รายงานวิจัยฉบับสมบูรณ์

การโคลนและการแสดงออกของโปรตีน VP19 และ VP26 ของไวรัสตัวแดงดวงขาวและการผลิตโมโนโคลนอลแอนติบอดี

Cloning and Expression of Structural Proteins VP19 and VP26 of White Spot Syndrome Virus (WSSV) and Monoclonal Antibodies Production

โดย ผศ. ดร. ปรินทร์ ชัยวิสุทธางกูร และคณะ

มกราคม 2553

สัญญาเลขที่ **RMU4980011**

รายงานวิจัยฉบับสมบูรณ์

การโคลนและการแสดงออกของโปรตีน VP19 และ VP26 ของไวรัสตัวแดงดวงขาวและการผลิตโมโนโคลนอลแอนติบอดี

Cloning and Expression of Structural Proteins VP19 and VP26 of White Spot Syndrome Virus (WSSV) and Monoclonal Antibodies Production

ผศ. ดร. ปรินทร์ ชัยวิสุทธางกูร
ภาควิชาชีววิทยา คณะวิทยาศาสตร์
มหาวิทยาลัยศรีนครินทรวิโรฒ
และคณะ

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
และสำนักงานคณะกรรมการการอุดมศึกษา

กิตติกรรมประกาศ

โครงการวิจัยเรื่อง “**การโคลนและการแสดงออกของโปรตีน VP19 และ VP26 ของไวรัสตัวแดงดวงขาวและการผลิตโมโนโคลนอลแอนติบอดี (Cloning and Expression of Structural Proteins VP19 and VP26 of White Spot Syndrome Virus (WSSV) and Monoclonal Antibodies Production)”** ได้รับทุนสนับสนุนจากสำนักงานคณะกรรมการการอุดมศึกษาและสำนักงานกองทุนสนับสนุนการวิจัย ตามสัญญาเลขที่ RMU4980011 ระยะเวลาดำเนินการ 3 ปี 6 เดือน ตั้งแต่วันที่ 20 กรกฎาคม 2549 ถึงวันที่ 19 มกราคม 2553 ผู้รับทุนขอขอบพระคุณสำนักงานฯ ทั้งสองที่ให้การสนับสนุนอย่างเต็มที่ทำให้โครงการวิจัยสำเร็จลุล่วงไปได้ และขอขอบคุณบุคคลต่อไปนี้ที่มีส่วนสำคัญให้โครงการสำเร็จได้แก่ ผู้ร่วมวิจัยคือ ผศ.ดร. ศิวาพร ลงยันต์ และ ศ.ดร. ไพศาล สิทธิกรกุล (ภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ) ผู้ช่วยวิจัยคือ นาย สมบัติ รักประทานพร (ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ) และ น.ส. ชุติมา ศรีสุข (ภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ) รวมทั้งภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ ในฐานะสถาบันต้นสังกัดที่ให้ความสนับสนุนและอำนวยความสะดวกในโครงการวิจัย

บทคัดย่อ

รหัสโครงการ: RMU4980011

ชื่อโครงการ: การโคลนและการแสดงออกของโปรตีน VP19 และ VP26 ของไวรัสตัวแดงดวงขาวและการผลิตโมโนโคลนอลแอนติบอดี

ชื่อนักวิจัย และสถาบัน: ผู้ช่วยศาสตราจารย์ ดร. ปรินทร์ ชัยวิสุทธางกูร
ภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ
E-mail Address: parin@swu.ac.th; parinc@yahoo.com

ระยะเวลาโครงการ: 20 กรกฎาคม 2549 ถึงวันที่ 19 มกราคม 2553

โครงการวิจัย:

ไวรัสจุดขาว (white spot syndrome virus; WSSV) เป็นไวรัสที่สำคัญที่ก่อให้เกิดโรคในกุ้งสกุล *Penaeus* ชนิดต่าง ๆ สามารถทำให้เกิดการตายได้ถึง 100% ภายในเวลา 3-10 วันหลังจากติดเชื้อ วัตถุประสงค์ของงานวิจัยนี้เป็นการพัฒนาวิธีตรวจวินิจฉัยทางด้านอิมมูโนโลยี เพื่อเป็นอีกทางเลือกหนึ่งแก่เกษตรกรในการตรวจการติดเชื้อ WSSV โดยอาศัยการโคลนและการแสดงออกของโปรตีนโครงสร้าง VP19 และ VP26 ของ WSSV เพื่อนำมาใช้เป็นแอนติเจนในการสร้างโมโนโคลนอลแอนติบอดี ที่สามารถช่วยเสริมกับแอนติบอดีต่อ VP28 ทำให้มีความไวในการตรวจวินิจฉัยสูงขึ้น

ได้ทำการโคลนยีน VP19 เข้าสู่เวกเตอร์ pMAL-C2 และกระตุ้นให้แสดงออกในรูปของ maltose binding protein (MBP)-VP19 จากนั้นนำ fusion protein ที่ได้ไปปลูกภูมิคุ้มกันในหนูขาวเพื่อสร้างโมโนโคลนอลแอนติบอดี (monoclonal antibody; MAb) พบว่า MAb สามารถตรวจการติดเชื้อ WSSV ตามธรรมชาติในกุ้งขาวได้โดยวิธี dot blotting, western blotting และ immunohistochemistry โดยไม่ทำปฏิกิริยาข้ามกับไวรัสชนิดอื่นได้แก่ Taura syndrome virus (TSV), yellow head virus (YHV), monodon baculovirus (MBV) และ hepatopancreatic parvovirus (HPV) MAb W25-8D ที่จำเพาะต่อ VP19 มีความไวประมาณ 1.2 fmole/จุด เมื่อใช้โปรตีน MBP-VP19 บริสุทธิ์ในการทดสอบโดยวิธี dot blotting เมื่อนำ MAb W25-8D นี้ไปใช้ร่วมกับ MAb W29 ที่จำเพาะต่อ VP28 ในการตรวจสารสกัดจากกุ้งที่ติดเชื้อ WSSV โดยวิธี dot blotting พบว่ามีความไวสูงขึ้น 2 เท่าแต่ยังคงมีความไวต่ำกว่าวิธี one-step PCR 25,000 เท่า เมื่อนำ MAb W25-8D มาใช้ตรวจการติดเชื้อในเนื้อเยื่อโดยวิธี immunohistochemistry พบว่าสามารถตรวจพบ VP19 ได้ทั้งในนิวเคลียสและไซโตพลาซึม ซึ่งแตกต่างจาก MAb W29 ที่สามารถตรวจพบ VP28 ได้เฉพาะในนิวเคลียสเท่านั้น

ในกรณีของ VP26 ได้ทำการโคลนยีน VP26 ที่ปราศจากส่วน transmembrane เข้าสู่เวกเตอร์ pQE30 และกระตุ้นให้แสดงออกในรูปของ 6xHis-VP26 จากนั้นนำ fusion protein ที่ได้ไปปลูกภูมิคุ้มกันในหนูขาวเพื่อสร้างโมโนโคลนอลแอนติบอดี พบว่า MAb สามารถตรวจการติดเชื้อ WSSV ตามธรรมชาติในกุ้งขาวได้โดยวิธี dot blotting, western blotting และ immunohistochemistry โดยไม่ทำปฏิกิริยาข้ามกับไวรัสชนิดอื่นได้แก่ TSV, YHV, MBV และ infectious hypodermal and hematopoietic necrosis virus (IHHNV) เมื่อนำ MAb W26-3 นี้ไปใช้ในการตรวจสารสกัดจากกุ้งที่ติดเชื้อ WSSV โดยวิธี dot blotting พบว่ามีความไวต่ำกว่า MAb W29 ที่จำเพาะต่อ VP28 ประมาณ 2 เท่า

เมื่อนำ MAb W26-3 มาใช้ตรวจการติดเชื้อในเนื้อเยื่อโดยวิธี immunohistochemistry พบว่ารูปแบบของ immunoreactivity เหมือนกับของ VP28 คือสามารถตรวจพบได้เฉพาะในนิวเคลียสเท่านั้น

คำหลัก: โมโนโคลนอลแอนติบอดี, ไวรัสจุดขาว, VP19, VP26, immunohistochemistry, การแสดงออกของโปรตีน

Abstract

Project Code: RMU4980011

Project Title: Cloning and Expression of Structural Proteins VP19 and VP26 of White Spot Syndrome Virus (WSSV) and Monoclonal Antibodies Production

Investigator: Assistant Professor Dr. Parin Chaivisuthangkura

Department of Biology, Faculty of Science, Srinakharinwirot University

E-mail Address: parin@swu.ac.th; parinc@yahoo.com

Project Period: 20 July 2006 – 19 January 2010

Project Description:

White spot syndrome virus (WSSV) is a serious pathogen causing white spot disease in various *Penaeus* shrimp species. The WSSV infected-shrimp could die within 3 to 10 days post infection. This research project aims to develop the antibody-based assay as an alternative method for WSSV detection. The VP19 and VP26 structural protein genes of WSSV were cloned and expressed and used for monoclonal antibodies production. These antibodies can be used to combine with the VP28-specific monoclonal antibody obtained from previous study to enhance the sensitivity of detection for WSSV.

The gene encoding the VP19 envelope protein of White Spot Syndrome Virus (WSSV) was cloned into pMAL-C2 expression vector and transformed into the BL21 *E. coli* strain. After induction, recombinant maltose binding protein (MBP)-VP19 fusion protein was produced, purified and electroeluted before use for immunization in Swiss mice for monoclonal antibody (MAb) production. MAbs specific to VP19 can be used to detect natural WSSV infection in *Penaeus vannamei* by dot blotting, western blotting and immunohistochemistry without cross-reaction to other shrimp tissues or other common shrimp viruses, including Taura syndrome virus (TSV), yellow head virus (YHV), *Penaeus monodon* nucleopolyhedrovirus (PemoNPV) formerly called monodon baculovirus (MBV) and *Penaeus monodon* densovirus (PmDNV) previously called hepatopancreatic parvovirus (HPV). The detection sensitivity of the VP19-specific W25-8D MAb generated in this study was approximately 1.2 fmole/spot of purified recombinant MBP-VP19 protein as determined by dot blotting while that of a VP28-specific W29 MAb obtained from a previous study was approximately 5 fmole/spot. Combining MAbs specific for VP19 and VP28 resulted in twofold higher sensitivity than use of either MAb alone. However, the sensitivity of the combined MAbs was 25,000 times lower than that of one-step PCR. Immunohistochemical analysis using MAbs specific to VP19 in WSSV-infected gill tissues and appendages demonstrated intense staining patterns in both the nucleus and cytoplasm compared to MAb specific to VP28. In conclusion, combination of VP19- and VP28-specific

MAbs could confirm and enhance the sensitivity of WSSV detection in shrimp in various types of antibody-based assays.

The DNA sequence encoding a truncated structural protein VP26 of white spot syndrome virus (WSSV) was cloned into pQE30 expression vector and transformed into *E. coli* M15(pREP4). After induction, the recombinant 6xHis-VP26 (rVP26) fusion protein was produced, purified and electroeluted before use in immunization of Swiss mice for monoclonal antibody (MAb) production. MAbs specific to VP26 can be used to detect natural WSSV infection in *Penaeus vannamei* by dot blotting, western blotting and immunohistochemistry without cross-reaction to other shrimp tissues or other common shrimp viruses, including TSV, YHV, MBV and *Penaeus stylirostris* densovirus (PstDNV) previously called infectious hypodermal and hematopoietic necrosis virus (IHHNV). The detection sensitivity by dot blotting of the VP26-specific W26-3 MAb generated in this study was approximately twofold less than that of MAb W29 specific to VP28. Immunohistochemical analysis using MAbs specific to VP26 in WSSV-infected tissues demonstrated similar staining pattern in the nucleus compared to MAb specific to VP28.

Keywords: monoclonal antibody, white spot syndrome virus, VP19, VP26, immunohistochemistry, protein expression

รายงานโครงการวิจัยฉบับสมบูรณ์

ชื่อโครงการ การโคลนและการแสดงออกของโปรตีน VP19 และ VP26 ของไวรัสตัวแดงดวงขาวสำหรับการผลิตโมโนโคลนอลแอนติบอดี

Cloning and Expression of Structural Proteins VP19 and VP26 of White Spot Syndrome Virus (WSSV) and Monoclonal Antibodies Production

1. Introduction and Rational

White spot syndrome virus (WSSV) is one of the most serious pathogens affecting the shrimp industry worldwide. WSSV is the sole member of the novel genus *Whispovirus* within the *Nimaviridae* family (Fauquet et al., 2005). At present, at least 40 WSSV structural proteins ranging from 68 to 6,077 amino acid residues in size have been identified (Huang et al., 2002; Zhang et al., 2004; Tsai et al., 2004; Xie et al., 2006; Leu et al., 2009). Six major structural proteins including VP664, VP28, VP24, VP26, VP19, and VP15 have been identified (Tsai et al., 2004). VP28 and VP19 were reported to be located in the viral envelope (van Hulten et al., 2000). However, the functions of VP19 in WSSV assembly and infection are not known. Recently, *in vitro* pull-down and yeast two-hybrid assays demonstrated interactions among several WSSV structural proteins (VP19-VP28, VP19-VP24, VP24-VP26 and VP24-VP24) confirming that these four major proteins can form a multiprotein complex (Zhou et al., 2009).

VP26, one of the structural proteins of WSSV was initially identified as a nucleocapsid protein (van Hulten et al., 2000). However, later investigations demonstrate that VP26 functions as a matrix-like linker protein, called tegument protein that is loosely associated with both the envelope and the nucleocapsid of virions (Xie and Yang, 2005; Tsai et al., 2006; Wan et al., 2008; Chang et al., 2008). VP26 has been shown to interact with actin by fluorescent probe method and coimmunoprecipitation (Xie and Yang, 2005). Further study using yeast two-hybrid assays, coimmunoprecipitation and colocalization indicated that VP26 acts as a linker protein in the formation of VP51A-VP26-VP28 complex (Chang et al., 2008). Nevertheless, crystal structure and immunoelectron microscopy revealed that the N-terminal transmembrane region of VP26 may anchor on the viral envelope membrane, making the core β-barrel protrude outside the envelope (Tang et al., 2007).

Several genome-based diagnostic methods such as *in situ* hybridization (Chang et al., 1996), one-step PCR (Takahashi et al., 1996), nested PCR (Lo et al., 1996; Kimura et al., 1996) and real-time PCR (Durand et al., 2003), have been developed for detection of WSSV.

Recently, loop-mediated isothermal amplification (LAMP) protocols were developed for WSSV detection (Kono et al., 2004; Mekata et al., 2008; Jaroenram et al., 2009). Although these molecular methods are sensitive and reliable, they are not feasible for pond-site detection. Therefore, an antibody-based assay presents an attractive alternative, providing a simple and low-cost detection system with high specificity and optimal sensitivity for disease monitoring during shrimp cultivation.

Polyclonal (Nadala and Loh, 2000; You et al., 2002) and monoclonal antibodies specific to WSSV proteins (Poulos et al., 2001; Anil et al., 2002; Lui et al., 2002; Chaivisuthangkura et al., 2004; Shih, 2004) have been developed. The MAbs recognizing WSSV envelope protein displayed the viral neutralizing activity in both cultured cells and in experimental shrimp (Shih, 2004). The W29 MAb, specific to VP28 of WSSV, was further developed into an immunochromatographic test strip to detect WSSV in shrimp (Sithigorngul et al., 2006). However, in most cases the detection limit was still inferior to PCR. In a previous study, a polyclonal antibody specific to VP19 and VP26 were developed (Chaivisuthangkura et al., 2006a; Chaivisuthangkura et al., 2006b). In this study, VP19- and VP26-specific monoclonal antibodies (MAbs) were generated. This antibody should prove useful in enhancing the sensitivity of various immunoassays for WSSV detection.

2. Objectives

1. To clone and express the structural protein genes VP19 and VP26 of white spot syndrome virus (WSSV)

2. To produce monoclonal antibodies specific to VP19 and VP26 using WSSV recombinant proteins as the antigens

3. To use generated monoclonal antibodies for enhancing WSSV detection sensitivity

3. Materials and Methods

Viral preparation

Naturally-WSSV-infected *P. vannamei* specimens were obtained from a farm in Chantaburi province, Thailand. Infection was verified by one-step PCR (Chaivisuthangkura et al., 2004). Pleopods from infected shrimp were homogenized in 2-fold PBS (0.3 M Phosphate buffered saline, pH 7.2) and centrifuged at 3,000 x *g* for 30 min. The supernatant was collected in aliquots and stored at –70^{o}C.

Preparation of recombinant VP19 and VP26 proteins

Escherichia coli (BL21strain) with the *VP19*-pMAL-C2 plasmid (Chaivisuthangkura et al., 2006a) and *Escherichia coli* M15(pREP4) with pQE30-VP26F109 plasmid (Chaivisuthangkura et al., 2006b) were cultured in Luria-Bertani (LB) broth to the exponential phase. Expression of each recombinant protein was induced with 1 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG) for 4 h. After centrifugation at 4,000 x *g* for 20 min at room temperature, the bacterial pellet was resuspended in a buffer containing 100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea pH 8, and 1 mM phenylmethylsulfonyl fluoride (PMSF) and sonicated until a clear lysate was obtained. The lysate was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After treatment with 0.3 M KCl, recombinant fusion protein called MBP-VP19 or 6xHis-VP26 was excised and collected in dialysis bags. Recombinant protein was eluted with a Transblot apparatus (BioRad) at 70 V for 6 h, dialyzed and concentrated using a vacuum concentrator (Savant). Protein concentration was determined by Bradford assay (Bradford, 1976). The protein solution was adjusted to 1 mg/ml, divided into small aliquots and stored at -70^{o}C.

