Production and Characterization of Polyclonal Antibody against Recombinant ORFs of Rock Bream Iridovirus (RBIV)

Yun-Im Kim and Sung-Koo Kim†

Rock bream iridovirus (RBIV) is a causative agent of epizootics among cultured rock bream (*Oplegnathus fasciatus*) in Korea. Despite the importance of virus infection in Korean aquacultural industry, researches on vaccines for RBIV have been limited and there are only few studies about antigen analysis of fish iridoviruses. In this study, transmembrane protein genes of the ORF 017L, ORF 049L and ORF 107L and ankyrin repeats gene of ORF 112L from RBIV were tested to develop protein vaccines against RBIV. Genes of RBIV were cloned into expression vector of pGEX-4T-1. The recombinant proteins were successfully expressed using *E. coli* BL21 (DE3). The soluble recombinant RBIV proteins were applied to affinity column for the purification of the proteins. BALB/c mice were immunized by the injection with purified recombinant proteins emulsified with an equal volume of Freund's adjuvant to produce polyclonal antibodies. Western blot was carried out to identify the immune reaction abilities of polyclonal antibodies to recombinant proteins. The effects of vaccines and antigenic properties of these proteins were evaluated by using neutralization test with BF-2 cells. In neutralization test, BF-2 cells infected with the mixture of RBIV and antisera obtained by anti-049L and anti-112L polyclonal antibody were healthy showing few CPE similar with the negative control (without RBIV). These studies suggest that it may be possible to develop an expressed genes-based vaccine and diagnostic kit against RBIV.

Key words : Iridovirus, rock bream, polyclonal antibody, protein vaccine

Introduction

Iridoviruses are recognized as causative agents of serious systemic diseases identified from more than 20 fish species in recent years[1,2]. Outbreaks of iridoviral diseases with high mortalities have been reported in worldwide.

The initial symptoms of the disease were the decrease of feed intake, lethargy and a darkened body with a typical swimming pattern at the edge of cages in the terminal stages of the disease. Characterization of iridoviruses has been hindered because of the difficulty in isolating and propagating the virus in tissue cultures[3,4].

Recently, iridoviral epizootics have occurred frequently among cultured fish in Korea. Iridovirus is a causative agent of epizootics among cultured rock bream (*Oplegnathus fasciatus*) in Korea[5], and the complete genome sequence of RBIV has been reported [6]. Despite the importance of virus infection in Korean aquacultural industry, researches on vaccines for RBIV have been limited and there are only few studies about antigen analysis of fish iridoviruses[7,8].

In this study, transmembrane protein genes of the ORF 017L, ORF 049L and ORF 107L and ankyrin repeats gene of ORF 112L from RBIV were tested to develop protein vaccines against RBIV. The selected genes were cloned into pGEX-4T-1. The effects of vaccines and antigenic properties of these proteins were evaluated by using neutralization test with BF-2 cells[8].

Materials and Methods

Virus sample

Tissue specimens of the spleen, kidney and liver

2007年 6 月18日受付. Received June 18, 2007.

Department of Biotechnology and Bioengineering, Pukyong National University, Busan 608-737, Korea

† Corresponding author: E-mail: skkim@pknu.ac.kr

obtained from the moribund rock bream infected by RBIV were homogenized in 10 volumes of Eagle's minimum essential medium. The tissue homogenate was centrifuged at 2,500×g for 5 min at 4℃. The supernatant was passed through a 0.2μm filter membrane. Permeate was used as a virus stock.

Antigen selection

The genome of RBIV was 112,080 bp long and contained at least 118 putative open reading frames (ORFs). The properties of antigen genes from the RBIV were investigated by using Blast P program (NCBI, http://www.ncbi.nlm.nih.gov/BLAST/) and TMHMM program (TMHMM Server v. 2.0, http://www.cbs.dtu.dk/services/TMHMM-2.0/).

Expression vector construction

The ORF 017L, ORF 049L, ORF 107L and ORF 112L genes were inserted into pGEX-4T-1 (Amersham Biosciences, UK). The recombinant plasmids were named as pGEX-017, pGEX-049, pGEX-107 and pGEX-112 and the host *E. coil* BL21 (DE3) were transformed with the recombinant plasmids.

Expression and purification of recombinant proteins

The expression of the fusion genes were induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The fusion proteins were purified by affinity chromatography according to the manufacturer's instructions. The purified proteins were analyzed by SDS-PAGE and the protein concentration was determined using Bradford assay.

