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Diagnosis of *Penaeus monodon*-type baculovirus by PCR and by ELISA of occlusion bodies

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ABSTRACT: The black tiger prawn *Penaeus monodon* is a valuable aquaculture product in Taiwan. Two specific diagnostic methods were established for *P. monodon*-type baculovirus, one using polymerase chain reaction (PCR) technology and the other enzyme-linked immunosorbent assay (ELISA) technology. Monodon-type baculovirus (MBV) was purified by sucrose gradient centrifugation from occlusion bodies of MBV-infected postlarvae of *P. monodon*. MBV DNA was subsequently purified from the occlusion bodies and its presence was confirmed by PCR using primers of the polyhedrin gene. Based on conserved sequences of the DNA polymerase genes of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) and *Lymantria dispar* nuclear polyhedrosis virus (LdMNPV), primers were designed and synthesized to yield a 714 bp PCR fragment from MBV. However, the sequence of this fragment revealed low homology with that of LdMNPV and AcMNPV. From the DNA sequence of this fragment, a second set of primers was designed, and using these primers, a 511 bp DNA fragment was amplified only when MBV DNA was the template. DNA templates from AcMNPV, white spot syndrome diseased shrimp, or PMO cells (a cell line derived from the Oka organ of *Penaeus monodon*) did not give any amplified DNA fragment. Therefore, this primer pair was specific for the diagnosis of MBV. By using intraspleenic immunization of rabbits with purified MBV occlusion bodies, a polyclonal rabbit antiserum against MBV was obtained. This antiserum could detect nanogram levels of MBV, but did not cross react with white spot syndrome virus (WSSV), homogenates of PMO cells, postlarvae, hepatopancreatic tissue or intestinal tissue of black tiger prawns by competitive ELISA. This sensitive method could detect MBV even in tissue homogenates.

KEY WORDS: MBV · PCR · ELISA

INTRODUCTION

Since the technological development of artificial fertilization, mass seed production, and feed formulation for penaeid shrimp, the shrimp culture industry has developed and expanded rapidly, particularly in Southeast Asia. In Taiwan, substantial losses in annual shrimp production have been reported since 1987 (Lightner et al. 1987, Chen et al. 1989a,b). Pathogens, such as bacteria, viruses, and fungi, along with environmental stressors such as temperature fluctuation, heavy rain, overfeeding, and industrial and agricultural pollutants have been considered to be the causes of these losses. In general, the incidence of virus infections increased rapidly as shrimp culture intensified, and secondary bacterial infections or environmental stress subsequently caused high mortality. To date, nearly 20 penaeid shrimp viral diseases have been discovered (Couch 1974, Lightner & Redman 1981, Sano et al. 1981, Lightner et al. 1983a, Tsing & Bonami 1984, Lightner & Redman 1985, Lu et al. 1991, Owens et al. 1991, Boonyaratpallin et al. 1993, Wang et al. 1995). Since these viral diseases cannot be cured by therapeutic reagents, rapid and effective diagnosis is essential to keep them under control.

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Investigations of postlarvae, juveniles, and brooders of black tiger prawns in Taiwan from 1984 to 1988 indicated that monodon-type baculovirus (MBV) was the causative agent of high mortality that occurred in 1987 (Chen et al. 1989a,b). MBV was first described in 1981 (Lightner & Redman 1981). MBV along with baculoviral midgut gland necrosis virus (BMNV), baculovirus penaei (BP), and white spot syndrome virus (WSSV) are considered to be important shrimp viruses since they have a very wide host range and distribution (Couch 1974, Sano et al. 1981, Lightner et al. 1983b).

In postlarval hatcheries of black tiger prawns, early detection of MBV infection is a very important issue for prevention of its spread, and screening of brooders to ensure that they are MBV-free is critical for the production of specific pathogen-free postlarvae. Several methods have been developed for diagnosis of MBV, including PCR of the polyhedrin gene (Chang et al. 1993, Vickers et al. 1993, Lu et al. 1995, Nunan & Lightner 1997). However, polyhedrin exists in all nucleopolyhedroviruses including the shrimp viruses, BP and MBV (Couch 1981, Lightner & Redman 1981) but not WSSV. The insect baculoviruses *Autographa california* nuclear polyhedrosis virus (AcMNPV), *Bombyx mori* single nucleopolyhedrovirus (BmNPV), *Lymantria dispar* nuclear polyhedrosis virus (LdMNPV), and *Orgyia pseudotsugata* multicapsid nucleopolyhedrovirus (OpMNPV) also belong to this group. In addition, Bjornson et al. (1992) reported that there was 80% sequence homology of polyhedrin genes but only 40 to 50% homology of DNA polymerase genes between insect baculoviruses. Therefore, the design for a set of MBV-specific primers based on its DNA polymerase gene should be more discriminatory. In this report, we describe the development of MBV-specific diagnoses using PCR of MBV genomic DNA and ELISA based on the protein in MBV occlusion bodies.

