Detection of Fish Virus by Using Immunomagnetic Separation and Polymerase Chain Reaction (IMS-PCR)

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Immunomagnetic separation of virus coupled with reverse transcription-polymerase chain reaction (IMS-PCR) was performed with infectious hematopoietic necrosis virus (IHNV). A DNA fragment of expected size was synthesized in the RT-PCR with total RNA extracted from IHNV inoculated CHSE-214. In a SDS-PAGE analysis, a protein band of over 70kDa was detected from non-infected cells and cells inoculated with IHNV and infectious pancreatic necrosis virus (IPNV). This protein was detected in the Western blot analysis probably because of non-specific reaction to monoclonal antibody against IHNV nucleocapsid protein. In the immunomagnetic separation, magnetic beads coated with monoclonal antibody against the IHNV nucleocapsid protein was incubated with supernatant from IHNV inoculated CHSE-214 cells. During this process, the non-specifically reacting protein could be removed by washing the magnetic bead with PBS in the presence of an external magnetic field, and viral proteins were detected from the remaining, cleaned magnetic beads. It was necessary to extract viral RNA from the captured virus particles before RT-PCR, and no DNA product was detected when the captured virus was only heated 5 min at 95℃. A PCR-product of expected size was synthesized from IMS-PCR with magnetic beads double coated either by goat anti-mouse IgG antibody - monoclonal antibody or streptavidin - biotin conjugated monoclonal antibody.

Key words : magnetic bead, monoclonal antibody, RT-PCR, IMS-PCR

Introduction

Viruses require live hosts and depend their replications on their hosts' machinery, and chemotherapeutic control methods such as application of antibiotics can not be used for the control of viral diseases. Contrast to viruses of land animals that transmitted mostly by insect vectors, fish viruses can be easily spread in water and can cause serious economic loss in very short time.

Although new control methods such as vaccine are under development, avoidance of the introduction of viruses is the best way of viral disease control at this moment. In order to achieve this, rapid and sensitive diagnostic techniques are required.

Some viruses cause specific symptom and this can be used for diagnosis. Diseased tissue can be observed with either light or electron microscope. Although virus particles can be seen with an electron microscope, it takes for few days to prepare the samples for observation, and sometimes it is hard to find the virus particles when the virus titer is low. Cultured fish cell can be inoculated with ground and filtered samples and cytopathic effect (CPE) can be observed. This procedure also takes for few days to a week, and some fish viruses replicate only in specific cell line or do not grow in cultured cell at all. Viral proteins can be detected by enzyme linked immunosorbent assay (ELISA) (Dixon and Hill, 1984; Way and Dixon, 1988), Western blot (Hsu and Leong, 1985), and a fluorescent antibody test (LaPatra et al., 1989a).

Recently, polymerase chain reaction (PCR) has been used for the detection of viral nucleic acids (Bruchhof et al., 1995; Arakawa et al., 1990). With this method, DNA of viral genome can be amplified with two primers that span the sequence selected for amplification. In case of viruses with RNA genome RNA should be converted in to DNA form by reverse transcriptase.

Because of the specificity and sensitivity of PCR, it is possible to amplify as few as one molecule of DNA, theoretically. However, viruses needed to be concentrated before PCR for better results. Especially, viruses with RNA genomes need to be concentrated and free from any contaminant that may inhibit the reverse transcription process. Polyethylene glycol has been used for the precipitation of Hepatitis A virus from oyster, water, and sediment samples (Lewis et al., 1988). Lees et al. (1994) has combined both polyethylene glycol precipitation and reverse transcription-polymerase chain reaction for the

detection of Hepatitis A virus. Coagglutination of virus particles with antibody bound to staphylococcus has been tried for the detection of IHNV from inoculated cell culture and inoculated rainbow trout (Bootland and Leong, 1992).

Magnetic beads coated with specific antibody was first used for the selection of specific lymphocytes and hybridoma cells producing monoclonal antibodies (Horton et al., 1989; Lea et al., 1985). Later, this method was applied for the capturing of bacteria and viruses from large volume sample (Grinde et al., 1995; Monceyron and Grinde, 1994; Christensen et al., 1992; Morgan et al., 1991).

