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Detection and Identification of Infectious Hematopoietic Necrosis Virus (IHNV) by Reverse Transcription (RT)-polymerase Chain Reaction (PCR)

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A method based on reverse transcription (RT)-polymerase chain reaction (PCR) was established for detection and identification of infectious hematopoietic necrosis virus (IHNV). A set of primers was prepared for amplification of a 510 bp nucleotide which encodes a region of IHNV nucleoprotein (N) gene. The PCR product was confirmed as the IHNV specific nucleotide by southern hybridization with an oligonucleotide probe synthesized from the N gene. The PCR was able to amplify the target sequence from five representative strains of IHNV from Japan and North America. There was no PCR product from five other fish rhabdoviruses. Viruses from ovarian fluid of masu salmon collected at Shibetsu River, Ichani River in Hokkaido and kidney tissue of rainbow trout in Aichi Prefecture were isolated and identified using the RT-PCR.

Key words: IHNV, RT-PCR, fish-rhabdoviruses, rhabdovirus

In recent years, the polymerase chain reaction (PCR) has proved to be a rapid, sensitive, and specific technique for the detection of fish disease viruses, such as IHNV, infectious pancreatic necrosis virus (IPNV), and striped jack nervous necrosis virus (SJNNV).¹⁻⁴ For detection of IHNV, there have been many reports which describe that the RT-PCR using the primer set from the G protein-specific gene could amplify the IHNV specific gene.⁵ The RT-PCR could detect the IHNV from paraffin sections of infected kidney tissue⁶ and pathological tissue of artificially infected fish.⁷ However, when hiramé rhabdo virus (HRV) was amplified on the RT-PCR of IHNV reported by Arakawa *et al.*,² two bands (430 and 280 bp) were obtained. In some researches, it is necessary to distinguish between IHNV and HRV. The PCR using the primer set from the N-gene was applicable to both messenger and genomic RNA. Therefore, in this study, a new primer set from the IHNV N gene which was bigger than the PCR product reported by Arakawa *et al.*² was designed for identification of different IHNV strains or fish rhabdoviruses. This paper describes the detecting conditions and sensitivity of the RT-PCR using the new primer set, and then attempts to identify the isolated viruses by RT-PCR.

Materials and Methods

Cells and Viruses

Rainbow trout embryo (RTE-2) cells were used for virus propagation and for determination of virus infectivity. The cells were maintained in Eagles' minimum essential medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, 0.15% NaHCO₃ and 1.6% Tris-buffer. The IHNV strain used in this study was chum salmon (*Oncorhynchus keta*) isolate from Hokkaido, Japan (ChAb strain). The specificity of the RT-PCR was tested using five different strains: HV-7601, North America standard (NA) type strain, Oregon MET-1, Oregon sockeye salmon virus (OSV), Sacramento

River chinook salmon virus (SRCV) and five other fish rhabdoviruses: hiramé rhabdovirus (HRV), spring viremia of carp virus (SVCV), pike fry rhabdovirus (PFRV), eel virus of America (EVA) and eel virus of Europe X (EVEX).

Nucleic Acid Extraction

Viral RNA was extracted from IHNV infected cells using the acid-guanidinium-thiocyanate-phenol-chloroform (AGPC) method.⁸ RTE-2 cells were inoculated with virus at a M.O.I. of 1.0 and after 24 h at 15°C, removed from the culture fluid and added to 500 µl denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M β-mercaptoethanol). The denatured cells were mixed, to which 50 µl 2 M sodium acetate and 500 µl saturated phenol and chloroform/isoamyl alcohol (49:1) were added with mixing after each addition.