Antibody production

BALB/c mice (4 mice/group, 4 to 5 weeks old) were used for the immunization of purified proteins. At the first immunization, mice inoculated by injection with 100μg of purified fusion proteins, emulsified with an equal volume of FCA. Then, three booster injections to all groups of the mice were given at 2 weeks intervals with the same amount of first immunization with an FIA. At 7 days after final injection, 1 ml of sarcoma cells containing 5×10^6 cells was injected intraperitoneally to all groups of mice[9]. Finally, antisera were obtained by the centrifugation of ascitic fluid in 2,000 rpm for 15 min at 4℃ and stored -80℃ until use.

Western blot analysis

Each expressed proteins were analyzed by SDS-PAGE. The gel was transferred onto a 0.45μm pore nitrocellulose membrane (BioTrace, PALL, USA) for western blot analysis. The blotted membrane was rinsed with TTBS (0.02 M Tris-HCl, 0.5 M NaCl, 0.05% Tween-20, pH 7.5) and then blocked in TBS (0.02 M Tris-HCl, 0.5 M NaCl, pH 7.5) containing 3% (w/v) BSA for 2 h at room temperature and then overnight at 4℃. Then, the membrane was rinsed with TTBS and then cut into several strips according to the sample lane and these strips were put into each polyclonal antisera diluted 1 : 500 in TTBS containing 1% BSA, and incubated for 2 h at room temperature. After three times washed with TTBS, the membrane was treated for 1 h with alkaline phosphatase conjugated anti-mouse IgG (1 : 2000, Santa Cruz Biotechnology, USA) in TTBS containing 1% BSA. Then, the membrane was washed in TTBS and developed by 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT, Sigma, USA) for 1 to 5 min. The developing was stopped by rinsing strips with distilled water[10].

Detection of RBIV using pGEX-049 polyclonal antibody

Virus sample For the detection of RBIV from virus-infected fish, tissue specimens of the spleen kidney and liver obtained from the moribund rock bream infected by RBIV were homogenized in 10 volumes of homogenizing buffer (20 mM Tris (pH 7.5), 1 mM EGTA, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride). The lysate was centrifuged at 1,000×g for 10 min at 4℃ to remove cell wall debris. The supernatant was collected and ultracentrifuged at 100,000×g for 1 h at 4℃. The supernatant (cytosol) was collected and the resulting pellet (crude membranes) was resuspended in homogenizing buffer with 1% Triton X-100 for 1 h at 4℃[11]. Three fractions of membrane extracts from virus-infected rock bream were obtained after centrifugation at 100,000×g for 1 h at 4℃. Top layer with white fraction (IM1), middle layer

with transparent solution (IM2) and bottom layer with turbidity (IM3) were obtained.

Enzyme linked immunosorbent assay (ELISA) The procedure used for the ELISA was as follows. Well plate was coated with quantities ranging from 300 μg to 30 ng of virus protein samples and incubated at room temperature for overnight. Membrane and cytosol proteins from uninfected rock bream and pGEX-049 recombinant protein were also used as negative and positive control. The plate was then rinsed with PBS-Tween and blocked with PBS containing 2 % (w/v) skimmed milk at 37 ℃ for 2 h. After washing with PBS-Tween, 100 $\mu\ell$ of 1 : 200 dilution of culture medium containing anti-RBIV-049L was added to each well. After incubation, plates were rinsed with PBS-Tween, and then 100 $\mu\ell$ of 1 :2000-diluted Horse Radish Peroxidase (HRP)-anti-mouse (Sigma, USA) was added to each well and incubated for 1 h at room temperature. After washing 8 times, the enzyme activity was determined by adding 100 $\mu\ell$ of 3, 3', 5, 5'-Tetramethylbenzidine substrate solution. Optical density was read at 630 nm with an ELISA plate reader after incubating for 30 min.

Dot blot analysis The procedure used for the dot blot analysis was followed by Lu et al.[12]. The nitrocellulose (NC) membrane was mounted onto a dot blot apparatus (model DP-96; Advantec, JAPAN). A serial dilution with phosphate-buffered saline from 300 μg to 3 ng of virus protein samples were spotted onto NC membrane and dried. The NC membrane was incubated in blocking solution (PBS containing 1 % gelatin) for 2 h at room temperature. The membrane was treated with the anti-RBIV-049L (1 :200 in blocking solution) for 2 h at room temperature. The membrane was washed with PBS-Tween, followed by incubation with alkaline phosphatase conjugated anti-mouse IgG for 1 h at room temperature. Then, the membrane was washed three times for 10 min in PBS-Tween and developed by BCIP/NBT for 1 to 2 min. The developing was stopped rinsing strips with distilled water.

