J. Fish. Sci. Tech. 4(1), 32~38, 2001

JOURNAL OF
FISHERIES SCIENCE AND TECHNOLOGY
© The Korean Fisheries Society

Comparison of IHNV Detection Limits by IMS-RT-PCR, Western Blot and ELISA

Soo-Jin Kim[1], Eun-Young Lee[1], Myung-Joo Oh[2] and Tae-Jin Choi[1]*

[1]*Department of microbiology, Pukyong National University, Pusan 608-737 Korea*
[2]*Department of Fish Pathology, Yosu National University, Yosu 550-749, Korea*

(Received November 2000, Accepted March 2001)

Several molecular biological techniques have been used to detect virus rapidly and accurately, but these methods have limitations in the early stage of viral infection with very low concentration of virus. We compared the detection limits of IMS-PCR, Western blot and ELISA with infectious hematopoietic necrosis virus (IHNV). Four antibodies, rabbit anti-IHNV polyclonal antibody, anti-IHNV nucleocapsid protein monoclonal antibody, anti-IHNV nucleocapsid protein polyclonal antibody, and anti-IHNV glycoprotein polyclonal antibody, were tested to find out the most effective antibody for each method. The detection limit with IMS-PCR was 2×10^6 pfu when the viral RNA was extracted before RT-PCR. In the western blot with rabbit anti-IHNV polyclonal antibody one pfu of virus could be detected. In ELISA, 10 pfu of virus particles were detected with the same antibody.

Key words: IHNV, IMS-RT-PCR, Western blot, ELISA

Introduction

Fish viruses can be easily spread through water but there are only limited chemotherapeutic agents and licensed vaccines available for prevention or control of viral diseases. Therefore, efforts to control the losses caused by fish viruses have relied upon avoidance through the examination of brood stock and destruction of infected fish and eggs. Techniques for the isolation and diagnosis of viral pathogens of fish have been traditionally involved virus isolation in cell culture and confirmation by serum neutralization tests, a process taking 14 days or more. Hence, an important priority for fish virus detection has been the development of methods for rapid screening and specific diagnoses (Deering et al., 1991).

Viral protein has been detected by enzyme linked immunosorbent assay (ELISA, Medina et al., 1992; Dixon and Hill, 1984), Western blot, fluorescent antibody test (Hsu et al., 1986). The nucleic acids of viral genomes have been detected by dot-blot hybridization (González et al., 1997) and DNA probe assay (Deering et al., 1991). Reverse transcription-polymerase chain reaction (RT-PCR) also has been employed in the detection of viral RNA. This is a very specific and sensitive detection method and the whole process takes about 6 h from the initial processing of the tissues. However, this method is hard to apply at the early stage of infection in which the virus titer is low. In addition, RT-PCR requires concentrated RNA free from any contaminant.

In order to concentrate virus that existed in large volume of sample and to remove contaminants in the sample, magnetic beads coated with specific antibody have been used. Magnetic beads coated with specific antibody were first used for the selection of specific lymphocytes and hybridoma cells producing monoclonal antibodies (Horton et al., 1989). Later, this method was applied for the capturing of bacteria and viruses from highly contaminated sample (López-

*Corresponding author: choitj@mail.pknu.ac.kr

Sabater et al., 1997; Kapperud et al., 1993).

In the previous study, immunomagnetic separation and reverse transcription-polymerase chain reaction (IMS-RT-PCR) has been proved to be effective in detection of infectious hematopoietic necrosis virus (IHNV), a well-known fresh water fish virus (Kim et al., 1997). In this study, the detection sensitivity by IMS-RT-PCR was compared with that of ELISA and Western blot, and the detection limit of each method was determined by plaque assay.

Materials and Methods

Cell and virus

IHNV RA (type 2) strain was kindly provided by Jo-Ann Leong in the Department of Microbiology, Oregon state university. Chinook salmon embryo cells (CHSE-214) were grown at 18℃ in minimal essential medium (MEM) in Hanks' salts (Sigma, USA) supplemented with fetal bovine serum (Hyclone Inc., USA) to 10%. The cells were cultured in 35 mm dish for RT-PCR and in 25 cm^2 culture flask for the recovery of fluid containing IHNV. After 24 h, the media were discarded and IHNV was infiltrated into cells at a multiplicity of infection of 0.001 for 1 h. MEM containing 5% FBS was added and the inoculated cells were incubated at 15℃.