Immunization

MBP-VP19 or 6xHis-VP26 protein mixed with complete Freund's adjuvant in a 1:1 ratio was injected intra-peritoneally into four Swiss mice at 0.05 mg protein per mouse. Mice were subsequently injected with the MBP-VP19 or 6xHis-VP26 protein mixed with incomplete Freund's adjuvant three more times at 2 week intervals. In the case of VP19, one week after the fourth injection, mouse antisera were collected and tested against lysates of *E. coli* containing either the pMAL-C2 or *VP19*-pMAL-C2 plasmid by western blotting. In the case of VP26, one week after the fourth injection, mouse antisera were collected and tested against lysates of *E. coli* containing either the pQE30 or *VP26*-pQE30 plasmid by western blotting.The best-performing mouse was subsequently boosted 3 days before hybridoma production.

Production of monoclonal antibody

A cell fusion protocol was adapted from the method developed by Köhler & Milestein (1976) with modifications described by Mosmann et al (1979). A P3X myeloma cell line was used as the fusion partner. Fusion products from one mouse were plated onto 30 microculture plates (96 wells/plate). After identification of positive cultures by screening methods including dot blotting, western blotting and immunohistochemistry as described below, cells were cloned by the limiting dilution method and stored in liquid nitrogen.

Specificity testing

Dot blotting

Lysates of *E. coli* BL21 containing either the pMAL-C2 or *VP19*-pMAL-C2 plasmid or pleopod homogenate samples (1 μl/spot) from uninfected or WSSV-infected shrimp, were applied to nitrocellulose membranes, baked at 60°C for 10 min and incubated in hybridoma conditioned medium from culture (diluted to 1:20 in 5% blotto blocking solution (5% nonfat drymilk, 0.1% Triton X-100 in PBS)) for 4 h. After extensive washing in 0.5% blocking solution, the membrane was incubated in horseradish peroxidase-labeled goat anti-mouse gamma immunoglobulin heavy and light chain-specific antibody (GAM-HRP, Bio-Rad) at 1:1000 dilution for 4 h. The membrane was then washed for 5 min in blocking solution and incubated in substrate mixture containing 0.03% diaminobenzidine (DAB), 0.006% hydrogen peroxide and 0.05% cobalt chloride in PBS (Sithigorngul et al., 2000).

In the case of VP26, lysates of *E. coli* (pREP4) containing either the pQE30 or *VP26*-pQE30 plasmid or pleopod homogenate samples (1 μl/spot) from uninfected or WSSV-infected shrimp, were applied to nitrocellulose membranes and processed as described above.

Western blotting

Lysates of *E. coli* containing either pMAL-C2 plasmid (MBP) or *VP19*-pMAL-C2 plasmid and pleopod homogenates from WSSV-infected shrimp were separated by 12% gel SDS-PAGE according to the method described by Laemmli (1970). Samples were electrophoresed for 3 hr at 60V and one part of the gel was stained using Coomassie brilliant blue R-250. For western blot analysis, the samples resolved by SDS-PAGE were transferred onto nitrocellulose membranes using Transblot apparatus (BioRad). Nitrocellulose membranes were incubated in 5% blocking solution for 10 min and treated with MAbs or mouse anti-recombinant VP19-antiserum (preabsorbed with *E. coli* lysate containing MBP) for 4 h. After extensive washing in 0.5% blocking solution, the membrane was incubated with GAM-HRP at 1:1000 dilution for 4 hr. The membrane was then washed extensively as before and incubated in a substrate mixture containing 0.006% hydrogen peroxide, 0.03% diaminobenzidine (DAB), 0.05% cobalt chloride in PBS. The membrane was also reprobed with the VP28-specific MAb (W29) obtained from a previous study (Chaivisuthangkura et al., 2004) and developed without cobalt chloride to obtain a brown precipitate for comparison.

In the case of VP26, pleopod homogenate samples (1 μl/spot) from uninfected or WSSV-infected shrimp, were applied to nitrocellulose membranes and processed as described above using MAbs or mouse anti-recombinant VP26-antiserum (preabsorbed with

lysate of *E. coli* containing pQE30 plasmid). The membrane was also reprobed with the VP28-specific MAb (W29) obtained from a previous study (Chaivisuthangkura et al., 2004) and developed without cobalt chloride to obtain a brown precipitate for comparison.

Immunohistochemistry

Cephalothoraces from *P. vannamei* specimens naturally infected with WSSV were fixed in Davidson's fixative solution for 24 hr before processing for paraffin sectioning. Serial sections (8 μm thickness) of tissues were prepared and processed for indirect immuno-peroxidase staining using MAb. Peroxidase activity was visualized by incubation with 0.03% DAB and 0.006% hydrogen peroxide in PBS. Preparations were counterstained with hematoxylin and eosin Y (H&E), dehydrated in graded ethanol series, cleared in xylene and mounted in Permount (Sithigorngul et al., 2000). Positive reactions were visualized as brown coloration against pink cytoplasm and purple nuclei.

MAb Class and subclass determination.

Classes and subclasses of the mouse immunoglobulins produced by hybridomas were determined by sandwich ELISA using Zymed's Mouse MonoAb ID Kit (HRP).

Cross-reactivity testing

Shrimp samples infected with *Penaeus monodon* densovirus (PmDNV), previously called hepatopancreatic parvovirus (HPV), *Penaeus monodon* nucleopolyhedrovirus (PemoNPV), previously called monodon baculovirus (MBV), *Penaeus stylirostris* densovirus (PstDNV) known previously as infectious hypodermal and hematopoietic necrosis virus (IHHNV), Taura syndrome virus (TSV) and yellow head virus (YHV) were processed for paraffin sectioning and immunohistochemistry using MAb specific to WSSV. Results were compared to those from MAbs specific to PmDNV (Rukpratanporn, et al., 2005), TSV (Longyant, et al., 2008), YHV (Sithigorngul, et al., 2002), PemoNPV (Boonsanongchokying, et al., 2006), and PstDNV (Sithigorngul et al., 2009).

Sensitivity testing with recombinant protein

Purified MBP-VP19 was serially diluted with PBS, spotted onto nitrocellulose membranes and processed for dot blotting using the VP19-specific MAb generated in this study. The last dilution yielding a clear positive result was determined. The sensitivity of the VP28-specific W29 MAb was also determined in the same fashion using purified 6xHis-VP28

protein from previous study (Chaivisuthangkura et al., 2004). Protein content was measured by Bradford protein assay (Bradford, 1976).

Comparison of sensitivity between MAb and one-step PCR using WSSV-infected shrimp sample

The sensitivity of WSSV detection in a naturally-infected shrimp was determined using MAbs W25-8D (VP19-specific), W26-3 (VP26-specific) and W29 (VP28-specific). The WSSV-infected shrimp sample, as verified by PCR (Chaivisuthangkura et al., 2004), was homogenized in PBS, serially diluted with normal shrimp homogenate and processed for dot blotting as describe above using MAb W29, W26-3, W25-8D and a combination of W29 and W25-8D in a 1:1 ratio. The last dilution of shrimp homogenate yielding a clear positive result was determined. DNA from the same shrimp homogenate were also extracted with a High Pure viral nucleic acid kit (Roche Molecular Biochemicals) and serially diluted with uninfected shrimp nucleic acid and tested for WSSV by PCR using primers VP28F (5'-CCG GAT CCA TGG ATG GGA TCT TTC TTT CAC CTT TCG-3') and VP28R (5'-TGC ACT GCA GTT ACT CGG TCT CAG TGC CAG-3') to yield an amplicon of 633 bp (Chaivisuthangkura et al., 2004).

Detection of WSSV in naturally infected shrimp specimens

For WSSV detection tests in wild shrimp specimens, 15 *P. vannamei* (5 g in weight) specimens infected with WSSV (collected from Chantaburi province, Thailand) verified by PCR (Chaivisuthangkura et al., 2004) and 14 *P. vannamei* (5 g weight) specimens infected with YHV (collected from Nakhon Pathom province, Thailand) verified by RT-PCR (Wongteerasupaya et al., 1997) were used. One pleopod from each shrimp was homogenized in 50 μl of PBS and spotted (1 μl/spot) on each square of a nitrocellulose membrane, which was processed for dot blotting using MAb against VP19 (W25-8D), VP26 (W26-3) or VP28 (W29) of WSSV or YHV (Y19) (Sithigorngul et al., 2002) as described above.

4. Results and Discussion

4.1 Generation of VP19-specific monoclonal antibody

4.1.1 MAb production

After hybridoma production, approximately 800 hybridoma-containing wells were obtained and approximately 80 wells gave positive binding results when initially screened with MBP-VP19 *E. coli* lysate. Hybridoma clones were further screened by dot blotting against *E. coli* lysates containing MBP or MBP-VP19 and pleopod homogenate samples from uninfected and WSSV-infected shrimps, by western blotting against pleopod homogenates from WSSV-infected shrimp and by immunohistochemical analysis of cephalothorax sections from WSSV-infected specimens. Five MAbs specific to VP19, namely W25-8D, W10-9D, W9-11C, W9-11D and W7-7H, were selected and cloned to established cell lines. All MAb belonged to 3 IgG subclasses (Table 1). Since MAbs W25-8D and W9-11D demonstrated the best results with the various immunoassays described above, one of these MAbs (W25-8D) was used as a representative MAb for further experiments.

Table 1. Monoclonal antibodies (MAb) obtained from mouse immunized with the MBP-VP19 protein in comparison with MAb W29 specific to VP28. IHC: immunohistochemistry; +++ = strong immunoreactivity; - = no immunoreactivity

MAb (subclass)	**Sensitivity (Dot blotting)**	**Western blotting**	**IHC**	**Specificity**
W25-8D (IgG1)	~70 pg/spot	+++	+++	VP19
W7-7H (IgG2b)	~280 pg/spot	+++	+++	VP19
W9-11C (IgG2a)	~140 pg/spot	+++	+++	VP19
W9-11D (IgG1)	~70 pg/spot	+++	+++	VP19
W10-9D (IgG2b)	~280 pg/spot	+++	+++	VP19
W29 (IgG1)	~140 pg/spot	+++	+++	VP28

4.1.2 Specificity of MAb

VP19-specifc W25-8D and VP28-specific W29 MAbs were used in dot blot assays as previously described (Chaivisuthangkura et al., 2004). As shown in Figure 1A, W25-8D bound intensely to *E. coli* lysates containing MBP-VP19 and pleopod homogenate from WSSV-infected shrimp but did not bind to *E. coli* lysate containing MBP or pleopod homogenate from uninfected shrimp. W29 bound specifically to WSSV-infected shrimp homogenate (Fig 1B).

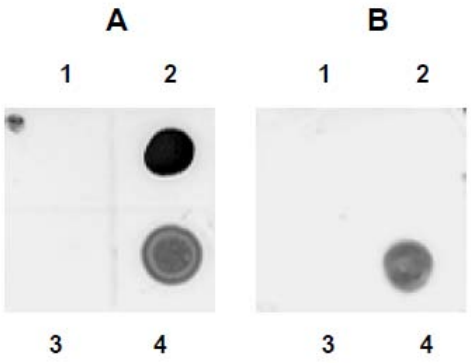

Figure 1. Dot blot analysis of monoclonal antibodies (MAb). Lysates of (1) BL21 *Escherichia coli* transfected with pMAL-C2 plasmid, (2) *E. coli* with *VP19*-pMAL-C2 plasmid, (3) pleopod homogenate from uninfected and (4) WSSV-infected *Penaeus vannamei* were spotted (1 µl spot^{-1}) onto a nitrocellulose membrane and treated with VP19-specific W25-8D (A) or VP28-specific W29 (B) MAbs.

VP19-specific W25-8D reacted with MBP-VP19 and yielded a single band of VP19 protein in pleopod homogenates from WSSV-infected shrimp (Fig. 2B, lane 2 and 3). The intensity of the VP19 band was comparable to that of VP28 as visualized using W29 (Fig. 2B, lane 3). This result demonstrates the specificity of W25-8D for recombinant or natural VP19 protein. All MAb specific to VP19 gave similar results demonstrating the specificity of the MAbs developed against the VP19 envelope protein.

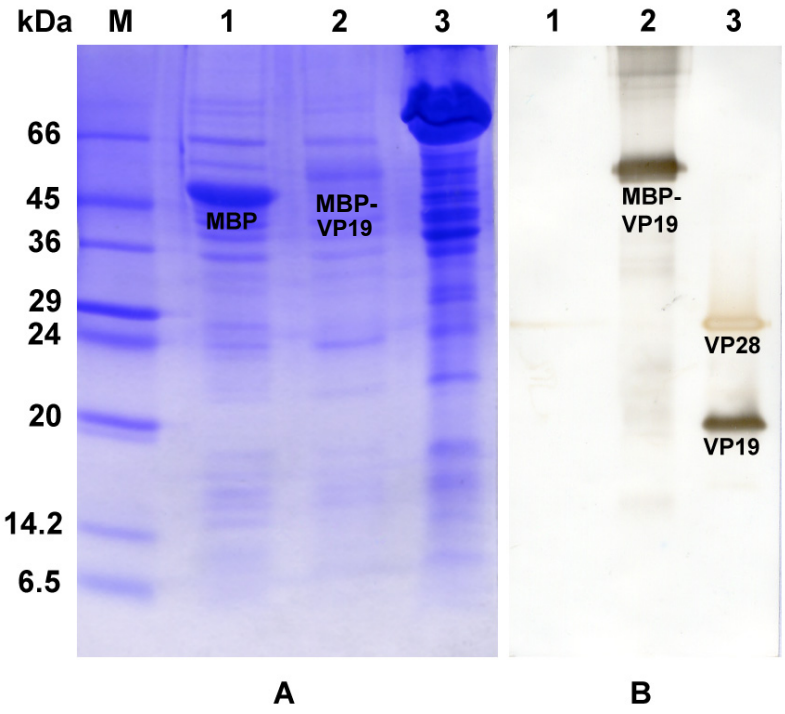

Figure 2. SDS-PAGE and western blotting. (1) lysate of *E. coli* expressing MBP, (2) lysate of *E. coli* expressing MBP-VP19, (3) pleopod homogenate of WSSV-infected *P. vannamei* were electrophoresed and (A) stained with Coomassie brilliant blue or (B) transferred to nitrocellulose membrane and treated with VP19-specific W25-8D MAb and retreated with VP28-specific W29 MAb (recognized only lane 3, upper band). M = standard marker proteins.

None of the VP19-specific MAbs exhibited cross-reactivity with tissues from shrimp infected with TSV, PmDNV, YHV or PemoNPV in immunohistochemical experiments (data not shown). However, the immunohistochemical analysis of WSSV-infected gill and appendage tissues using MAbs specific to VP19 yielded intense staining with distinct patterns different from those seen following staining with VP28-specific MAb. Interestingly, the W25-8D MAb not only reacted with VP19 Ag in the nucleus, showing Cowdry type A inclusion, but also reacted with Ag in the cytoplasm of the infected cells (Fig. 3). This immunoreactivity was specific to VP19 none of the immunoreactivity was observed in nearby areas such as the haemocoel (H) of the same stained gill tissue (Fig. 3B). The presence of VP19 Ag in the cytoplasm may result from WSSV virions released from disrupted nuclei of epidermal cells which are readily budded from the cytoplasmic membrane as reported earlier (Chou et al., 1995). The lack of observed immunoreactivity in the cytoplasm of infected cells in this experiment using VP28-specific MAb

remain puzzling even though the immunoreactivity of the VP28 Ag in the nucleus was clearly observed (Fig. 3A, column 2).

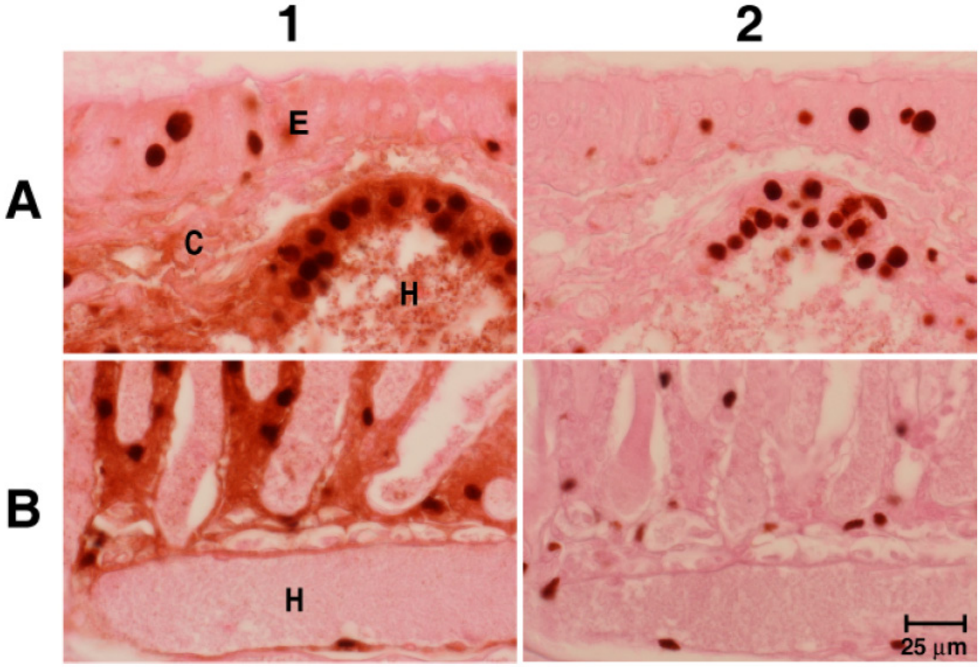

Figure 3. Immunohistochemical analysis of tissues from WSSV-infected *P. vannamei* using VP19-specific W25-8D (column 1) and VP28-specific W29 (column 2) MAbs. Consecutive sections were counterstained with eosin for clear visualization of immunoreactivity. Strong immunoreactivity was exhibited in subcutaneous epithelium of mouth appendage (A) and gill tissues (B). E = subcutaneous epithelium; C=connective tissue; H = hemocoel.

