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Production of Monoclonal Antibodies to Polyhedrin of Monodon Baculovirus (MBV) from Shrimp

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ABSTRACT: Monodon baculovirus (MBV) is a member of the nucleopolyhedrosis virus (NPV) group that produces polyhedral protein occlusion bodies that contain virions in epithelial cells of the hepatopancreas of the peinaeid shrimp *Penaeus monodon*. The major constituent protein of occlusion bodies is polyhedrin. Polyhedrin protein of MBV was extracted from the infected hepatopancreas of *P. monodon* post larvae by hydroforce and partially purified using Urografin gradient ultracentrifugation. A fraction at 50% Urografin was examined by electron microscopy and shown to be dominated by polyhedrin protein particles of 20-23 nm diameter. By SDS-PAGE, this fraction yielded a single protein band at a molecular weight of 58 kDa corresponding to the published size of MBV polyhedrin. This fraction was used to produce 17 monoclonal antibodies (MAb) that were specific to MBV and without cross-reactivity to other common shrimp viruses (i.e, hepatopancreatic parvovirus or HPV, yellow head virus or YHV and white spot syndrome virus or WSSV) by immunohistochemistry. These antibodies could be used to detect purified-polyhedrin with high sensitivity up to 0.2 µg/ml by dot blot immunoassay. These MAb are candidates for sensitive MBV immunodetection methods.

KEYWORDS: monodon baculovirus, MBV, polyhedrin, monoclonal antibodies.

INTRODUCTION

Penaeus monodon-type baculovirus (MBV) is an occluded baculovirus that contains double-stranded DNA as its nucleic acid.[1,2,3] MBV was one of the earliest viruses found in shrimp, but its economic impact on shrimp farming has not yet been determined. However, it is considered to be a potentially serious pathogen in larvae, post larvae (PL) and early juvenile stages of cultivated shrimp. MBV infections are characterized by the presence of prominent, polyhedral, intranuclear occlusion bodies in affected epithelial cells of the hepatopancreas and midgut, or free within lysed cell debris in the feces.[2,4] Acute MBV causes loss of hepatopancreatic tubule and midgut epithelia and, consequently, dysfunction of these organs may be followed by secondary bacterial infections. Crowding or environmental stress may enhance the pathogenicity and increase the prevalence of MBV in its hosts.[5,6,7] MBV transmission occurs exclusively by the oral route, principally by cannibalism and faecal-oral contamination.[8,9]

Polyhedrin is a crystalline protein characteristic of viruses in the nuclear polyhedrosis virus (NPV) group of the family Baculoviridae.[10] Occlusion bodies (OB) consist of polyhedrin protein matrices that appear to stabilize virions and maintain their viability under damaging environmental conditions.[11,12] For MBV, the molecular weight of polyhedrin has been estimated at 58 kDa.[13]

MBV diagnosis can be accomplished by several methods. The simplest is based on the microscopic observation of the characteristic polyhedral occlusion in whole hepatopancreatic tissue mounts or smears.[2] Molecular methods based on gene probes used for *in situ* DNA hybridization, simple polymerase chain reaction (PCR) and nested-PCR are also available and are satisfactory for surveillance applications.[14] An antibody-based enzyme-linked immunosorbent assay (ELISA) for diagnosis of MBV has been reported,[15] but the method used polyclonal antibodies and the antibodies are not commercially available. Monoclonal antibodies for detection of MBV have not been reported. Since MBV produces polyhedrin in large quantity, it is a good candidate for viral detection assays and an attempt to produce polyclonal antibodies against MBV polyhedrin using a synthetic fragment of the protein has been reported.[16] However, the sensitivity of the

reagent was low and it was, thus, unsuitable for diagnostic purposes. The aims of this study were to purify MBV polyhedrin from infected shrimp and use it to produce specific monoclonal antibodies (MAb) for use in developing a simple immunodiagnostic test for MBV infection in shrimp.

