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Production of monoclonal antibody against ORF72 of koi herpesvirus isolated in Taiwan

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Abstract A monoclonal antibody (MAb) was generated against the capsid protein (ORF 72) of koi herpesvirus (KHV) isolated from diseased koi *Cyprinus carpio* in Taiwan. The clone of MAb-B2 was obtained by immunizing mice with whole virus particles and further identified using indirect enzyme-linked immunosorbent assay and Western blot assay. In addition, it detected KHV in KHV-infected cells but not in those of mock-infected cells as demonstrated by indirect immunofluorescence assay. The neutralization test showed that MAb-B2 neutralized KHV. Furthermore, we uncovered that MAb-B2 recognizes the ORF72 of KHV as revealed by liquid chromatography–tandem mass spectrometry and Western blot assays. Additionally, MAb-B2 has been used as a diagnostic tool for detection of KHV in clinical samples by immunohistochemistry. Collectively, our results indicated that MAb-B2 could be used in the development of a diagnostic kit for diagnosis of KHV infections and ORF72 protein of KHV might be a candidate for future vaccine development.

Abbreviations

MAb	Monoclonal antibody
KHV	Koi herpesvirus
ELISA	Enzyme-linked immunosorbent assay
CyHV-3	Cyprinid herpesvirus-3
IFA	Indirect immunofluorescence assay
IHC	Immunohistochemistry
CCB	Common carp brain cell
CPE	Cytopathic effect

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Introduction

KHV, also known as cyprinid herpesvirus-3 (CyHV-3), is a fatal pathogen which causes mass mortality in juvenile and adult carp. Thus, a rapid and simple diagnostic tool is needed in the koi industry. Koi herpesvirus (KHV) infections were first reported in Israel and USA in 1998 (Hedrick et al. 2000) and spread worldwide rapidly through fish trade and exhibitions (Neukirch et al. 1999; Oh et al. 2001; Grimmett et al. 2006; Dong et al. 2011). Since KHV was first detected in northern Taiwan in 2003 (Tu et al. 2004), it has spread to koi and common carp and become enzootic in fish farms from 2003 through 2005 (Cheng et al. 2011). The sporadic outbreaks usually occur after introduction of newly-bought fish from other farms. To solve this recurrent problem, we encourage fish farmers to establish their own self-defense system in addition to receiving periodic KHV surveillance as part of a government program. The monoclonal antibody for KHV is required for developing an easily-performed and time-saving chromatographic strip assay for farmers' KHV self-detection in Taiwan.

To date, three groups have developed antibodies against ORF 81, ORF92, ORF136, and ORF 68 of KHV. In these groups, they used the recombinant antigenic proteins of KHV to prepare polyclonal and monoclonal antibody (MAb), respectively (Rosenkranz et al. 2008; Dong et al. 2011; Aoki et al. 2011).

In this report, we have prepared a neutralizing MAb-B2 against ORF72 of KHV by immunizing mice with whole

virus particles purified from cell cultures. MAb-B2 was verified by Western blot and indirect immunofluorescence assays (IFA) in KHV-infected cell cultures. The prepared MAb-B2 has been used as a diagnostic tool for immunohistochemical detection in KHV-infected tissues. To our knowledge, MAb-B2 is the first reported protective neutralizing antibody induced by the structural protein of KHV. The ORF72 protein of KHV might be a candidate for future vaccine development.

Materials and methods

Virus preparation for immunization KHV isolate was first isolated in a laboratory-developed koi fin cell (unpublished data) in 2008, then adapted and propagated in common carp brain (CCB) cell line (a kind gift from Dr. Roland Riebe), which were cultured with minimum essential medium (Earle's salts) containing 1 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 10 % fetal calf serum. The 175 cm^2 flask of confluent CCB cell was infected with 1×10^5 median tissue culture infective dose ($TCID_{50}$) per milliliter of KHV at 25 °C. Ten days post-infection, the infected cell lines were frozen and thawed three times after 80 % of them showed cytopathic effect (CPE) with typical vacuoles in cytoplasm. Thirty 175-cm^2 flasks of cells were harvested and centrifuged at 10,000×*g* for 10 min at 4 °C to remove cell debris. The supernatants were further centrifuged at 100,000×*g* at 4 °C for 50 min (Hitachi P45AT rotor) and pellets were resuspended and centrifuged at 110,000×*g* at 4 °C for 60 min (Hitachi P40ST rotor) in a discontinuous 20–60 % (*w*/*v*) sucrose gradient prepared in phosphate buffered saline (PBS). The visualized bands were aspirated with needles and syringes, diluted in tenfold PBS, and repelleted at 100,000×*g* for 50 min. The aliquots of PBS-suspended viral pellet were frozen at −80 °C.