MATERIALS AND METHODS

Experimental animals. Postlarvae (PL) of black tiger prawns from various hatcheries were checked randomly by eosin-staining of specimen smears for characteristic MBV occlusion bodies. Positive MBV-infected PL were subsequently purchased from one hatchery located at Pingtung County in southern Taiwan, frozen and stored on dry ice.

Chemicals and enzymes. Buffer-saturated phenol and phenol/chloroform/isoamyl alcohol were purchased from Amresco Co. (Solon, OH, USA). Cetyltrimethylammonium bromide (CTAB) and proteinase K were obtained from Sigma Co. (St. Louis, MO, USA). Xba I restriction enzyme, T4 DNA ligase, and IsoTherm TM DNA sequencing kits were products of Epicentre Technologies (Madison, WI, USA). Agarose gel extraction kits were from Boehringer Mannheim (Germany). Dynazyme and its 10X buffer for PCR were purchased from Finnzymes (Espoo, Finland). Wizard TM DNA purification kits and pGEM.7Zf(-) plasmid vector were obtained from Promega (Madison, WI, USA). All other reagent grade chemicals were purchased from T. T. Baker (Phillipsburg, NJ, USA).

Isolation and purification of MBV from PL. Frozen MBV-infected PL (about 500 g) were placed in a 1 l beaker and quickly thawed in a 37°C water bath. They were homogenized with a polytron homogenizer in 20 mM Tris-HCl (pH 7.8) at 4°C. The homogenate was filtered through 300-mesh nylon, and the filtrate was centrifuged at 1000 × *g* for 10 min at 4°C. The pellet was resuspended and emulsified in an organic solvent mixture (20 mM Tris-HCl:n-butanol:n-hexane = 10:4:1). After centrifugation again at 2000 × *g* for 20 min at 4°C, the pellet was extracted once again with the organic solvent mixture, before being resuspended in Tris buffer and layered on the top of a 36 to 64% (W/V) continuous sucrose gradient. The gradient was centrifuged at 30 000 × *g* for 1 h at 18°C, and a white band containing occlusion bodies was collected and suspended in Tris-EDTA buffer (10 mM Tris-HCl, 0.1 mM EDTA). The suspension was centrifuged at 2500 × *g* for 30 min, and the presence of MBV occlusion bodies in the pellet was confirmed by eosin staining. Purified MBV occlusion bodies were stored in Tris-EDTA buffer at –20°C.

Isolation of MBV genomic DNA. An aliquot of the MBV occlusion body suspension containing 20 µg of protein as determined by Bradford assay was added to 1 ml of TN buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl), and viral proteins were digested with proteinase K (100 µg ml^{-1}) at 37°C for 30 min. Concentrated sodium lauryl sarkosome solution (10% w/v) (25 µl) and 100 µl of 5 M NaCl were added to the reaction mixture in a microfuge tube and then mixed on a vortex mixer. Then 150 µl of CTAB/NaCl (10% CTAB in 0.7 M NaCl) was mixed in and heated at 65°C for 30 min. The reaction mixture was extracted once with an equal volume of phenol/chloroform. The supernatant was further extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Viral DNA in the supernatant was then precipitated with 0.6 volumes of isopropanol at room temperature. After centrifugation at 13 000 × *g* for 15 min, the viral DNA pellet was washed with 70% ethanol and dissolved in 10 mM Tris-HCl and 1 mM EDTA, pH 8.0.

WSSV and AcMNPV sample preparation. The purification of WSSV DNA was carried out as described in Wang et al. (1995). AcMNPV strain E2 (Smith & Summers 1978) was propagated in IPLB SF-21 AE cells. The isolation and purification of polyhedra and virus

particles, and DNA preparation were as described by Summers & Smith (1987).