4.1.3 Sensitivity testing

To determine the sensitivity of VP19-specific MAbs, dot blotting against purified recombinant MBP-VP19 protein was performed. Sensitivity ranged from 70-280 ng/ml, which is equivalent to 70-280 pg/spot or 1.2-5 fmole/spot. Conversely, the sensitivity of VP28-specific W29 MAb was 140 pg/spot or 5 fmole/spot (Table 1). In a previous study, the detection limits of MAbs specific for VP28 were 500 pg (Anil et al., 2002) and 400 pg (Liu et al., 2002). Therefore, the VP19-specific MAb generated in this study had sensitivity approximately 1.5-5 times higher than those of MAbs from previous reports. In antigen-capture ELISA experiments, the sensitivity for VP28 was as low as 20 pg (Anil et al., 2002). This may be due to the fact that the sample volumes used in that study (50 to 100 µl) was larger than those used for dot blotting in this study (1 µl).

To determine the ability of MAbs to detect WSSV infection in field samples, a pleopod homogenate sample of naturally-infected shrimp was serially diluted with normal shrimp pleopod homogenate for dot blot assays. As shown in Figure 4A, the WSSV detection limit of VP19-specific W25-8D and VP28-specific W29 MAbs were approximately 1:200. However, the combination of both W29 and W25-8D MAbs increased the detection limit to 1:400, two fold higher than that of a single antibody. These data indicate that the mixture of MAbs specific to different antigens could increase the sensitivity of WSSV detection. Even though the W25-8D MAb seemed to demonstrate stronger immunohistochemical staining than that of W29, they possessed identical detection limits as measured by dot blotting. This could be due to the fact that VP19 Ag might be lost during the centrifugation step in dot blotting assays, co-precipitating with the cell debris.

To compare the sensitivity of WSSV detection between dot blotting and one-step PCR, nucleic acids were extracted from the same shrimp homogenate used for dot blotting and used for PCR detection. As shown in Figure 4B, at a dilution of 10^{-7}, a 633 bp band was clearly observed. Therefore, in comparison with one-step PCR the sensitivity of dot blotting using a combination of both MAb was approximately 25,000 times less sensitive (Fig. 4).

Even though the sensitivity of our antibody-based assay was lower than that of PCR, its high specificity could be regarded as an advantage. In other report, the high specificity (100%) of immunodot assay was identical to that of 1-step PCR and the immunodot test could be used to replace 1-step PCR assay for WSSV disease monitoring. In that study, six farms with the shrimp samples that yielded negative result for WSSV by immunodot and 1-step PCR at various times post stocking, the successful crops were obtained. Whereas, the other six farms that were positive for WSSV by both immunodot test and 1-step PCR, their crops failed. In contrast, four farms that gave positive results for WSSV by 2-step PCR at various time post stocking, they were successfully harvested at 105 d post stocking (Patil et al., 2008).

In another case, the limitation of PCR due to inhibiting factors present in some tissues such as eyestalk with eye could be prevailed with the use of immunodot test that gave positive result reaction (Shekhar et al., 2006).

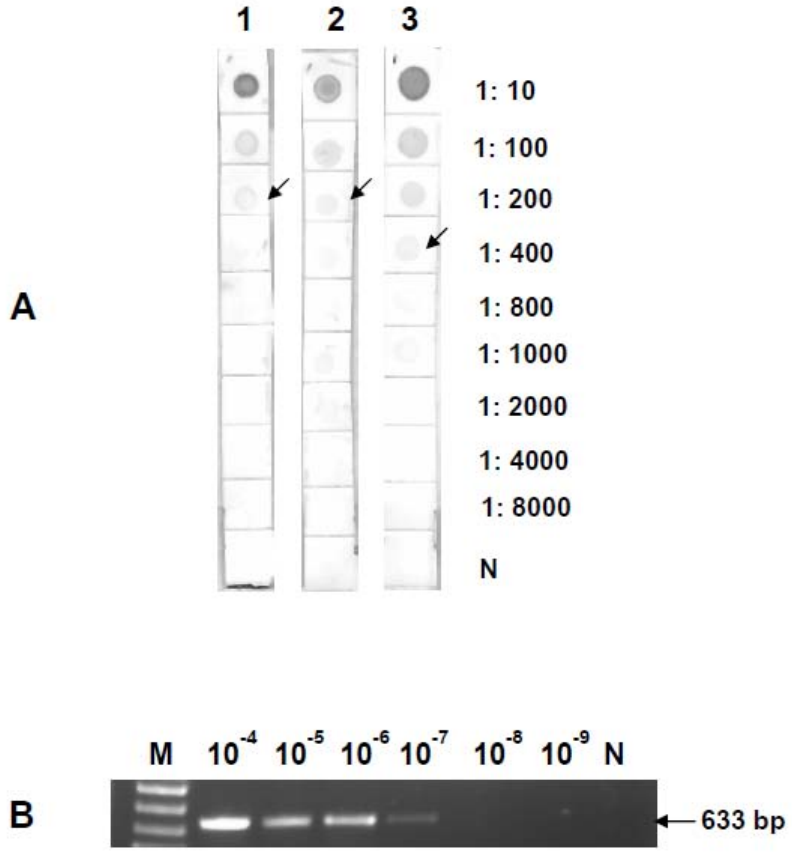

Figure 4. Sensitivity of WSSV detection by dot blotting and PCR. (A) Dot blotting; pleopod homogenate from a naturally-infected *P. vannamei* specimen was serially diluted with healthy shrimp homogenate and spotted onto nitrocellulose membrane (1 ul/spot) and processed for dot blotting using VP19-specific W25-8D (1) VP28-specific W29 (2) MAbs and a combination of W29 and W25-8D (3) MAbs. The last square of each column was spotted with pleopod homogenate from uninfected shrimp (N). (B) PCR detection; nucleic acids extracted from the pleopod homogenate from a naturally-infected *P. vannamei* speciemen used in the above dot blotting experiment were serially diluted with normal shrimp nucleic acids and used as a template for PCR amplification. The various dilutions are indicated on top of the gel. Lane M: molecular weight marker, lane N: no template control (negative control).

4.1.4 Detection of WSSV infection in naturally infected shrimp

A total of 15 WSSV-infected shrimp samples (verified by dot blotting using VP28-specific W29 MAb and 1-step PCR) (Fig. 5B) and 14 YHV-infected shrimp samples (verified by dot blotting using MAb Y19 and RT-PCR) (Fig. 5D) were used to demonstrate the ability of VP19-specific MAbs to detect naturally-occuring WSSV infection in shrimp. As shown in Figure 5A, VP19-specific W25-8D MAb bound specifically to to all 15 WSSV-infected *P. vannamei*, while no cross-reactivity with YHV-infected shrimp was observed. The combination of W29 and W25-8D (1:1 ratio) yielded stronger immunoreactivity (Fig. 5C). In many cases of light infections, immunoreactivity was enhanced by W25-8D compared to W29 (Fig. 5C, row 2 shrimp no. 9; row 3 shrimp no. 12, 13). To provide the quantitative data, the ImageJ software (http://rsbweb.nih.gov/ij/) was used to quantify the dot intensity. The results revealed that the relative intensity of signal in dot blotting using the combination of W29 and W25-8D compared to those of W29 alone increased approximately 23 to 27 % (shrimp no. 9 (23%); shrimp no. 12 (25%) and shrimp no. 13 (27%)). These data indicate that antibodies against different antigens can be used to augment WSSV detection in naturally-infected shrimp samples. In the case of Taura syndrome virus (TSV) detection, the combination of MAbs specific to VP2 and VP3 could be used to increase the sensitivity of TSV detection and to confirm TSV infection in *Penaeus vannamei* using dot blotting and immunohistochemistry (Chaivisuthangkura et al., 2010).

In conclusion, we have demonstrated that MAbs specific for different WSSV epitopes can be used in concert to enhance the detection of WSSV infection in shrimp samples by immunohistochemistry. The detection limit of dot blotting using a combination of MAbs against VP19 and VP28 was two-fold higher than that of a single MAb. It is our belief that the VP19-specific MAb generated in this study can be used to develop a simple WSSV detection kit similar to the immunochromatographic strip used in WSSV and YHV detection kits (Sithigorngul, et al., 2006; 2007).

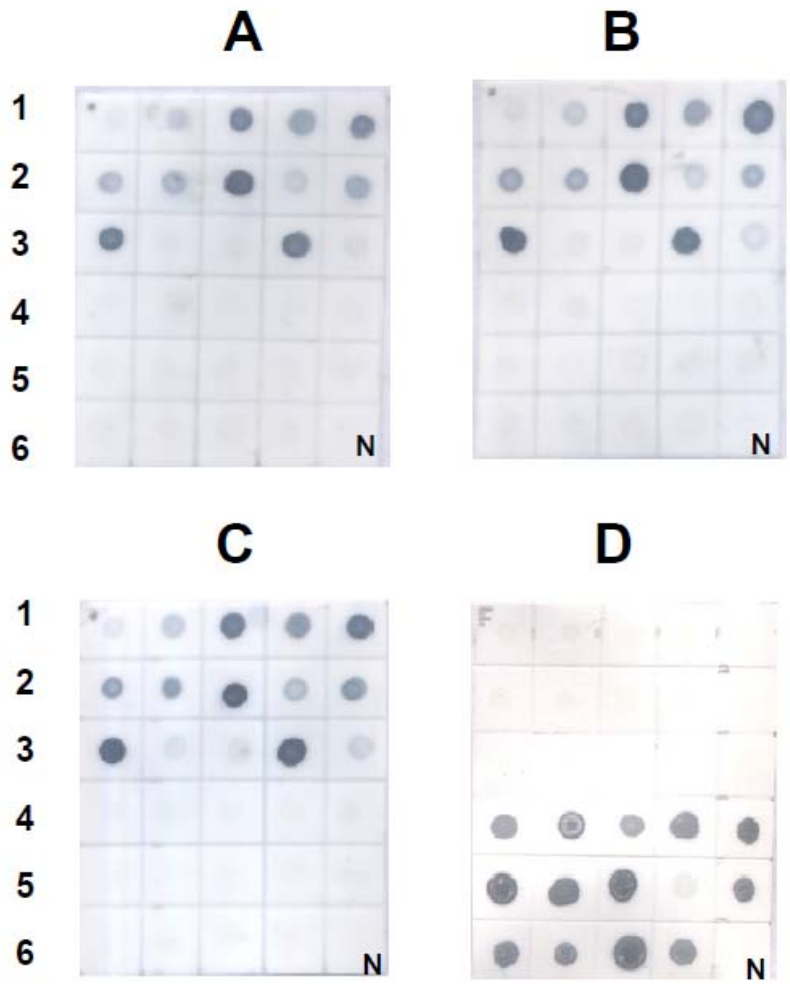

Figure 5. Dot blot detection of natural WSSV infection. Pleopod homogenates from 15 WSSV-infected *P. vannamei* specimens (row 1-3; row 1: shrimp no. 1 to 5; row 2: shrimp no. 6 to 10; row 3: shrimp no. 11 to 15) and 14 YHV infected *P. vannamei* specimens (row 4 -6) and uninfected *P. vannamei* specimens (N) were spotted onto nitrocellulose membranes and treated with VP19-specific W25-8D (A) and VP28-specific MAb W29 (B) MAbs as well as with a combination of W29 and W25-8D (C) and Y19 MAb specific to YHV (D).

4.2 Generation of VP26-specific monoclonal antibody

4.2.1 MAb production

After hybridoma production, approximately 600 hybridoma-containing wells were obtained and approximately 60 wells gave positive binding results when initially screened with 6xHis-VP26 *E. coli* lysate. Hybridoma clones were further screened by dot blotting against lysate of *E. coli* containing pQE30 or *VP26*-pQE30 and pleopod homogenate samples from uninfected and WSSV-infected shrimps, by western blotting against pleopod homogenates from WSSV-infected shrimp and by immunohistochemical analysis of cephalothorax sections from WSSV-infected specimens. Three MAbs specific to VP26, designated W17-5B, W26-2, and W26-3 were selected and cloned to established cell lines. All MAb belonged to IgG1 subclass (Table 2). Since MAb W26-3 demonstrated the best results with the various immunoassays described above, it was used as a representative MAb for further experiments.

Table 2. Monoclonal antibodies (MAb) obtained from mouse immunized with the 6xHis-VP26 protein in comparison with MAb W29 specific to VP28. IHC: immunohistochemistry; +++ = strong immunoreactivity; ++ = moderate immunoreactivity; - = no immunoreactivity

MAb (subclass)	**Western blotting**	**IHC**	**Specificity**
W17-5B (IgG1)	++	++	VP26
W26-2 (IgG1)	++	++	VP26
W26-3 (IgG1)	+++	+++	VP26
W29 (IgG1)	+++	+++	VP28

4.2.2 Specificity of MAb

VP26-specifc W26-3 and VP28-specific W29 MAbs were used in dot blot assays as previously described (Chaivisuthangkura et al., 2004). As shown in Figure 6A, W26-3 bound intensely to *E. coli* lysates containing 6xHis-VP26 and pleopod homogenate from WSSV-infected shrimp but did not bind to *E. coli* lysate containing 6xHis or pleopod homogenate from uninfected shrimp. W29 bound specifically to WSSV-infected shrimp homogenate (Fig 6B).

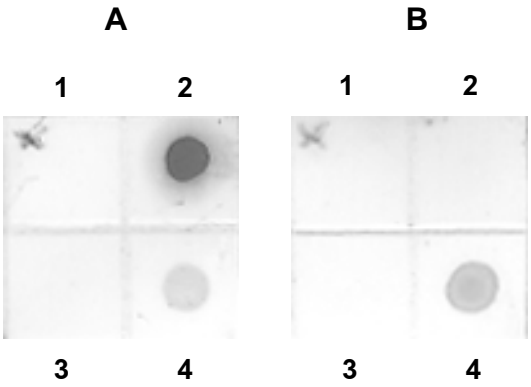

Figure 6. Dot blot analysis of monoclonal antibodies (MAb). Lysates of (1) M15 *Escherichia coli* transfected with pQE30 plasmid, (2) *E. coli* with *VP26*-pQE30 plasmid, (3) pleopod homogenate from uninfected and (4) WSSV-infected *Penaeus vannamei* were spotted (1 μl spot^{-1}) onto a nitrocellulose membrane and treated with VP26-specific W26-3 (A) or VP28-specific W29 (B) MAbs.

VP26-specific W26-3 reacted with 6xHis-VP26 and yielded a single band of VP26 protein in pleopod homogenates from WSSV-infected shrimp (Fig. 7B, lane b). The intensity of the VP26 band was relatively less than that of VP28 as visualized using W29 (Fig. 7B, lane b). However, this result demonstrates the specificity of W26-3 for recombinant or natural VP26 protein. All MAb specific to VP26 gave similar results demonstrating the specificity of the MAbs developed against the VP26 structural protein.

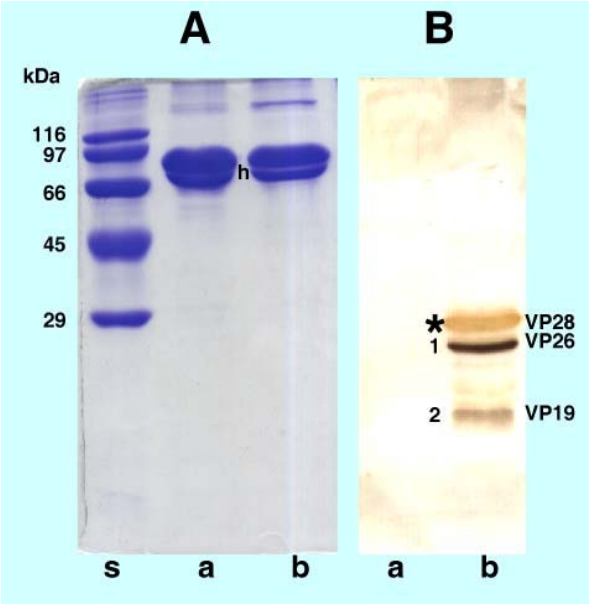

Figure 7. SDS-PAGE and western blotting. (a) pleopod homogenate of uninfected *P. vannamei* (b) pleopod homogenate of WSSV-infected *P. vannamei* were electrophoresed and (A) stained with Coomassie brilliant blue or (B) transferred to nitrocellulose membrane and treated with VP26-specific W26-3 MAb and retreated with VP28-specific W29 MAb and VP19-specififc W25-8D MAb. s = standard marker proteins.

None of the VP26-specific MAbs exhibited cross-reactivity with tissues from shrimp infected with TSV, PstDNV, YHV or PemoNPV in immunohistochemical experiments (data not shown). However, the immunohistochemical analysis of WSSV-infected tissues using MAbs specific to VP26 yielded intense staining with similar patterns with those seen following staining with VP28-specific MAb. (Fig. 8).