Electron microscope examination The diluted purified virions were centrifuged at 16,000×*g* for 10 min by Airfuge (Beckman, USA) and resuspended pellets were stained with an equal volume of 2 % phosphotungstic acid for 2 min. The stained virions were absorbed to the grids (Agar, UK) and observed using a transmission electron microscope (JEOL JEM-1400, Japan) at 100 kV.

Immunization of mice and screening for MAb against KHV Injected intraperitoneally into Balb/c mice (6–8 weeks old) was 0.05 mL (50 μg) of purified virus mixed with an equal volume of Freund's complete adjuvant; the same volume of KHV mixed with Freund's incomplete adjuvant was used as booster shots at 7- to 8-day intervals twice. Two weeks later, the mice were boosted with a fourth injection of 50 μg purified virus without adjuvant via tail vein injection. After 4 days of the fourth injection, each mouse was bled and blood samples were tested using enzyme-linked immunosorbent assay (ELISA) in a 96-well ELISA plate coating with 1 ng of purified KHV per well as previously described (Aoki et al. 2011). When the titer of the immunized mouse had reached or was higher than 10,000-fold, the mouse was euthanized and its spleen cells were fused with Sp2/o tumor cells with 38 % polyethelene glycol 1,500, and centrifuged at 1,200×*g* for 10 min. The hybridomas were resuspended in Iscove's modified Dulbecco's medium (IMDM)/20 % fetal calf serum (F20) and plated out in six 96-well plates. After 10 days, the hybridomas which were selected using hypoxanthin, aminopterin, and thimidine medium were then fed with hypoxanthin and thimidine medium. On day 13, the medium was changed to IMDM/F20. The antibody-secreting hybridomas were screened by ELISA, identified by immunofluorescence assay, and cloned by limiting dilution on a feeder layer of thymus cells. The production of ascitic fluid of hybridoma were done as previously described (Johnstone and Thorpe 1987)

Western blot assay The purified virus sample was diluted at 1:5 with reducing sample buffer (2 % (*w*/*v*) sodium dodecylsulphate (SDS), 0.05 % (*v*/*v*) β-mercaptoethanol, 0.01 % bromophenol blue, 0.0625 mmol/L Tris–HCl, pH 6.8), treated at 95 °C for 5 min, and analyzed with 12 % SDS polyacrylamide gel electrophoresis (PAGE). The purified viral samples were loaded onto and separated by 12 % SDS-PAGE. The gel was run at 100 V for 1 h and one part stained with silver staining kit (Invitrogen, USA) and the other transferred onto a nitrocellulose sheet for 1 h at 0.45 V using a transfer buffer (0.192 mol/L glycine, 0.025 mol/L Tris). After blotting, the transfer sheet was washed in PBS containing 0.05 % Tween 20 (PBST) for 10 min and blocked with 5 % skim milk in PBST at 37 °C for 1 h. After PBST washing, the sheet was incubated with hybridoma-derived ascetic fluid of MAb-B 210,000× diluted in PBST containing 5 % skim milk at 37 °C for 1 h, washed three times with PBST, and then incubated at 37 °C for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, USA) 100,000× diluted in PBST containing 5 % skim milk. The sheet was washed three times with PBST and soaked with Amersham Select Western blotting detection ECL reagent (GM Healthcare, USA) and detected with chemiluminescence system (Vilber Lourmat, France).

