

## Immunological significance of recombinant VP2 and VP3 proteins of aquabirnavirus in olive flounder, *Paralichthys olivaceus*

Hyoung Jun Kim\*, Ki Hong Kim\*\*, Jeong Su Park\*\*\*, Haeng Lim Lee\*\*\*, Hyuk Chu Kwon\*\*\* and Se Ryun Kwon†

\*National Fishery Products Quality Management Services, Busan 606-080, Korea

\*\*Department of Aquatic Life Medicine, Pukyong National University, Busan 608-737, Korea

\*\*\*Department of Aquatic Life Medical Sciences, Sunmoon University, Asan-si, Chungcheongnam 336-708, Korea

Full-length recombinant VP2 and VP3 proteins of aquabirnavirus isolated from olive flounder were expressed successfully in *E. coli* expression system. After rats were immunized with these proteins, antisera were used for *in vitro* and *in vivo* neutralization test. In *in vitro* test, VP2 antibody titers were higher than that of VP3. In *in vivo* assays, fish challenged with aquabirnavirus neutralized with VP2 antibody survived longer than other fish.

**Key words:** Aquabirnavirus, Recombinant VP2, Recombinant VP3, Neutralization

Aquabirnavirus is a member of the Birnaviridae family. The birnavirus virions are consists of an unenveloped, icosahedral capsid and a bisegmented, double-stranded RNA (dsRNA) genome (Dobos & Roberts, 1983). The genome segment B encodes an internal polypeptide VP1, the putative virion-associated RNA-dependent RNA polymerase. Genome segment A contains 2 partially overlapping open reading frames (ORF) (Duncan *et al.*, 1987). The larger ORF encodes a polyprotein that is cleaved by mature viral proteins VP2, VP3 and VP4. VP2 and VP3 are the major structural proteins of the virion. VP2 is the major outer capsid protein of the virion, whereas VP3 is through to be an internal virion protein. Also, VP3 is two small polypeptides (VP3; 31kDa, VP3a; 29 kDa) (Chang *et al.*, 1978). VP4 is nonstructural (NS) polypeptide, cleaved at the VP2-NS and NS-VP3 junctions by the protease activity. All the epitopes

recognized by neutralizing monoclonal antibodies are located on VP2 (Christie *et al.*, 1988; Tarrab *et al.*, 1995). VP3 is second major structural protein, also neutralization epitopes have been suggested (Tarab *et al.*, 1995).

In vaccination study, commercially available IPNV vaccines for the salmon industry are presently based on either inactivated cell culture-propagated virus, or *Escherichia coli*-expressed structural virus proteins such as immunogens (Christie, 1997). Moreover, Moon *et al.* (2004) reported that the recombinant truncated fragment of VP2 protein and recombinant VP3 protein induced anti-sera in fish and neutralized the birnavirus infection, although they failed to over-express recombinant protein of full-length VP2 of MABV.

The aim of the present study was to elucidate the immunological significance of VP2 and VP3 proteins of aquabirnavirus. For this purpose, antisera against recombinant proteins of full-length VP2 and VP3 were produced, and analyzed the strength of each antiserum in neutralization of aquabirnavirus *in vitro*

†Corresponding author: Se Ryun Kwon  
Tel: 82-41-530-2289; Fax: 82-41-530-2917  
E-mail: srkwon@sunmoon.ac.kr

and *in vivo*.

## Materials and Methods

### Virus

Aquabirnavirus isolated from olive flounder (*Paralichthys olivaceus*) was kindly provided by Prof. M.-J. Oh (Chonnam National University). The chinook salmon embryo cell line (CHSE-214) was used for virus propagation. Cells were grown at 20°C in minimal essential medium (MEM; Sigma) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 IU/ml penicillin (Gibco), and 100 µg/ml streptomycin (Gibco). The virus was harvested when the cytopathic effect was complete and quantified by titration against CHSE-214 cells in 96-well microtitre plates in tissue culture infective doses of 50% (TCID<sub>50</sub>).