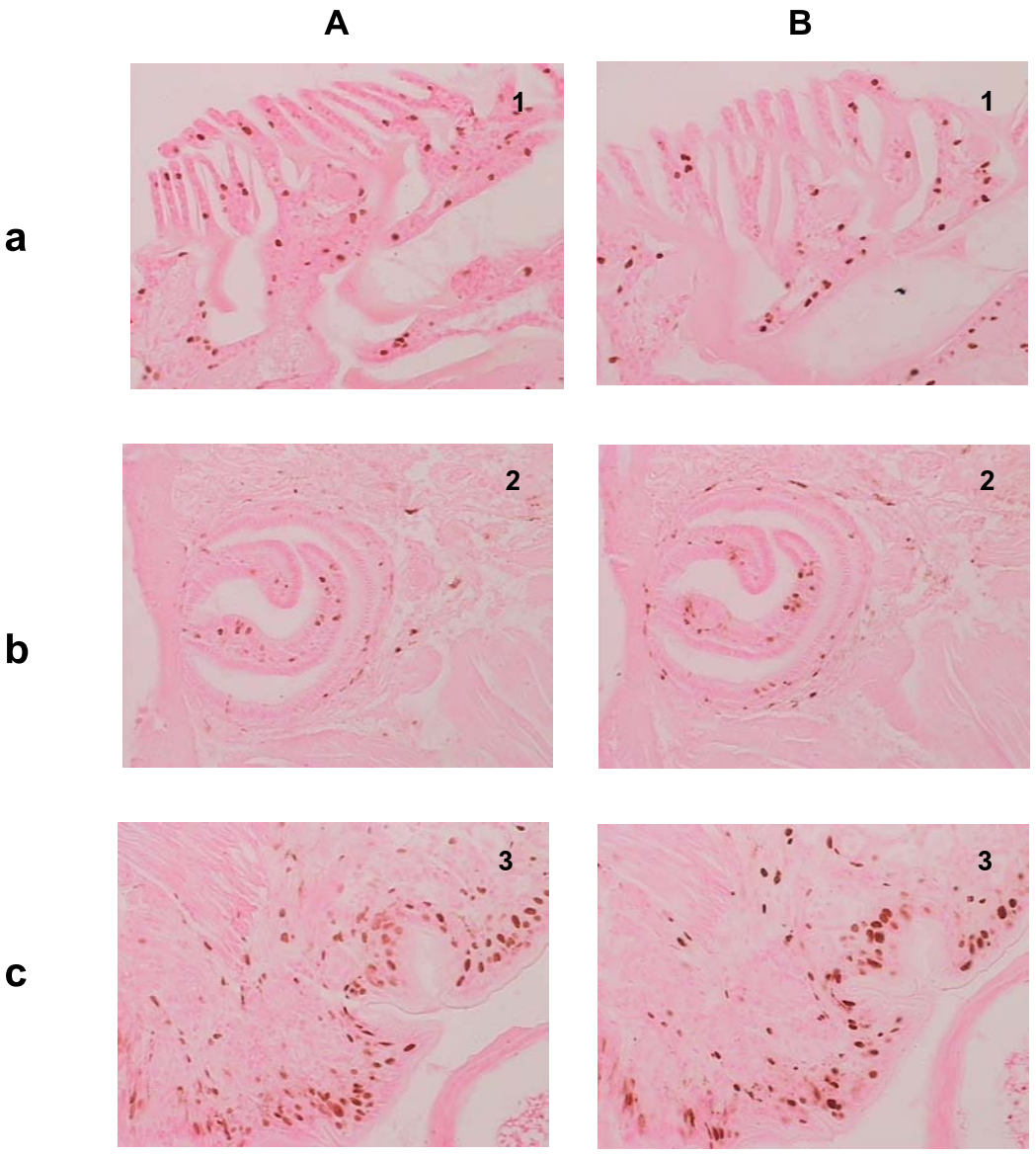

Figure 8. Immunohistochemical analysis of tissues from WSSV-infected *P. vannamei* using VP26-specific W26-3 (column A) and VP28-specific W29 (column B) MAbs. Consecutive sections were counterstained with eosin for clear visualization of immunoreactivity. Strong immunoreactivity was exhibited in gill tissues (a), mid gut (b), and subcuticular epithelium (c).

4.2.3 Sensitivity testing

To determine the ability of MAbs to detect WSSV infection in field samples, a pleopod homogenate sample of naturally-infected shrimp was serially diluted with normal shrimp pleopod homogenate for dot blot assays. As shown in Figure 9, the WSSV detection limit of VP26-specific W26-3 was 1:80 whereas that of VP28-specific W29 MAbs was 1:160. Therefore, the sensitivity of VP26-specific W26-3 was twofold less than that of VP28-specific W29 MAbs. This could be due to the fact that the amount of VP26 Ag may be less than that of VP28 Ag as demonstrated by SDS-PAGE of purified WSSV particles in other reports (Xie et al., 2006; Wan et al., 2008).

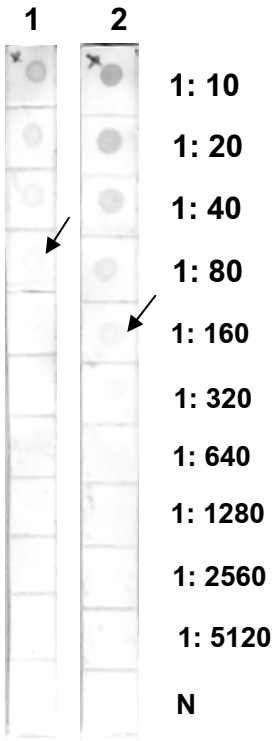

Figure 9. Sensitivity of WSSV detection by dot blotting. A pleopod homogenate from a naturally-infected *P. vannamei* specimen was serially diluted with healthy shrimp homogenate and spotted onto nitrocellulose membrane (1 μl/spot) and processed for dot blotting using VP26-specific W26-3 (1) and VP28-specific W29 (2) MAbs. The last square of each column was spotted with pleopod homogenate from uninfected shrimp (N).

4.2.4 Detection of WSSV infection in naturally infected shrimp

A total of 15 WSSV-infected shrimp samples (verified by dot blotting using VP28-specific W29 MAb and 1-step PCR) (Fig. 10B) and 14 YHV-infected shrimp samples (verified by dot blotting using MAb Y19 and RT-PCR) (Fig. 10D) were used to demonstrate the ability of VP26-specific MAbs to detect naturally-occuring WSSV infection in shrimp. As shown in Figure 10A, VP26-specific W26-3 MAb bound specifically to to all 15 WSSV-infected *P. vannamei*, while no cross-reactivity with YHV-infected shrimp was observed. However, in WSSV-light infection samples (numbers 1, 12, and 13), the immunoreactivity was difficult to be observed with each of the MAb. (Fig. 10A, 10B, and 10C).

In conclusion, we have demonstrated that MAbs specific for VP26 can be used to detect WSSV infection in shrimp samples by various immuno-based assays. However, the detection limit of dot blotting using a MAb against VP26 was still two-fold less than that of a VP28-specific W29 MAb. This could limit the use of VP26-specific MAb to enhance the sensitivity of WSSV detection. Nevertheless, the VP26-specific MAb can be used to confirm the WSSV infection in the shrimp sample.

For future research, the VP19-specific MAb generated in this study will be used in concert with the VP28-specific MAb to develop a higher sensitive detection kit for diagnosis of WSSV infection in shrimp.

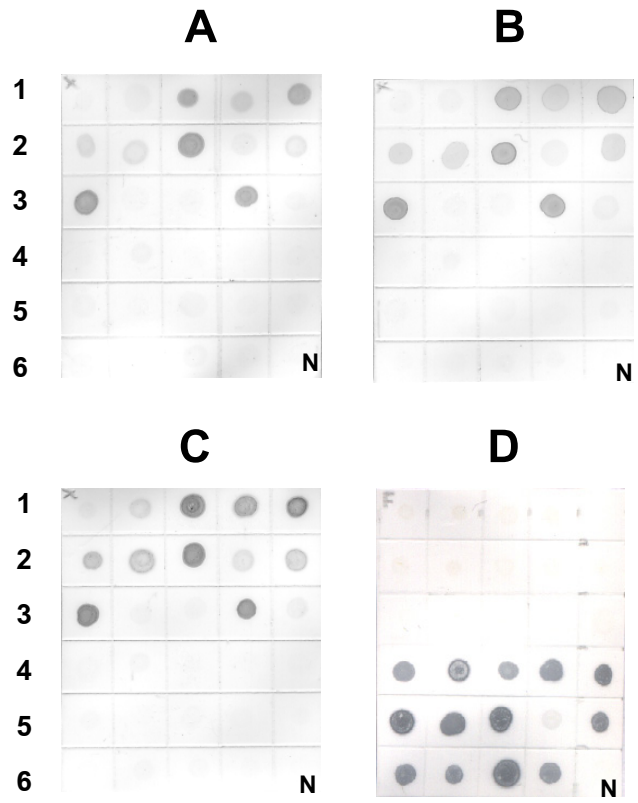

Figure 10. Dot blot detection of natural WSSV infection. Pleopod homogenates from 15 WSSV-infected *P. vannamei* specimens (row 1-3; row 1: shrimp no. 1 to 5; row 2: shrimp no. 6 to 10; row 3: shrimp no. 11 to 15) and 14 YHV infected *P. vannamei* specimens (row 4 -6) and uninfected *P. vannamei* specimens (N) were spotted onto nitrocellulose membranes and treated with VP26-specific W26-3 (A), VP28-specific MAb W29 (B), and VP19-specific MAb W25-8D (C) and Y19 MAb specific to YHV (D).

5. References

Anil, T.M., Shankar, K.M., Mohan, C.V., 2002. Monoclonal antibodies developed for sensitive detection and comparison of white spot syndrome virus isolates in India. Dis. Aquat. Org. 51, 67-65.

Boonsanongchokying, C., Sang-oum, W., Sithigorngul, P., Sriurairatana, S., Flegel, T.W., 2006. Production of monoclonal antibodies to polyhedrin of monodon baculovirus (MBV) from shrimp. ScienceAsia 32, 371-376.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248-254.

Chaivisuthangkura, P., Tangkhabuanbutra, J., Longyant, S., Sithigorngul, W., Rukpratanporn, S., Menasveta, P., Sithigorngul, P., 2004. Monoclonal antibodies against a truncated viral envelope protein (VP28) can detect white spot syndrome virus (WSSV) infections in shrimp. Sci. Asia. 30, 359-363.

Chaivisuthangkura, P., Phattanapaijitkul, P., Thammapalerd, N., Rukpratanporn, S., Longyant, S., Sithigorngul, W., Sithigorngul, P., 2006a. Development of a polyclonal antibody specific to VP19 envelope protein of white spot syndrome virus (WSSV) using a recombinant protein preparation. J Virol. Meth. 133, 180-184.

Chaivisuthangkura, P., Phattanapaijitkul, P., Thammapalerd, N., Rukpratanporn, S., Longyant, S., Sithigorngul, W., Sithigorngul, P., 2006b. Production of Polyclonal Antibodies against Recombinant VP26 Structural Protein of White Spot Syndrome Virus (WSSV). Sci. Asia. 32, 201-204.

Chaivisuthangkura, P., Longyant, S., Hajimasalaeh, W., Sridulyakul, P., Rukpratanporn, S., Sithigorngul, P., 2010. Improved sensitivity of Taura syndrome virus immunodetection with a monoclonal antibody against the recombinant VP2 capsid protein. J Virol. Meth. 163, 433-439.

Chang, P.S., Lo, C.C., Wang, Y.C., Kou, G.H., 1996. Identification of white spot syndrome associated baculovirus (WSBV) target organs in the shrimp *Penaeus monodon* by *in situ* hybridization. Dis. Aquat. Org. 27, 131-139.

Chang, Y.S., Liu, W, J., Chou, T.L., Lee, Y.T., Lee, T.L., Huang, W.T., Kou, G.H., Lo, C.F., 2008. Characterization of white spot syndrome virus envelope protein VP51A and its interaction with viral tegument protein VP26. J. Virol. 82, 12555-12564.

Chou, H.Y., Huang, C.Y., wang, C.H., Chiang, H.C., Lo, C.F., 1995. Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan. Dis. Aquat. Org. 23, 165-173.

Durand, S.V., Redman, R.M., Mohney, L.L., Tang-Nelson, K., Bonami, J.R., Lightner, D.V., 2003. Qualitative and quantitative studies on the relative virus load of tails and heads of shrimp acutely infected with WSSV. Aquaculture 216, 9-18.

Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A., 2005. Virus Taxonomy. Classification and Nomenclature of Viruses. Eighth Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, San Diego.

Huang, C., Zhang, X., Lin, Q, Xu, X., Hew, C.L., 2002. Characterization of a novel envelope protein (VP281) of shrimp white spot syndrome virus by mass spectrometry. J. Gen. Virol. 83, 2385-2392.

Jaroenram, W., Kiatpathomchai, W., Flegel, T.W., 2009. Rapid and sensitive detection of white spot syndrome virus by loop-mediated isothermal amplification combined with a lateral flow dipstick. Mol. Cell. Probes 23, 65-70.

Kimura, T., Yamano, K., Nakano, H., Momoyama, K., Hiraoka, M., Inouye, K., 1996. Detection of penaeid rod-shaped DNA virus (PRDV) by PCR. Fish Pathol. 31, 93– 98.

Köhler, G., Milstein, C., 1976. Derivative of specific antibody producing tissue culture and tumor cell fusion. Eur. J. Immunol. 6, 511–519.

Kono, T., Savan, R., Sakai, M., Itami, T., 2004. Detection of white spot syndrome virus in shrimp by loop-mediated isothermal amplification. J. Virol. Meth. 115, 59–65.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-85.

Leu, J.H., Yang, F., Zhang, X., Xu, X., Kou, G.H., Lo, C.F., 2009. Whispovirus. Curr. Top. Microbiol. Immunol. 328, 197-227

Lo, C.F., Ho, C.H., Peng, S.E., Chen, C.H., Hsu, H.C., Chiu, Y.L., Chang, C.F., Lui, K.F., Su, M.S., Wang, C.H., Kou, G.H., 1996. White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods. Dis. Aquat. Org. 27, 215-225.

Longyant, S., Poyoi, P., Chaivisuthangkura, P., Tejankura, T., Sithigorngul, W., Sithigorngul, P., Rukpratanporn, S., 2008. Specific monoclonal antibodies raised against Taura syndrome virus (TSV) capsid protein VP3 detect TSV in single and dual infections with white spot syndrome virus (WSSV). Dis. Aquat. Org. 79:75-81.

Liu, W., Wang, Y.T., Tian, D.S., Yin, Z.C., Kwang, J., 2002. Detection of white spot syndrome virus (WSSV) of shrimp by means of monoclonal antibodied (MAbs) specific to an envelope protein (28 kDa). Dis. Aquat. Org. 49, 11-18.

Mekata, T., Sudhakaran, R., Kono, T., Supamattaya, K., Linh, N.T., Sakai, M., Itami, T., 2009. Real-time quantitative loop-mediated isothermal amplification as a simple method for detecting white spot syndrome virus. Lett. Appl. Microbiol. 48, 25-32.

Mosmann, T.R., Bauman, R., Williamson, A.R., 1979. Mutations affecting immunoglobulin light chain secretion by myeloma cells I. Functional analysis by cell fusion. Eur. J. Immunol. 9, 511–516.

Nadala, E.C.B., Loh, P.C., 2000. Dot blot enzyme immunoassays for the detection of white spot virus and yellow-head virus of penaeid shrimp. J. Virol. Meth. 84, 175-79.

Patil, R., Palaksha, K.J., Anil, T.M., Guruchannabasavanna, Patil, P., Shankar, K.M., Mohan, C.V., Sripada, A., 2008. Evaluation of an immunodot test to manage white spot syndrome virus (WSSV) during cultivation of the giant tiger shrimp *Penaeus monodon*. Dis. Aquat. Org. 79, 157-161.

Poulos, B.T., Pantoja, C.R., Bradley-Dunlop, D., Aguilar, J., Lightner, D.V., 2001. Development and application of monoclonal antibodies for the detection of white spot syndrome virus of penaeid shrimp. Dis. Aquat. Org. 47, 13-23.

Rukpratanporn, S., Sukhumsirichart, W., Chaivisuthngkura, P., Longyant, S., Sithigorngul, W., Menasveta, P., Sithigorngul, P., 2005. Generation of monoclonal antibodies specific to hepatopancreatic parvovirus (HPV) from *Penaeus monodon*. Dis. Aquat. Org. 65, 85-89.

Shekhar, M.S., Azad, I.S., Ravichandran, P., 2006. Comparison of dot blot and PCR diagnosis techniques for detection of white spot syndrome virus in different tissues of *Penaeus monodon*. Aquaculture. 261, 1122-1127.

Shih , H.H., 2004. Neutralization of white spot syndrome virus by monoclonal antibodies against viral envelope proteins. Taiwania. 49, 159-165.

Sithigorngul, P., Chauychuwong, P., Sithigorngul, W., Longyant, S., Chaivisuthangkura, P., Menasveta, P., 2000. Development of a monoclonal antibody specific to yellow head virus (YHV) from *Penaeus monodon*. Dis. Aquat. Org. 42, 27-34.