Indirect immunofluorescence assay The assay was employed in CCB cells growing on glass cover slips in 24-well plates (Corning, USA; Aoki et al. 2011). Briefly, $2–4\times10^5$ cells mL^{-1} were added to each well of the 24-well plate. After 18–24 h, the cells were inoculated with KHV at $2–4\times10^4$ $TCID_{50}mL^{-1}$. The glass cover slips were removed at 3, 5,

7, 10 days post-infection, fixed in iced 80 % acetone in distilled water for 10 min, air dried, incubated with hybridoma cell supernatant of MAb-B2 diluted 1:5 for 1 h at 37 °C, and washed with PBS twice. The cover slips were incubated with fluorescein isothiocyanate-labeled goat anti-mouse IgG serum (KPL, USA) diluted at 1:1,000 for 1 h at 37 °C and washed with PBS twice. The cover slips were covered with 50 % glycerol/50 % PBS and observed with a fluorescent microscope.

Identification of MAb-reacted protein by 1D gel/nanoLC-MS/MS Nano-liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis was performed on an integrated nanoLC-MS/MS system (QSTAR XL) comprising a LC Packings NanoLC system with an autosampler and a QSTAR XL Q-Tof mass spectrometer (Applied Biosystems) fitted with a nano-LC sprayer. After electrophoresis, the stained band in SDS-PAGE corresponding to that detected by MAb was cut, washed, digested with a sequencing-grade trypsin, and extracted. The prepared samples were first trapped and desalted on a LC-Packings PepMap™ C18 μ-Precolumn™ Cartridge (5 μm, 30 μm I.D.×5 mm; Dionex, Sunnyvale, CA, USA). After that, the peptides were eluted off the precolumn and separated on an analytical LC-Packings PepMap C18 column (3 μm, 150 mm×75 μm I.D.) connected inline to the mass spectrometer at 200 nL-min^{-1} using a 40-min gradient of 5–60 % acetonitrile in 0.1 % formic acid.

Online nano-electrospray ionization mass spectrometry (ESI-MS) survey scan and data-dependent acquisition of CID MS/MS were fully automated and synchronized with the nanoLC running under the full software control of Analyst QS. Prior to online analysis, the nanoLC sprayer source parameters were tuned and optimized. Argon was used as the collision gas for CID MS/MS. Calibration was done using the product ions generated from fragmentation of the doubly charged molecular ion of renin.

For routine protein identification analysis, 1-s survey scans were acquired over the mass range *m/z* 400–1,600 and a maximum of two concurrent MS/MS acquisitions were triggered for 2+, 3+, and 4+ charged precursors detected at an intensity above the predefined threshold. Each MS/MS acquisition would be completed and switched back to the survey scan when the precursor intensity felt below a predefined threshold or after a maximum of a 6-s acquisition.

After data acquisition, the individual MS/MS spectra acquired for each of the precursors within a single LC run were combined and the output was a single Mascot-searchable peak list file. The peak list files were used to query the Swiss-Prot database using the Mascot program with the following parameters: peptide mass tolerance, 150 ppm; MS/MS ion mass tolerance, 0.15 Da; and allowing up to one missed cleavage. Only significant hits defined by Mascot probability analysis will be considered initially. In addition, a minimum total score of 20, comprising of at least a peptide match of ion score more than 20, was arbitrarily set as the threshold for acceptance.

Neutralizing test with MAb-B2 The 175 cm^2 of confluent koi gill (KoG) cells (unpublished data) were digested and diluted to 88 mL with minimum essential medium containing 1-mmol/L HEPES and 10 % fetal calf serum with antibiotics. One milliliter of the mixture was added into each of the 24-well culture plates. Ascitic fluid secreted by hybridoma cell of MAb-B2 was diluted 1:2 and 1:10 in L-15 medium. A $1\times 10^{4.7}$ $TCID_{50}mL^{-1}$ of KHV was diluted from 10^{-1} to 10^{-4}. The 0.5 mL of MAb was mixed with 0.5 mL of each of the virus dilutions and incubated for 2 h at 25 °C. Of the mixture, 0.2 mL was added into duplicate wells KoG cells on 24-well plates and shaken slowly for 1 h at 25 °C. Then, the cultures were overlaid with 1 mL of 1.5 % methylcellulose in 2× L-15 medium and incubated for 2–7 days at 25 °C. When CPEs were evident, the overlays were removed and the cells were stained with crystal violet solution (0.1 % crystal violet, 9.5 % ethanol, and 18 % formaldehyde) for 5 min. The plaques in the wells were counted. The neutralization index (NI) was calculated as follows (Rovozzo and Burke 1973). NI indicates the difference between the $\log_{10}$ number of plaques found on control wells and the $\log_{10}$ numbers of plaques found with the neutralizing antibody. NI greater than 1.7 is significant.