### RNA extraction and cDNA synthesis

Total RNA was extracted from spleen and kidney using TRIzol reagent (Invitrogen), according to the manufacturer's recommendations. Concentration and purity of the extracted RNA was measured spectrophotometrically at 260 and 280 nm. cDNA was obtained by reverse transcription by incubating 1 µg of total RNA preparation with 1 µl of random hexamer primers (1.25 mM random primer; Promega) and 8.5 µl of nuclease-free water at 70°C for 5 min, followed by cooling on ice for 5 min. The sample was brought to a final volume of 20 µl with a reverse transcription mixture consisting of 4 µl of reverse transcriptase (RT) buffer, 1 µl of reverse transcriptase (Promega), 0.5 µl of RNasin (Promega), and 4 µl of 2.5mM dNTP mix. The cDNA was synthesized at 42°C for

1 h, and the reaction was terminated by heating to 95°C for 10 min.

### Polymerase chain reaction (PCR) and sequencing

The reaction primer sets were shown in Table 1. The primer sets were contained an restriction enzyme site (underlined). The synthesized cDNA was used in a 20 µl of PCR reaction containing 10 pmol of each primer, TE buffer, and 0.5 U of Taq DNA polymerase (TaKaRa). PCR was performed with 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min using an automated thermal cycler (iCycler, BioRad). The PCR products were separate on 0.7% agarose gel and stained with ethidium bromide. The amplified product was purified using spin column (Nucleogen) and cloned into pGEM-T easy vector (Promega). The recombinant clones of correct size were selected, and the inserts were sequenced using ABI377 automatic sequencer (Applied Biosystems).

### Expression of recombinant VP2 and VP3 proteins

pGEM-T easy vector harboring VP2 and VP3 were double-digested with *Sal* I and *Not* I) and inserted into *Sal* I-*Not* I digested pET28a vector (Novagen). The recombinant plasmids were prepared by transforming *Escherichia coli* BL21 (DE3) competent cells.

The expression of recombinant VP2 and VP3 was then induced by 1mM IPTG (isopropyl-β-D-thiogalactoside). The insoluble VP2 protein was dissolved using denaturation buffer (8M urea), and soluble VP3

Table 1. Specific primers for construction of recombinant protein expression vector

Gene	Sequence	Enzyme	Size (bp)
VP2	F 5' - <u>GTCGACATGAACACAACAAAGGCAACCG</u> - 3'	<i>Sal</i> I	1,362
	R 5' - <u>GCGGCCGCTTTTGTAGGTTGG</u> - 3'	<i>Not</i> I	
VP3	F 5' - <u>GTCGACAATCAGGATGGACGAAGAGCT</u> - 3'	<i>Sal</i> I	726
	R 5' - <u>GCGGCCGCTTACACTTCTCCGTTGTC</u> - 3'	<i>Not</i> I	

protein was dissolved using binding buffer (5mM imidazole), subsequently purified using a Ni-NTA His-Bind<sup>®</sup> Resin (Novagen, USA) open column. and then refolded in binding buffer (5mM imidazole). The concentration of the purified proteins were determined using the BCA protein assay (Sigma) and then analyzed by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

#### Production of rat polyclonal antibody against recombinant VP2 or VP3 protein

Specific-pathogen-free male Wistar rats were selected for this immunization experiment and they were 4 weeks old at the time of immunization. At the first immunization, three rats inoculated by intraperitoneal injection with 120 µg of purified recombinant VP2 or VP3 proteins, emulsified with an equal volume of complete Freund's adjuvant (FCA, Sigma). Three rats of control group 1 inoculated by intraperitoneal injection with 100 µl of PBS. Three rats of control group 2 inoculated by intraperitoneal injection with complete Freund's adjuvant (FCA, Sigma). On 2 weeks post-first immunization, boosting was performed 120 µg of recombinant proteins, emulsified with an equal volume of incomplete Freund's adjuvant (IFA, Sigma). On 4 weeks post-first immunization, all rats in the immunization experiments described above were bled to obtain serum. These sera were stored -80°C until analysis.