Neutralization test

Fish cell line The Bluegill Fry-2 (BF-2) cells were maintained in Eagle's minimum essential medium (EMEM, Sigma, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and antibiotic antimycotic solution. The cells were cultured at 25℃ and the medium was changed daily.

Virus neutralization test in BF-2 cell culture The neutralization test was carried out with 24-well cell culture plates. BF-2 cells were cultured in 24-well plates for 2 days for forming cell monolayers and the antisera were treated at 56℃ for 30 min then 400 μl of the antisera with a log dilution range from 10^0 to 10^3 in EMEM medium were mixed with 400 μl of RBIV stock. For neutralization reaction, the virus-antisera mixture was incubated at 25℃ for 1 h. Then, 200 μl of each mixture was added to BF-2 monolayers and incubated at 25℃ for 1 h. After incubation with the mixture, EMEM containing 5 % of FBS was added to the plates. The plate was incubated at 25℃ for 5 days. The plates were observed under the microscope everyday for the detection of CPE.

Results and Discussion

Expression and purification of recombinant RBIV proteins

Selected genes of RBIV were cloned into expression vector of pGEX-4T-1. As shown in Fig. 1, lane 1 was the band of one cut recombinant DNA digested by one restriction enzyme. Lane 2 shows the separated vector and RBIV selected gene digested by two restriction enzymes. This indicates that selected genes were correctly inserted into expression vector. The recombinant proteins were successfully expressed using *E. coli* BL21 (DE3). The whole cell lysate obtained from the induced *E. coli* strain containing recombinant plasmid DNA contained a soluble fusion protein with the predicted molecular weight. The induced control bacterial extract containing only expression vector did not show the expressed fusion protein band. From the analysis of SDS-PAGE, correct size recombinant proteins were observed as 40 kDa, 42 kDa, 37 kDa and 72 kDa for fusion proteins of ORF 017L, ORF 049L, ORF 107L and ORF 112L as shown in Fig. 2. The soluble recombinant RBIV proteins were applied to GSTrapFF affinity column chromatography. The band of purified fusion proteins showed high purities and correct sizes by SDS-PAGE (Fig. 3).

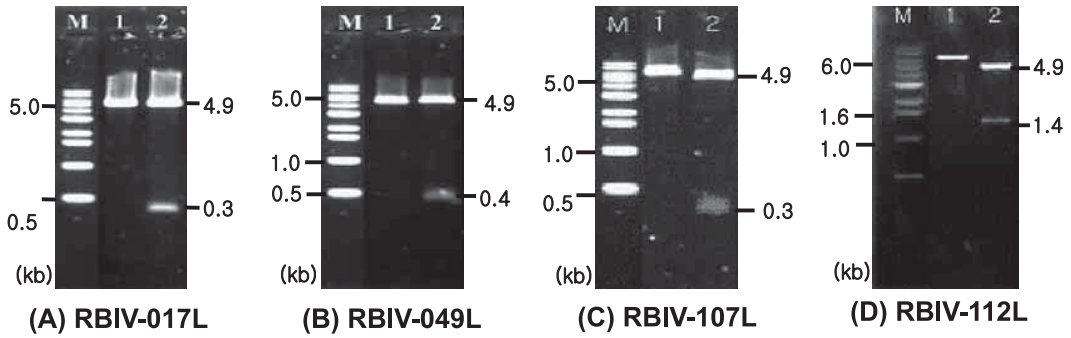

Fig. 1. Cloning of the selected genes of RBIV into expression vector of pET-28a or pGEX-4T-1 M: 1 kb DNA ladder, lane 1 : recombinant DNA was digested with one restriction enzyme (one cut), lane 2 : recombinant DNA was digested with two restriction enzyme (two cut). Each bands are expression vector and separated inserted gene.