In this study immunomagnetic separation and reverse transcription-polymerase chain reaction (IMS-PCR) has been developed for the detection of fish virus with infectious hematopoietic necrosis virus (IHNV), a well known fresh water fish virus of Korea (Park et al., 1993).

Materials and Methods

Cell line and Virus

The chinook salmon embryo (CHSE-214) cell line was propagated in minimum essential medium (MEM, GIBCO BRL, Grand Island, NY, USA) supplemented with fetal bovine serum (FBS) to 10%, penicillin to 50 I.U/mℓ, streptomycin to 50 μg/mℓ and buffered to pH7.5 with sodium bicarbonate and Tris-HCl. IHNV Round Butte strain (RB-76) was kindly provided by Dr. M. A. Park of National Fisheries Research and Development Agency. Cells were grown at 20℃ for routine propagation and kept 5~7 days at 18℃ after virus inoculation. Infectious pancreatic necrosis virus (IPNV) provided by Dr. M. A. Park was used as control in virus capturing analysis.

Magnetic bead and antibodies

Magnetic bead coated with goat anti-mouse antibody, the Dynabeads M-280, was purchased from Dynal (Oslo, Norway). The diameter of this bead is 2.8 μm ± 0.2 μm and contains $6\sim7+10^8$ beads/mℓ (10 mg/mℓ). Magnetic bead coated with streptavidin, the Streptavidin MagneSphere Paramagnetic Particles, was purchased from Promega (Madison, Wisconsin, USA). The diameter of this bead is 5.0 μm and contains 5×10^{11} bead/mg. Monoclonal antibodies against IHNV nucleocapsid protein were purchased from DiagXotics (Wilton, Connecticut, USA). Monoclonal antibody used for the coating of the Strepatavidin MagneSphere was supplied as biotin conjugated form.

Oligonucleotides for cDNA synthesis and PCR

The primers used for the cDNA synthesis and PCR amplification were designed based on the published IHNV glycoprotein gene sequence (Koener et al, 1987). The 5' primer, (5'-CAAGGATCCACATCCAC-3') is located at the nucleotides 331~348 of the glycoprotein gene. A BamH I restriction site (underlined) was introduced by replacing adenine with thymine for convenient cloning of the PCR product. The 3' primer (5'-TCATCGGATCCATCATGC-3') is complementary to the nucleotide of 1300~1318. A BamH I restriction site (underlined) was introduced by replacing thymine with adenine. The resulting PCR product was 988 nucleotides (nts) and could be divided into fragments of 615nts and 373nts by Xba I digestion.

Extraction of total RNA and RT-PCR

Monolayer of CHSE-214 cell on 35 mm diameter culture dish was inoculated with the virus by using standard procedure and incubated at 18℃ for 3~4 days. Right before any cytopathic effect (CPE) develops, the medium was removed and the cell was liquefied with 1 mℓ of Trizol (GIBCO BRL). The lysate was then transferred to a 1.5 mℓ microcentrifuge tube and incubated at room temperature for 5 min. Two hundred microliter of chloroform was added and the tube was vortexed for 15 sec, and stood for two min. After 15 min centrifugation with microcentrigufe at 12,000×g, the upper aqueous phase (about 600 μℓ) was mixed with 500 μℓ isoprophyl alcohol. After 10 min incubation at room temperature, the RNA was precipitated for 10 min at 12,000×g. The pellet was washed with 1 mℓ of 75% ethanol by centrifugation at 7,500×g for 5 min, and dried with vacuum, and resuspended with 15 μℓ of RNase free distilled water.