#### Western blotting

Electrophoretically separated proteins were electro-blotted onto nitrocellulose membrane (Pall Corporation). For immunodetection, the filter was blocked in 3% bovine serum albumin (BSA, Sigma) in TTBS (0.05% Tween-20, 10 mM Tris-HCL, 150 mM NaCl, pH7.5) at 4°C for 16 h, followed by incubating with 1 : 500 rat antisera against recombinant proteins for 90 min at room temperature. After three washes with TTBS, the filter was incubated with 1 : 2,000 of goat anti-rat IgG alkaline phosphate conjugates for 1 h

(SantaCruz). The filter was washed with TTBS and incubated with substrate NBT (nitroblue tetrazolium) and BCIP (5-bromo-4-chloro-3-indoyl-phosphate) for color development at room temperature.

#### *In vitro* neutralization test

Rat antisera were previously heat-inactivated (56°C for 30 min) for neutralization test. In brief, the anti-serum were serially diluted in two-fold steps with MEM in 96-well plates. A virus preparation 10<sup>2</sup> TCID 50/50 µl was added to each well. Plates were incubated at room temperature for 1 h with continuous shaking on rocker. To each well 100 µl of CHSE-214 cells at 1×10<sup>5</sup> cells/ml was added and the plates were further incubated at 20 °C for six days. The neutralizing antibody titer of the antisera was expressed as the reciprocal of the highest dilution of antisera protecting 50% of the cells inoculated.

#### *In vivo* neutralization test

The complement of anti-sera were previously heat-inactivated (56°C for 30 min) for neutralization test. Each anti-sera 50 µl were mixed with equal volume of virus solution in 1.5 ml E-tube (10<sup>5</sup> TCID 50/50 µl). The tubes were incubated at room temperature for 2 h with continuous shaking on rocker. Used fish were 7-8 cm rock bream. Before challenge, fish were divided into 4 groups of 5 fish. Each group were consist of anti-VP2, anti-VP3, anti-FCA and only virus. Infection were intraperitoneally injected with 100 µl reaction mixture. The injected fish were maintained at 17-18°C and daily dead fish sampled.

## Results

#### Production of recombinant VP2 and VP3 proteins

The recombinant VP2 and VP3 proteins were successfully expressed using *E. coli* BL21(DE3). The recombinant proteins were purified as His-tagged proteins and were analyzed using SDS-PAGE (Fig. 1).

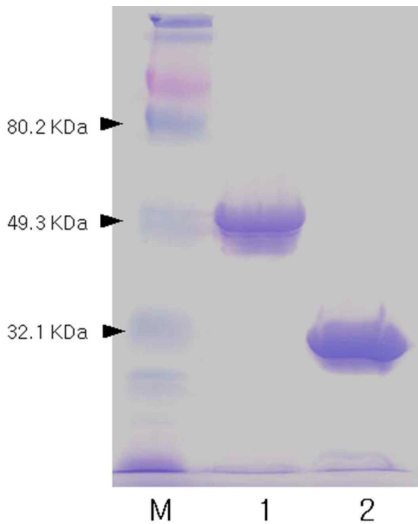


Fig. 1. SDS-PAGE analysis for purification of induced VP2 and VP3. M: protein molecular marker, Lane 1: Recombinant VP2 protein is approximately 52 kDa, Lane 2: Recombinant VP3 protein is approximately 30 kDa.

The molecular weights of the VP2 and VP3 were approximately 52 kDa and 30 kDa, respectively.