PCR amplification using primers based on DNA polymerase genes. Two primers were designed according to conserved amino acid sequences of the DNA polymerase gene of 2 insect baculoviruses LdMNPV and AcMNPV (Bjornson et al. 1992). These primers were: Primer 1, 5'-TCT AGA GTI AAT CGC GTI CAC ATG CAI A-3', and Primer 2, 5'-TCT AGA ATC IAT GTG AAT GTA ITA-3'. An Xba I recognition site (TCT AGA) was added to the 5' end of each primer.

PCR amplifications were performed in 100 µl reactions containing 10 ng of viral DNA, 1.6 µM each of the 2 primers, 10 µl of 10X Dynazyme buffer (supplied by the manufacturer), 20 mM mixture of all 4 deoxynucleotides, and 5 units of Dynazyme. The reactions were overlaid with mineral oil and heated to 94°C for 10 min before the start of the PCR cycle in a Perkin-Elmer DNA Thermal Cycler (Norwalk, CT, USA). The first 5 cycling parameters were: denaturing for 2 min at 94°C, annealing for 1 min at 42°C, extension for 1 min at 72°C. This was followed by 30-cycle PCR using the following parameters: denaturing for 2 min at 94°C, annealing for 1 min at 59°C, and extension for 1 min at 72°C. During the final cycle, extension lasted 10 min. PCR reactions were stopped at 4°C, and the presence of the PCR product was examined on a 2% agarose gel and molecular size was estimated.

PCR amplification using primers based on polyhedrin genes. For comparative purposes, PCR primers based on the sequence of the MBV polyhedrin gene (Chang et al. 1993) were also used with MBV and AcMNPV genomic DNA as the template. The size of the expected PCR fragment was 674 bp. The conditions for the PCR reaction were the same as those described above for the primers designed from DNA polymerase sequences.

Subcloning and sequencing of PCR products. PCR-amplified DNA was extracted from a 2% agarose gel with an agarose gel extraction kit, digested with Xba I, and ligated to a linearized pGEM.7Zf(-) plasmid. The ligated plasmid (2 µg) was mixed with 0.21 ml of transformation-competent JM109 cells, and the plasmid DNA was transformed into the bacterial host with heat shock at 42°C for 90 s using standard transformation procedures. Plasmid minipreparations were purified with a Wizard TM DNA cleanup system kit before sequencing. DNA sequencing was performed following annealing of appropriate primers (20 ng μl^{-1}) to alkali-denatured plasmid DNA (5 µg in 8 µl H_2O), by using an IsoTherm TM sequencing kit. Based on the sequencing results, the actual sequences for Primers 1 and 2 were 5'-TCT AGA GTG AAT CGC GTG CAC ATG CAG A-3' and 2: 5'-TCT AGA ATC GAT GTG AAT GTA GTA-3'. The sequence of one 714 bp cloned amplicon was used to design internal primers for specific MBV detection.

PCR detection of MBV using primers designed from the 714 bp clone. From the selected 714 bp PCR product clone, a forward internal primer, pA1, 5'-GTG CTG CAA CGA CTG GAC TGT CC-3', and a reverse internal primer, pA2, 5'-TGA TCC TAG GCG TGC CTG TAT TGG G-3', were designed for specific detection of MBV. The size of the expected amplicon from these primers was 511 bp. For comparative purposes, PCR primers based on the sequence of the MBV polyhedrin gene as described by Chang et al. (1993) were used. These primers yielded a 674 bp amplicon. Total DNA isolated from a shrimp cell line (PMO cell line) or from various tissues of normal and WSSV-infected penaeid shrimp were incubated with different groups of primers and subjected to PCR analysis. Total DNA from AcMNPV was also tested with the PCR primers described. PCR products were examined by electrophoresis using 2% agarose gels.

ELISA for MBV occlusion bodies. Polyclonal antibodies against purified occlusion bodies (OB) of MBV (100 µg) were produced in a New Zealand rabbit via intraspleenic immunization. Then an ELISA test was conducted to detect antibody titers each week up to 3 wk after the final booster. Purified OB of MBV (0.2 µg) in 0.05 M sodium carbonate (pH 9.6) were coated onto a 96-well microtiter plate at 4°C overnight followed by the addition of 3% bovine serum albumin as a blocking agent at 37°C for 1 h. Plates were washed 3 times. Next rabbit anti-MBV serum (1/1000 dilution or more) was added at 37°C for 1 h followed by goat anti-rabbit IgG-horseradish peroxidase serum (1/200 dilution) at 37°C for 1 h and finally TMB (3, 3', 5, 5'-tetramethyl benzidine) for color development. The color reaction was stopped with 1 N H_2SO_4. The optical density (OD) of wells in the microtiter plate was measured at 450 nm with an ELISA reader (Dynatech MR5000). For competitive ELISA, rabbit anti-MBV serum was first incubated with various amounts of competing agents at 37°C for 1 h. The mixture was then added to a 96-well plate coated with 0.2 µg of MBV OB. The rest of the procedure was the same as previously described. The OD obtained in the presence of a competing antigen (A) was normalized against the OD obtained in its absence (A_0).

