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Simple immunoblot and immunohistochemical detection of Penaeus stylirostris densovirus using monoclonal antibodies to viral capsid protein expressed heterologously

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A B S T R A C T

Penaeus stylirostris densovirus (PstDNV), called formerly infectious hypodermal and hematopoietic necrosis virus (IHHNV), is an important shrimp pathogen which can cause mortality in the blue shrimp *Penaeus* (*Litopenaeus*) *stylirostris* and stunting in the whiteleg shrimp *Penaeus* (*Litopenaeus*) *vannamei*. Five monoclonal antibodies (MAbs) were produced against the 37 kDa capsid protein 3 (CP3) of PstDNV expressed heterologously in the form of a fusion protein with glutathione-S-transferase called GST-CP3. All MAbs belonged to the IgG2b subclass and could bind to GST-CP3 at 300 pg/spot in immunodot-blot tests. They could detect CP3 in naturally infected shrimp extracts by Western blotting and dot blotting and in shrimp tissues by immunohistochemistry without cross-reactivity to extracts from uninfected shrimps or shrimps infected with several other viruses. Although dot blot assay sensitivity was approximately 1000 times lower than that of one step PCR for PstDNV, it easily detected PstDNV infections in field samples of *Penaeus monodon* and *Penaeus vannamei*.

1. Introduction

Penaeus stylirostris densovirus (PstDNV) (Tattersall et al., 2005) was called formerly infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Lightner et al., 1983). It is an important shrimp pathogen which can cause mortality in the blue shrimp *Penaeus* (*Litopenaeus*) *stylirostris* and stunting (called runt deformity syndrome) in the whiteleg shrimp *Penaeus* (*Litopenaeus*) *vannamei* (Bell and Lightner, 1984; Castille et al., 1993). The virus is endemic in the range of the giant tiger shrimp *Penaeus monodon* in Africa, Australia and Asia (Primavera and Quinitio, 2000; Tang et al., 2003; Krabsetsve et al., 2004; Flegel, 2006; Teng et al., 2006; Yang et al., 2007; Rai et al., 2009). However, this virus was first discovered in the Americas after it spreaded from normal imported, experimental stocks *P. monodon* to cultured stocks of *P. stylirostris* (Lightner et al., 1983). It spread subsequently widely in *P. stylirostris* and *P. vannamei* in the Americas (Garver et al., 2003; Motte et al., 2003; Braz et al., 2009). Despite the negative affect on American shrimp species, PstDNV infection in *P. monodon* has not been associated with serious disease outbreaks during the cultivation of Asian shrimp species (Chayaburakul et al., 2005; Withyachumnarnkul et al., 2006). There are two counter reports, one previous report that proposed PstDNV as the cause of runt deformity syndrome in *P. monodon* broodstock in the Philippines (Primavera and Quinitio, 2000) and another recent report suggesting that PstDNV is associated with slow growth in *P. monodon* (Rai et al., 2009).

In early studies, PstDNV detection was achieved mainly by histological examination (Lightner et al., 1983; Bell and Lightner, 1984; Flegel et al., 1999). Later, molecular techniques such as *in situ* DNA hybridization assays (Mari et al., 1993; Carr et al., 1996), single polymerase chain reaction (PCR) assays (Anon., 2006; Yang et al., 2007), duplex PCR assays (Yang et al., 2006) or multiplex PCR assays (Xie et al., 2007; Khawsak et al., 2008) were developed. Other nucleic acid amplification methods developed included ramification amplification (Teng et al., 2006), loop-mediated isothermal amplification (Sun et al., 2006) and quantitative real-time PCR in either single (Tang and Lightner, 2001) or duplex assays (Dhar et al., 2001). However, recent results with PCR-based detection have shown false positive results for PstDNV detection in some

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P. monodon specimens from Africa and Australia, due to the presence of non-infectious, PstDNV-related sequences inserted into the *P. monodon* genome (Tang and Lightner, 2006). Subsequently, a PCR method was developed to distinguish infectious PstDNV from the non-infectious, inserted sequences (Tang et al., 2007).

Although PCR-based methods are used widely for shrimp virus detection in the laboratory, they are not suitable for pond-side detection. By contrast, simpler immunological-based methods using monoclonal antibodies (MAbs) have been developed for detection of shrimp pathogens such as white spot syndrome virus or WSSV (Poulos et al., 2001; Anil et al., 2002; Chaivisuthangkura et al., 2004), yellow head virus or YHV (Sithigorngul et al., 2000, 2002), Penaeus monodon densovirus (PmDNV), called formerly hepatopancreatic parvovirus or HPV (Rukpratanporn et al., 2005), Penaeus monodon nucleopolyhedrovirus (PemoNPV) called formerly monodon baculovirus or MBV (Boonsanongchokying et al., 2006), Taura syndrome virus or TSV (Longyant et al., 2008), *Vibrio harveyi* (Phianphak et al., 2005) and *Vibrio alginolyticus* (Sithigorngul et al., 2006a). Although immunological methods have lower detection sensitivity than PCR methods, they provide a field-friendly, inexpensive way to confirm infections with high accuracy and speed. Examples are rapid strip tests suitable for use at the pond side by unskilled personnel (Powell et al., 2006; Sithigorngul et al., 2006b, 2007a,b).

An early report described the use of 6 IgM MAbs generated against purified PstDNV for its immunodetection in shrimp by ELISA (Poulos et al., 1994). Unfortunately, detection was not reliable since some of the shrimp samples that gave negative results by ELISA gave positive results for PstDNV by histopathology or DNA hybridization and *vice versa*. Here, we describe a new attempt to develop MAbs specific to a recombinant capsid fusion protein of PstDNV to detect PstDNV-infected shrimp by immunodot, Western blot and immunohistochemical assays.