Aquabirnavirus cell culture supernatant and virus-free cell culture supernatant were separated by SDS-PAGE. VP2 and VP3 proteins of virula fluids were recognized by each antiserum (Fig. 2). The band

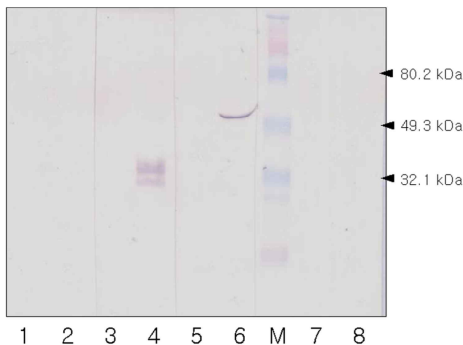


Fig. 2. Antigen detection using western blotting. Lane 1, 3, 5, 7 : virus-free cell culture supernatant and Lane 2, 4, 6, 8 : aquabirnavirus cell culture supernatant. M, protein molecular marker; Lane 1 and 2, anti-FCA serum; Lane 3 and 4, anti-VP3 serum; Lane 5 and 6, anti-VP2 serum; Lane 7 and 8, anti-PBS serum.

of VP2 was approximately 52 kDa. VP3 was detected as 2 bands, approximately 30 and 32 kDa. Any bands were not detected in the virus-free cell culture supernatant.

#### *In vitro* neutralization test

The antisera against VP2 and VP3 showed significantly higher *in vitro* neutralization antibody titers than the control serum. The titer of VP2 antiserum was higher than that of VP3 antiserum (Fig. 3).

#### *In vivo* neutralization test

Although all experimental groups showed 100% mortality by artificial challenge with aquabirnavirus, VP2 group showed longer time to 100% death than VP3 and control groups (Fig. 4). During 14-day observation period fish injected with neutralized aquabirnavirus with VP2 antiserum had significantly lower mortality than other fish by RPS value of 40%.

## Discussion

In the present study, full-length recombinant VP2 and VP3 proteins were expressed successfully using *E. coli* expression system. Moon *et al.* (2004) reported that truncated fragment of VP2 protein was used to

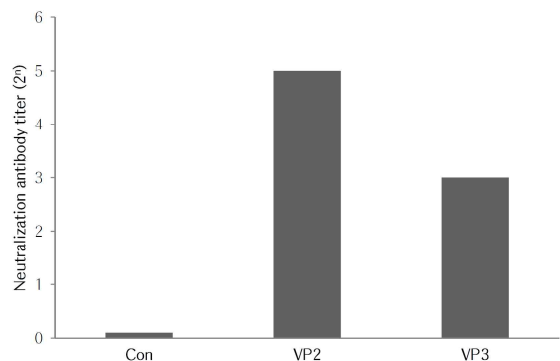


Fig. 3. *In vitro* neutralization antibody tests against aquabirnavirus. Neutralization effect in the sera from rat immunized with recombinant VP2 and VP3 proteins. Control was used serum from rat immunized with only FCA.

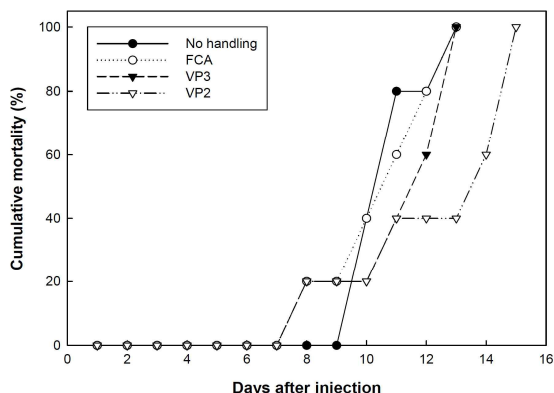


Fig. 4. *In vivo* (rock bream) neutralization effect in the sera from rat immunized with recombinant FCA, VP2 and VP3 proteins. Only virus injection group is positive control.

produce antiserum in fish because of failure in production of recombinant full-length VP2 of MABV. Although we could not know the cause of their failure, in this study, we achieved over-expression of full-length recombinant VP2 protein (52 kDa) and recombinant VP3 protein (30 kDa) of aquabirnavirus. In the present western blot analysis, antiserum against recombinant VP2 protein recognized a 52 kDa viral capsid protein, but antiserum against recombinant VP3 protein recognized 2 polypeptides (30 and 32 kDa). Dobos and Rowe (1977) indicated that VP3 of IPNV is comprised of two small polypeptides (VP3 & VP3a) and divided during virus maturation. We firstly described in this study that VP3 of aquabirnavirus consists of two polypeptides (30 and 32 kDa) as in IPNV.