Materials and Methods

Determination of MBV Infection

Wet-mounts of fresh tissue were used to screen lots of 5,000-6,000 black tiger shrimp (*Penaeus monodon*) post larvae (PL) at stages PL10-15 for the presence of MBV. Approximately 100 PL from each lot were subjected to microscopic checks for occlusion bodies in hepatopancreatic tissue squashes stained with 0.05% malachite green.[2] Infection of MBV was confirmed in histologically positive lots by PCR. Briefly, DNA from the hepatopancreas was extracted using a QIAamp® DNA mini kit following the protocol from the QIAamp® DNA mini kit handbook (QIAGEN, Hilden, Germany) and a nested-PCR assay was carried out using MBV533/361-IC NESTED-PCR READY TO USE KIT following the protocol from the manufacturer (Ezee Gene®, SBBU (Shrimp Biotechnology Business Unit, Bangkok, Thailand). The first step primers generated a 533 bp fragment while the second nested-PCR step generated a 361 bp fragment of MBV DNA plus a 175 bp internal control fragment. The PCR amplicons were visualized on an UV light box. The presence of expected PCR products of 533 and 361 bp were considered positive for MBV. PCR positive batches were used for preparation of polyhedrin protein.

Purification of MBV Polyhedrin

After confirmation of MBV infection, approximately 5,000 MBV-infected PL were stunned in ice water and then placed in a beaker containing 50 ml of distilled water. The plunger was removed from a 50 ml plastic syringe with no needle and a thoroughly mixed portion of the PL water mixture was poured into the syringe with the syringe opening held closed. Then the plunger was added and the mixture was forced through the syringe opening into a receiving beaker on ice. This disruption procedure was repeated 10 times in rapid succession. The homogenate was then filtered twice through a 100 µm Nitex-mesh. The filtrate was centrifuged at 4,000 g for 20 minutes at 4° C. The pellet was resuspended in TN buffer (0.01 M Tris-HCl, 0.1 M NaCl, pH 7.4) and layered onto the top of a discontinuous Urografin™ gradient (20% to 60% in steps of 10%). Then the Urografin™ gradients were ultracentrifuged at 120,000 x g for 16 h at 4° C. After ultracentrifugation, sample bands were removed, resuspended in TN buffer and pelleted by centrifugation twice at 120,000 x g for 2 h at 4º C. The final pellets were resuspended in a minimum amount of TN buffer and stored at -20º C. The concentration of proteins was determined by Bradford's method (BioRad protein assay kit, Bio Rad Corp., Hercules, CA, USA). The purity of the MBV polyhedrin was checked by a transmission electron microscopy (Hitachi H-600, Hitachi Corp., Tokyo, Japan) using the negative staining technique. For transmission electron microscopy (TEM), a droplet of the purified MBV polyhedrin was placed on a copper grid coated with Formvar and carbon (Electron Microscopy Sciences, PA, USA). After drying, a droplet of staining solution (2% phosphotungstic acid solution in distilled water, pH 7.4) was placed on the grid for about 1 min before removal of excess liquid.[17] After air drying overnight, the grids were examined for the presence of MBV polyhedrin protein by transmission electron microscopy (Hitachi H-600).

Immunization of Mice and Antiserum Assays

Four, 6-week old Swiss albino mice were first immunized intraperitoneally with 0.8 mg/ml of fractions containing polyhedrin mixed with Freund's complete adjuvant (Sigma®) for a total volume of 100 µl per mouse (antigen: adjuvant 1:1 v/v; 40 µg/mouse). The mice were boosted twice; first, 2 weeks after the first immunization and, second, 1 month after the first boost with the same preparation mixed with incomplete Freund's adjuvant (Sigma®). One week after the third injection, antisera were collected, absorbed with normal hepatopancreas homogenate in PBS overnight at 4º C and tested for immunoreactivity by dot blot, western blot, and immunohistochemistry. Hybridomas were produced according to Mosmann et al. (1979) with slight modification.[18] The mouse was then euthanized by cervical dislocation. The spleen was placed and washed in two sterile Petri dishes containing phosphate buffered saline (PBS), then centrifuged for 5 min at 200 xg. The supernatant was discarded as completely as possible. Non-secreting murine myeloma P3X cells were fused with spleen cells of the spleen donor. To facilitate fusion, one ml of pre-warmed (37ºC) 40% (w/v) polyethylene glycol (PEG 1500, Sigma®) was added to the cell suspension using a pipettor and the tube was simultaneously agitated slightly. Thirty-nine millilitres of HT medium (Gibco BRL, USA) was added to the suspension, and it was then incubated in a humidified incubator for 2 h. The cell suspension was centrifuged for 5 min at 200 x g and the supernatant was discarded. The cell pellet was suspended in HAT medium (80 ml RPMI medium, 20 ml fetal calf serum, 1X HT supplement, 1X aminopterin and 1% mouse red blood cell). One hundred microlitres of this mixed cell suspension was dispensed into each well of the thirty 96-well culture plates, and they were kept in a humidified incubator.