Immunohistochemistry Tissues from a clinically KHV-infected fish were confirmed by PCR (Bercovier et al. 2005) and tissues from a healthy fish were used for immunohistochemistry (IHC) detection. The 10 % neutral buffered formalin-fixed tissues were trimmed; dehydrated in 75, 80, 90, and 95 % ethanol each for 1 h, then 100 % ethanol for 1 h three times; immersed in 100 % xylene 1 h twice; embedded in molten paraffin for 2 h; and cut into 4–6 μm sections. Sections were floated in a 40 °C water bath and collected onto silane-coated slides; incubated in 60 °C for 30 min; deparaffined in xylene; rehydrated in 100, 95, and 70 % ethanol each for 5 min; and rinsed in distilled water. The prepared sections were digested in ProTaqs Pepsin Digest (ProTaqs, Germany) for 25 min, washed three times with PBS, incubated with 3 % hydrogen peroxide for 20 min, washed with PBS three times, incubated with goat serum (diluted 1:10 in PBS) for 10 min, incubated with the MAb-B2 for 30 min at room temperature, and washed three times with PBS. For the secondary antibody, sections were incubated with HRP goat-anti mouse IgG conjugate (1:100 dilution, Jackson Immuno Research Laboratories, USA) for 30 min, washed three times with PBS, rinsed with distilled water, incubated with AEC buffer (5 mg 3-amino-9-ethyl