After suspensions were left on ice for 15 min, then centrifuged at 14,000 g for 20 min, the aqueous phase was transferred to a fresh tube containing 500 µl of isopropanol. Following 10 min incubation at room temperature, nucleic acid was pelleted by centrifugation at 14,000 g for 15 min and the pellet was dissolved in 150 µl denaturing solution prior to reprecipitation with an equal volume of isopropanol at -20°C for 30 min. Finally, the nucleic acid pellet was dissolved in 10 µl DEPC (diethylpyrocarbonate) treated distilled water. We used 1 µl of it as the PCR template.

Primers and RT-PCR Procedure

Two DNA oligonucleotide primers were designed using a published sequence of the N gene of the Round Butte strain of IHNV.⁹ Primers were synthesized on Applied Biosystems (Foster City, CA) model 392 DNA synthesizer using established techniques, and purified using ABI oligonucleotide purification cartridges (Applied Biosystems). The first primer (5'-TCAT-TGCAGAGACGGTCCAT-3') and the second primer (5'-TGTTGAACAGTCCCACCAT-3') were located at

nucleotides 212–231 and 669–688, respectively in the open reading frame of the N gene. The primers bracketed a 510 nucleotide region of the N gene and the T_m was 64.2 (primer 1) and 65.0°C (primer 2). The second primer was used to generate first-strand cDNA in a reverse transcription reaction mixture (10 μ l) containing 2.5 U AMV reverse transcriptase XL (Life Science Inc.), 1.0 U RNase Inhibitor (TaKaRa), and 1 mM each of the 4 deoxynucleotide triphosphates in PCR buffer supplied with the TaKaRa RT-PCR kit. The reaction mixture was incubated for 30 min at 50°C. The cDNA was subsequently amplified by adding 2.5 U of Taq polymerase (TaKaRa) to the reaction mixture. The reaction mixtures were incubated for 40 cycles in an automatic thermal cycler (Perkin Elmer Model 480) with each cycle consisting of 30 s at 94°C, 30 s at 45°C, and 1 min at 72°C. The amplified product was analyzed for purity and size by electrophoresis (50 V, 1 h) in 2% agarose gels and stained with ethidium bromide.

Southern Hybridization

DNA fragments in agarose gels were transferred overnight to nitrocellulose membrane essentially as described by Southern.¹⁰ Oligonucleotide probe (5'-CTTGTTTTGGC AGTATGTGGCCATCTTGTC-3')¹¹ was synthesized and labeled at the 3' end using the DIG Oligonucleotide Tailing Kit (Boehringer Mannheim). Hybridization and detection were carried out using protocols of the kit.

Sensitivity of the RT-PCR

RTE-2 were inoculated with IHNV ChAb at a M.O.I. of 1.0 and after 24 h at 15°C, removed from the culture fluid and added to 500 μ l denaturing solution and mixed. Then, a 10-fold dilute series was made by adding denaturing solution. IHNV RNA from each dilution (10^{-1} ~ 10^{-7}) was extracted using the AGPC method. Extracted RNA was dissolved in 10 μ l DEPC treated water and 1 μ l of it used as the template in the RT-PCR.

Identification of Isolated Viruses

The three viruses used in this study were isolated from ovarian fluid of masu salmon collected from the Shibetsu River and the Ichani River in Hokkaido in September, 1995 and from kidney tissue of rainbow trout (weight 70 g) from Aichi Prefecture, respectively. Each virus was inoculated onto RTE-2 cells in 24 well plates. After CPE appeared, infected cells were removed the culture fluid and denaturing solution added. RNA was extracted from the homogenates using the AGPC method and used as the template in the RT-PCR.

Results

PCR Products and Southern Hybridization

The oligonucleotide primers directed the synthesis of a 510 base-pair (bp) segment of DNA from the IHNV ChAb strain. A single band of DNA of the expected molecular weight was observed after agarose gel electrophoresis and ethidium bromide staining (Fig. 1A). The 510 bp PCR products hybridized with the DIG labeled probe by Southern hybridization (Fig. 1B).