Six days after fusion, macroscopic growth of cell cultures was examined. If cell growth was visible, cell supernatants were screened for anti-MBV MAb activity as described below. Selected positive hybridomas were cloned in new 96-well culture plates by the limiting dilution method to obtain single clones per well. Wells that contained a single colony were further propagated in 20% FCS RPMI 1640 medium and cell supernatants were tested by dot blot ELISA, western blotting and immunohistochemistry.

Western Blot Analysis

Gels containing proteins separated by SDS-PAGE were blotted onto nitrocellulose membranes. The membranes were then exposed to hybridoma culture fluid (MAb) for 5 h at RT with continuous rocking. After washing 4 times with PBS, the membranes were treated with 1:1,500 diluted goat anti-mouse immunoglobulin conjugated with horseradish peroxidase (GAM-HRP) (Bio Rad) in PBS. Upon completing the washing step, the membranes were developed by adding freshly prepared substrate solution (0.03% DAB, 0.006% H_2O_2 and 0.05% $CoCl_2$) 5 min before washing with sterile water to stop the enzymatic reaction.

Dot Blot ELISA

Nitrocellulose membranes were used as the solid phase in dot blot ELISA tests. The polyhedrin band derived from the Urograffin gradient (0.8 mg/ml) diluted (1:20) in PBS was applied to the nitrocellulose membrane at 1 ml per spot. The membrane was allowed to air dry for 10 min and then placed in a plastic box containing 30 ml of 5% skim milk in PBS for 30 min. After blocking, membranes were incubated for 1 h with supernatants containing each MAb in blocking solution. After extensive washing in PBS-T, the membranes were incubated with 1:1,500 diluted GAM-HRP (Bio Rad) in PBS. The membranes were washed extensively before color development using HRP substrate, as described in the western blot analysis.

Immunohistochemistry

Shrimp cephalothoraxes were cut longitudinally and fixed in Davidson's fixative for paraffin sectioning according to the methods described by Lightner (1996).[1] Serial sections were prepared and processed for indirect immunoperoxidase staining using various dilutions of antisera previously adsorbed with formalin-fixed hepatopancreatic homogenate from uninfected shrimp. This was followed by treatment with GAM-HRP diluted at 1:1000 with 10% calf serum in PBS (pH 7.4) and visualization by incubation with HRP substrate. Preparations were counterstained with hematoxylin and eosin Y,[1] dehydrated in a graded ethanol series, cleared in xylene and mounted in permount. A positive immunoreaction was visualized as brown coloration at the site of MBV infection against the purple blue of basophilic hepatopancreatic tubule epithelial cells and nuclei. Archived material infected with other common shrimp viruses, including hepatopancreatic parvovirus (HPV), yellow head virus (YHV) and white spot syndrome virus (WSSV), were used to test for immunochemical cross-reactivity with the anti-MBV polyhedrin MAb. For these tests, positive control antibodies comprised MAb HPV16-9C for HPV[19], MAb Y19 for YHV[20], and MAb W28 for WSSV[21].

Determination of Antibody Isotypes and Subclasses using Sandwich ELISA

The heavy chain classes and light chain types of MAb were determined using Mouse Monoclonal-ID Kit (Zymed Lab, USA). The assay is based on an indirect sandwich ELISA principle. Rabbit anti-mouse class and subclass specific antibodies were employed in isotyping the mouse immunoglobulins secreted into the culture supernatant. Peroxidase-labelled goat anti-mouse IgG served as the signal generating reagent. The procedure was conducted following the manufacturer's recommendations.