RESULTS AND DISCUSSION

Verification of occlusion bodies and MBV genomic DNA

The identity of purified MBV occlusion bodies was confirmed by eosin staining and examination by light microscopy (2.5 to 7.5 µm diameter) (Fig. 1). They were

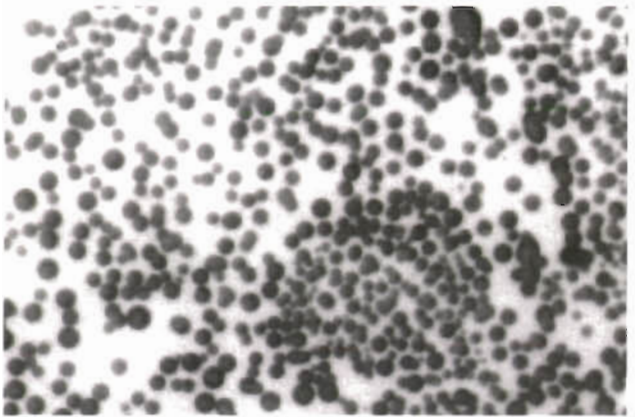

Fig. 1. Occlusion bodies of monodon-type baculovirus. Photography of purified eosin-stained occlusion bodies of MBV by light microscopy (400×)

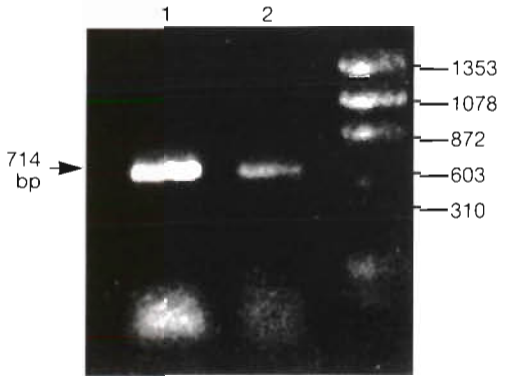

Fig. 3. Effect of preliminary annealing temperature on the PCR yield from MBV using primers based on conserved regions of baculovirus DNA polymerase genes. Lane 1: annealing temperature for the first 5 PCR cycles was 42°C, lane 2: annealing temperature for the first 5 PCR cycles was 45°C

stored at 4°C before genomic DNA extraction to provide PCR template. By using the pair of primers designed for MBV polyhedrin, a 674 bp product was amplified from both AcMNPV and MBV DNA (Fig. 2, lanes 1 and 2). No PCR products were obtained from WSSV, and PMO cells or healthy, uninfected hepatopancreatic tissue or intestinal tissue of adult shrimp (Fig. 2, lanes 3, 4, 5, 6, and 7). Obviously these primers were unable to distinguish between MBV and AcMNPV.

PCR products using MBV with DNA polymerase primers

By using Primers 1 and 2 designed from conserved amino acid sequences of insect baculovirus DNA polymerase, a 714 bp PCR product was amplified from MBV DNA (Fig. 3, lane 1; Fig 4, lane 1). By contrast, these primers gave a 621 bp fragment with AcMNPV DNA (Fig. 4, lane 2). The best annealing temperature for the first 5 PCR cycles was 42°C (Fig. 3, Lane 1). At 45°C, the amount of PCR product obtained was much less (Fig. 3, lane 2) and at 49°C, it became a smear (data not shown). Sequencing of the 714 bp fragment (Fig. 5) revealed the complete sequences of the primers, 3 TAG stop codons at the 5′ end and 2 at the 3′ end. However, there were no stop codons in the 465 bp middle region. From sequence comparison (NCBI/BLAST/fasta), only the designed primers from the DNA polymerase gene gave high homology to LdMNPV and AcMNPV sequences. There was very little homology in the remainder of the 714 bp fragment. When different PCR reaction conditions and different