cDNA synthesis and PCR

The extracted RNA was heated 60℃ for 10 min and used for cDNA synthesis. Five microliter of the RNA was added to 20 μℓ reaction mixture [100 pmol of IGC primer, 200 μM dNTP, 40U RNase inhibitor, AMV

reverse transcriptase buffer, 10U of AMV reverse transcriptase (Promega)]. The reaction mixture was incubated at 42℃ for an hour and then the enzyme was heat inactivated at 95℃ for 10 min. To 50 $\mu\ell$ PCR mixture containing 100 pmol IGN primer, 10X Taq polymerase buffer, 2.5U Taq DNA polymerase (Takara, Otsu, Japan), 20 $\mu\ell$ of the synthesized cDNA was added and polymerase chain reaction was performed by using a Perkin-Elmer 480 DNA thermal Cycler. The reaction was composed of heating of the mixture for 2 min at 95℃ followed by 35 cycles of reactions composed of 15 sec denaturation at 95℃, 30 sec annealing at 52℃, and 1 min extension at 72℃. After 10 min extension at 72℃, 10 $\mu\ell$ of the PCR product was analyzed on an 1% agarose gel.

Coating of magnetic bead with Antibody

Before coating, the magnetic bead was washed three times with 1X phosphate buffered saline (PBS, 10 mM phosphate buffer, pH7.4, 150 mM NaCl). 5×10^7 particles of goat anti-mouse IgG coated magnetic bead or 5×10^{10} particles of streptavidin coated magnetic beads were mixed with monoclonal antibody to the final dilution of 1 : 200. The mixtures were incubated at room temperature for one hour with a rotator running at 50 rpm. Unbound antibody was removed by using a magnetic separation stand. On this stand, the magnetic beads coated with antibody move to the side of the tube and the supernatant can be removed by using a pipette. The beads were resuspended with PBS by removing the external magnet and washed three times with same method.

Capturing of virus with antibody coated magnetic beads

The supernatant was harvested 7 days after virus infection when the cell was completely destroyed. Two hundred microliter of the supernatant was equilibrated to 1X PBS in 1 mℓ and mixed with the prepared magnetic bead, and then incubated 2 hours at room temperature. The unbound virus and cell debris was removed by washing the beads with 1X PBS three times as above. In order to test the binding of virus particles to the beads and efficiency of cleaning, the cleaned magnetic bead was resuspended with 30 $\mu\ell$ of PBS, 15 $\mu\ell$ of the resuspended bead and 15 $\mu\ell$ of the supernatant was analyzed by using a 10% SDS-PAGE and Western blotting.

RT-PCR analysis of the captured virus

RT-PCR of the captured virus was conducted in two different methods. In the first method, the concentrated virus was washed one more time with RNase free water and resuspended in 48 $\mu\ell$ of reverse transcription mixture described above. The mixture was boiled at 95℃ for 5 min and cooled on ice. Forty units of RNase inhibitor and 10U of AMV reverse transcriptase were added to the mixture, and cDNA synthesis and PCR were performed as described above. In second method, RNA was extracted by using Trizol and used for RT-PCR. The concentrated virus was washed once with RNase free water and resuspended in 100 $\mu\ell$ Trizol without addition of any buffer. The RNA was extracted and RT-PCR was conducted as above. In order to confirm the amplification of the targeted sequence, the PCR product was eluted from the agarose and used for sequencing of the both ends and digestion with Xba I.

Results and Discussion

Detection of Viral RNA from Inoculated Cell.

One of the classical methods for virus detection is inoculation of cultured cell with ground and filtered sample, and observation of CPE development. In some cases, it takes over one week to the CPE developed and it is not easy to identify the virus with the CPE. Therefore, it is necessary to use more than one method to identify the virus, and RT-PCR is one of them.

In order to test the primers synthesized for the RT-PCR, we extracted total RNA from IHNV inoculated CHSE-214 cell 4 days post infection and used for the RT-PCR. As shown in Fig. 1, a DNA fragment of expected size was obtained. When the amplified DNA was purified and sequenced at the both ends up to 150nts, the sequence was identical to the published IHNV glycoprotein sequence (Koener et al, 1987, sequences are not shown). Also, the purified PCR product produced two DNA fragments of expected size when it was digested with Xba I (data not shown). In order to introduce a BamH I sites,