Results and Discussion

Antigen Preparation

MBV polyhedrin particles were successfully extracted at high purity by the method based on hydroforce. This was very simple and rapid and allowed the preparation of relatively large quantities by Urografin™ gradient ultracentrifugation. By TEM, the 50% Urografin™ fraction was shown to contain 20-23 nm diameter particles with the appearance of full and

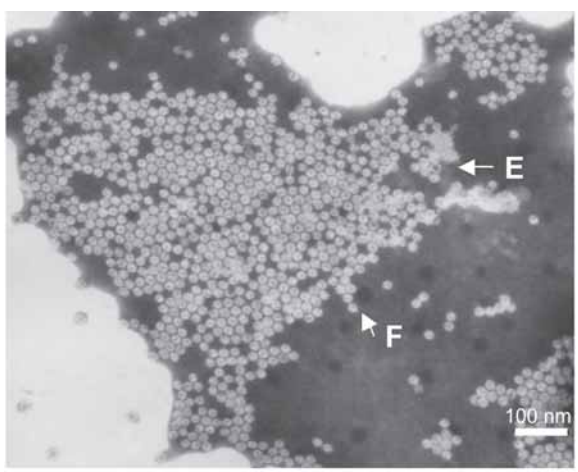

Fig 1. Transmission electron micrograph of purified polyhedrin protein particles negatively stained with 2% PTA. They appear to be in full (F) and empty (E) forms.

empty subunits (Fig. 1) similar to the MBV polyhedrin particles described by Bonami *et al.*[13] Complete virions and their components (nucleocapsids and envelopes) were not found in this fraction. Gradient ultracentrifugation is the usual method used for purification of viral particles and their components. Sucrose gradient centrifugation is another method that has been successfully used to isolate occlusion bodies and complete virions.[15]

Using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining, the 50% Urografin™ fraction gave a single major protein band corresponding to approximately 58 kDa, as previously reported for MBV polyhedrin.[13, 22] (Fig. 2).

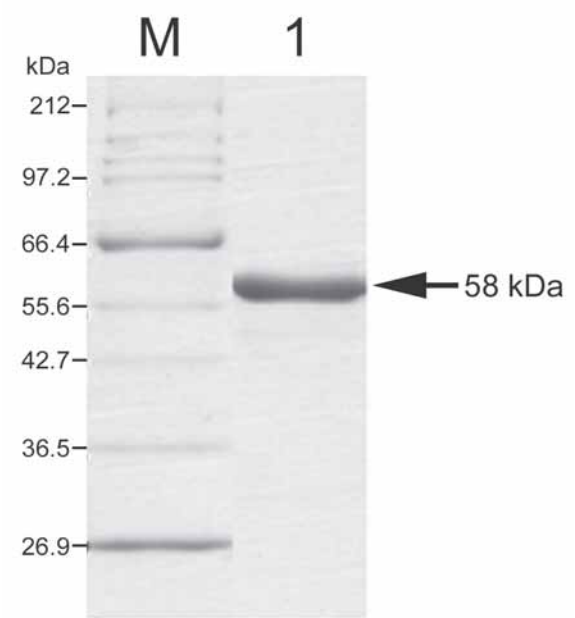

Fig 2. SDS-PAGE gel of the 50% Urografin™ fraction stained with Coomassie blue. Lane M: Molecular weight standard in kDa is indicated. Lane 1 contains the Urografin fraction.

Production of MAb and Type Determination

One week after completion of the second booster of purified polyhedrin, Swiss mice sera were checked to be positive for antibodies against purified MBV polyhedrin. Western blot analysis at an antiserum dilution of 1:5000 revealed that the antibodies gave strong immunoreactivity to the 58 kDa protein. Immunohistochemistry at antiserum dilutions of 1:500, 1:1,000, and 1:2,500 revealed strong specific binding to MBV intra-nuclear occlusion bodies in hepatopancreatic tubule epithelial cells of *P. monodon* infected with MBV. There was no cross reactivity with uninfected tissues (data not shown). This sensitivity was higher than that (1:50) obtained by means of immunohistochemistry with a previously reported polyclonal antibody prepared against a synthetic fragment of MBV polyhedrin.[16]

One fusion trial with 30 microculture plates yielded approximately 2000 culture wells, each giving 1 to 5 colonies of hybridoma cells. The first screening by dot-blot revealed 144 hybridoma clones that gave strong reactions with purified-polyhedrin of MBV (data not shown). The positive culture fluid was further screened by western blot and immunohistochemistry. Supernatants from 39 clones showed specific and intense bands and specific binding to MBV intra-nuclear