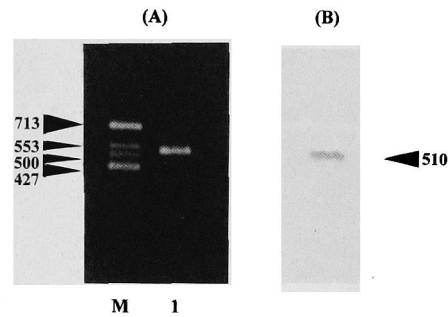


Fig. 1 (A) Agarose gel electrophoresis of RT-PCR amplification products from the IHNV ChAb strain.

Gel was stained with EtBr. Lanes: M; DNA molecular weight marker, 1; IHNV ChAb.

(B) Southern blot hybridization of RT-PCR products with DIG-labeled oligonucleotide probe which is the specific N-gene of the IHNV RB-76 strain.

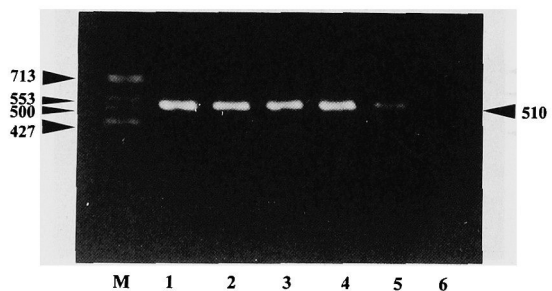


Fig. 2. Agarose gel electrophoresis of RT-PCR amplification products from diluted virus suspension of IHNV ChAb. Gel was stained with EtBr.

Lanes: M; DNA molecular weight marker, 1; $10^{5.8}$ TCID₅₀/ml, 2; $10^{4.8}$ TCID₅₀/ml, 3; $10^{3.8}$ TCID₅₀/ml, 4; $10^{2.8}$ TCID₅₀/ml, 5; $10^{1.8}$ TCID₅₀/ml, 6; $10^{0.8}$ TCID₅₀/ml.

Sensitivity of the RT-PCR

The sensitivity of the RT-PCR assay was examined using 10-fold dilutions of denatured infected cells. Although the 510 bp DNA fragment was detected at $10^{1.8}$ TCID₅₀/ml, the PCR template was used at 1/10 volume of the dissolved nucleic acid pellet, so the sensitivity of the RT-PCR was $10^{0.8}$ TCID₅₀/ml.

Specific Response of the RT-PCR

To confirm that the RT-PCR detected different strains of IHNV but not other fish rhabdoviruses, five isolates of IHNV and five other fish rhabdoviruses were used in the amplification reaction. A product of the same size as that obtained from the ChAb strain was obtained from the other five isolates of IHNV: HV-7601, SRCV, NA type strain, OSV and MET (Fig. 3). A PCR product was not obtained from the five other fish rhabdoviruses: HRV, PFRV, EVA, EVEX and SVC (Fig. 4).

Identification of Isolated Viruses

A product of the same size as that obtained from the ChAb strain was obtained from all the isolated viruses (Fig. 5). This result indicates that these viruses were IHNV.

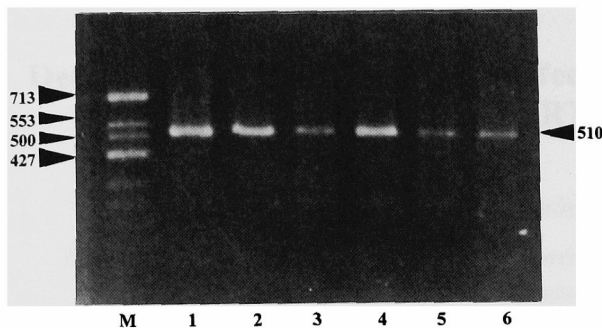


Fig. 3. Agarose gel electrophoresis of RT-PCR amplification products from different IHN strains. Gel was stained with EtBr.