Sithigorngul, P., Rukpratanporn, S., Longyant, S., Chaivisuthangkura, P., Sithigorngul, W., Menasveta, P., 2002. Monoclonal antibodies specific to yellow-head virus (YHV) of *Penaeus monodon*. Dis. Aquat. Org. 49, 71-76.

Sithigorngul, W., Rukpratanporn, S., Pecharaburanin, N., Longyant, S., Chaivisuthangkura, P., Sithigorngul, P., 2006. A simple and rapid immunochromatographic test strip for detection of white spot syndrome virus (WSSV) of shrimp. Dis. Aquat. Org. 72, 101-106.

Sithigorngul, W., Rukpratanporn, S., Sittidilokratna, N., Pecharaburanin, N., Longyant, S., Chaivisuthangkura, P., Sithigorngul, P., 2007. A convenient immunochromatographic test strip for rapid diagnosis of yellow head virus infection in shrimp. J. Virol. Meth. 140, 193-199.

Sithigorngul P, Hajimasalaeh W, Longyant S, Sridulyakul P, Rukpratanporn S, Chaivisuthangkura P., 2009. Simple immunoblot and immunohistochemical detection of Penaeus stylirostris densovirus using monoclonal antibodies to viral capsid protein expressed heterologously. J. Virol. Meth. 162, 126-132.

Takahashi,Y., Itami,T., Maeda, M., Suzuki, N., Kasornchandra, J., Supamattaya, K., Khongpradit, R., Boonyaratpalin, S., Kondo, M., Kawai, K., Kusuda, R., Hirono, I., Aoki, T., 1996. Polymerase chain reaction (PCR) amplification of bacilliform virus (RV-PJ) DNA in *Penaeus japonicus* Bate and systemic ectodermal and mesodermal baculovirus (SEMBV) DNA in *Penaeus monodon* Fabricius. J. Fish Diseases 19, 399–403.

Tang, X., Wu, J., Sivaraman, J., Hew, C.L., 2007. Crystal structure of major envelope proteins VP26 and VP28 from white spot syndrome virus shed light on their evolutionary relationship. J. Virol. 81, 6709-6717.

Tsai, J.M., Wang, H.C., Leu, J.H., Hsiao, H.H., Wang, A.H., Kou, G.H., Lo, C.F., 2004. Genomic and proteomic analysis of thirty-nine structural proteins of shrimp white spot syndrome virus. J. Virol. 78, 11360-11370.

Tsai, J.M., Wang, H.C., Leu, J.H., Wang, A.H., Zhuang, Y., Walker, P.J., Kou, G.H., Lo, C.F., 2006. Identification of the nucleocapsid, tegument, and envelope proteins of the shrimp white spot syndrome virus virion. J. Virol. 80, 3021-3029.

van Hulten, M. C. W., Westenberg, M., Goodall S.D., Vlak, J.M., 2000. Identification of two major virion protein genes of White Spot Syndrome virus of shrimp. Virology 266, 227-236.

Wan, Q., Xu, L., Yang, F., 2008. VP26 of white spot syndrome virus functions as a linker protein between the envelope and nucleocapsid of virions by binding with VP51. J. Virol. 82, 12598-12601.

Wongteerasupaya, C., Thongcheua, W., Boonsaeng, V., Panyim, S., Tassanakajon, A., Withyachumnarnkul, B., Flegel, T.W., 1997. Detection of yellow-head virus (YHV) of *Penaeus monodon* by RT-PCR amplification. Dis Aquat Org 31, 181–186.

Xie, X., Xu, L.M., Yang, F., 2006. Proteomic analysis of the major envelope and nucleocapsid proteins of white spot syndrome virus. J. Virol. 80, 10615-10623.

Xie, X., Yang, F., 2005. Interaction of white spot syndrome virus VP26 protein with actin. Virology 336, 93-99.

You, Z., Nadala, Jr.E.C.B., Yang, J., van Hulten, M.C.W., Loh, P.C., 2002. Production of polyclonal antiserum specific to the 27.5 kDa envelope protein of white spot syndrome virus. Dis. Aquat. Org. 51, 77-80.

Zhang, X., Huang, C., Tang, X., Zhuang, Y., Hew, C.L., 2004. Identification of structural proteins from shrimp white spot syndrome virus (WSSV) by 2DE-MS. Proteins. 55, 229-235.

Zhou, Q., Xu, L., Li, H., Qi, Y.P., Yang, F., 2009. Four major envelope proteins of white spot syndrome virus bind to form a complex. J. Virol. 83, 4709-4712.

Outputs ที่ได้จากงานวิจัย

ในขณะนี้มีผลงานวิจัยที่ตีพิมพ์ในวารสารระดับนานาชาติจำนวน 2 เรื่องคือ

1. Chaivisuthangkura P, Phattanapaijitkul P, Thammapalerd N, Rukpratanporn S, Longyant S, Sithigorngul W, Sithigorngul P. Production of Polyclonal Antibodies against Recombinant VP26 Structural Protein of White Spot Syndrome Virus (WSSV). ScienceAsia. 2006; 32: 201-204.
2. Chaivisuthangkura P, Longyant S, Rukpratanporn S, Srisuk C, Sridulyakul P, Sithigorngul P. Enhanced white spot syndrome virus (WSSV) detection sensitivity using monoclonal antibody specific to heterologously expressed VP19 envelope protein. Aquaculture. 2010; 299: 15-20. (impact factor = 1.678)

มีผลงานที่กำลังจัดเตรียมเป็น manuscript 1 เรื่องคือ

1. Chaivisuthangkura P, Longyant S, Rukpratanporn S, Srisuk C, Sridulyakul P, Sithigorngul P. Generation of a monoclonal antibody specific to VP26 structural protein of white spot syndrome virus (WSSV) using a heterologously expressed protein preparation. (in preparartion)

.

มีผลงานนำเสนอในรูปแบบโปสเตอร์ในการประชุมวิชาการ 1 เรื่องคือ

1. Chaivisuthangkura P, Longyant S, Rukpratanporn S, Phattanapaijitkul P, Thammapalerd N, Sithigorngul W, Sithigorngul P. Generation of monoclonal antibody against recombinant VP26 structural protein of white spot syndrome virus (WSSV). Second Annual Symposium pf Protein Society of Thailand, Chulabhorn Research Institute Conference Center, Bangkok, Thailand. September 20-21, 2007, p114.

ภาคผนวก

บทความสำหรับการเผยแพร่

โครงการเรื่อง การโคลนและการแสดงออกของโปรตีน VP19 และ VP26 ของไวรัสตัวแดงดวงขาวและการผลิตโมโนโคลนอลแอนติบอดี

ไวรัสตัวแดงดวงขาว (white spot syndrome virus; WSSV) เป็นไวรัสที่สำคัญที่ก่อให้เกิดโรคในกุ้งสกุล *Penaeus* ต่าง ๆ โดยเฉพาะกุ้งกุลาดำ สามารถทำให้เกิดการตายได้ 100% ภายในเวลา 3-10 วัน หลังจากติดเชื้อ WSSV มีรายงานการพบโรคครั้งแรกในไต้หวันปี 1992 จากนั้นมีรายงานระบาดทั่วไปในส่วนต่าง ๆ ของโลก WSSV สามารถติดเชื้อในครัสตาเซียที่สำคัญทางเศรษฐกิจทุกชนิด โดยสามารถตรวจพบได้ในครัสตาเซียที่อาศัยในน้ำจืดและทะเล สัตว์เหล่านี้สามารถเป็นพาหะนำโรคและก่อให้เกิดความเสียหายกับอุตสาหกรรมการเลี้ยงกุ้งเป็นจำนวนมหาศาล ดังนั้นจึงมีการพัฒนาวิธีการตรวจ WSSV โดยอาศัยการตรวจ nucleic acid เช่น PCR ซึ่งมีความไวในการตรวจไวรัสสูง

จากการศึกษาโปรตีนโครงสร้างของ WSSV พบว่ามีโปรตีนโครงสร้างหลัก 5 ชนิดด้วยกันคือ 28 kD (VP28) 26 kD (VP26) 24kD (VP24) 19kD (VP19) และ 15 kD (VP15) โดยเชื่อว่า ส่วน VP 28 และ VP 19 เป็นองค์ประกอบของ envelope และคาดว่า VP26 อาจเป็นองค์ประกอบของ envelope หรืออาจเป็น tegument protein ต่อมาได้มีการใช้ VP28 recombinant protein ในการผลิต monoclonal antibody (MAb) ซึ่ง MAb เหล่านี้มีประโยชน์สามารถใช้ตรวจสอบการติดเชื้อ WSSV ได้ตั้งแต่ 12 ช.ม. หลังจากฉีด WSSV ให้กุ้งกุลาดำโดยการตรวจด้วยวิธี immunohistochemistry และ dot blot และต่อมาได้มีการพัฒนาเป็นชุดตรวจไวรัสอย่างง่าย (strip-test)

ดังนั้นวัตถุประสงค์ของงานวิจัยนี้เป็นการพัฒนาวิธีตรวจวินิจฉัยทางด้านอิมมูโนโลยี เพื่อเป็นอีกทางเลือกหนึ่งแก่เกษตรกรในการตรวจการติดเชื้อ WSSV โดยอาศัยการโคลนและการแสดงออกของโปรตีนโครงสร้าง VP19 และ VP26 ของ WSSV เพื่อนำมาใช้เป็นแอนติเจนในการสร้างโมโนโคลนอลแอนติบอดี ที่สามารถช่วยเสริมกับแอนติบอดีต่อ VP28 ทำให้มีความไวในการตรวจวินิจฉัยสูงขึ้น

ได้ทำการโคลนยีน VP19 เข้าสู่เวกเตอร์ pMAL-C2 และกระตุ้นให้แสดงออกในรูปของ maltose binding protein (MBP)-VP19 จากนั้นนำ fusion protein ที่ได้ไปปลูกภูมิคุ้มกันในหนูขาวเพื่อสร้าง MAb พบว่า MAb สามารถตรวจการติดเชื้อ WSSV ตามธรรมชาติในกุ้งขาวได้โดยวิธี dot blotting, western blotting และ immunohistochemistry โดยไม่ทำปฏิกิริยาข้ามกับไวรัสชนิดอื่นได้แก่ Taura syndrome virus (TSV), yellow head virus (YHV), monodon baculovirus (MBV) และ hepatopancreatic parvovirus (HPV) MAb W25-8D ที่จำเพาะต่อ VP19 มีความไวประมาณ 1.2 fmole/จุด เมื่อใช้โปรตีน MBP-VP19 บริสุทธิ์ในการทดสอบโดยวิธี dot blotting เมื่อนำ MAb W25-8D นี้ไปใช้ร่วมกับ MAb W29 ที่จำเพาะต่อ VP28 ในการตรวจสารสกัดจากกุ้งที่ติดเชื้อ WSSV โดยวิธี dot blotting พบว่ามีความไวสูงขึ้น 2 เท่าแต่ยังคงมีความไวต่ำกว่าวิธี one-step PCR 25,000 เท่า เมื่อนำ MAb W25-8D มาใช้ตรวจการติดเชื้อในเนื้อเยื่อโดยวิธี immunohistochemistry พบว่าสามารถตรวจพบ

VP19 ได้ทั้งในนิวเคลียสและไซโตพลาซึม ซึ่งแตกต่างจาก MAb W29 ที่สามารถตรวจพบ VP28 ได้เฉพาะในนิวเคลียสเท่านั้น

ในกรณีของ VP26 ได้ทำการโคลนยีน VP26 ที่ปราศจากส่วน transmembrane เข้าสู่เวกเตอร์ pQE30 และกระตุ้นให้แสดงออกในรูปของ 6xHis-VP26 จากนั้นนำ fusion protein ที่ได้ไปปลูกภูมิคุ้มกันในหนูขาวเพื่อสร้างโมโนโคลนอลแอนติบอดี พบว่า MAb สามารถตรวจการติดเชื้อ WSSV ตามธรรมชาติในกุ้งขาวได้โดยวิธี dot blotting, western blotting และ immunohistochemistry โดยไม่ทำปฏิกิริยาข้ามกับไวรัสชนิดอื่นได้แก่ Taura syndrome virus (TSV), yellow head virus (YHV), monodon baculovirus (MBV) และ infectious hypodermal and hematopoietic necrosis virus (IHHNV) เมื่อนำ MAb W26-3 ที่จำเพาะต่อ VP26 ไปใช้ในการตรวจสารสกัดจากกุ้งที่ติดเชื้อ WSSV โดยวิธี dot blotting พบว่ามีความไวต่ำกว่า MAb W29 ที่จำเพาะต่อ VP28 ประมาณ 2 เท่า เมื่อนำ MAb W26-3 มาใช้ตรวจการติดเชื้อในเนื้อเยื่อโดยวิธี immunohistochemistry พบว่ารูปแบบของ immunoreactivity เหมือนกับของ VP28 คือสามารถตรวจพบได้เฉพาะในนิวเคลียสเท่านั้น

สำหรับการวิจัยในอนาคตคาดว่า จะนำ MAb W25-8D ที่จำเพาะต่อ VP19 มาใช้ร่วมกันกับ MAb W29 ที่จำเพาะต่อ VP28 ในการพัฒนาเป็นชุดตรวจไวรัสอย่างง่าย (strip-test) เพื่อเสริมให้มีความไวสูงขึ้นในการตรวจวินิจฉัยการติดเชื้อ WSSV ในกุ้ง

ผลงานวิจัยที่ได้รับทุนสนับสนุนจาก สกว. และ สกอ. ในโครงการวิจัยชุดนี้ ได้รับการตีพิมพ์จำนวน 2 บทความในวารสารนานาชาติ ดังนี้:

1. Chaivisuthangkura P, Phattanapaijitkul P, Thammapalerd N, Rukpratanporn S, Longyant S, Sithigorngul W, Sithigorngul P. Production of Polyclonal Antibodies against Recombinant VP26 Structural Protein of White Spot Syndrome Virus (WSSV). ScienceAsia. 2006; 32: 201-204.

2. Chaivisuthangkura P, Longyant S, Rukpratanporn S, Srisuk C, Sridulyakul P, Sithigorngul P. Enhanced white spot syndrome virus (WSSV) detection sensitivity using monoclonal antibody specific to heterologously expressed VP19 envelope protein. Aquaculture. 2010; 299: 15-20. (impact factor = 1.678)

SHORT REPORT
doi: 10.2306/scienceasia1513-1874.2006.32.201

ScienceAsia 32 (2006): **201-204**

Production of Polyclonal Antibodies against Recombinant VP26 Structural Protein of White Spot Syndrome Virus (WSSV)

Parin Chaivisuthangkura[a], Phiromsak Phattanapaijitkul[b], Nitaya Thammapalerd[b], Sombat Rukpratanporn[c], Siwaporn Longyant[a], Weerawan Sithigorngul[a] and Paisarn Sithigorngul[a]*

[a] Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok 10110, Thailand.
[b] Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand.
[c] Center of Excellence for Marine Biotechnology at Chulalongkorn University, National Center for Genetic Engineering and Biotechnology (BIOTEC), Bangkok 10330, Thailand.

* Corresponding author, E-mail: paisarn@swu.ac.th

Received 24 Aug 2005
Accepted 30 Nov 2005

ABSTRACT: A portion of the VP26 gene (VP26F109) encoding a structural protein of white spot syndrome virus was cloned into an expression vector and transformed into *E. coli*. The objective was to produce a truncated VP26 structural protein lacking the N-terminal transmembrane region. After induction, the recombinant protein rVP26F109 was produced, purified by SDS-PAGE and used to immunize Swiss mice for polyclonal antibody production. The mouse anti VP26 antiserum demonstrated specific immunoreactivity to viral antigen in white spot syndrome virus (WSSV) infected *Penaeus monodon*, as verified by immunohistochemistry and western blot. This constitutes the first step in producing monoclonal antibodies against rVP26F109 that can be combined with anti-VP28 monoclonal antibodies to enhance the sensitivity in WSSV immunological assays.

KEYWORDS: immunohistochemistry, polyclonal antibody, VP26, Western blot, WSSV.

INTRODUCTION

White spot syndrome virus (WSSV) is one of the most virulent pathogens that causes major losses in shrimp farming. Genome based diagnostic methods, such as *in situ* hybridization[1], PCR[2] and real time PCR[3] have been developed for WSSV detection. Immunological based diagnostic methods using polyclonal antibodies[4] and monoclonal antibodies specific to the VP28 envelope protein have also been developed.[5-8] However, in most cases the detection limit of the immunodiagnostic methods is inferior to that of PCR. This study aimed to clone a structural protein gene, VP26 in order to produce antigen and then antibody. The antibody against VP 26 was expected to be used in combination with an antibody specific to VP28 in order to improve the sensitivity of various immunoassays for WSSV.

MATERIALS AND METHODS

Viral Preparation

Natural white spot syndrome virus (WSSV) infected *P. monodon* was obtained from a farm at Nakhon Srithamarat Province, Thailand. Gills from the infected shrimp were homogenized in 2X PBS (phosphate buffered saline, pH 7.2), then centrifuged at 3,000 g for 30 min. Aliquots of the supernatant were collected and stored at –70°C.

WSSV DNA Preparation

Gills from naturally WSSV-infected *P. monodon* were homogenized in lysis buffer (50 mM Tris-HCl, pH 9, 100 mM EDTA, 50 mM NaCl, 2% SDS; Timothy Flegel personal communication). DNA from 200 µl of the the homogenate was prepared using a High pure viral nucleic acid kit from Roche Molecular Biochemicals as described in the product manual.