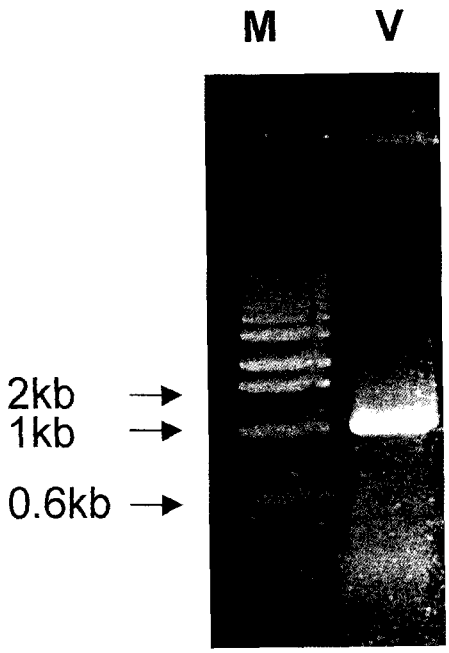

Fig. 1. RT-PCR of total RNA extracted from IHNV inoculated CHSE-214. Total RNA was extracted with Trizol from CHSE-214 cells inoculated with IHNV RB-76 strain 4 days post inoculation. Part of the RNA was used for the reverse transcription with IGN primer followed by 35 cycles of polymerase chain reaction with IGN and IGC primers. The expected size of the PCR product (lane V) was 988 nucleotides.

two primers were designed as one nucleotide from each primer was replaced (A→T in IGN T→A in IGC), and these changes did not affect the specificity and efficiency of the primers. Although, total RNA was extracted from artificially inoculated cell line, this methods might be applied for the detection of virus from small sample such as organs or tissues or mucus from infected fish (LaPatra et al., 1989). It is known that IHNV is transmitted from the mother fish to eggs (Mulcahy and Pascho, 1985), and this method also could be used for the detection of virus from the infected eggs. However, virus in a sample of large volume such as water needs to be concentrated and freed from contaminants before RT-PCR.

Binding of IHNV to antibody coated magnetic bead.

Many methods have been developed for the precipitation of virus particles from samples by acids (Sobsey et al., 1978), organic flocculation (Katzenelson et al., 1976), polyethylene glycol (Lewis and Metcalf, 1988), and Staphylococcal coagglutination (Bootland and Leong, 1992). Tubes coated with antibody against hepatitis A virus were used for the capturing of the virus from fecal specimen before PCR (Jansen et al., 1990). However, above methods are usually tedious, involve multiple manipulations, and the sample can be contaminated during these processes.

Magnetic beads coated with antibodies were first used to select specific cells from blood (Lea et al., 1985). In this method, polymer particles of unique size with magnetic core were coated with antibodies and used to select specific target of the antibody. Alternatively, the beads can be coated with two layers of antibodies such as anti-mouse immunoglobulin first and a second layer of monoclonal antibody. Most of the magnetic beads are supermagnetic, that is, they only exhibit magnetic properties in the presence of an external magnetic field. Any particles bound to the antibody can be concentrated with external magnet and the supernatant can be removed for the cleaning of the sample. Also, the magnetic bead itself can be removed from the suspension, too.

Before conducting the IMS-PCR, specific binding of virus particles to the antibody coated beads was tested with SDS-PAGE and Western blotting. After removal of unbound antibody, the beads were reacted with supernatant from IHNV inoculated CHSE-214 cell. As shown in Fig. 2, a protein of molecular mass of about 70 kDa was detected from non-infected cells, IHNV and IPNV infected cells. Although it was not clear whether this protein originated from the cell or supplemented serum, this protein could be inhibitory for the detection of the virus with other method such as ELISA. This possibility could be seen in the Western blot in Fig. 3. The protein observed in the SDS-PAGE showed non-specific reaction to the monoclonal antibody against the IHNV nucleocapsid protein (lanes 1, 2, 3). However, this protein could be removed from the sample by using the magnetic bead coated with monoclonal antibody.