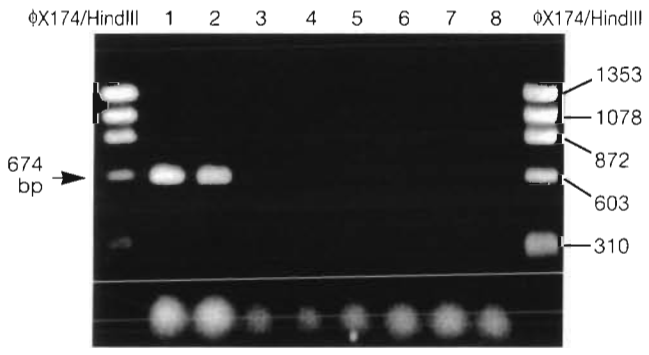

Fig. 2. Genome confirmation of monodon-type baculovirus by PCR using primers based on insect baculovirus polyhedrin genes. DNA templates used were lane 1. MBV, lane 2: AcMNPV, lane 3: WSSV, lane 4: PMO cells, lane 5: normal tiger prawn PL, lane 6: hepatopancreatic tissue of an adult tiger prawn, lane 7: intestinal tissue of an adult tiger prawn, lane 8: no DNA template. Size markers (*Hind* III cut of phage X174 DNA) are shown on both sides

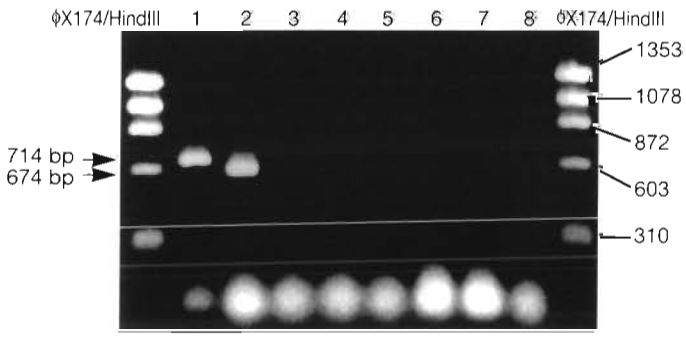

Fig. 4. Diagnosis of MBV by PCR using primers based on conserved regions of the baculoviral DNA polymerase gene. DNA templates were lane 1. MBV, lane 2: AcMNPV, lane 3: WSSV, lane 4: PMO cells, lane 5: normal tiger prawn PL, lane 6: hepatopancreatic tissue from an adult tiger prawn, lane 7: intestinal tissue of an adult tiger prawn, lane 8: no DNA template. Size markers are shown on both sides

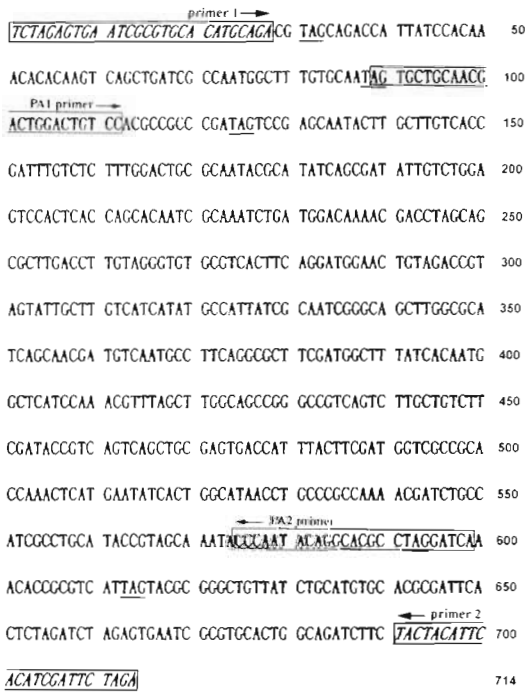

Fig. 5. DNA sequence of the 714 bp PCR fragment from MBV. Primers are shown in rectangular boxes. TAG stop codons are underlined. Positions of the inner primers designed specifically for MBV are noted as PA1 and PA2

thermo-cyclers were used, these 5 stop codons were always present, so they truly existed in the sequence. The consistency of the amplicon sequence from only extracts containing MBV confirmed that the sequence was part of the MBV genome and so it was used to further develop a specific PCR assay.