Construction of recombinant IHNV N and G protein expression system

The complete open reading frame of the IHNV nucleoprotein (N) and the C-terminal half of the glycoprotein (G) genes were cloned into the NdeI/Bam-HI site of the pET-11c vector. The open reading frames of N and G protein were amplified by using RT-PCR with the primers 5'-CGGATCACGACATATGACAAGCGC-3', 5'-GGAGGATCCTGGCTCACTG-3' and 5'-GACGTCTACCATATGCACAAAGG-3', 5'-CGGGATCCTTTAGGACCGGTTT-3', respectively. RNA templates were extracted from IHNV inoculated CHSE-214 with TRIzol (Gibco-BRL, USA). Briefly, cells in a 35 mm diameter dish were lysed directly by adding 1 mℓ of TRIzol reagent and 200 μℓ of chloroform was added. After 15 min centrifugation with microcentrifuge at 12,000 g (4℃), the upper aqueous phase was mixed with 500 μℓ of isoprophyl alcohol per 1 mℓ of Trizol reagent. The samples were incubated at room temperature for 10 min and centrifuged at 12,000 g for 10 min at 4℃. The pellets were washed once with 1 mℓ of 75% ethanol and centrifuged at 7,500 g for 5 min at 4℃. The RNA pellets were dried for 5 min under vacuum and resuspended in DEPC treated water.

Preparation of antibodies

E. coli transformed with the clones containing the N gene and G gene were induced with IPTG (Isopropyl-β-D-thiogalactoside) to 0.6 mM final concentration. The cells were harvested by centrifugation at 5,000 rpm in GS-3 rotor (SORVALL) for 10 min at 4℃ and resuspended in 15 mℓ lysis buffer [10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF]. The mixture was sonicated with Vibra cell™ (Sonics and materials Inc.) for 2 min with 50% output in ice-bath and centrifuged at 8,000 rpm in SS-34 rotor (SORVALL) for 5 min at 4℃. The pellet was resuspended in 20 mℓ of lysis buffer containing lysozyme (200 μg/mℓ), 0.1% Triton X-100, deoxycholic acid (1 mg/mℓ) and keep on ice for 30 min. After centrifugation at 8,000 rpm for 5 min, the pellet was washed with lysis buffer containing 0.1% Triton X-100, deoxycholic acid (1 mg/mℓ) and centrifuged at 8,000 rpm for 5 min. The pellet was washed one more time with lysis buffer and centrifuged pellet was resuspended in 5 mℓ of lysis buffer. 0.5 mℓ of the sample was denatured and loaded on 20×16×0.15 cm 10% or 12.5% polyacrylamide gel. After electrophoresis, the gels were stained with 0.3 M $CuCl_2$ and viral protein band was sliced. Protein in the sliced gel was eluted by electro-separation system (S&S ELUTRAP, Schleicher & Schuell) and quantified by Braford assay (Boyer, 1993).

Purified antigen (100 μℓ, 1 mg/mℓ) was mixed with an equal volume of Freund's complete adjuvant (Sigma) and the emulsion was injected subcutaneously into four 6-week-old female BALB/c mice. Three booster injections with 25 μg of antigen mixed with Freund's incomplete adjuvant (Sigma) were given at one week intervals. One week after the last booster, 1×10^7 sarcoma cells in 0.5 mℓ of PBS were injected intraperitoneally (Harlow and Lane, 1988). Ascitics fluid was collected after 10 days. The fluid was incubated for 1 h at 37℃, overnight at 4℃ and clarified by centrifugation at 3,000 rpm in MF-80 centrifuge (Han-il Co.) for 20 min. Anti-IHNV nuc-

leocapsid protein monoclonal antibody was purchased from DiagXotics Inc. (USA). Rabbit anti-IHNV virion polyclonal antibody was kindly provided by Myong Ae Park in the Pathology Division of National Fisheries Research & Development agency, Pusan, Korea.

IMS-RT-PCR

The procedures for IMS-RT-PCR of IHNV are described in the previous report (Kim et al., 1997). Briefly, goat anti-mouse IgG coated magnetic beads (PerSeptive Biosystems, USA) were conjugated with anti-IHNV nucleocapsid protein monoclonal antibody (DiagXotics Inc.). After removal of unbound antibodies, virus particles in 200 μℓ~1 mℓ of fluid from IHNV infected cell culture were mixed and incubated for 2 h at room temperature with rotation. Unbound viruses and cell debris were removed and RNA from the immunomagnetically-separated virus was extracted with TRIzol reagent, and used for RT-PCR with G gene specific forward primer (5'-CAAGGGATCCACATCCAC-3') and reverse primer (5'-TCATCGGATCCATCATGC-3').