Cloning and Expression of Truncated VP26

Primers, VP26F109 (5'- CG GGA TCC CGT GTT GGA AGA AGC GTC GTC-3'; 109 nucleotides downstream of the ATG start site) and VP26RPST (5'T GCA CTG CAG TTA CTT CTT GAT TTC G-3') with added restriction sites (underlined) were used to amplify a truncated VP26 gene by polymerase chain reaction (PCR) using *pfx* polymerase (GIBGO BRL). The PCR product was cloned into the pQE30 expression vector at the *Bam*HI and *Pst*I sites and transformed into *E. coli* strain M15 (pREP4). The integrity of the open reading frame of the recombinant plasmid was verified by DNA sequencing.

Preparation of Recombinant VP26F109

E. coli with pQE30-VP26F109 plasmid was cultured in LB broth to the exponential phase and expression of the recombinant proteins was induced with 1 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG) for 4 h. After centrifugation at 4,000 g for 20 min, the bacterial pellet was dissolved 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 separated by SDS-PAGE with a 15% gel. After staining with Coomassie brilliant blue, the recombinant protein bands of rVP26F109 were cut out and destained until the gels were clear. They were collected in dialysis bags and the protein was eluted with a transblot apparatus (BioRad) at 70 V for 6 h. The protein solution was dialysed to eliminate SDS and salt before determining the protein content by Bradford protein assay.[9] The protein solution was divided into small aliquots and stored at −70°C.

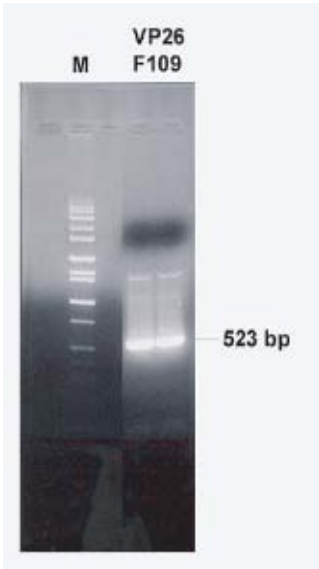

Fig 1. Ethidium bromide stained gel of VP26F109 PCR product. M=DNA marker.

Polyclonal Antibody Production

Three Swiss mice were injected intra-peritoneally with purified rVP26F109 (0.05 mg/mouse) mixed with complete Freund's adjuvant in a 1:1 ratio. Mice were subsequently injected three more times with the protein mixed with incomplete Freund's adjuvant at two weekly intervals. One week after the fourth injection, mouse antisera were collected and tested against *E. coli* lysate, purified rVP26F109 and gill extract from WSSV infected *P. monodon* by western blot. They were also tested against head tissues from WSSV infected *P. monodon* by immunohistochemistry.

Western Blot Analysis

Lysate of *E. coli* M15 (pREP4) with pQE30 or with pQE30-VP26F109, purified rVP26F109 and gill extract from WSSV infected *P. monodon* were separated by 15% SDS-PAGE according to method described by Laemmli.[10] Samples were electrophoresed for 6 h at 30 V and gels were stained using Coomassie brilliant blue. For western blot analysis , samples resolved by SDS-PAGE were electroblotted onto nitrocellulose membranes using a Transblot apparatus (BioRad) then incubated for 4 h with mouse anti rVP26F109 antiserum at dilution of 1:3000 in 5% Blotto (5% nonfat dry milk, 0.1% Triton X-100 in PBS). After extensive washing in 0.5% Blotto, the membrane was incubated in horseradish peroxidase conjugated goat anti-mouse IgG heavy and light chain specific antibody (GAM-HRP; BioRad) at 1:1000 in 5% Blotto for 4 h. The membranes were then washed extensively as before and incubated for 5 min in a substrate mixture containing 0.006% hydrogen peroxide, 0.03% diaminobenzidine (DAB), and 0.05% cobalt chloride in PBS and washed extensively in distilled water. Immunoreactive protein appeared as dark gray bands. The membrane was also reprobed with W29 monoclonal antibody (specific to the VP28 enveloped protein) obtained from previous work[8] for comparison with the immunoreactivity from the antiserum.

Immunohistochemistry

Cephalothoraces from WSSV infected *P. monodon* were cut and fixed in Davidson's fixative solution for 24 h before processing for paraffin sectioning. Serial sections (8 μm thickness) were prepared and processed for indirect immunoperoxidase staining using mouse anti-rVP26F109 antiserum at 1:1000 dilution and GAM-HRP at 1:1000 dilution in 10% calf serum in PBS for 5 h at 37 °C for each step. After extensive washing with PBS, peroxidase activity was revealed by incubation with 0.03% DAB, 0.006 % hydrogen peroxide in PBS for 5 min. Preparations were counterstained with haematoxylin and eosinY, dehydrated in a graded ethanol series, cleared in xylene and mounted in Permount.[11] Positive reactions were visualized as brown coloration against the pink and purple colors of haematoxylin and eosin. A nearby section was also treated with W29 monoclonal antibody in the same fashion for comparison.

Results and Discussion

The truncated VP26 gene, VP26F109, of WSSV structural protein could be amplified as a 523 bp PCR product (Fig 1.). This was cloned, expressed in *E. coli*, and visualized by Coomassie blue staining as a band

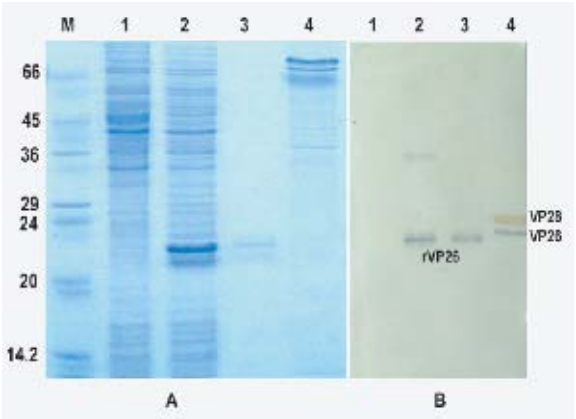

Fig 2. SDS-PAGE and Western blot analyses. (A) Coomassie blue stained gel, (B) Western blot using polyclonal antibody against rVP26F109 (lane 1-4) and reprobed with W29 monoclonal antibody specific to VP28 (lane 4, upper band). M = protein marker, 1 = lysate of *E. coli* strain M15 (pREP4) containing pQE30, 2= lysate of *E. coli* strain M15 (pREP4) containing pQE30-VP26F109, 3 = purified rVP26F109 protein, 4 = gill homogenate of *P. monodon* infected with WSSV.

with molecular mass of ~23 kDa that was slightly smaller than natural VP26 (Fig 2A lane 2). After this band was cut and eluted, high purity rVP26F109 was obtained (Fig 2A lane 3) and adjusted to 1 mg/ml protein before storage in small aliquots. The truncated VP26 structural protein was in the form of a recombinant fusion protein with a 6-histidine tag at the N-terminus.

After immunization of Swiss mice with rVP26F109 protein, antisera obtained from 3 mice displayed very strong immunoreactivities and specificities on western blot to purified rVP26F109 protein and lysate of *E. coli* containing VP26F109-pQE30 (Fig 2B lane 2 and 3) but not to the lysate of *E. coli* containing only pQE30 (Fig 2B lane 1). Immunoreactivities against VP26 (Fig 2B lane 4, lower band) and VP28 (Fig 2B lane 4, upper band) were observed in gill homogenates from *P. monodon* infected with WSSV. By immunohistochemistry, the immunoreactivity of anti-VP26 antibody occurred in a similar pattern to that of W29 monoclonal antibody specific to VP28 of WSSV (Fig 3). Therefore, this result confirmed that the rVP26 protein obtained from transformed *E. coli* shared similar epitopes with natural VP26 of WSSV.

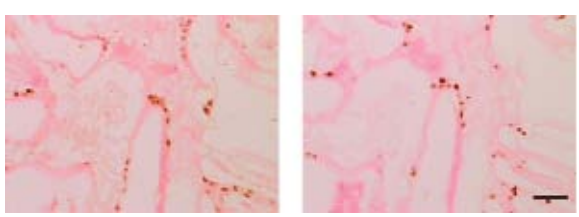

Fig 3. Immunohistochemistry of gill tissues from WSSV infected *P. monodon* using polyclonal antibody against rVP26F109 (A) and W29 monoclonal antibody specific to VP28 (B). Bar = 50 μm.

In western blot analysis, the content of VP26 structural protein in WSSV appeared to be slightly less than that of VP28. However, the tissue immunoreactivity demonstrated by the antibody against VP26 was comparable to that of the monoclonal antibody specific to VP28, as revealed by immunohistochemistry (Fig 3). Therefore, the antibody against VP26 could be used well for localization of WSSV infection in tissues similarly to antibodies specific for VP28. Hydrophobicity analysis of the VP26 protein demonstrated that there was a strong hydrophobic region at the N terminus suggesting that it is a membranous protein. Immunogold electron microscopy revealed that VP26 is an envelope protein.[12] Therefore, the combination of antibodies against VP26 and VP28 should be able to enhance the sensitivity for detection of WSSV. Recently, recombinant VP28 and VP19 were applied as injected [13] or oral [14] protectants against WSSV. The recombinant VP26 may be potentially applied in the same fashion for additional efficacy in protection against WSSV.

Acknowledgements

This work was supported by Thailand Research Fund and Office of Commission on Higher Education, Thailand.

References

1. Chang PS, Lo CC, Wang YC and Koou GH (1996) Identification of white spot syndrome associated baculovirus (WSBV) target organs in the shrimp *Penaeus monodon* by *in situ* hybridization. *Dis Aquat Org* **27**, 131-9.
2. Lo CF, Ho CH, Peng SE, Chen CH, Hsu HC, Chiu YL, Chang CF and Lui KF et al (1996) White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods. *Dis Aquat Org* **27**, 215-25.
3. Durand SV, Redman RM, Mohney LL, Tang-Nelson K, Bonami JR and Lightner DV (2003) Qualitative and quantitative studies on the relative virus load of tails and heads of shrimp acutely infected with WSSV. *Aquaculture* **216**, 9-18.
4. Nadala ECB, and Loh PC (2000) Dot blot enzyme immunoassays for the detection of white spot virus and yellow-head virus of penaeid shrimp. *J Virol Meth* **84**, 175-79.
5. Poulos BT, Pantoja CR, Bradley-Dunlop D, AguilarJ and Lightner DV (2001) Development and application of monoclonal antibodies for the detection of white spot syndrome virus of penaeid shrimp. *Dis Aquat Org* **47**, 13-23.
6. Anil TM, Shankar KM and Mohan CV (2002) Monoclonal antibodies developed for sensitive detection and comparison of white spot syndrome virus isolates in India. *Dis Aquat Org* **51**, 67-5.
7. Lui W, Wang YT, Tian DS, Yin ZC, and Kwang J (2002)

Detection of white spot syndrome virus (WSSV) of shrimp by means of monoclonal antibodied (MAbs) specific to an envelope protein (28 kDa). *Dis Aquat Org* **49**, 11-8.

8. Chaivisuthangkura P, Tangkhabuanbutra J, Longyant S, Sithigorngul W, Rukpratanporn S, Menasveta P and Sithigorngul P (2004) Monoclonal antibodies against a truncated viral envelope protein (VP28) can detect white spot syndrome virus (WSSV) infections in shrimp. *ScienceAsia* **30**, 359-63.
9. Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248-54.
10. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680-85.
11. Sithigorngul P, Rukpratanporn S, Longyant S, Chaivisuthangkura P, Sithigorngul W and Menasveta P (2002) Monoclonal antibodies specific to yellow-head virus (YHV) of *Penaeus monodon*. *Dis Aquat Org* **49**, 71-6.
12. Zhang X, Huang C, Xu X, and Hew CL (2002) Transcription and identification of an envelope protein gene (p22) from shrimp white spot syndrome virus. *J Gen Virol* **83**, 471-7.
13. Witteveldt J, Vlak JM and van Hulten MCV (2004) Protection of *Penaeus monodon* against white spot syndrome virus using a WSSV subunit vaccine. *Fish Shellfish Immunol* **16**, 571-9.
14. Witteveldt J, Cifuentes CC, Vlak JM and van Hulten MCV (2004) Protection of *Penaeus monodon* against white spot syndrome virus by oral vaccination. *J Virol* **78**, 2057-61.

Aquaculture 299 (2010) 15–20

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Enhanced white spot syndrome virus (WSSV) detection sensitivity using monoclonal antibody specific to heterologously expressed VP19 envelope protein

Parin Chaivisuthangkura [a,*], Siwaporn Longyant [a], Sombat Rukpratanporn [b], Chutima Srisuk [a], Pattarin Sridulyakul [a], Paisarn Sithigorngul [a]

[a] Department of Biology, Faculty of Science, Srinakharinwirot University, Sukhumvit 23, Bangkok 10110, Thailand
[b] Center of Excellence for Marine Biotechnology at Chulalongkorn University, National Center for Genetic Engineering and Biotechnology (BIOTEC), Bangkok 10330, Thailand

ARTICLE INFO

Article history:
Received 21 September 2009
Received in revised form 15 November 2009
Accepted 5 December 2009

Keyword:
Immunohistochemistry
Monoclonal antibody
VP19
Western blot
WSSV

ABSTRACT

The gene encoding the VP19 envelope protein of white spot syndrome virus (WSSV) was cloned into pMAL-C2 expression vector and transformed into the BL21 *Escherichia coli* strain. After induction, recombinant maltose binding protein (MBP)-VP19 fusion protein was produced, purified and electroeluted before use for immunization in Swiss mice for monoclonal antibody (MAb) production. MAbs specific to VP19 can be used to detect natural WSSV infection in *Penaeus vannamei* by dot blotting, western blotting and immunohistochemistry without cross-reaction to other shrimp tissues or other common shrimp viruses, including Taura syndrome virus (TSV), yellow head virus (YHV), *Penaeus monodon* nucleopolyhedrovirus (PemoNPV) formerly called monodon baculovirus (MBV) and *P. monodon* densovirus (PmDNV) previously called hepatopancreatic parvovirus (HPV). The detection sensitivity of the VP19-specific W25-8D MAb generated in this study was approximately 1.2 fmol/spot of purified recombinant MBP-VP19 protein as determined by dot blotting while that of a VP28-specific W29 MAb obtained from a previous study was approximately 5 fmol/spot. Combining MAbs specific for VP19 and VP28 resulted in two-fold higher sensitivity than use of either MAb alone. However, the sensitivity of the combined MAbs was 25,000 times lower than that of one-step PCR. Immunohistochemical analysis using MAbs specific to VP19 in WSSV-infected gill tissues and appendages demonstrated intense staining patterns in both the nucleus and cytoplasm compared to MAb specific to VP28. In conclusion, combination of VP19- and VP28-specific MAbs could confirm and enhance the sensitivity of WSSV detection in shrimp in various types of antibody-based assays.

1. Introduction

White spot syndrome virus (WSSV) is one of the most serious pathogens affecting the shrimp industry worldwide. WSSV is the sole member of the novel genus *Whispovirus* within the *Nimaviridae* family (Fauquet et al., 2005). At present, at least 40 WSSV structural proteins ranging from 68 to 6077 amino acid residues in size have been identified (Huang et al., 2002; Zhang et al., 2004; Tsai et al., 2004; Xie et al., 2006; Leu et al., 2009). Six major structural proteins including VP664, VP28, VP24, VP26, VP19, and VP15 have been identified (Tsai et al., 2004). VP28 and VP19 were reported to be located in the viral envelope (van Hulten et al., 2000). However, the functions of VP19 in WSSV assembly and infection are not known. Recently, *in vitro* pull-down and yeast two-hybrid assays demonstrated interactions among several WSSV structural proteins (VP19–VP28, VP19–VP24, VP24–VP26 and VP24–VP24) confirming that these four major proteins can form a multiprotein complex (Zhou et al., 2009).

Several genome-based diagnostic methods such as *in situ* hybridization (Chang et al., 1996), one-step PCR (Takahashi et al., 1996), nested PCR (Lo et al., 1996; Kimura et al., 1996) and real-time PCR (Durand et al., 2003), have been developed for detection of WSSV. Recently, loop-mediated isothermal amplification (LAMP) protocols were developed for WSSV detection (Kono et al., 2004; Mekata et al., 2009; Jaroenram et al., 2009). Although these molecular methods are sensitive and reliable, they are not feasible for pond-site detection. Therefore, an antibody-based assay presents an attractive alternative, providing a simple and low-cost detection system with high specificity and optimal sensitivity for disease monitoring during shrimp cultivation.

Polyclonal (Nadala and Loh, 2000; You et al., 2002) and monoclonal antibodies specific to WSSV proteins (Poulos et al., 2001; Anil et al., 2002; Liu et al., 2002; Chaivisuthangkura et al., 2004; Shih, 2004) have been developed. The MAbs recognizing WSSV envelope protein displayed the viral neutralizing activity in both cultured cells and in experimental shrimp (Shih, 2004). The W29 MAb, specific to VP28 of WSSV, was further developed into an immunochromatographic test strip to detect WSSV in shrimp (Sithigorngul et al., 2006). However, in most cases the detection limit was still inferior to PCR. In a previous

* Corresponding author. Tel.: +662 664 1000x8511; fax: +662 260 0127.
E-mail address: parin@swu.ac.th (P. Chaivisuthangkura).

0044-8486/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquaculture.2009.12.005

study, a polyclonal antibody specific to VP19 was developed (Chaivisuthangkura et al., 2006). In this study, a VP19-specific monoclonal antibody (MAb) was generated. This antibody should prove useful in enhancing the sensitivity of various immunoassays for WSSV detection.

