7812 strain was observed in 96.67% (29/30) of the total fish tested. Only one fish tested was negative for OMV both by PCR and Cell cultivation. Not all samples from individual fish tested were positive for OMV. Brain samples showed the highest results (90%) followed by rear nerve (80%), liver (76.7%), front nerve (70%), kidney (66.7%) and middle nerve (46.7%) tissues. For 10 fish the middle nerve tissue was not tested, thus the result for middle nerve was lower than the other two nerves (Fig. 5). Amplification of a 439 bp DNA fragment from the diseased fish confirmed our initial suspicion based on clinical signs and the past OMV history of the station that the causative agent of the disease was OMV.

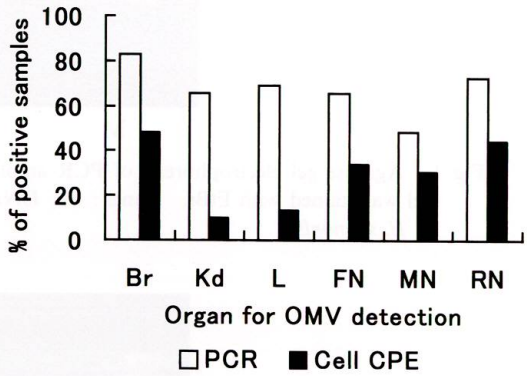

Fig. 5. Detection of OMV in different of organs of infected fish by PCR and Cell CPE observation. Organs tested were: Brain; Br, Kidney; Kd, Liver; L, Front Nerve; FN, Middle Nerve; MN, Rear Nerve; RN.

Discussion

Salmonid herpesvirus is divided into salmonid herpesvirus type 1 (SaHV-1) and type 2 (SaHV-2) based on serological (Yoshimizu et al., 1995, Jung et al., 1996) and DNA homology studies (Hedrick et al., 1987, Eaton et al., 1991 and Gou et al., 1991). SaHV-1 comprises USA isolates with *H. salmonis* as the reference strain while SaHV-2 comprises Japan isolates with OMV 00-7812 as the reference strain.

Although OMV was first isolated in Japan in 1978 and found to be pathogenic to salmonids, economic losses due to infection were experienced in net cage culture of coho salmon in 1988 (Kumagai et al., 1994). During 1992, massive mortalities of rainbow trout were noticed in Hokkaido and the central part of Japan from 1995. Yearlings of pond-reared rainbow trout were affected (Suzuki 1994, Yoshimizu and Nomura, 2001), causing serious economic loss on the industry. Kumagai et al. (1995) found that an indirect fluorescent antibody technique (IFAT) using polyclonal antibody to detect coho salmon herpesvirus (CHV) in kidney, liver, heart and spleen tissue imprints of diseased fish and found it to be a simple, rapid and reliable diagnosis of OMVD. Currently, a fluorescent antibody test (FAT) using a polyclonal or monoclonal antibody is the most effective and routinely used method for diagnosis of OMVD. In coho salmon culture, FAT is used to detect OMV infected juvenile fish when transferring them from freshwater ponds to net-pen cages in seawater. This has effectively eradicated OMV from the pen cage culture of coho salmon (Kumagai et al., 1995).

The present study is the first report of a PCR method that can be used to detect OMV. Since its inception, PCR has gained wide acceptance in the field of molecular biology, where genetic research involving the analysis and identification of new genes is now routinely carried out using PCR (Deacon and Lah, 1989). Recently PCR has become popular in the detection and identification of the causative agent of fish diseases. For instance, Gray et al., (1999a, b) used PCR to detect channel catfish virus (CCV), a fish herpesvirus, in blood and tissues of acutely and latently infected catfish. They were able to confirm using PCR that CCV establishes latent infection in channel catfish. PCR has now been established for the detection of important fish viruses, such as IHN (Arakawa et al., 1990), IPN (Lopez-Lastra et al., 1994), CCV disease (Boyle and Blackwell, 1991), viral nervous necrosis (Nishizawa et al., 1994), and red sea bream irridovirus disease (Kurita et al., 1998). Although OMV diagnosis takes longer using PCR (about 8 hours) than using IFAT or FAT (about one hour), unlike IFAT or FAT, PCR analysis can be standerdized. PCR is sensitive and specific and can detect minute quantities of antigen in flesh and is more sensitive than other nucleic acid probes (Boyle and Blackwell, 1991).

The established OMV PCR was able to amplify a 439 bp product from four strains of OMV at unknown concentrations and OMV strain 00-7812 at a concentration of $10^{0.8}TCID_{50}$/ml. No amplification product was observed for the other seven fish viruses tested. Moreover, it was able to detect and distinguish between SaHV-1 and SaHV-2. A PCR amplification product of about 800 bp was observed for *H. salmonis*, the reference strain of SaHV-1, whereas SaHV-2 strains showed a 439 bp product.

We applied this PCR to test 30 diseased rainbow trout fingerlings thought to be infected by OMV from the Nagano Prefectural Fisheries Experimental Station. The PCR yielded DNA amplification products of the same molecular weight as that of OMV 00-7812. PCR results were 50% higher that those obtained for cell CPE observation using the same samples. The PCR detected a higher number of positive samples from the brain and rear nerve than it did for the routine cell inoculation organs (liver and kidney) for CPE observation. According to Tanaka et al. (1984), the kidney and liver are the appropriate organs for OMV detection, however our results showed lower results for these two organs. This is probably due to their toxic nature where inoculated cells are killed before CPE is observed.

Since OMV diagnosis methods now commonly used require either a long analysis time or specialized skills, and are difficult to standardize, the PCR reported in the present paper could be a useful substitute for serological confirmation of isolated viruses or for direct detection of OMV during disease outbreaks. It is more sensitive and OMV specific than serological confirmation of isolated viruses. However, before the PCR is widely accepted as a standard OMV detection method, additional work to decrease costs and simplify the procedure is necessary.

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