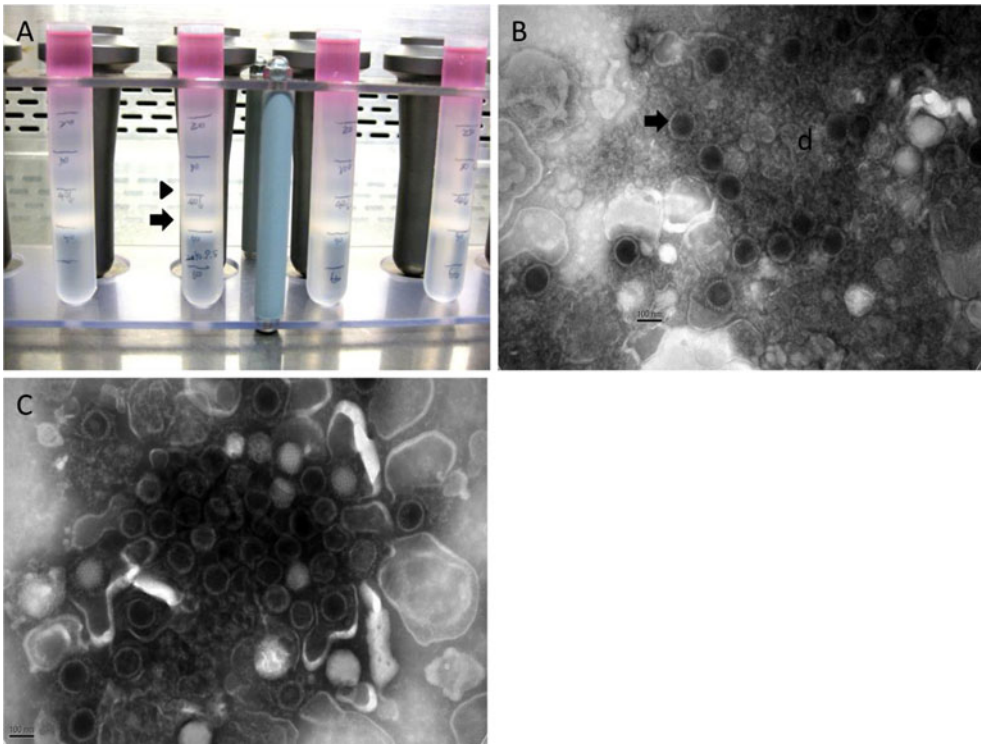

Fig. 1 Purity of KHV for immunization after centrifugation. **a** Two visible bands were observed after sucrose gradient centrifugation. *Arrow* indicates band between 40 and 50 % sucrose; *arrowhead* indicates band between 30 and 40 % sucrose. **b** Electron micrograph of the banded viruses. Virions (*arrow*) and cell debris (*d*) are observed at the interface between 30 and 40 % sucrose. **c** Electron micrograph of the banded viruses. There are abundant virions and viral envelopes present at the interface between 40 and 50 % sucrose

carbazole diluted in 10 mL distilled water and 10 μL of 30 % hydrogen peroxide, pH 5.1) for 10 min, and washed with tap water twice. Sections were counterstained with Mayer's hematoxylin for 3 min, washed with tap water for 5 min, washed with PBS for 10 min, sealed with Aquatic Mounting Medium, and observed with a light microscope.

Isotype determination The MAb class and subclass were determined with the IsoStrip mouse monoclonal antibody isotyping kit (Roche Applied Science, Germany). The ascites sample was diluted 1: 5,000 and 150 μL of the diluted sample was used in the test per manufacturer's instruction.

Results

The KHV-infected CCB cells showing 70–80 % CPE were collected and the supernatants were further centrifuged. The banded viruses were observed at two sites; one was at the interface between 40 and 50 % and the other was between 30 and 40 % sucrose (Fig. 1a). The sample purity was further examined by electron microscope. The 40/50 % band showed abundant virions (Fig. 1b) compared to the 30/40 % band with cell debris and less virions (Fig. 1c). The virions from the 40/50 % band were used for immunization in the production of MAb. Only one clone of MAb-B2 was generated in this study.

Using Western blot analysis, a 40-kDa protein band was detected in purified KHV with MAb-B2 (Fig. 2). Furthermore, the stained band in SDS-PAGE corresponding to that detected

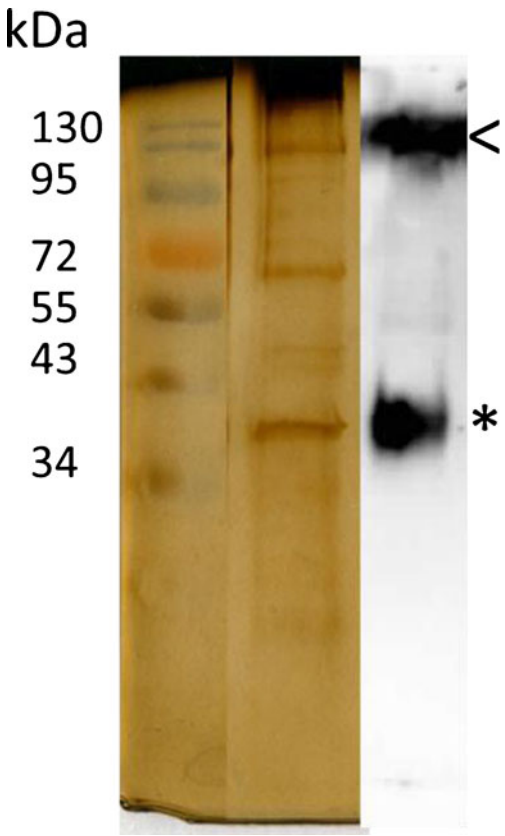

Fig. 2 Western blot analysis of KHV with MAb-B2. The approximately 40-kDa-sized protein (*asterisk*) was detected with MAb-B2 in 75 ng of purified KHV (*3*). Molar mass markers (*1*) and silver-stained PAGE of purified KHV (*2*) are indicated to the *left*. The nonspecific background signals are shown as *arrowhead*

Table 1 KHV protein identified by LC-MS/MS in Western blot analysis

ORF	% Coverage	Mascot score	Tolerance ppm	Protein Molecular weight (Da)/pI	Species	Protein
72	53	668	17	40,476/5.6	Cyprinid herpesvirus 3	gi:131840098 Capsid triplex protein 2

by MAb was cut, washed, digested with trypsin, extracted, and analyzed by nanoLC-MS/MS. Through the search in the Swiss-Prot database using the Mascot program, the MAb-reacted protein was identified as ORF72 of KHV by the LC-MS/MS (Table 1). The specificity of MAb-B2 was verified in the experimental infection in vitro using IFA and the positive signals were detected at KHV-infected CCB cells but not in the mock-infected cells (Fig. 3).