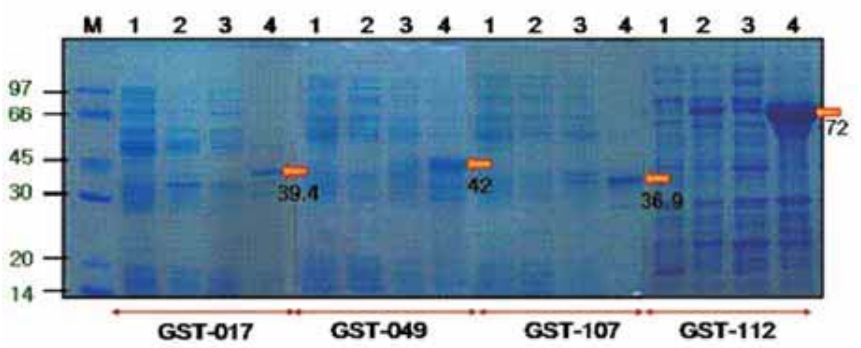

Fig. 2. SDS-PAGE analysis of GST-fusion protein expressed in *E. coli* BL21 (DE3) soluble and insoluble fraction of the cell lysate. M: low molecular weight marker, 1 : non-induction total protein, 2 : induction total protein, 3 : induction soluble fraction, 4 : induction insoluble fraction

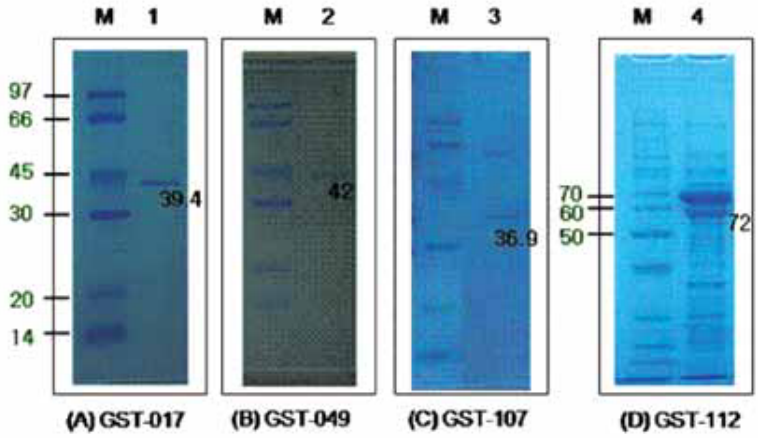

Fig. 3. Purification of GST-fusion protein M: Low molecular marker, 1 : GST-017L, 2 : GST-049L, 3 : GST-107L, 4 : GST-112L

Production and detection of RBIV-specific polyclonal antibody

BALB/c mice were immunized by the injection with purified recombinant proteins emulsified with an equal volume of Freund's adjuvant. Western blot was carried out to identify the reaction abilities of polyclonal antibodies to recombinant proteins.

GST-fusion proteins were separated by SDS-PAGE and recognized by each antiserum. The band of GST-017 was approximately 52 kDa, GST-049 was approximately 52

kDa, GST-107 was approximately 52 kDa and GST-112 was approximately 32 kDa (Fig. 4, lane 1-4), respectively. All polyclonal antibodies successfully detected recombinant proteins.

Detection Sensitivity for RBIV

Detection and isolation of plasma membrane proteins using antibodies have become standard approaches leading to their detailed structural and functional characterization[13]. In addition, in spite of various diagnostic tests for detection of viruses that are available today, PCR, though a very sensitive technique, can lead to false positive results if handling of samples is incorrect[14]. The objective of the present study was to compare dot blot and ELISA to know the sensitivity of these diagnostic techniques to detect RBIV from RBIV-infected rock breams. RBIV in membrane fraction 1 from RBIV-infected rock breams (Fig. 5, lane 7, IM 1) could be detected by dot blot analysis using antiserum to GST-049L as the positive control. The strongest reaction was observed in lane 1 of GST-049L. Strong signals were also observed in Lane 5, 7 and 8 of total protein, membrane fraction 1 and 2 from RBIV-infected rock breams, respectively.

To investigate the specificity and sensitivity of anti-RBIV-049L serum against RBIV, the ELISA assay was performed in total, cytosol and membrane fractions from RBIV-infected rock breams. As shown in Fig. 6, the ELISA using anti-RBIV-049L serum recognized the total protein and membrane fraction 1 from RBIV-infected rock breams.

Immunoblot detection system would be useful to investigate the specificities and sensitivities of antibodies against fish viruses[15].