Christie *et al.* (1988) reported that neutralizing monoclonal antibodies could recognize all the epitopes located on VP2. Nagy and Dobos (1987) have prepared monoclonal antibodies against IPNV and reported that all the neutralizing monoclonal antibodies developed were VP2 specific. VP3 is second major structural protein, also neutralization epitopes have been suggested (Tarab *et al.*, 1995). In the present results, antiserum against VP2 protein showed higher *in vitro* and *in vivo* neutralization titer than antiserum

against VP3 protein. Moon *et al.* (2004) suggested that VP3 protein is more immunogenic than VP2 protein in artificially epitopes exposed conditions.

In our preliminary experiments, in contrast to olive flounder, which showed no symptoms by artificial challenge with aquabirnavirus, rock bream was very susceptible to artificial challenge with aquabirnavirus isolated from olive flounder (Kim *et al.*, 2007). Therefore, rock bream fingerlings were used as an experimental fish for aquabirnavirus challenge in this study. As the results *in vivo* neutralization experiment, fish group injected with aquabirnavirus which was neutralized with recombinant VP2 protein showed higher survival rate than other fish groups during 14-day observation period (Fig. 4). These results suggested that VP2 proteins of aquabirnavirus has epitopes which can induce protective antibodies.

## References

- Chang, N., Macdonald, R. D., and Yamamoto, T. (1978). Purification of infectious pancreatic necrosis (IPN) virus and comparison of peptide composition of different isolates. *Can. J. Microbiol.* 24: 19-27.
- Christie, K. E. (1997). Immunization with viral antigens: infectious pancreatic necrosis. *J. Gen. Virol.* 76: 1165-1172.
- Christie, K. E., Havarstein, L. S., Djupvik, H. O., Ness, S., and Endresen, C. (1988). Characterization of a new serotype of infectious pancreatic necrosis virus isolated from Atlantic salmon. *Arch. Virol.* 103: 167-177.
- Dobos, P., and Roberts, T. E. (1983). The molecular biology of infectious pancreatic necrosis virus: a review. *Can. J. Microbiol.* 29: 377-384.
- Dobos, P., and Rowe, D. (1977). Peptide map comparison of infectious pancreatic necrosis virus-sepicific polypeptides. *J. Virol.* 24: 805-820.
- Duncan, R., Nagy, E., Krell, P. J., and Dobos, P. (1987). Synthesis of the infectious pancreatic necrosis virus polyprotein, detection of a virus-encoded protease, and the fine structure mapping of the genome segment A coding regions. *J. Virol.* 61(12): 3655-3664.
- Kim, H. J., Kwon, S. R., Lee, E. H., Nam, Y. K., Kim, S. K., and Kim, K. H. (2007). Pathogenicity of ma-

rine birnavirus (MABV) on fingerlings of rock bream (*Oplegnathus fasciatus*). *Aquaculture*. 272: 762-766.

Moon, C. H., Do, J. W., Cha, S. J., Bang, J. D., Park, M. A., Yoo, D. J., Lee, J. M., Kim, H. G., Chung, D. K., and Park, J. W. (2004). Comparison of the immunogenicity of recombinant VP2 and VP3 of infectious pancreatic necrosis virus and marine birnavirus. *Arch. Virol.* 149: 2059-2068.

Tarrab, E., Berthiaume, L., Grothe, S., O'Connor-McCourt, M., Heppell, J., and Lecomte, J. (1995). Evidence of a major neutralizable conformational epitope region on VP2 of infectious pancreatic necrosis virus. *J. Gen. Virol.* 76: 551-558.

---

Manuscript Received : Dec 5, 2014

Revised : Aug 17, 2015

Accepted : Aug 17, 2015