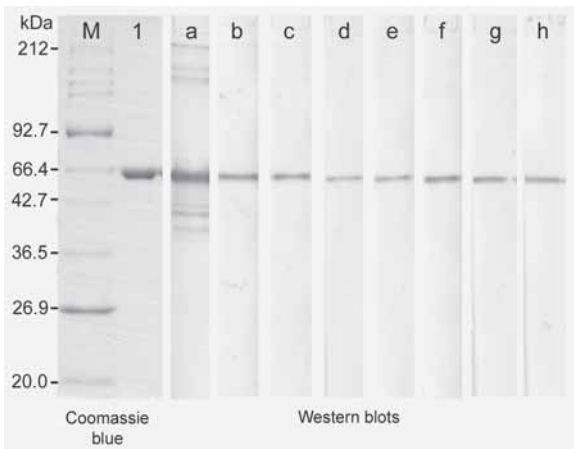

Fig 3. SDS-PAGE of purified polyhedrin stained with Coomasie blue and Western blots of purified polyhedrin (9.33 ng/lane) reacted with one representative PAb (a) and several representative MAb (b, 5-3H; c, 5-6F; d, 9-2F; e, 10-5F; f, 14-1G; g, 16-3G; h, 27-8F). M = Molecular weight standard with kDa is indicated.

Table 1. Specificities of monoclonal antibodies tested by dot blot ELISA, Western blot analysis and immunohistochemistry.

MAb	Dot Blot (ng/ml)	Western blot (kDa)	Immunohisto-chemistry	Subtype
MBV9-1D, MBV9-2F, MBV21-4B, MBV25-4G	0.4	58	+++	IgG_1
MBV6-9G, MBV14-1G, MBV16-3G, MBV21-11B, MBV24-5E	0.2	58	+++	IgG_1
MBV10-5F	0.4	58	+++	IgG_{2a}
MBV10-11H, MBV21-8A, MBV27-8F	0.4	58	+++	IgG_{2b}
MBV5-3H, MBV5-6F, MBV8-5G, MBV13-10E	0.2	58	+++	IgG_{2b}

occlusion bodies in hepatopancreatic tubule epithelial cells of *P. monodon* infected with MBV. All monoclonal antibodies bound specifically to both denatured and native forms of purified polyhedrin, based on western blot analysis and dot blot ELISA, respectively. By western blotting, all antibodies reacted strongly with the 58 kDa purified polyhedrin of MBV (Fig. 3). These antibodies detected purified-polyhedrin in dot blot assays with different sensitivities, ranging from 0.2 – 0.4 µg/ml (Table 1). Furthermore, the MAb showed no cross-reactivity to other viruses (HPV, YHV, and WSSV) (Fig. 4), normal hepatopancreas and other shrimp tissues (heart and gill) by means of immunohistochemistry (Fig. 5).

Twelve MAb were selected based on strong binding to MBV polyhedrin in dot blot assays. By indirect ELISA using a mixture of 2 MAb/well in 96 well plates coated with purified-polyhedrin (0.133 µg/well). MAb MBV5-3H showed the highest sensitivity with purified polyhedrin. The results also suggested that MBV5-3H, MBV14-1G, and MBV16-3G bound to the same or overlapping epitopes because MAb MBV14-1G and MBV16-3G showed lower sensitivity when competitively bound with MBV5-3H. By contrast, mixing MAb MBV5-3H with MBV5-6F, MBV8-5G, MBV10-5F, MBV10-11H, MBV13-10E, MBV21-4B, MBV21-8A, MBV21-11B and MBV27-8F resulted in increased OD values. Thus, mixtures of MAb MBV5-3H with these MAb could be used to increase MBV polyhedrin detection sensitivity. Results from antibody isotyping showed that isotypes and subisotypes of MAb obtained were IgG_1, IgG_{2a}, and IgG_{2b} (Table 1).