Although these primers gave PCR products for both MBV (714 bp) and AcMNPV (621 bp) (Fig. 4, lanes 1 and 2), they gave none with purified WSSV DNA (Fig. 4, lane 3) or DNA extracted from WSSV-infected shrimp. Other negative controls such as DNA templates from PMO cells, normal PL and hepatopancreatic tissue or intestinal tissue from adult *Penaeus monodon* gave no PCR products (Fig. 4, lanes 4, 5, 6, and 7). These data indicated that the designed primers might perhaps be used for PCR diagnosis of MBV in the absence of other baculoviral DNA. On the other hand, the cross reaction of Primers 1 and 2 with MBV and AcMNPV was apparently fortuitous and not a result of common sequence in DNA polymerase genes, as expected when this work was begun. The function of this MBV sequence remains to be determined.

MBV-specific primers derived from the 714 bp fragment

Based on the sequence of the 714 bp MBV genomic DNA amplicon (Fig. 5), a new pair of primers (PA1 and

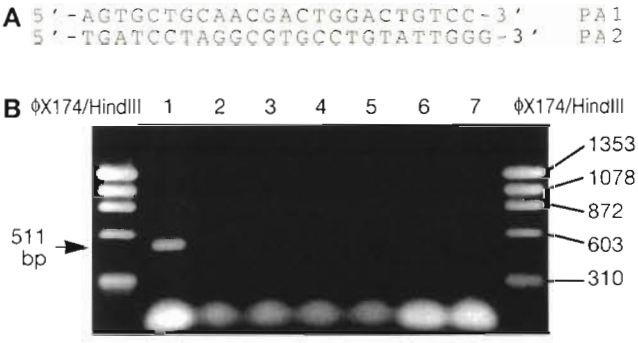

Fig. 6. MBV-specific PCR using primers PA1 and PA2 (A) designed from the 714 bp fragment. (B) Lane 1: MBV, lane 2: AcMNPV, lane 3: WSSV, lane 4: PMO cells, lane 5: normal tiger prawn PL, lane 6: hepatopancreatic tissue of an adult tiger prawn, lane 7: intestinal tissue of an adult tiger prawn. Size markers are shown on both sides

PA2) were designed (Fig. 6A). These primers yielded a 511 bp PCR product only with MBV DNA (Fig. 6B, lane 1) and not with DNA of AcMNPV, WSSV, PMO, PL or hepatopancreatic tissue or intestinal tissue of tiger prawns (Fig. 6B, lanes 2, 3, 4, 5, 6, and 7). Thus, they may be used as a species specific assay for MBV that yields a 511 bp PCR amplicon.

ELISA of MBV

The highest antibody titer appeared 4 wk after the booster and this serum was therefore used for all experiments. The sensitivity of the polyclonal antibody was measured by ELISA. Serial dilutions (10-fold) of viral antigen (OB of MBV) and antiserum were prepared for these tests. The antiserum could detect MBV antigen at nanogram levels (Fig. 7).

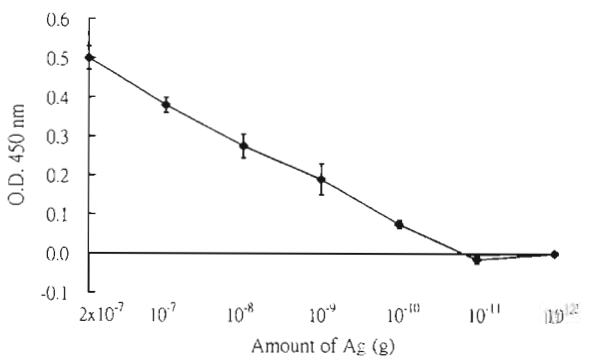

Fig. 7. ELISA test for MBV. Sensitivity of polyclonal antibodies to MBV. Ten-fold dilutions of purified occlusion bodies of MBV were coated onto 96-well microtiter plates and polyclonal antibodies were added. Data points represent means with standard deviation bars