The detection sensitivity of MAb-B2 is determined by the detection limit of ORF72 protein, which has a minimum of 75 ng purified virus sample per gel lane. It is known that ORF72 protein possesses conserved homologues in IcHV-1 ORF27, RaHV-1 ORF95, and RaHV-2 ORF131(Michel et al. 2010), and might share similar epitopes with CyHV-1 and CyHV-2 (Adkinson et al. 2005). These findings suggest that examination of cross-reactivity of MAb-B2 between KHV and other aquatic herpesviruses may provide more information for assessing common antigens between them. However, due to government regulations in Taiwan, we were not permitted to import viruses which have not been isolated locally in Taiwan. Thus, we did not determine the reaction of MAb-B2 against other aquatic herpesviruses in this study.

In neutralizing activity test, the 1:2 dilution MAb-B2 (Fig. 4a) showing an NI value equal to 2.0 ($\log 61\times10^3 - \log 59\times10^1$) was found to neutralize KHV. However, when tenfold dilution MAb-B2 was used (Fig. 4b), NI value declined to 1.0 ($\log 40\times10^3 - \log 40\times10^2$). This indicated that MAb-B2 in high concentrations could inactivate KHV and protect the cells from infection.

To realize the application of MAb-B2 to immunohistochemistry, two main targets (gills and kidney) were chosen to react with MAb. In KHV-infected fish, the prominent positive signals were observed in the infected and necrotic epithelia of the gill filament and secondary lamella (Fig. 5a). Few signals were found in the necrotic foci in kidneys (Fig. 5b). In healthy fish, no signals were found in the gills and kidney (data not shown). In isotype determination, the MAb-B2 was IgG1 subclass with kappa light chain.

Discussions

Forty structural proteins have been identified, including 3 capsid, 13 envelope, and two tegument proteins and 22 unclassified proteins in CyHV-3 (Michel et al. 2010). Possessing the antibodies as a tool would be convenient to investigate the role of those proteins played in the pathogenesis of KHV in the host. Until now, the antigenicity of four CyHV-3 proteins, ORF 81 (envelope protein), ORF68 (integument protein), ORF92 (major capsid protein), and ORF136 (envelope-like protein) have been studied by the expression of recombinant proteins and confirmed by the