There are several reports on the production of MAb against specific antigens of shrimp viral pathogens for diagnostic purposes. Poulos *et al.*[23] produced and identified monoclonal antibodies against viral proteins of Taura syndrome virus (TSV) and used them to compare specificities for 3 structural proteins of the virus. The same group described production of MAb to

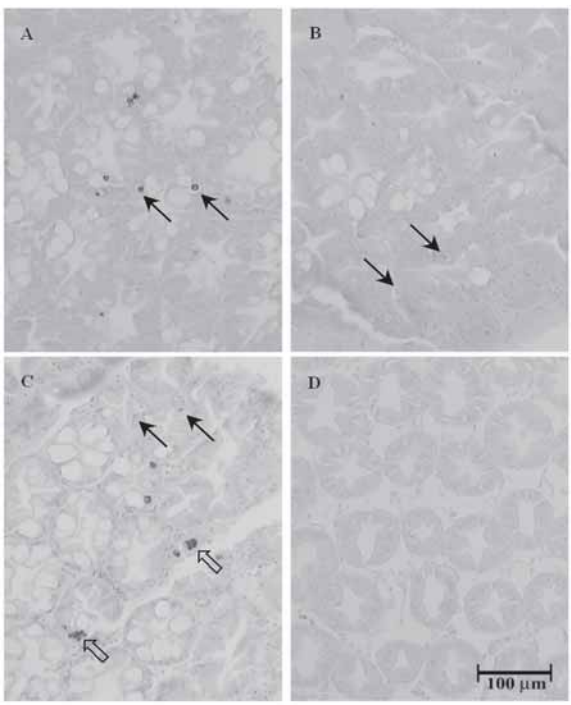

Fig 4. Immunohistochemistry of *P. monodon* hepatopancreatic tissue infected singly with HPV (A, B), infected dually with MBV and HPV (C) and uninfected with either virus (i.e., normal hepatopancreatic tissue) (D). (A) Section of HPV infected tissue reacted with anti-HPV MAb HPV16-9C as a positive (arrows) HPV control. (B) Similar section to (A) but reacted with anti-MBV polyhedrin MAb MBV5-3H and showing no reactivity (arrows) with HPV inclusions. (C) Dually infected tissue reacted with anti-MBV polyhedrin MAb MBV5-3H and showing strong positive reactions (open arrows) with MBV polyhedra but no reaction with HPV inclusions (solid arrows). (D) Normal shrimp hepatopancreatic tissue reacted with anti-MBV polyhedrin MAb MBV5-3H. Sections A, B and D were counter stained only with eosin Y while C was counter stained with H&E.

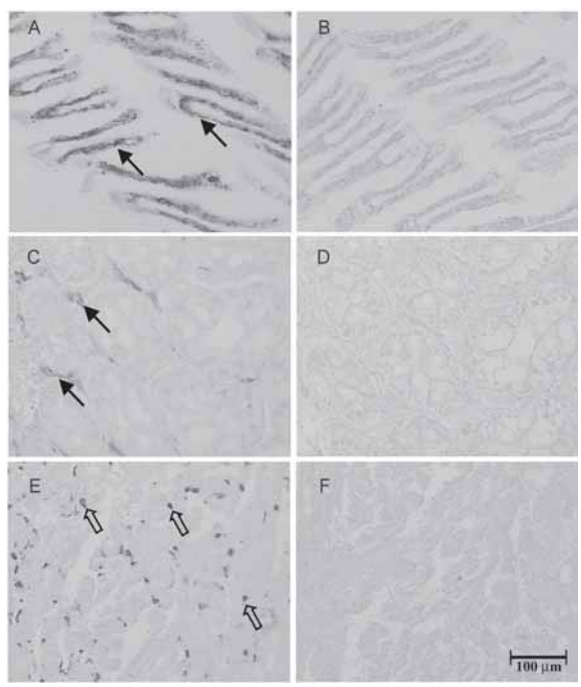

Fig 5. Immunohistochemistry examples of lack of cross-reactivity of anti-MBV polyhedrin MAb to other shrimp viruses in *P. monodon*. Column one (A, C, E) shows positive controls of tissues infected with various shrimp viruses reacted with specific MAb while column 2 (B, D, F) shows similar sections reacted with anti-MBV polyhedrin MAb MBV5-3H; (A, B) gills infected with YHV; (C, D) hepatopancreas infected with YHV; (E, F) heart infected with WSSV.

the 28 kDa envelope protein of white spot syndrome virus (WSSV) and its use for immunodiagnosis.[24] Sithigorngul *et al*.[20] reported the production of monoclonal antibodies specific to 22, 64, and 135 kDa proteins of yellow-head virus (YHV) and they have been used for viral detection[20] and typing[25]. We can now add MBV to the list of shrimp viruses for which MAb detection reagents are available. They should be useful for the development of rapid detection kits and perhaps for characterization of geographical types of MBV.

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