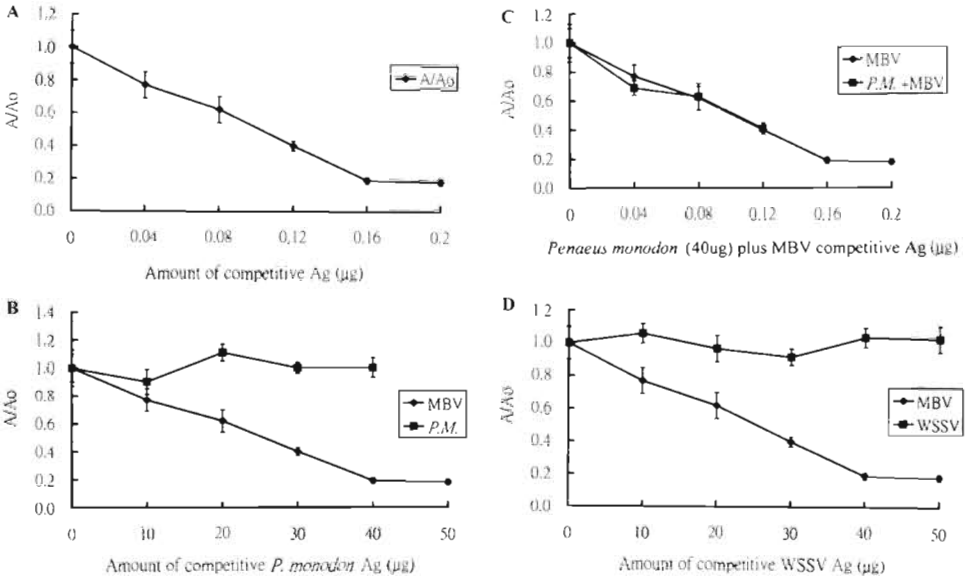

Fig. 8. Competitive ELISA test for MBV. (A) Standard curve for the competitive ELISA. A 1000-fold dilution of rabbit anti-MBV antiserum was mixed with different concentrations of purified occlusion bodies of MBV at 37°C for 1 h; then the mixture was added to MBV-coated (0.2 μg) microtiter plates. (B) Competitive ELISA for MBV and tiger prawn. A 1000-fold dilution of antibodies was mixed with different concentrations of MBV or with homogenates of the tiger prawn. Then a competitive ELISA was performed as in (A). *P.M.*: *Penaeus monodon*. (C) Competitive ELISA for MBV and MBV + tiger prawn. Different concentrations of MBV or MBV + 40 μg tissue homogenate were used as antigens. (D) Competitive ELISA for MBV and WSSV. Different concentrations of MBV and WSSV were used as antigens in a competitive ELISA

Competitive ELISA

Dilutions (1000-fold) of anti-MBV serum were mixed with various OB concentrations (0, 0.04, 0.08, 0.12, 0.16 and 0.20 μg) at 37°C for 1 h. The mixtures were then added to wells of an MBV-coated (0.2 μg) in 96-well microtiter plates. HRP-goat anti-rabbit IgG was then added and color reactions were conducted as described in the 'Materials and methods'. The standard curve for competitive ELISA with MBV OB is shown in Fig. 8A. To test for cross reactions between MBV antiserum and hpatopancreatic tissue of the tiger prawn, a competitive ELISA for MBV was conducted using hepatopancreatic tissue (Fig. 8B) and OB mixed with hepatopancreatic tissue (Fig. 8C). The anti-MBV polyclonal antiserum reacted only with MBV. In a competitive ELISA using WSSV and MBV, purified OB (0, 0.04, 0.08, 12 and 0.16 μg) and WSSV samples (10, 20, 30, and 40 μg) were mixed individually with 1/1000 dilution of rabbit antiserum at 37°C for 1 h. Then these mixtures were added individually to an MBV-coated 96-well microtiter plate for competitive ELISA. The rabbit antiserum reacted only with MBV, and not with WSSV (Fig. 8D).

Thus, polyclonal rabbit antiserum prepared from MBV OB could specifically detect OB of MBV in 40 μg homogenates of tiger prawn tissue at nanogram levels using ELISA. It might find wider application than PCR as a specific diagnostic tool for MBV detection in the field.

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LITERATURE CITED

Bjornson RM, Glocker B, Rohrmann GF (1992) Characterization of the nucleotide sequence of the *Lymantria dispar* nuclear polyhedrosis virus DNA polymerase gene region. J Gen Virol 73:3177–3183

Boonyaratpalin S, Supamattaya K, Kasornchandra J, Direkbusaracon S, Aelpainthanpong U, Chantanachooklin C (1993) Non-occluded baculo-like virus, the causative agent of yellow head disease in the black tiger shrimp (*Penaeus monodon*). Gyobyo Kenkyu 28:103–109