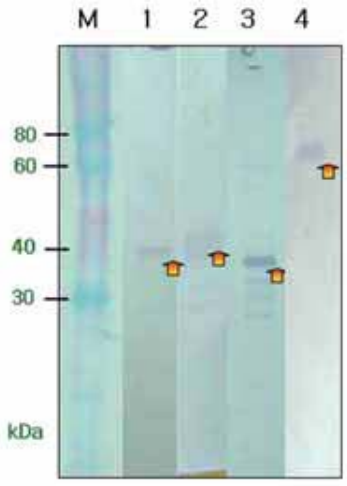

Fig. 4. Polyclonal antibody production were confirmed by western blot. M: 20kDa prestained marker, Expressed proteins of GST-017(1), GST-049(2), GST-107(3) and GST-112(4) reacted with anti-RBIV-017(1), anti-RBIV-049(2), anti-RBIV-107(3) and anti-RBIV-112(4) polyclonal antibody

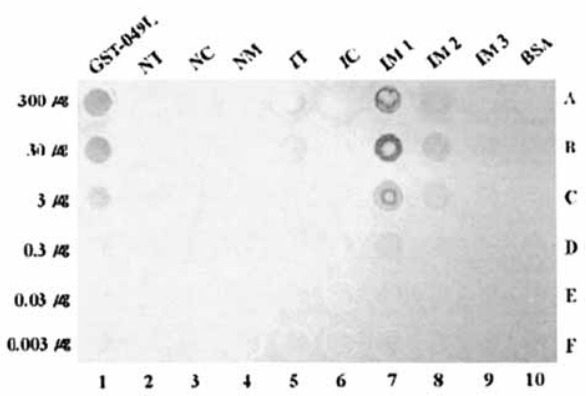

Fig. 5. Sensitivity of the anti-RBIV-049L in dot blot analysis.
lane 1 : positive control, purified protein of GST-049L, lane 2 : total protein of normal (uninfected) rock bream, lane 3 : cytosol of normal rock bream, lane 4 : membrane extracts of normal rock bream, lane 5 : total protein of RBIV-infected rock bream, lane 6 : cytosol of RBIV-infected rock bream, lane 7 : membrane extract 1 (virus band) of RBIV-infected rock bream, lane 8 : membrane extract 2 of RBIV-infected rock bream, lane 9 : membrane extract 3 of RBIV infected rock bream, lane 10 : negative control.

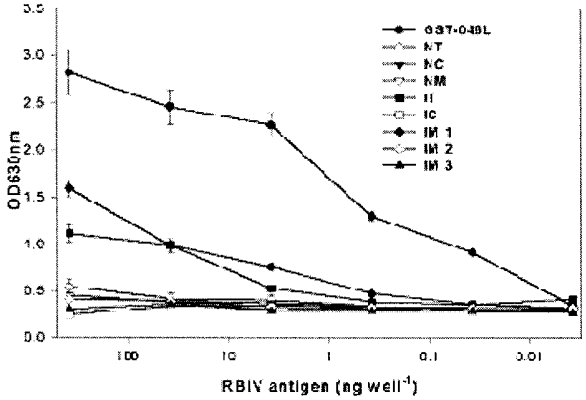

Fig. 6. Detection of RBIV by ELISA from virus-infected rock bream

Virus neutralization test in BF-2 cell culture

Neutralization test was carried out with antisera obtained by mice and rabbit immunized with PBS and GST proteins, respectively. Cytopathic effect (CPE) was detected from BF-2 cells infected by the RBIV in 2 to 3 days of post-infection (Fig. 7, B). All of the infected cells were died in 4 to 5 days of post-infection. BF-2 cells infected with the mixture of RBIV and anti-PBS or anti-GST polyclonal antibody showed CPE in all plates (Fig. 7, C-D). BF-2 cells infected with the mixture of RBIV and anti-RBIV-017L and anti-RBIV-107L polyclonal antibodies also showed CPE (Fig. 7, E, G). Whereas, BF-2 cells infected with the mixture of RBIV and antisera obtained by anti-RBIV-049L and anti-RBIV-112L polyclonal antibodies were healthy showing few CPE (Fig. 7, F, H) similar to the negative control (without RBIV, Fig. 7, A). As above, BF-2 cells infected with the mixture of RBIV and antisera obtained by anti-RBIV-049L and anti-RBIV-112L polyclonal antibodies were able to neutralize cytotoxic activity of RBIV (Fig. 7, F, H).