Western blot

Serially diluted fluid containing IHNV was mixed with equal volume of sample loading buffer [1 mM EDTA, 250 mM Tris-Cl (pH 6.8), 4% SDS, 2% beta mercaptoethanol, 0.2% bromophenol blue, 50% glycerol]. After 5 min boiling and centrifagation, the supernatant was electrophoresed on a 10% SDS-poly acrylamide gel under reducing conditions. Electrophoretically separated polypeptides were transferred to nitrocellulose (NC) membrane at 250 mA for 1 h in transfer buffer [25 mM Tris, 192 mM Glycine, 20% methanol]. The NC membrane was blocked with 5% skim milk in TTBS [0.05% Tween-20 contained TBS]. The membrane was incubated with mouse anti-IHNV antibodys, diluted 1:3,000 in 5% skim milk in TTBS for 1 h and, unbound antibodies were removed by three times washing with TTBS. The membrane was treated with secondary antibodies, alkaline phosphatase-conjugated goat anti-mouse IgG antibodies for 1 h. Unbound antibodies were removed as above. Bound antibodies were detected by addition of 66 μℓ BCIP (5-bromo-4-chloro-3-indolyl phosphate, 0.15 mg/mℓ) and 33 μℓ NBT (ρ-nitro blue tetrazolium chloride, 0.3 mg/mℓ) phosphatase substrate in 10 mℓ alkaline phosphatase buffer [0.1 M Tris-Cl (pH 9.5), 0.1 M NaCl, 5 mM $MgCl_2$].

ELISA

A solid phase indirect ELISA system was employed using equal volumes (100 μℓ) of each reagent in following manner: Fluid from IHNV infected cell cultures, serially diluted (10^{-1}~10^{-12}) in 0.1 M carbonate-bicarbonate buffer (pH 9.6) was added to wells of a 96 well assay plate (Corning, USA). The plate was incubated at 4℃ for 16 h and the plate was washed 3 times at 10 min intervals with PBS containing 0.08% Tween-20 (PBS-T). Blocking buffer (PBS-T plus 2% bovine serum albumin) was deposited into each well and the plate was incubated for 30 min at 37℃. After removal of the blocking buffer, rabbit anti-IHNV serum or mouse anti-IHNV/N serum (diluted 1:500 and 1:100 in PBS-T) was added and the plate was incubated at 37℃ for 2 h. Intervening rows received equally diluted negative 'spent' cell culture fluid. The plate was washed and blocked as described above. Alkaline phosphatase conjugated goat anti-rabbit or anti-mouse IgG serum diluted 1:31,000 in PBS-T was added to each well and incubated at 37℃ for 2 h. The plate was washed 3 times for 10 min intervals with PBS-T, followed by one wash with distilled water for 5 min. Substrate (10 mg ρ-nitrophenyl phosphate dissolved in 10 mℓ of Diethanolamine buffer, pH 9.8) was added to each well. The plate was incubated at room temperature for 30 min and the reaction was stoped with 100 μℓ of 0.1 M EDTA. The optical density (OD_{405}) of the reactions at 405 nm was measured with CERES uv 900c (Bio-Tek instruments Inc.).

Plaque assay

IHNV containing fluid was 10-fold (10^{-2}~10^{-12}) diluted in MEM. One hundred microliter of the diluted virus was added to the center of the monolayer without agitating the plates. At least 1 h was allowed for virus adsorption, followed by addition of 2 mℓ methylcellulose overlay media [0.75% methylcellulose (4,000 centipoise, sig-ma), 1X MEM, 5% FBS, 14 mM HEPES, penicillin 100 units/mℓ, streptomycin 100 μg/mℓ, Amphotericin B 0.5 μg/mℓ, sodium desoxycholate 0.41 μg/mℓ, Gentamycin 0.5 mg/mℓ, 1 X L-Glutamin, buffed pH 7.2 with 7.5% sodium bicarbonate]. When discrete clear plaques were observed in

an inverted microscope, 2 mℓ of staining solution [3.7 % formaldehyde, 0.5% crystal violet] was added the dishes. The overlay media and staining solution was discarded after 24 h and dishes were washed with distilled water. The cell layer was allowed to dry and the plaques were counted. The PFU was calculated as follow: plaques in dish×dilution factor×correction for 0.1 mℓ inoculation volume=titer (pfu/mℓ)