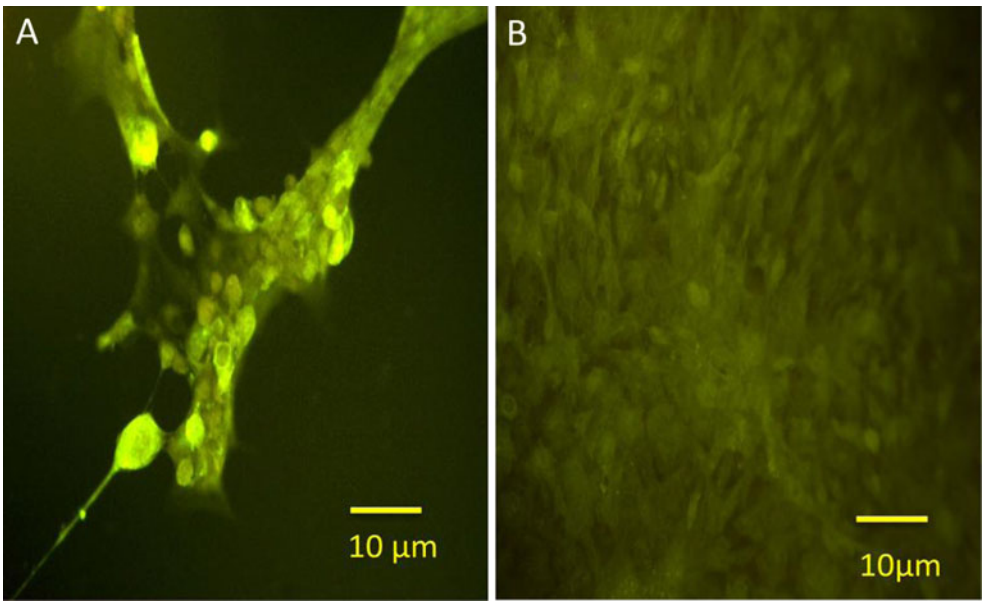

Fig. 3 Immunofluorescence assay with MAb-B2 against CCB cells infected with KHV (**a**) and mock-infected CCB cells (**b**)

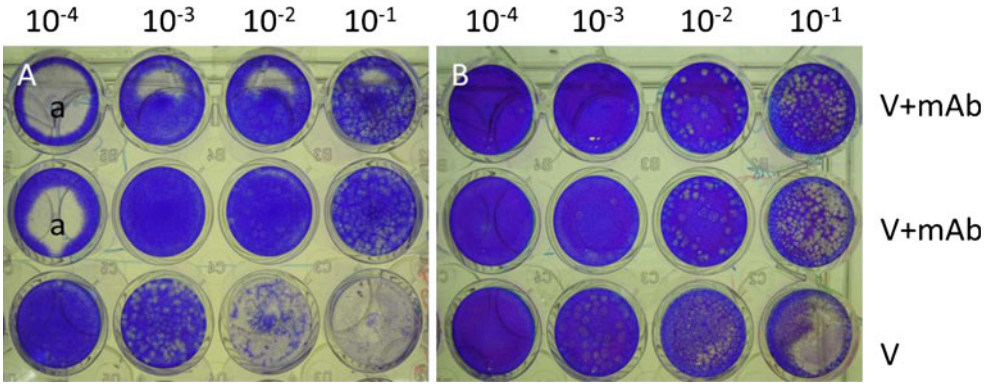

Fig. 4 The neutralizing test with two distinct constant concentrations of MAb-B2 (1:2 in **a** and 1:10 in **b**) against varying titers of KHV (e.g., 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}). *a* Artifact

specific antibodies (Rosenkranz et al. 2008; Aoki et al. 2011; Dong et al. 2011). Two truncated (ORF81 and ORF92), one full-length (ORF136), and one truncated structural proteins (ORF68) were used to produce three polyclonal antibodies and a monoclonal antibody, respectively, and all antibodies were applied in IFA, immunoelectron microscopy, or IHC. The goal of this study was to produce a specific monoclonal antibody by immunizing mice using virions and to define the reacted antigen. Our results showed that a monoclonal antibody was produced in mice by immunizing purified KHV virions. It could detect both denatured and native KHV proteins via Western blot, IFA, and IHC. The MAb-B2 was demonstrated to recognize the capsid protein ORF72 of KHV by LC-MS/MS, a component in mature KHV (Michel et al. 2010). These results suggested that MAb-B2 is specific for KHV ORF72 protein and cannot only help detect KHV in cell cultures for early diagnosis but also be used to develop an easily-performed and time-saving diagnostic kit for farmers in Taiwan. In the future, we plan to use MAb-B2 coupled with a polyclonal antibody against KHV to develop a pond-side rapid strip test for farmers. The rapid diagnosis can help farmers make an early decision in treatment during an outbreak and control the disease.

Although earlier, the gills showed pathological changes at the time of experimental infection (Pikarsky et al. 2004), the common, multiple factor-causing gill lesions, such as hyperplasia and lamellar fusion, make diagnosis of KHV difficult in histopathology. In this study, we showed that application of MAb to histopathology can easily detect KHV-infected epithelia in gills even without observing pathognomonic nuclear inclusions. In kidneys, only the necrotic foci of interstitial cells showed weak signals. Thus, gills are suggested for histopathology examinations in KHV infection compared with other findings (Hedrick et al. 2000; Pikarsky et al. 2004). The results showed that MAb-2B can be used to enhance the diagnosis of KHV in histopathology and to study pathogenesis in the future.