Lanes: M; DNA molecular weight marker, 1; IHN HV-7601 strain, 2; IHN SRCV strain, 3; IHN NA standard strain, 4; IHN OSV strain, 5; IHN MET strain, 6; IHN ChAb strain.

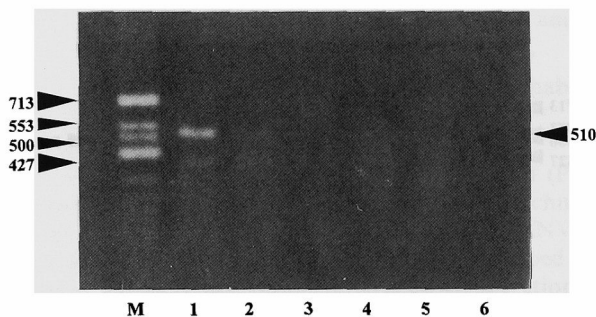


Fig. 4. Agarose gel electrophoresis of RT-PCR amplification products from different fish rhabdoviruses. Gel was stained with EtBr.

Lanes: M; DNA molecular weight marker, 1; IHN, 2; HRV, 3; PFRV, 4; SVCV, 5; EVA, 6; EVEX.

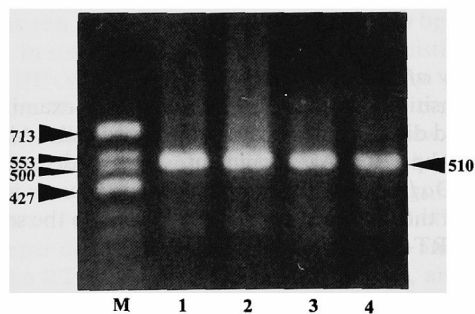


Fig. 5. Agarose gel electrophoresis of RT-PCR amplification products from virus strains cultured in RTE-2 cells. Gel was stained with EtBr.

Lanes: M; DNA molecular weight marker, 1; Virus isolated from ovarian fluid of masu salmon collected at Shibetsu R., 2; Virus isolated from ovarian fluid of masu salmon collected at Ichani R., 3; Virus was isolated from rainbow trout in Aichi Prefecture, 4; IHN ChAb strain.

Discussion

In this study, we designed a set of primers for amplification of the portion of the N gene of IHN. Confirmation that the target sequence was the N gene of IHN was

made by hybridization of the amplification product with an IHN specific probe in a southern hybridization. Further evidence for the specificity of the RT-PCR for IHN was demonstrated by amplification of the 510 bp product from five isolates of IHN and no amplification of the target sequence from the five other fish rhabdoviruses. The sensitivity of the RT-PCR using the specified reaction conditions was $10^{0.8}$ TCID₅₀/ml.

The RT-PCR has not yet been applied to the detection of virus in carrier fish because of the inhibitory effect of fish components on the extraction of viral RNA. However, the assay may be useful as a substitute for serological confirmation of isolated viruses. The serum neutralization test, which is the standard diagnostic method, may take up to 10 days to verify the results. To confirm that the RT-PCR can be used as a substitute for serological methods, the three viruses that were isolated from ovarian fluids from infected masu salmon and kidney tissue from a viral-infected rainbow trout were inoculated onto RTE-2 cells. After CPE appeared, the cells were harvested and the extracted RNA was used as the template in the RT-PCR. The PCR product of the same size as IHN was obtained from these three isolated viruses within 8 hours. At the same time, neutralization tests were performed after 10 days, and the identity of these viruses was confirmed as IHN. Currently, the quickest diagnostic method is the direct fluorescent antibody test using monoclonal antibody for an imprint specimen from disease fish. However, diagnosis by PCR is quicker and more accurate than serological confirmation of isolated viruses from cultured cells. Therefore, it is effective to substitute neutralization tests for PCR for identification. Although the PCR method is applicable to the detection and identification of viruses, it is evident that additional work to decrease costs and simplify the procedure is necessary before the assay is widely accepted.

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