Results and Discussion

Production of polyclonal antibody against nucleocapsid protein and glycoprotein

Polyclonal antiserum has greater sensitivity whereas monoclonal antibody has greater specificity, and polyclonal antibody has the advantage of virus detection. For this reason, polyclonal antibodies were made from viral protein expressed in *E. coli.* The nucleocapsid protein and glycoprotein were selected for antibody production because nucleocapsid protein is most prevalent and glycoprotein presents on the surface of the virus particles.

RNA template for the amplification of the nucleocapsid protein gene and glycoprotein gene was extracted from IHNV infected CHSE-214 with TRIzol. The yield of viral RNA was maximum when cytopathic effect (CPE) just appeared. From the PCR with respective primers (see methods), 1173 bp of N gene fragments and 509 bp of G gene fragments were obtained (Fig. 1).

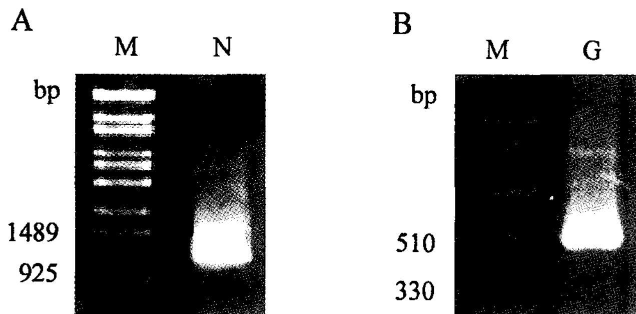

Fig. 1. Amplification of N- and G-specific cDNA from IHNV RNA by RT-PCR. Total RNA was extracted with TRIzol from CHSE-214 cells inoculated with IHNV 3 days post infection. Parts of the RNA were used for reverse transcription of nucleocapsid protein (A) and glycoprotein (B) followed by 35 cycles of polymerase chain reaction. The expected sizes of the PCR products were 1170 bp (Lane N) and 509bp (Lane G).

Expression of viral protein in *E. coli* could be toxic to the bacterial cells (Choi et al., 1999). Therefore, part of the glycoprotein was expressed and used for antibody production. Recombinant plasmid containing the C-terminal half (nucleotide of 4033-4533 of the published IHNV glycoprotein sequence; Schutze et al., 1995) of the glycoprotein gene was generated rather than intact glycoprotein gene (nucleotide 3007-4533 of the published IHNV glycoprotein sequence).

The PCR generated fragments were digested with NdeI and BamHI prior to ligation into pET-11c. *E. coli* strain BL21 (DE3) transformants were selected from plate containing ampicillin and the plasmid DNA was purified. The DNAs were digested with NdeI and BamHI and clones, pTIHN and pTIHG containing the nucleocapsid protein gene and glycoprotein gene fragment, respectively were selected by agarose gel electrophoresis of the digested plasmid.

Expression of inserted gene was induced by addition of IPTG up to the final concentration of 0.6 mM. Bacterial lysates were prepared and the proteins were analyzed by SDS-PAGE. Coomassie blue staining of these gels showed that expressed N and G protein had predicted molecular weight; N protein of 42 kDa and G protein fragment of 18 kDa. These proteins were proved as proteins of IHNV by reaction with rabbit anti-IHNV polyclonal antibodies on western blot (Fig. 2).

Antibodies against different viral antigens were obtained to compare the detection sensitivity of these antibodies. However, this was apparently unsuccessful because the reactivity of antibodies to virus particles in the fluid was too low as shown in Fig. 3. The correlation between the neutralization titer of an antiserum and its efficacy in immunoblots suggests that an antiserum needs to have a homologous 50% plaque-neutralization titer of 1:50,000 or preferably greater to be suitable for immunoblots (Dixon et al., 1984). The reason of low reactivity to viral protein is not clear. However, it seems not related to the quality of antibodies because the antibodies reacted well to the antigens used for immunization. The antigens were proved as viral proteins by western blot with rabbit anti-IHNV polyclonal antibody and nucleotide sequence of cloned genes were confirmed viral genes by sequencing (data not shown).