It is crucial for researchers to develop a safe and efficacious vaccine to control KHV in the koi industry. Although attenuated live vaccine and "naturally infected" fish produced a prolonged protection to KHV (Ronen et al. 2003; Perelberg et al. 2008), those applications in koi aquaculture are still limited to a few countries in consideration of virus shedding. In aquaculture, two capsid antigens have been evaluated as vaccines. One was VP2, the major capsid protein of infectious pancreatic necrosis virus (Christie. 1997), and the other was T2, a capsid protein of striped jack nervous necrosis virus (Husgard et al. 2001). Therefore, the capsid

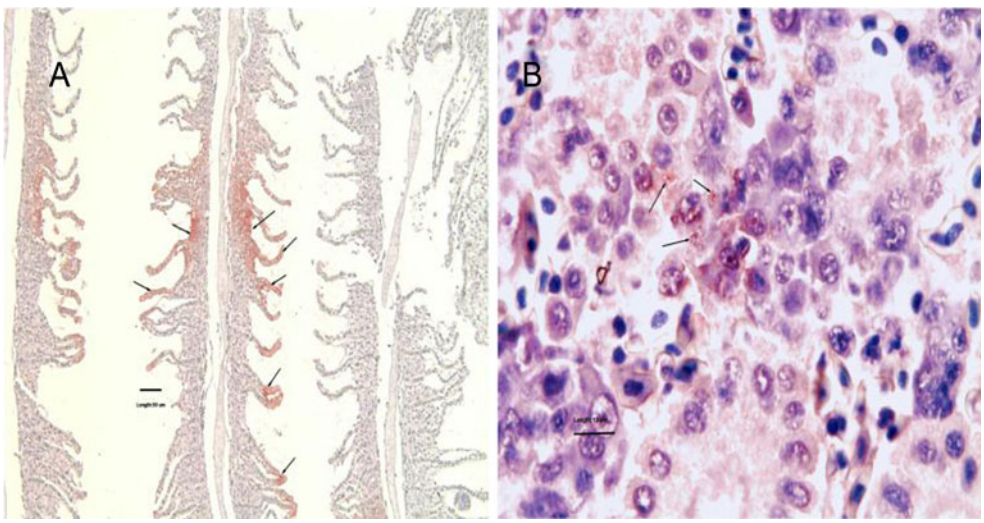

Fig. 5 Immunochemistry with MAb-B2 against KHV in gill (**a**) and kidney (**b**) of infected fish. (**a**) In the gill filaments, the signals (*arrow*) are present within the epithelia in the affected and hyperplastic secondary lamellae. (**b**) In the kidney, affected hemapoietic cells and cell debris display signals (*arrow*) within the necrotic foci

proteins in KHV might have a good potential for vaccine development. However, three capsid proteins, pORF92, pORF72, and pORF78, have been revealed in mature virions in KHV (Michel et al. 2010) but there are no reports demonstrating the protective neutralizing antibody induced by individual structure protein of KHV. Additionally, none of the neutralizing capability of four antibodies against ORF81, ORF68, ORF92, and ORF136 protein was available (Rosenkranz et al. 2008; Aoki et al. 2011; Dong et al. 2011). In this work, MAb-B2 exhibits a neutralizing ability to neutralize KHV indicating that ORF72 protein of KHV might possess neutralizing epitopes for inducing protective immunity in fish. The low titer of neutralizing activity in vitro was unexpected because the capsid of KHV is surrounded by a tegument with an outer envelope (Miyazaki et al. 2008). Several explanations for this result are possible. The high concentration of antibody may not bind to the envelope of KHV but intercalate the fusion interface between the virus envelope and the cell membrane suggesting that the fusion process is blocked by interactions of virus and cell membrane by conformational changes or by the hindrance of contact between virus and cell membrane (Klasse and Sattentau. 2002). To our knowledge, this is the first report describing the neutralizing capacity induced by individual capsid protein of KHV. These results suggested that ORF72 can be a potential candidate for development in genetically recombinant vaccines.

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