2. Materials and methods

2.1. Viral preparation

Naturally-WSSV-infected *P. vannamei* specimens were obtained from a farm in Chantaburi province, Thailand. Infection was verified by one-step PCR (Chaivisuthangkura et al., 2004). Pleopods from infected shrimp were homogenized in 2-fold PBS (0.3 M Phosphate buffered saline, pH 7.2) and centrifuged at 3000×*g* for 30 min. The supernatant was collected in aliquots and stored at −70 °C.

2.2. Preparation of recombinant VP19 protein

Escherichia coli (BL21strain) were transformed with the *VP19*-pMAL-C2 plasmid (Chaivisuthangkura et al., 2006) and cultured in Luria–Bertani (LB) broth to the exponential phase. Expression of recombinant protein was induced with 1 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG) for 4 h. After centrifugation at 4000×*g* for 20 min at room temperature, the bacterial pellet was resuspended in a buffer containing 100 mM NaH_2PO_4, 10 mM Tris–HCl, 8 M urea pH 8, and 1 mM phenylmethylsulfonyl fluoride (PMSF) and sonicated until a clear lysate was obtained. The lysate was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After treatment with 0.3 M KCl, recombinant fusion protein called MBP-VP19 was excised and collected in dialysis bags. Recombinant protein was eluted with a Transblot apparatus (BioRad) at 70 V for 6 h, dialyzed and concentrated using a vacuum concentrator (Savant). Protein concentration was determined by Bradford assay (Bradford, 1976). The MBP-VP19 protein solution was adjusted to 1 mg/ml, divided into small aliquots and stored at −70 °C.

2.3. Immunization

MBP-VP19 protein mixed with complete Freund's adjuvant in a 1:1 ratio was injected intra-peritoneally into four Swiss mice at 0.05 mg protein per mouse. Mice were subsequently injected with the MBP-VP19 protein mixed with incomplete Freund's adjuvant three more times at 2 week intervals. One week after the fourth injection, mouse antisera were collected and tested against lysates of *E. coli* containing either the pMAL-C2 or *VP19*-pMAL-C2 plasmid by western blotting. The best-performing mouse was subsequently boosted 3 days before hybridoma production.

2.4. Production of monoclonal antibody

A cell fusion protocol was adapted from the method developed by Köhler and Milstein (1976) with modifications described by Mosmann et al. (1979). A P3X myeloma cell line was used as the fusion partner. Fusion products from one mouse were plated onto 30 microculture plates (96 wells/plate). After identification of positive cultures by screening methods including dot blotting, western blotting and immunohistochemistry as described below, cells were cloned by the limiting dilution method and stored in liquid nitrogen.

2.5. Specificity testing

2.5.1. Dot blotting

Lysates of *E. coli* BL21 containing either the pMAL-C2 or *VP19*-pMAL-C2 plasmid or pleopod homogenate samples (1 μl/spot) from uninfected or WSSV-infected shrimp, were applied to nitrocellulose membranes, baked at 60 °C for 10 min and incubated in hybridoma conditioned medium from culture (diluted to 1:20 in 5% blotto blocking solution (5% nonfat drymilk, 0.1% Triton X-100 in PBS)) for 4 h. After extensive washing in 0.5% blocking solution, the membrane was incubated in horseradish peroxidase-labeled goat anti-mouse gamma immunoglobulin heavy and light chain-specific antibody (GAM-HRP, BioRad) at 1:1000 dilution for 4 h. The membrane was then washed for 5 min in blocking solution and incubated 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* containing either pMAL-C2 plasmid (MBP) or *VP19*-pMAL-C2 plasmid and pleopod homogenates from WSSV-infected shrimp were separated by 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, the samples resolved by SDS-PAGE were transferred onto nitrocellulose membranes using Transblot apparatus (BioRad). Nitrocellulose membranes were incubated in 5% blocking solution for 10 min and treated with MAbs or mouse anti-recombinant VP19-antiserum (preabsorbed with *E. coli* lysate containing MBP) for 4 h. After extensive washing in 0.5% blocking solution, the membrane was incubated with GAM-HRP at 1:1000 dilution for 4 h. The membrane was then washed extensively as before and incubated in a substrate mixture containing 0.006% hydrogen peroxide, 0.03% DAB, 0.05% cobalt chloride in PBS. The membrane was also reprobed with the VP28-specific MAb (W29) obtained from a previous study (Chaivisuthangkura et al., 2004) and developed without cobalt chloride to obtain a brown precipitate for comparison.

2.5.3. Immunohistochemistry

Cephalothoraces from *P. vannamei* specimens naturally infected with WSSV were fixed in Davidson's fixative solution for 24 h before processing for paraffin sectioning. Serial sections (8 μm thickness) of tissues were prepared and processed for indirect immuno-peroxidase staining using MAb. Peroxidase activity was visualized by incubation with 0.03% DAB and 0.006% hydrogen peroxide in PBS. Preparations were counterstained with hematoxylin and eosin Y (H&E), dehydrated in graded ethanol series, cleared in xylene and mounted in Permount (Sithigorngul et al., 2000). Positive reactions were visualized as brown coloration against pink cytoplasm and purple nuclei.

2.6. MAb class and subclass determination

Classes and subclasses of the mouse immunoglobulins produced by hybridomas were determined by sandwich ELISA using Zymed's Mouse MonoAb ID Kit (HRP).

2.7. Cross-reactivity testing

Shrimp samples infected with *P. monodon* densovirus (PmDNV), previously called hepatopancreatic parvovirus (HPV), *P. monodon* nucleopolyhedrovirus (PemoNPV), previously called monodon baculovirus (MBV), Taura syndrome virus (TSV) and yellow head virus (YHV) were processed for paraffin sectioning and immunohistochemistry using MAb specific to WSSV. Results were compared to those from MAbs specific to PmDNV (Rukpratanporn et al., 2005), TSV (Longyant et al., 2008), YHV (Sithigorngul et al., 2002) and PemoNPV (Boonsanongchokying et al., 2006).

2.8. Sensitivity testing with recombinant VP19 protein

Purified MBP-VP19 protein was serially diluted with PBS, spotted onto nitrocellulose membranes and processed for dot blotting using the VP19-specific MAb generated in this study. The last dilution yielding a clear positive result was determined. The sensitivity of the VP28-specific

W29 MAb was also determined in the same fashion using purified 6xHis-VP28 protein from previous study (Chaivisuthangkura et al., 2004). Protein content was measured by Bradford protein assay (Bradford, 1976).

2.9. Comparison of sensitivity between MAb and one-step PCR using WSSV-infected shrimp sample

The sensitivity of WSSV detection in a naturally-infected shrimp was determined using MAbs W25-8D (VP19-specific) and W29 (VP28-specific). The WSSV-infected shrimp sample, as verified by PCR (Chaivisuthangkura et al., 2004), was homogenized in PBS, serially diluted with normal shrimp homogenate and processed for dot blotting as describe above using MAb W29, W25-8D and a combination of W29 and W25-8D in a 1:1 ratio. The last dilution of shrimp homogenate yielding a clear positive result was determined. DNA from the same shrimp homogenate were also extracted with a High Pure viral nucleic acid kit (Roche Molecular Biochemicals) and serially diluted with uninfected shrimp nucleic acid and tested for WSSV by PCR using primers VP28F (5′-CCG GAT CCA TGG ATG GA TCT TTC TTT CAC CTT TCG-3′) and VP28R (5′-TGC ACT GCA GTT ACT CGG TCT CAG TGC CAG-3′) to yield an amplicon of 633 bp (Chaivisuthangkura et al., 2004).

2.10. Detection of WSSV in naturally-infected shrimp specimens

For WSSV detection tests in wild shrimp specimens, 15 *P. vannamei* (5 g in weight) specimens infected with WSSV (collected from Chantaburi province, Thailand) verified by PCR (Chaivisuthangkura et al., 2004) and 14 *P. vannamei* (5 g weight) specimens infected with YHV (collected from Nakhon Pathom province, Thailand) verified by RT-PCR (Wongteerasupaya et al., 1997) were used. One pleopod from each shrimp was homogenized in 50 μl of PBS and spotted (1 μl/spot) on each square of a nitrocellulose membrane, which was processed for dot blotting using MAb against VP19 (W25-8D) or VP28 (W29) of WSSV or YHV (Y19) (Sithigorngul et al., 2002) as described above.

3. Results and discussion

3.1. MAb production

After hybridoma production, approximately 800 hybridoma-containing wells were obtained and approximately 80 wells gave positive binding results when initially screened with MBP-VP19 *E. coli* lysate. Hybridoma clones were further screened by dot blotting against *E. coli* lysates containing MBP or MBP-VP19 and pleopod homogenate samples from uninfected and WSSV-infected shrimps, by western blotting against pleopod homogenates from WSSV-infected shrimp and by immunohistochemical analysis of cephalothorax sections from WSSV-infected specimens. Five MAbs specific to VP19, namely W25-8D, W10-9D, W9-11C, W9-11D and W7-7H, were selected and cloned to established cell lines. All MAb belonged to 3 IgG subclasses (Table 1). Since MAbs W25-8D and W9-11D demonstrated the best results with the various immunoassays described above, one of these MAbs (W25-8D) was used as a representative MAb for further experiments.

3.2. Specificity of MAb

VP19-specifc W25-8D and VP28-specific W29 MAbs were used in dot blot assays as previously described (Chaivisuthangkura et al., 2004). As shown in Fig. 1A, W25-8D bound intensely to *E. coli* lysates containing MBP-VP19 and pleopod homogenate from WSSV-infected shrimp but did not bind to *E. coli* lysate containing MBP or pleopod homogenate from uninfected shrimp. W29 bound specifically to WSSV-infected shrimp homogenate (Fig 1B).

Table 1
Monoclonal antibodies (MAbs) obtained from mouse immunized with the MBP-VP19 protein in comparison with MAb W29 specific to VP28. IHC: immunohistochemistry; +++ = strong immunoreactivity; − = no immunoreactivity.

MAb (subclass)	Sensitivity (dot blotting)	Western blotting	IHC	Specificity
W25-8D (IgG1)	~70 pg/spot	+++	+++	VP19
W7-7H (IgG2b)	~280 pg/spot	+++	+++	VP19
W9-11C (IgG2a)	~140 pg/spot	+++	+++	VP19
W9-11D (IgG1)	~70 pg/spot	+++	+++	VP19
W10-9D (IgG2b)	~280 pg/spot	+++	+++	VP19
W29 (IgG1)	~140 pg/spot	+++	+++	VP28

VP19-specific W25-8D reacted with MBP-VP19 and yielded a single band of VP19 protein in pleopod homogenates from WSSV-infected shrimp (Fig. 2B, lanes 2 and 3). The intensity of the VP19 band was comparable to that of VP28 as visualized using W29 (Fig. 2B, lane 3). This result demonstrates the specificity of W25-8D for recombinant or natural VP19 protein. All MAb specific to VP19 gave similar results demonstrating the specificity of the MAbs developed against the VP19 envelope protein.

None of the VP19-specific MAbs exhibited cross-reactivity with tissues from shrimp infected with TSV, PmDNV, YHV or PemoNPV in immunohistochemical experiments (data not shown). However, the immunohistochemical analysis of WSSV-infected gill and appendage tissues using MAbs specific to VP19 yielded intense staining with distinct patterns different from those seen following staining with VP28-specific MAb. Interestingly, the W25-8D MAb not only reacted with VP19 Ag in the nucleus, showing Cowdry type A inclusion, but also reacted with Ag in the cytoplasm of the infected cells (Fig. 3). This immunoreactivity was specific to VP19 because none of the immunoreactivity was observed in nearby areas such as the haemocoel (H) of the same stained gill tissue (Fig. 3B). The presence of VP19 Ag in the cytoplasm may result from WSSV virions released from disrupted nuclei of epidermal cells which are readily budded from the cytoplasmic membrane as reported earlier (Chou et al., 1995). The lack of observed immunoreactivity in the cytoplasm of infected cells in this experiment using VP28-specific MAb remains puzzling even though the immunoreactivity of the VP28 Ag in the nucleus was clearly observed (Fig. 3A, column 2).

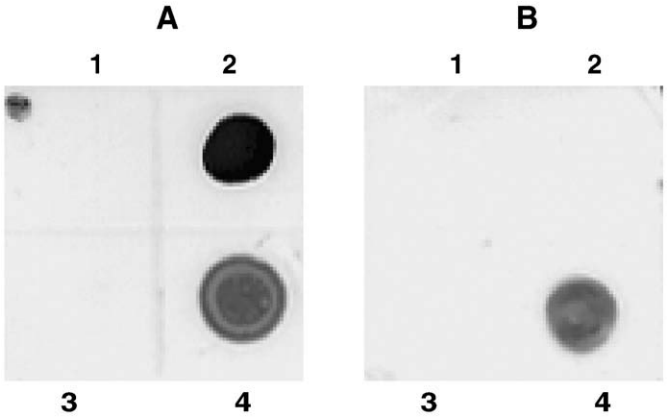

Fig. 1. Dot blot analysis of monoclonal antibodies (MAbs). Lysates of (1) BL21 *Escherichia coli* transfected with pMAL-C2 plasmid, (2) *E. coli* with *VP19*-pMAL-C2 plasmid, (3) pleopod homogenate from uninfected and (4) WSSV-infected *Penaeus vannamei* were spotted (1 μl spot^{-1}) onto a nitrocellulose membrane and treated with VP19-specific W25-8D (A) or VP28-specific W29 (B) MAbs.

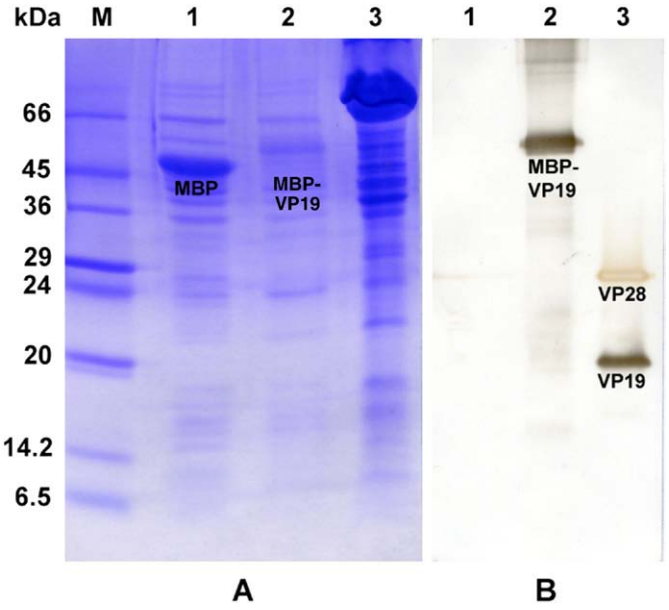

Fig. 2. SDS-PAGE and western blotting. (1) Lysate of *E. coli* expressing MBP, (2) lysate of *E. coli* expressing MBP-VP19, (3) pleopod homogenate of WSSV-infected *P. vannamei* were electrophoresed and (A) stained with Coomassie brilliant blue or (B) transferred to nitrocellulose membrane and treated with VP19-specific W25-8D MAb and retreated with VP28-specific W29 MAb (recognized only lane 3, upper band). M = standard marker proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

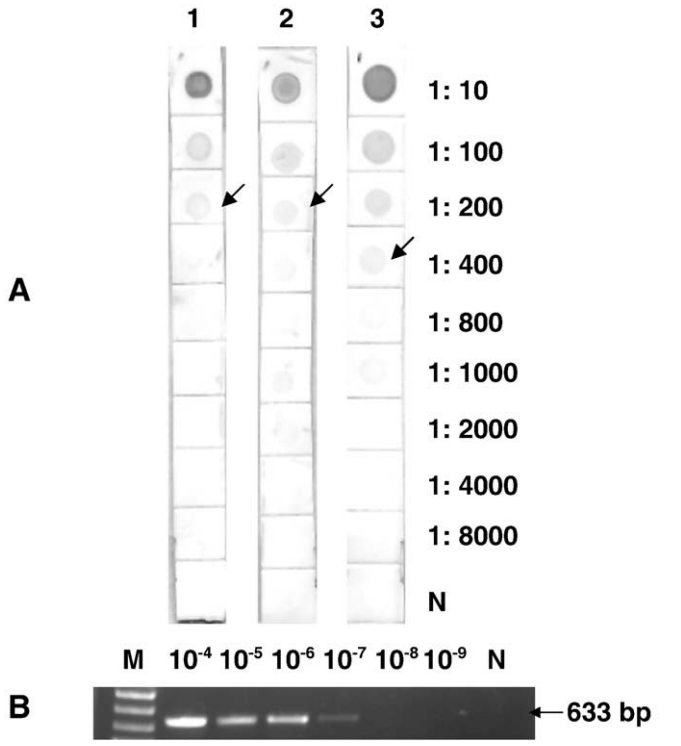

Fig. 4. Sensitivity of WSSV detection by dot blotting and PCR. (A) Dot blotting; pleopod homogenate from a naturally-infected *P. vannamei* specimen was serially diluted with healthy shrimp homogenate and spotted onto nitrocellulose membrane (1 μl/spot) and processed for dot blotting using VP19-specific W25-8D (1) VP28-specific W29 (2) MAbs and a combination of W29 and W25-8D (3) MAbs. The last square of each column was spotted with pleopod homogenate from uninfected shrimp (N). (B) PCR detection; nucleic acids extracted from the pleopod homogenate from a naturally-infected *P. vannamei* specimen used in the above dot blotting experiment were serially diluted with normal shrimp nucleic acids and used as a template for PCR amplification. The various dilutions are indicated on top of the gel. Lane M: molecular weight marker; lane N: no template control (negative control).