Therefore, rabbit anti-IHNV polyclonal antibody which showed higher titer, and monoclonal antibody

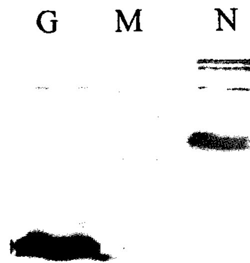

Fig. 2. Western blot analysis of nucleocapsid protein and glycoprotein expressed in *E. coli* with polyclonal rabbit anti-IHNV serum. *E. coli* lysates were electrophoresed in a 12.5% polyacrylamide gel, transferred to nitrocellulose, and detected with the rabbit anti-IHNV polyclonal bodies. lane G: IPTG induced pTIHG containing cells; lane M, broad range prestained protein standard; lane N, IPTG induced pTIHN containing cells.

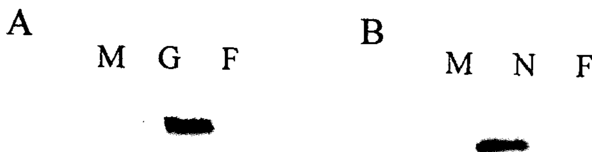

Fig. 3. Western blot assay of polyclonal antibodies against IHNV N and G protein. IHNV inoculated CHSE-214 cell extract (lane F) and purified IHNV (lane G and N) were electrophoresed in a 12.5% polyacrylamide gel, transferred to nitrocellulose, and detected with the mouse anti-IHNV G protein polyclonal antibody (A, 1:1000 dilution), mouse anti-IHNV N protein polyclonal bodies (B, 1:1000 dilution). lane M, broad range prestained protein standard; G, glycoprotein (56 kDa); N, nucleocapsid protein (42 kDa).

against the N protein were used for virus detection.

Detection of IHNV by IMS-RT-PCR

The magnetic beads are supermagnetic so that beads can be concentrated with external magnet and the supernatant can be removed for the cleaning of the sample (Safarik et al., 1995). Magnetic beads coated with antibodies have previously been used for the capture of various viruses such as the hepatitis A virus and group A rotaviruses (Grinde et al., 1995; López-Sabater et al., 1997). In these studies, viral RNA was exposed by boiling, and the detection limit was less than 10 pfu. However, IHNV could not be detected when virus was boiled to expose the RNA from the virus particle (Kim et al., 1997) probably because of the viral envelope, and the RT-PCR product was obtained when the IHNV RNA was extracted with TRIzol (Kim et al., 1997). However, there could be some loss of viral RNA during the RNA extraction process, which might reduce the sensitivity of this detection method. In fact, the detection limit of this method was 10^6 pfu, which is much lower than the results with hepatitis A virus or rotavirus. Therefore, the IMS-RT-PCR methods look inappropriate for the detection of enveloped virus such as IHNV. However, this method could be used for the detection of other fish viruses that are not enveloped. Also, the immunocapturing of enveloped fish virus can be applied for virus separation and concentration. If immunomagnetic separation is performed prior to several detection methods such as dot-blot, ELISA, and western blot, very low titer of virus could be detected.

Detection of IHNV by Western blot

Western blot is independent of biological activity and sensitive enough to measure very small amounts of protein. Antibodies are adequate reagents because those react specifically with the corresponding antigens. The immunological detection of virus proteins have offered advantages in cost, accuracy, speed and, in the case of the enzyme conjugated antibodies reagent, the stability and ease in handling of a nonradioactive compound (Dixon et al., 1984).

Rabbit anti-IHNV virion polyclonal antibody and anti-IHNV N protein monoclonal antibody were used in western blot for IHNV detection because the titers of these antibodies were higher than those of other antibodies. As shown in Fig. 4, the detection limit with rabbit anti-IHNV virion polyclonal antibody was 1 pfu and that with anti-IHNV N protein monoclonal antibody 3×10^3 pfu. One strange observation in Fig. 4B is the detection of N protein only by polyclonal antibody raised against purified virion. However, this could be related to the antibody itself because this antibody reacted only to the N protein of purified