Conclusions

In this study, transmembrane protein genes of the ORF 017L, ORF 049L and ORF 107L and ankyrin repeats gene of the ORF 112L from RBIV were selected to develop protein vaccines against RBIV. Genes of RBIV were cloned into expression vector of pGEX-4T-1. The recombinant proteins were successfully expressed using *E. coli* BL21 (DE3). The bands of purified fusion proteins showed high purities and correct sizes by SDS-PAGE.

BALB/c mice were immunized by the injection with purified recombinant proteins emulsified with an equal volume of Freund's adjuvant to produce polyclonal antibodies. Western blot was carried out to identify the immune reaction abilities of polyclonal antibodies to recombinant proteins. All polyclonal antibodies successfully detected recombinant proteins.

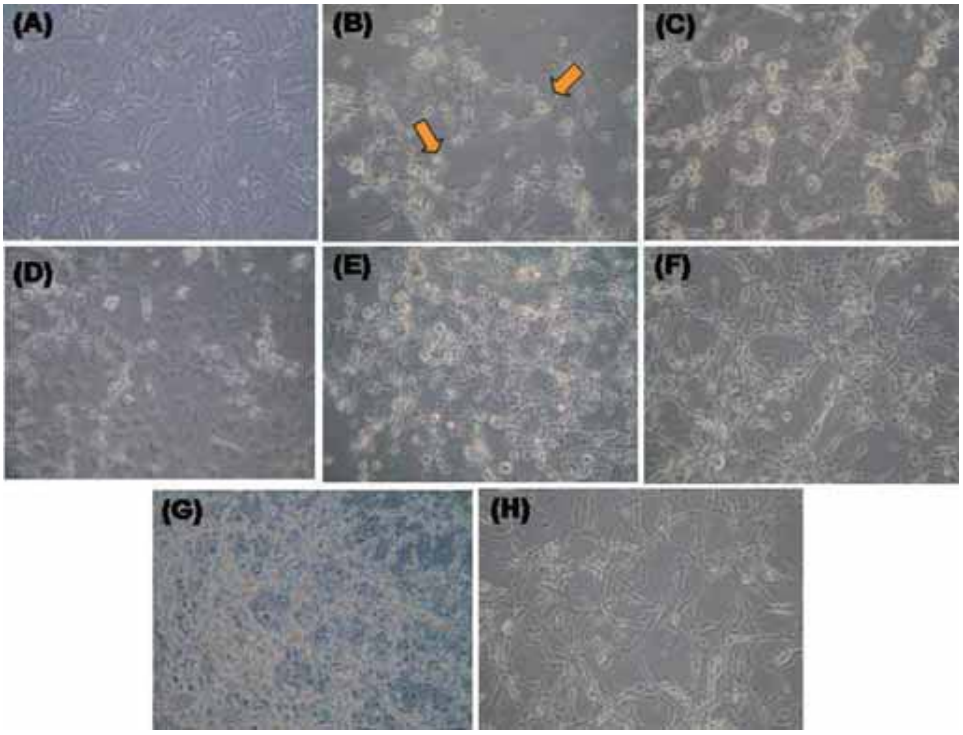

Fig. 7. *In vitro* test of neutralization in BF-2 cell culture (A): cells infected by medium-only as a negative control, (B): cells infected by RBIV-only as a positive control, (C): cells infected with the mixture of RBIV and anti-PBS polyclonal antibody, (D): cells infected with the mixture of RBIV and anti-GST polyclonal antibody, (E): cells infected with the mixture of RBIV and anti-GST-017L polyclonal antibody, (F): cells infected with the mixture of RBIV and anti-GST-049L polyclonal antibody, (G): cells infected with the mixture of RBIV and anti-GST-107L polyclonal antibody, (H): cells infected with the mixture of RBIV and anti-GST-112L polyclonal antibody (dilution of 100, × 200).

Neutralization test was carried out with antisera obtained by mice that were boosted with PBS, GST protein and purified recombinant fusion protein, respectively. Whereas, BF-2 cells infected with the mixture of RBIV and antisera obtained by anti-RBIV-049L and anti-RBIV-112L polyclonal antibodies were healthy showing few CPE similar to the negative control.