In order to remove this non-specifically reacting protein, the tube containing the coated bead and the sample was placed on a magnetic stand. The magnetic bead coated with antibody and virus moved toward the side of the tube on this stand. The aqueous of the tube,

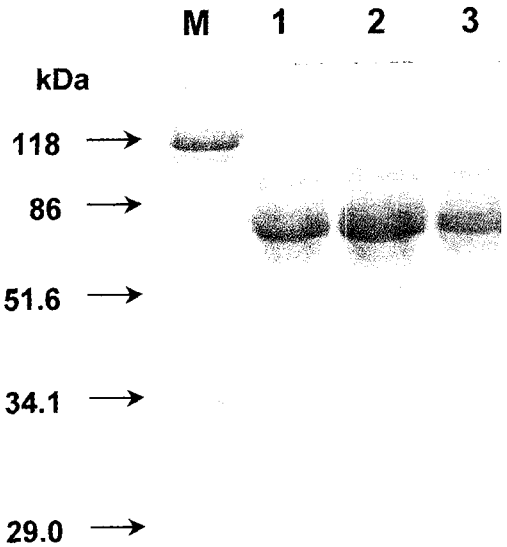

Fig. 2. SDS-PAGE analysis of CHSE-214 cell infected with IHNV and IPNV. Cells were harvested after complete lysis and used for the analysis. Lame M; Protein size marker, Lane 1; cells infected with IHNV, lane 2; non-infected cells, lane 3; cells infected with IPNV.

Fig. 3. Capturing of virus particles with antibody coated magnetic beads. Cell lysate of IHNV or IPNV infected CHSE-214 and supernatant after binding reaction, captured virus particles were analyzed with Western blotting by using monoclonal antibody against IHNV nucleocapsid protein. Lane M: protein size marker, Lane 1; lysate from IHNV infected cell, lane 2; non-infected cell, lane 3; lysate from IPNV infected cell, Lane S; supernatant from binding mixture after magnetic concentration of the captured virus, lane C; virus-bound magnetic beads after three washes with PBS. Proteins showing non-specific reaction (NS) and viral nucleocapsid protein (N) are marked.

which is designated as S in Fig. 3, was carefully removed without disturbing the magnetic bead pellet. One milliliter of washing buffer (1X PBS) was added to the tube and the pellet was resuspended by removing the tube from the magnetic stand. The washing buffer removed with same method and the pellet was washed twice. The supernatant and the final pellet were analyzed by Western blotting. As shown in Fig. 3, the 70kDa protein was detected only from the supernatant and was not detected from the concentrated magnetic bead pellet.

The IHNV nucleocapsid (N) protein was not detected from SDS-PAGE gel stained with Coomassie brilliant blue (Fig. 2, lane 1). However, it was detected in the Western blotting (Fig. 3, lane 1). The N protein was also detected from the supernatant and the pellet in the immunomagnetic separation. The N protein in the supernatant might come from virus particles not captured by the magnetic beads. Because the pellet was washed three times, any non-bound virus particles should be removed from the pellet. Therefore, the N protein detected from the pellet should come from virus particles captured by the monoclonal antibody on the magnetic bead. In the western blot analysis of the pellet, two protein bands appeared above and below the N protein (Fig. 3, Lane C). Although the exact nature of these two proteins has not been determined, these proteins came from the antibody coated magnetic beads because they were detected when the double-coated magnetic bead was analyzed before virus capturing (data not shown). From the Western blot, it was concluded that the virus bound to the beads and other cellular or non-specific protein could be removed.

RT- PCR with immunocaptured virus.

After introduction, the magnetic beads coated with antibody have been used for the capturing of bacteria from water, sediment, food before enrichment of the bacteria (Safarik et al., 1995; Cudjoe et al., 1994; Kapperud et al., 1993; Morgan et al., 1991; Skjerve and Olsvik, 1991; Christensen et al., 1992). This method also has been used for the capturing of viral RNA by coating the bead with specific oligonucleotides (Beaulieux et al., 1997; Albretsen et al, 1990). In case of Hepatitis A virus, virus captured with antibody coated in a microcentrifuge tube was used for RT-PCR after boiling (Jansen et al., 1990). However, in our experiment with IHNV,

no PCR product was detected when the captured virus was used for RT-PCR after boiling (Fig. 4). The RT-PCR product was only detected when the viral RNA was extracted with Trizol before RT-PCR. This difference might be due to the structure of the virus. Contrast to the hepatitis A virus that belongs to the picornaviridae family and does not have an envelope outside of the ribonucleoprotein, IHNV belongs to the rhabdoviridae family and has outer membrane derived from the host.