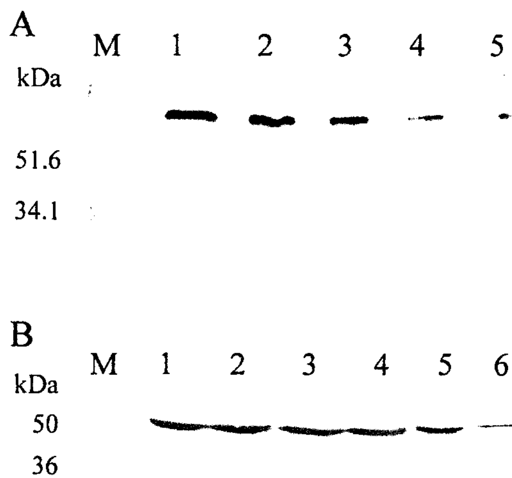

Fig. 4. Detection of IHNV by Western blot. (A) IHNV containing fluid was separated on a 10% acrylamide gel, transferred to nitrocellulose, and detected with the anti-IHNV N protein monoclonal antibody. lane M, broad range prestained protein standard; lane 1~5, 5×10^4 pfu, 3×10^4 pfu, 1×10^4 pfu, 5×10^3 pfu, 3×10^3 pfu. (B) IHNV was detected with the rabbit anti-IHNV virion polyclonal antibody. lane M, broad range pre-stained protein standard; lane 1~6, 1×10^6 pfu, 1×10^5 pfu, 1×10^4 pfu, 1×10^3 pfu, 1×10^2 pfu, 1×10 pfu, 1 pfu.

virion (data not shown). The lower detection limit with western blot compared to that of IMS-RT-PCR methods reflects the nature of plaque assay. In the plaque assay, only live virus particles with intact genome produce plaques. However, the Western blot detects any viral protein whether they are in complete virus particle or that are in free or disassembled stage. Therefore, 1 pfu in plaque assay or IMS-RT-PCR mean one complete virus particle, but there could be a lot of viral protein.

Detection of IHNV by ELISA

Several techniques including fluorescent antibody detection in cell culture, immuno-blot assay, detection of viral nucleic acids by means of polymerase chain reaction and biotinylated DNA probe have been used for IHNV detection (Hsu and Leong, 1985; González et al., 1997; Arakawa et al., 1990; Deering et al., 1991). Although these techniques are sensitive, they are technically intensive. An alternative method, the enzyme linked immunosorbent assay (ELISA), is generally less expensive and more expedient (Medina et al., 1992). ELISA was performed to compare the detection limit of antibodies and techniques used in this study.

A test reading was considered positive when the OD value of sample minus the OD value of matched dilution of uninfected 'spent' cell culture fluids (negative control) was exceed 0. Virus was detectable at 10 pfu when rabbit anti-IHNV virion polyclonal antibody was used at 1:500 dilution (Table 1), while 10^3 pfu of virus was detectable when mouse anti-IHNV N protein monoclonal antibody was used. Although monoclonal antibodies provide a high degree of specificity, the use of polyclonal antisera in ELISA can allow detection of a greater range of virus types and antigenic variants, which would be an advantage for field test. Detection sensitivity of ELISA was lower than that of Western blot.

Table 2 summarizes the results of this study. Western blot is the most sensitive method among three IHNV detection method followed by IMS-RT-PCR and ELISA. Combination of Western blot that has excellent virus detection capacity and immunomagnetic separation that has excellent virus concentration capacity, will be good to detect fish virus in field.

Table 1. Sensitivity of IHNV detection by ELISA

Virus conc.[a]		10^4	10^3	10^2	10^1	<10
Anti-IHNV pAb	1:500	+[b]	+	+	+	−

a: Virus concentration: plaque forming unit
b: +, OD of sample; −, OD of negative control>0
negative control: matched dilution of uninfected 'spent' cell culture fluids

Table 2. Comparison of IHNV detection methods

	IMS-RT-PCR	ELISA	Western blot
Used antibody	Anti-IHNV mAb	Anti-IHNV pAb	Anti-IHNV pAb
Detection	10^6 pfu	10 pfu	1 pfu
Required time	6 hr	9 hr	6 hr

Acknowledgments

The authors wish to acknowledge the financial support of the Korea Research Foundation made in the Program Year 1996.

References

Arakawa, C.K., R.E. Deering, K.H. Higman, K.H. Oshima, P.J. Óhara and J.R. Winton. 1990. Polymerase chain reaction (PCR) amplification of a nucleoprotein gene sequence of infectious hematopoietic necrosis virus. Dis. Aquat. Org., 8, 165~170.