These studies suggest that it may be possible to develop an expressed genes-based vaccine and diagnostic kit against RBIV. Further studies are carried out for the evaluation of increasing titer of RBIV-ORF expression for the mass production of antigen as the vaccine.

Acknowledgement

This research was supported by Regional Research Fund from the Ministry of Commerce, Industry and Energy.

References

1) Hyatt AD, Gould AR, Zupanovic Z, Cunningham AA, Hengstberger S, Whittington RJ, Kattenbelt J, Coupar BEH: Comparative studies of piscine and amphibian iridoviruses. *Arch Viol*, **45**, 301-331 (2000)

2) Langdon JS, Humphrey JD, Williams LM, Hyatt AD, Westbury HA: First virus isolation from Australian fish: an iridovirus-like pathogen from redfin perch, *Perca fluviatilis*. *J Fish Dis*, **9**, 263-268 (1986)

3) Chua FHC, Ng ML, Ng KL, Loo JJ, Wee JY: Investigation of outbreaks of a novel disease, 'Sleepy Grouper Disease', affecting the brown-spotted grouper, *Epinephelus tauvina* Forskal. *J Fish Dis*, **17**, 417-427 (1994)

4) Kasornchandra J, Khongpradit R: Isolation and preliminary characterization of a pathogenic iridovirus-like agent in nursing grouper, *Epinephelus malabaricus*. *Asian Aquac Health News*, **45** (1995)

5) Jung SJ, Oh MJ: Iridovirus-like infection associated with high mortalities of striped beakperch, *Oplegnathus fasciatus* (Temminck et Schlegel), in southern coastal areas of the Korean peninsula. *J Fish Dis*, **23**, 223-226 (2000)

6) Do JW, Moon CH, Kim HJ, Ko MS, Kim SB, Son JH, Kim JS, An EJ, Kim MK, Lee SK, Han MS, Cha SJ, Park MS, Park MA, Kim YC, Kim JW, Park JW: Complete genomic DNA sequence of rock bream iridovirus. *Virology*, **325**, 351-363 (2004)

7) Hyatt AD, Gould AR, Zupanovic Z, Cunningham AA, Hengstberger S, Whittington RJ, Kattenbelt J, Coupar BEH: Comparative studies of piscine and amphibian iridoviruses. *Arch Viol*, **145**, 301-331 (2000)

8) Nakajima K, Maeno Y, Yokoyama K, Kaji C, Manabe S: Antigen analysis of red sea bream iridovirus and comparison with other fish iridoviruses. *Fish Pathol*, **33**, 73-78 (1998)

9) Kaufman RJ, Brown PC, Schimke RT: Loss and stabilization of amplified dihydrofolate reductase genes in mouse sarcoma s-180 cell lines. *Mol Cell Biol*, **1**, 1084-1093 (1981)

10) Seo JY: Protective immunogenicity of the G protein of hirame rhabdovirus (HIRRV) in flounder using DNA vaccine. Graduate school, Pukyong Natl. Univ., Korea (2003)

11) Stahelin RV, Ananthanarayanan B, Blatner NR, Singh S, Bruzik KS, Murray D, Cho W: Mechanism of membrane binding of the phospholipase D 1 PX domain. *J Biol Chem*, **279**, 54918-54926 (2004)

12) Lu Y, Tapay LM, Loh PC: Development of a nitrocellulose-enzyme immunoassay for the detection of yellow-head virus from penaeid shrimp. *J Fish Dis*, **19**, 9-13 (1996)

13) Ilangumaran S, Arni S, Chicheportiche Y, Briol A, Hoessli DC: Evaluation by Dot-Immunoassay of the Differential Distribution of Cell Surface and Intracellular Proteins in Glycosylphosphatidylinositol-Rich Plasma Membrane Domains. *Anal Biochem*, **235**, 49-56 (1996)

14) Shekhar MS, Azad IS, Ravichandran P: Comparison of dot blot and PCR diagnostic techniques for detection of white spot syndrome virus in different tissues of *Penaeus monodon*. *Aquaculture*, **261**, 1122-1127 (2006)

15) Choi SK, Kwon SR, Nam YK, Kim SK, Kim KH: Organ distribution of red sea bream iridovirus (RSIV) DNA in asymptomatic yearling and fingerling rock bream (*Oplegnathus fasciatus*) and effects of water temperature on transition of RSIV into acute phase. *Aquaculture*, **256**, 23-26 (2006)