In the IMS-PCR, the antibody can be bound to the bead directly or by using a mediator. In this experiment, magnetic beads were double coated with goat anti-mouse immunoglobulin and monoclonal antibody against IHNV nucleocapsid protein, and double coated with streptavidin and biotin conjugated monoclonal antibody were used. As shown in Fig. 5, there was no difference in the capturing and specificity if the IMS-PCR reaction in both cases.

There are many methods for virus detection and some of the important requisites for the good detection methods are rapidity, simplicity, low cost, sensitivity and specificity. With IMS-PCR, detection of virus could be done within a day. Increasing number of companies are making the magnetic beads, and the test can be conducted in low cost soon. Specificity is the strong point of IMS-PCR. The target virus is first selected with antibody and then the specific viral genome is amplified with RT-PCR or direct PCR in case of DNA viruses. Although reasonable sensitivity was observed in our preliminary experiment compare to ELISA and Western blot (data not shown), methods are being developed to increase the sensitivity of IMS-PCR.

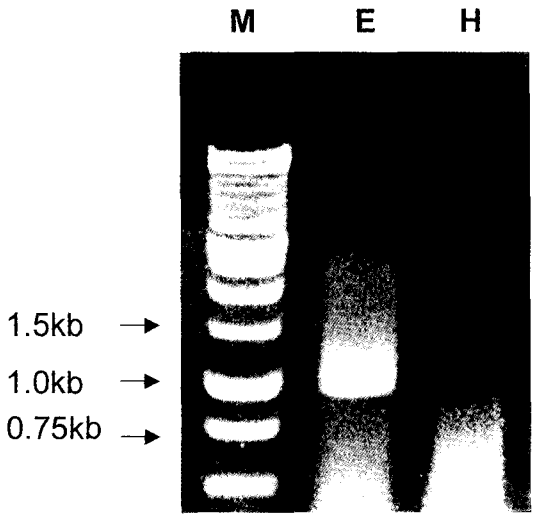

Fig. 4. Preparation of RNA templates from the captured virus particles. After washing and concentration, the RNA was prepared for the RT-PCR either by heating at 95°C for 5 min (lane H) or extraction with Trizol followed by ethanol precipitation (lane E).

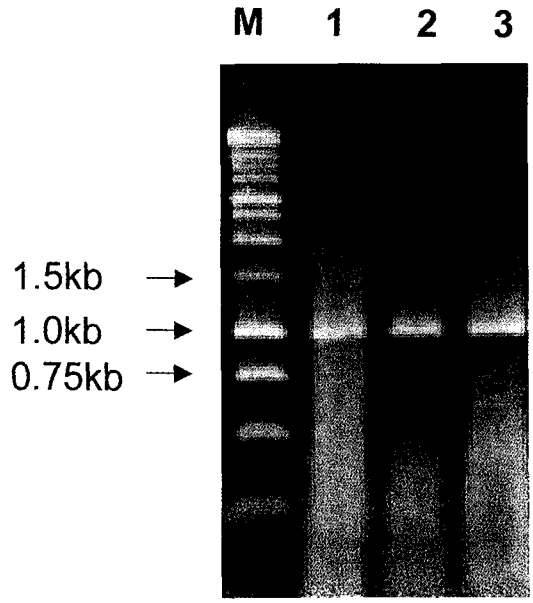

Fig. 5. Different methods of magnetic bead coating. Lane M; DNA size marker, Lane 1; magnetic beads double coated with goat anti-mouse Ig antibody and monoclonal antibody against IHNV nucleocapsid protein, lane 2; magnetic beads double coated with streptavidin and biotin conjugated monoclonal antibody. After concentration, the RNA was extracted with Trizol and used for RT-PCR. RNA extracted from IHNV inoculated CHSE-214 was used as control (lane 3).

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