Boyer, R.F. 1993. Measurement of protein solutions. Modern experimental biochemistry. 2nd edition. The Benjamin/ Cummings Publishing Company, Menlo Park, CA. p. 54.

Choi, T.-J., S.-C. Kim and T.L. German. 1999. 12. Toxicity of tomato spotted wilt virus glycoproteein signal peptide and promoter activity of the 5′ UTR. Plant Pathol. J., 15, 313~318.

Deering, R.E., C.K. Arakawa, K.H. Oshima, P.J. Óhara and J.R. Winton. 1991. Development of a biotinylated DNA probe for detection and identification of infectious hematopoietic necrosis virus. Dis. Aquat. Org., 11, 57~65.

Dixon, P.F. and B.J. Hill. 1984. Rapid detection of fish rhabdoviruses by the enzyme-linked immunosorbent assay (ELISA). Aquaculture 42, 1~12.

González, M.P., X. Sánchez, M.A. Ganga, M. López-Lastra, M. Jashés and A.M. Sandino. 1997. Detection of the infectious hematopoietic necrosis virus derectly from infected fish tissues by dot blot hybridization with a non-radioactive probe. J. Virol. Methods, 65, 273~279.

Grinde, B., T.O. Jonassen and H. Ushijima. 1995. Sensitive detection of group A rotaviruses by immunomagnetic separation and reverse transcription-polymerase chain reaction. J. Virol. Methods, 55, 327~338.

Harlow, E. and D. Lane. 1988. Immunizationa. In Antibodies: A laboratory manual. Cold Spring Harbor Laboratory Press. Plainview, New York, pp. 53~135.

Horton, J.K., O.M. Evans, K. Swann and S. Swinburne. 1989. A new and rapid method for the selection and cloning of antigen-specific hybridomas with magnetic microspheres. J. Immunol. Methods, 124, 225~30.

Hsu, Ya-Li. and J.C. Leong. 1985. A comparison of detection methods for infectious hematopoietic necrosis virus. J. Fish Dis., 8, 1~12.

Hsu, Ya-Li., H.M. Engelking and J.C. Leong. 1986. Occurrence of different types of infectious hematopoietic necrosis virus in fish. Appl. Environ. Microbiol., 52, 1353~1361.

Kapperud, G., T. Vardund, E. Skjerve, E. Hornes and T.E. Michaelsen. 1993. Detection of pathogenic *Yersinia enterocolitica* in foods and water by immunomagnetic separation, nasted polymerase chain reaction, and colorimetric detection of amplified DNA. Appl. Environ. Microbiol., 59, 2938~2944.

Koener, J.F., C.W. Passavant, G. Kurath and J.C. Leong. 1987. Nucleotide sequence of a cDNA clone carrying the glycoprotein gene of infectious hematopoietic necrosis virus, a fish rhabdovirus. J. Virol., 61, 1342~1349.

Kim, S.J., H.K. Oh and T.-J. Choi. 1997. Detection of fish virus by using immunomagnetic separation and polymerization chain reaction (IMS~PCR). J. Korean Fish. Soc., 30, 948~955.

López-Sabater, E.I., M.Y. Deng and D.O. Cliver. 1997. Magnetic immunoseparation PCR assay (MIPA) for detection of hepatitis A virus (HAV) in American oyster (*Crassostrea virglnica*). Lett. Appl. Microbiol., 24, 101~104.

Medina, D.J., P.W. Chang, T.M. Bradley, M.-T. Yeh and E.C. Sadasiv. 1992. Diagnosis of infectious hematopoietic necrosis virus in Atlantic salmon *Salmo salar* by enzyme-linked immunosorbent assay. Dis. Aquat. Org., 13, 147~150.

Park, J.W., J.J. Lee, G. Jeong and Y.C. Hah. 1989. Characterization of the infectious pancreatic necrosis virus (IPNV) isolated from cultured rainbow trout in Korea. Kor. J. Microbiol., 27, 225~230.

Safarik, I., M. Safaříková and S.J. Forsythe. 1995. The application of magnetic separations in applied microbiology. J. Appl. Microbiol., 78, 575~585.

Schütze, H., P.-J. Enzmann, E. Mundt, H. Niemann and T.C. Mettenleiter. 1995. Complete genomic sequence of the fish rhabdovirus infectious hematopoietic necrosis virus. J. Gen. Virol., 76, 2519~2527.