Chang PS, Lo CF, Kou GH, Lu CC, Chen SN (1993) Purification and amplification of DNA from *Penaeus monodon*-type baculovirus (MBV). J Invertebr Pathol 62:116–120

Chen SN, Chang PS, Kou GH (1989a) Observation on pathogenicity and epizootiology of *Penaeus monodon* Baculovirus (MBV) in cultured shrimp in Taiwan. Fish Pathol 24: 189–195

Chen SN, Chang PS, Kou GH, Lightner DV (1989b) Studies on virogenesis and cytopathology of *Penaeus monodon* Baculovirus (MBV) in the giant tiger prawn (*Penaeus monodon*) and the red tail prawn (*Penaeus penicillatus*). Fish Pathol 24:89–100

Couch JA (1974) An enzootic nuclear polyhedrosis virus of pink shrimp: ultrastructure, prevalence and enhancement. J Invertebr Pathol 24:311–331

Couch JA (1981) Viral disease of invertebrates other than insects. In: Davidson EW (ed) Pathogenesis of invertebrate microbial diseases. Allanheld, Osmun Publishers, Totowa, NJ, p 125–160

Lightner DV (1985) A review of the disease of cultured penaeid shrimps and prawns with emphasis in recent discoveries and developments. In: Taki Y, Primavera JH, Llobrera JA (eds) Proceedings of the first international conference on the culture of penaeid prawns/shrimps. Published by aquaculture department Southeast Asian Aquaculture Development Center, Ilolo, Philippines, p 79–103

Lightner DV, Redman RM (1981) A baculovirus-caused disease of the penaeid shrimp *Penaeus monodon*. J Invertebr Pathol 38:299–302

Lightner DV, Redman RM (1985) A parvo-like virus disease of penaeid shrimp. J Invertebr Pathol 45:47–53

Lightner DV, Redman RM, Bell TA (1983a) Infectious hypodermal and hematopoietic necrosis, a newly recognized virus disease of penaeid shrimp. J Invertebr Pathol 42: 62–70

Lightner DV, Redman RM, Bell TA (1983b) Observation on the geographic distribution, pathogenesis and morphology of the baculovirus from *Penaeus monodon* Fabricius. Aquaculture 32:209–233

Lightner DV, Hedrick RP, Fryer JL, Chen SN, Liao IC, Kou GH (1987) A survey of cultured penaeid shrimp in Taiwan for viral and other diseases. Fish Pathol 22:127–140

Lu Y, ECBN Jr, Brock JA, Loh PC (1991) A new virus isolate from infectious hypodermal and hematopoietic necrosis virus (IHHNV) infected penaeid shrimps. J Virol Methods 31:189–196

Lu CC, Tang KFJ, Kou GH, Chen SN (1995) Detection of *Penaeus monodon*-type baculovirus (MBV) infection in *Penaeus monodon* Fabricius by in situ hybridization. J Fish Dis 18:337–345

Nunan LM, Lightner DV (1997) Development of a non-radioactive gene probe by PCR for detection of white spot syndrome virus (WSSV). J Virol Methods 63:193–201

Owens LS, De Beer S, Smith J (1991) Lymphoidal parvovirus-like particles in Australian penaeid prawns. Dis Aquat Org 11:129–134

Sano T, Nishimura T, Oguma K, Momoyama K, Takeno N (1981) Baculovirus infection of cultured kuruma shrimp, *Penaeus japonicus* in Japan. Fish Pathol 15:185–191

Smith GE, Summers MD (1978) Analysis of baculovirus genomes with restriction endonucleases. Virology 89: 517–527

Summers MD, Smith GE (1987) A manual of methods for baculovirus vectors and insect cell culture procedures. Tex Agric Exp Stn Bull 1555:1–57

Tsing A, Bonami JR (1984) A new virus disease in the shrimp *Penaeus japonicus*. Premier collogue international de pathologieen aquaculture marine, Laboratoire de pathologie comparee, Montpellier Cedex

Vickers JE, Paynter JL, Spradbrpw PB, Lester RJG (1993) An impression smear method for rapid detection of *Penaeus monodon*-type baculovirus (MBV) in Australian prawns. J Fish Dis 16:507–511

Wang CH, Lo CF, Leu JH, Chou CM, Yeh PY, Chou HY, Tung MC, Chang CF, Su MS, Kou GH (1995) Purification and genomic analysis of baculovirus associated with white spot syndrome (WSBV) of *Penaeus monodon*. Dis Aquat Org 23:239–242

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