2. Materials and methods

2.1. Viral preparation

P. vannamei (ca. 25 g body weight) infected naturally with PstDNV and showing gross signs of runt deformity syndrome were obtained from a shrimp farm in Trang Province, Southern of Thailand. *P. monodon* (approximately 20 g body weight) infected naturally with PstDNV and showing darkened body color was provided by Dr. A. Udomkit, Institute of Molecular Biology and Genetics, Mahidol University. Pleopods from infected shrimp (1 piece/50 μl) were homogenized in 2× PBS (0.3 M phosphate buffered saline, pH 7.2), then centrifuged at 3000 × *g* for 30 min; 0.5 ml aliquots of the supernatant were stored at −70 °C. The PstDNV infection was verified by PCR using primers specific to PstDNV capsid protein gene as described below.

2.2. PstDNV DNA preparation

Pleopods from *P. vannamei* infected naturally with PstDNV were homogenized in lysis buffer (50 mM Tris–HCl, pH 9, 100 mM EDTA, 50 mM NaCl, 2% SDS; Flegel, pers. Comm.) and DNA was extracted from 200 μl of homogenate by ion-exchange purification using a High Pure Viral Nucleic Acid Kit (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.), as described in the product manual. The extracted nucleic acid was stored at −70 °C until use.

2.3. Cloning and expression of PstDNV capsid protein gene

DNA extracts from PstDNV-infected shrimps (above) were used as the template to isolate PstDNV capsid protein gene 3 (*CP3*) by PCR amplification using *Pfx* polymerase (Invitrogen, Carlbad, California, U.S.A.) with primers IHHNVF (5′-CG GGA TCC ATG TGC GCC GAT TCA ACA AG-3′) and IHHNVR (5′-CCG CTC GAG TTA GTT AGT ATG CAT AAT ATA AC-3′) containing added restriction sites (underlined). The 1007 bp PCR product was cloned into *Bam*HI and *Xho*I sites of the pGEX-6P-1 expression vector to yield *CP3*-pGEX-6P-1 for transformation into *Escherichia coli* strain BL21. The integrity of the open reading frame of the recombinant plasmid was verified by DNA sequencing.

2.4. Preparation of recombinant PstDNV capsid protein

E. coli BL21 harboring *CP3*-pGEX-6P-1 was cultured in Luria–Bertani (LB) broth to exponential phase and expression of recombinant fusion protein GST-CP3 (26 kDa + 37 kDa = 63 kDa) was induced with 1 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG) for 4 h. After centrifugation at 3000 × *g* for 20 min the bacterial pellet was suspended in 100 mM NaH_2PO_4, 10 mM Tris–HCl, 8 M urea, pH 8, containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and sonicated until a clear lysate was obtained. The lysate was resolved using 12% SDS-PAGE, 80 V for 2 h. After treatment with 0.3 M KCl, recombinant GST-CP3 protein bands were cut, collected in dialysis bags, and the protein was eluted with Trans-Blot apparatus (BioRad, Hercules, California, U.S.A.) at 70 V for 6 h. The GST-CP3 solution was dialyzed and concentrated in a vacuum concentrator (Savant) before determining the protein content by Bradford protein assay (Bradford, 1976). The GST-CP3 solution was adjusted to 1 mg/ml and divided into 0.5 ml aliquots before storage at −70 °C.

2.5. Production of monoclonal antibodies

The immunization and cell fusion protocols for production of hybridoma were similar to those described by Longyant et al. (2008). The selection of hybridoma clones producing MAbs specific to recombinant PstDNV capsid protein was done by dot blotting, Western blotting and immunohistochemistry as described below.

2.5.1. Dot blotting

Lysates of *E. coli* BL21 containing pGEX-6P-1 expression vector (GST), of *E. coli* containing *CP3*-pGEX-6P-1 (i.e., with GST-CP3 fusion protein) at 10 μg/ml, of pleopod homogenates from uninfected shrimps or of PstDNV-infected *P. monodon* were applied to nitrocellulose membranes (1 μl/spot) followed by heating at 60 °C for 10 min and incubation in full grown hybridoma-conditioned medium from the culture diluted to 1:20 dilution in 1% blotto (1% nonfat drymilk, 0.1% Triton X-100 in PBS) for 5 h. After extensive washing in 0.1% blotto, the membrane was incubated in horseradish peroxidase labeled goat anti-mouse gamma immunoglobulin heavy and light-chain specific antibody (GAM-HRP, BioRad) at 1:1500 dilution for 3 h. The membrane was then washed for 5 min in 0.1% blotto and incubated for 5 min in substrate mixture containing 0.03% diaminobenzidine (DAB), 0.006% hydrogen peroxide, and 0.05% cobalt chloride in PBS (Sithigorngul et al., 2000).

2.5.2. Western blotting

Lysates of *E. coli* BL21 with pGEX-6P-1 plasmid, of *E. coli* with *CP3*-pGEX-6P-1 plasmid, pleopod homogenates from uninfected shrimps or of PstDNV-infected *P. monodon* were resolved using 12% gel SDS-PAGE according to the method described by Laemmli (1970). Samples were electrophoresed for 3 h at 60 V and one part of the gel was stained using Coomassie brilliant blue R-250. For Western blot analysis, samples resolved by SDS-PAGE were electroblotted onto nitrocellulose membranes using a Trans-Blot apparatus. The nitrocellulose membrane was incubated