3.3. Sensitivity testing

To determine the sensitivity of VP19-specific MAbs, dot blotting against purified recombinant MBP-VP19 protein was performed. Sensitivity ranged from 70 to 280 ng/ml, which is equivalent to 70–280 pg/spot or 1.2–5 fmol/spot. Conversely, the sensitivity of VP28-specific W29 MAb was 140 pg/spot or 5 fmol/spot (Table 1). In a previous study, the detection limits of MAbs specific for VP28 were 500 pg (Anil et al., 2002) and 400 pg (Liu et al., 2002). Therefore, the VP19-specific MAb generated in this study had sensitivity approximately 1.5–5 times higher than those of MAbs from previous reports. In antigen-capture ELISA experiments, the sensitivity for VP28 was as low as 20 pg (Liu et al., 2002). This may be due to the fact that the

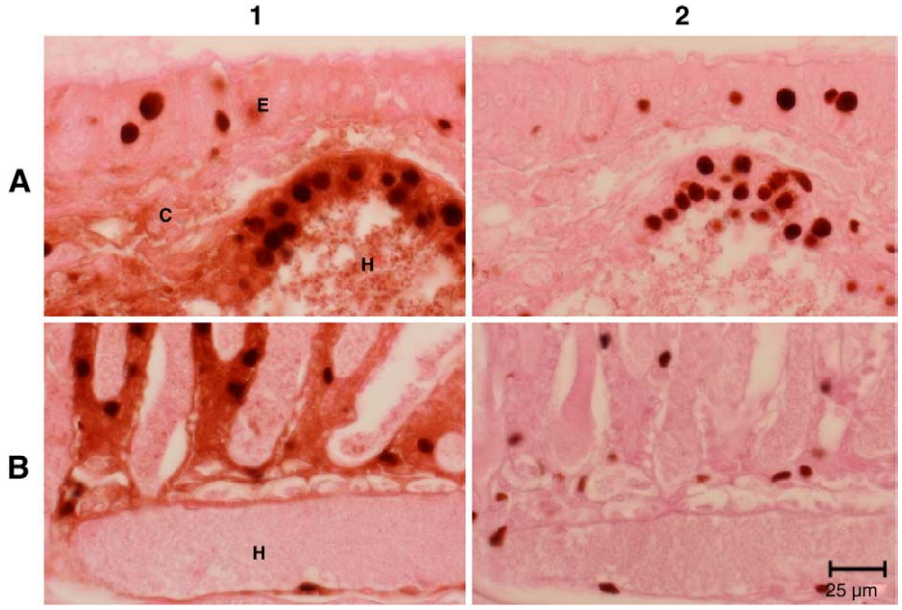

Fig. 3. Immunohistochemical analysis of tissues from WSSV-infected *P. vannamei* using VP19-specific W25-8D (column 1) and VP28-specific W29 (column 2) MAbs. Consecutive sections were counterstained with eosin for clear visualization of immunoreactivity. Strong immunoreactivity was exhibited in subcutaneous epithelium of mouth appendage (A) and gill tissues (B). E = subcutaneous epithelium; C = connective tissue; H = hemocoel.

sample volumes used in that study (50 to 100 μl) was larger than those used for dot blotting in this study (1 μl).

To determine the ability of MAbs to detect WSSV infection in field samples, a pleopod homogenate sample of naturally-infected shrimp was serially diluted with normal shrimp pleopod homogenate for dot blot assays. As shown in Fig. 4A, the WSSV detection limit of VP19-specific W25-8D and VP28-specific W29 MAbs were approximately 1:200. However, the combination of both W29 and W25-8D MAbs increased the detection limit to 1:400, two-fold higher than that of a single antibody. These data indicate that the mixture of MAbs specific to different antigens could increase the sensitivity of WSSV detection. Even though the W25-8D MAb seemed to demonstrate stronger immunohistochemical staining than that of W29, they possessed identical detection limits as measured by dot blotting. This could be due to the fact that VP19 Ag might be lost during the centrifugation step in dot blotting assays, co-precipitating with the cell debris.

To compare the sensitivity of WSSV detection between dot blotting and one-step PCR, nucleic acids were extracted from the same shrimp homogenate used for dot blotting and used for PCR detection. As shown in Fig. 4B, at a dilution of 10^{-7}, a 633 bp band was clearly observed. Therefore, in comparison with one-step PCR the sensitivity of dot blotting using a combination of both MAb was approximately 25,000 times less sensitive (Fig. 4).

Even though the sensitivity of our antibody-based assay was lower than that of PCR, its high specificity could be regarded as an advantage. In other report, the high specificity (100%) of immunodot assay was identical to that of 1-step PCR and the immunodot test could be used to replace 1-step PCR assay for WSSV disease monitoring. In that study, six farms with the shrimp samples that yielded negative result for WSSV by immunodot and 1-step PCR at various times post stocking, the successful crops were obtained. Whereas, the other six farms that were positive for WSSV by both immunodot test and 1-step PCR, their crops failed. In contrast, four farms that gave positive results for WSSV by 2-step PCR at various time post stocking, they were successfully harvested at 105 days post stocking (Patil et al., 2008).

In another case, the limitation of PCR due to inhibiting factors present in some tissues such as eyestalk with eye could be prevailed with the use of immunodot test that gave positive result reaction (Shekhar et al., 2006).

3.4. Detection of WSSV infection in naturally-infected shrimp

A total of 15 WSSV-infected shrimp samples (verified by dot blotting using VP28-specific W29 MAb and 1-step PCR) (Fig. 5B) and 14 YHV-infected shrimp samples (verified by dot blotting using MAb Y19 and RT-PCR) (Fig. 5D) were used to demonstrate the ability of VP19-specific MAbs to detect naturally-occurring WSSV infection in shrimp. As shown in Fig. 5A, VP19-specific W25-8D MAb bound specifically to all 15 WSSV-infected *P. vannamei*, while no cross-reactivity with YHV-infected shrimp was observed. The combination of W29 and W25-8D (1:1 ratio) yielded stronger immunoreactivity (Fig. 5C). In many cases of light infections, immunoreactivity was enhanced by W25-8D compared to W29 (Fig. 5C, row 2 shrimp no. 9; row 3 shrimp nos. 12 and 13). To provide the quantitative data, the ImageJ software (http://www.

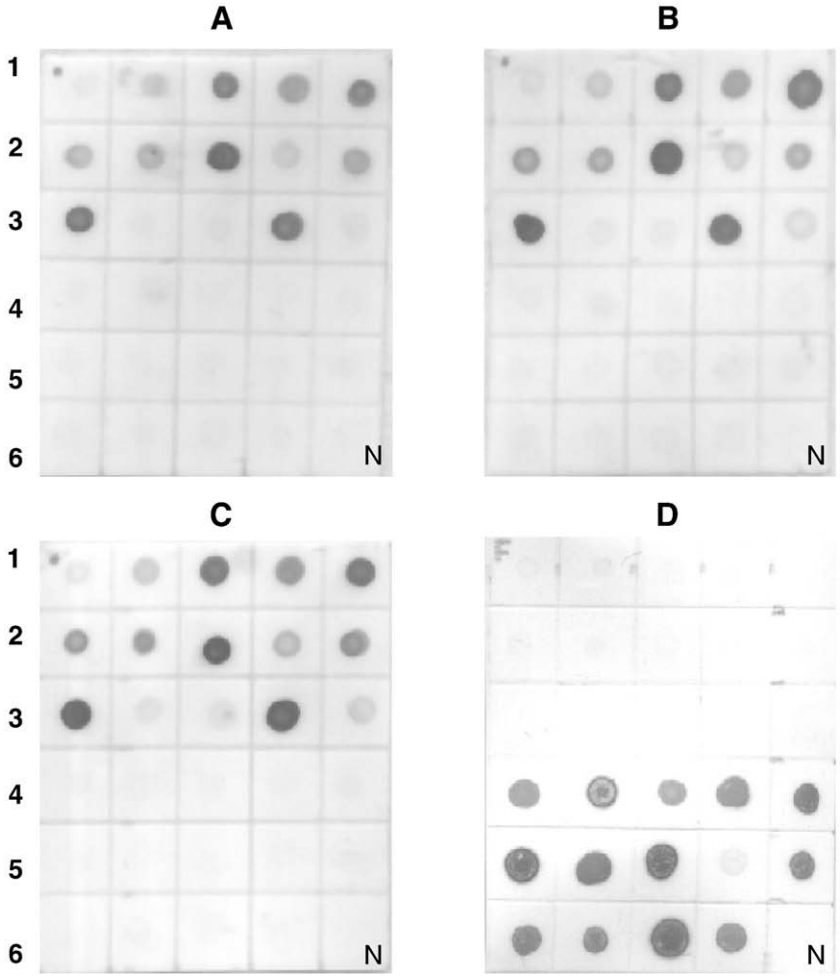

Fig. 5. Dot blot detection of natural WSSV infection. Pleopod homogenates from 15 WSSV-infected *P. vannamei* specimens (rows 1–3; row 1: shrimp nos. 1 to 5; row 2: shrimp nos. 6 to 10; and row 3: shrimp nos. 11 to 15) and 14 YHV-infected *P. vannamei* specimens (rows 4–6) and uninfected *P. vannamei* specimens (N) were spotted onto nitrocellulose membranes and treated with VP19-specific W25-8D (A) and VP28-specific MAb W29 (B) MAbs as well as with a combination of W29 and W25-8D (C) and Y19 MAb specific to YHV (D).

rsbweb.nih.gov/ij/) was used to quantify the dot intensity. The results revealed that the relative intensity of signal in dot blotting using the combination of W29 and W25-8D compared to those of W29 alone increased approximately 23 to 27 % (shrimp no. 9 (23%); shrimp no. 12 (25%) and shrimp no. 13 (27%)). These data indicate that antibodies against different antigens can be used to augment WSSV detection in naturally-infected shrimp samples. In the case of Taura syndrome virus (TSV) detection, the combination of MAbs specific to VP2 and VP3 could be used to increase the sensitivity of TSV detection and to confirm TSV infection in *P. vannamei* using dot blotting and immunohistochemistry (Chaivisuthangkura et al., 2009).

In conclusion, we have demonstrated that MAbs specific for different WSSV epitopes can be used in concert to enhance the detection of WSSV infection in shrimp samples by immunohistochemistry. The detection limit of dot blotting using a combination of MAbs against VP19 and VP28 was two-fold higher than that of a single MAb. It is our belief that the VP19-specific MAb generated in this study can be used to develop a simple WSSV detection kit similar to the immunochromatographic strip used in WSSV and YHV detection kits (Sithigorngul et al., 2006, 2007).

Acknowledgements

This work was supported by the Thailand Research Fund (TRF) and the Office of Commission on Higher Education, Thailand. We would like to thank the research division of Srinakharinwirot University for Excellent Center funding, the farmers at Kung Krabaen, Chantaburi Province for providing us with WSSV-infected shrimp and the farmers at Bang Len, Nakhon Pathom Province for providing us with YHV-infected shrimp.

References

Anil, T.M., Shankar, K.M., Mohan, C.V., 2002. Monoclonal antibodies developed for sensitive detection and comparison of white spot syndrome virus isolates in India. Dis. Aquat. Org. 51, 67-65.

Boonsanongchokying, C., Sang-oum, W., Sithigorngul, P., Sriurairatana, S., Flegel, T.W., 2006. Production of monoclonal antibodies to polyhedrin of monodon baculovirus (MBV) from shrimp. Sci. Asia 32, 371–376.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Chaivisuthangkura, P., Tangkhabuanbutra, J., Longyant, S., Sithigorngul, W., Rukpratanporn, S., Menasveta, P., Sithigorngul, P., 2004. Monoclonal antibodies against a truncated viral envelope protein (VP28) can detect white spot syndrome virus (WSSV) infections in shrimp. Sci. Asia 30, 359–363.

Chaivisuthangkura, P., Phattanapaijitkul, P., Thammapalerd, N., Rukpratanporn, S., Longyant, S., Sithigorngul, W., Sithigorngul, P., 2006. Development of a polyclonal antibody specific to VP19 envelope protein of white spot syndrome virus (WSSV) using a recombinant protein preparation. J Virol. Methods 133, 180–184.

Chaivisuthangkura, P., Longyant, S., Hajimasalaeh, W., Sridulyakul, P., Rukpratanporn, S., Sithigorngul, P., 2009. Improved sensitivity of Taura syndrome virus immunodetection with a monoclonal antibody against the recombinant VP2 capsid protein. J Virol. Methods. doi:10.1016/j.viromet.2009.11.007.

Chang, P.S., Lo, C.C., Wang, Y.C., Kou, G.H., 1996. Identification of white spot syndrome associated baculovirus (WSBV) target organs in the shrimp *Penaeus monodon* by *in situ* hybridization. Dis. Aquat. Org. 27, 131–139.

Chou, H.Y., Huang, C.Y., Wang, C.H., Chiang, H.C., Lo, C.F., 1995. Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan. Dis. Aquat. Org. 23, 165–173.

Durand, S.V., Redman, R.M., Mohney, L.L., Tang-Nelson, K., Bonami, J.R., Lightner, D.V., 2003. Qualitative and quantitative studies on the relative virus load of tails and heads of shrimp acutely infected with WSSV. Aquaculture 216, 9–18.

Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A., 2005. Virus Taxonomy. Classification and Nomenclature of Viruses. Eighth Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, San Diego.

Huang, C., Zhang, X., Lin, Q., Xu, X., Hew, C.L., 2002. Characterization of a novel envelope protein (VP281) of shrimp white spot syndrome virus by mass spectrometry. J. Gen. Virol. 83, 2385–2392.

Jaroenram, W., Kiatpathomchai, W., Flegel, T.W., 2009. Rapid and sensitive detection of white spot syndrome virus by loop-mediated isothermal amplification combined with a lateral flow dipstick. Mol. Cell. Probes 23, 65–70.

Kimura, T., Yamano, K., Nakano, H., Momoyama, K., Hiraoka, M., Inouye, K., 1996. Detection of penaeid rod-shaped DNA virus (PRDV) by PCR. Fish Pathol. 31, 93–98.

Köhler, G., Milstein, C., 1976. Derivative of specific antibody producing tissue culture and tumor cell fusion. Eur. J. Immunol. 6, 511–519.

Kono, T., Savan, R., Sakai, M., Itami, T., 2004. Detection of white spot syndrome virus in shrimp by loop-mediated isothermal amplification. J. Virol. Methods 115, 59–65.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Leu, J.H., Yang, F., Zhang, X., Xu, X., Kou, G.H., Lo, C.F., 2009. Whispovirus. Curr. Top. Microbiol. Immunol. 328, 197–227.

Lo, C.F., Ho, C.H., Peng, S.E., Chen, C.H., Hsu, H.C., Chiu, Y.L., Chang, C.F., Lui, K.F., Su, M.S., Wang, C.H., Kou, G.H., 1996. White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods. Dis. Aquat. Org. 27, 215–225.

Longyant, S., Poyoi, P., Chaivisuthangkura, P., Tejankura, T., Sithigorngul, W., Sithigorngul, P., Rukpratanporn, S., 2008. Specific monoclonal antibodies raised against Taura syndrome virus (TSV) capsid protein VP3 detect TSV in single and dual infections with white spot syndrome virus (WSSV). Dis. Aquat. Org. 79, 75–81.

Liu, W., Wang, Y.T., Tian, D.S., Yin, Z.C., Kwang, J., 2002. Detection of white spot syndrome virus (WSSV) of shrimp by means of monoclonal antibodied (MAbs) specific to an envelope protein (28 kDa). Dis. Aquat. Org. 49, 11–18.

Mekata, T., Sudhakaran, R., Kono, T., Supamattaya, K., Linh, N.T., Sakai, M., Itami, T., 2009. Real-time quantitative loop-mediated isothermal amplification as a simple method for detecting white spot syndrome virus. Lett. Appl. Microbiol. 48, 25–32.

Mosmann, T.R., Bauman, R., Williamson, A.R., 1979. Mutations affecting immunoglobulin light chain secretion by myeloma cells I. Functional analysis by cell fusion. Eur. J. Immunol. 9, 511–516.

Nadala, E.C.B., Loh, P.C., 2000. Dot blot enzyme immunoassays for the detection of white spot virus and yellow-head virus of penaeid shrimp. J. Virol. Methods 84, 175–179.

Patil, R., Palaksha, K.J., Anil, T.M., Guruchannabasavanna, Patil, P., Shankar, K.M., Mohan, C.V., Sripada, A., 2008. Evaluation of an immunodot test to manage white spot syndrome virus (WSSV) during cultivation of the giant tiger shrimp *Penaeus monodon*. Dis. Aquat. Org. 79, 157–161.

Poulos, B.T., Pantoja, C.R., Bradley-Dunlop, D., Aguilar, J., Lightner, D.V., 2001. Development and application of monoclonal antibodies for the detection of white spot syndrome virus of penaeid shrimp. Dis. Aquat. Org. 47, 13–23.

Rukpratanporn, S., Sukhumsirichart, W., Chaivisuthngkura, P., Longyant, S., Sithigorngul, W., Menasveta, P., Sithigorngul, P., 2005. Generation of monoclonal antibodies specific to hepatopancreatic parvovirus (HPV) from *Penaeus monodon*. Dis. Aquat. Org. 65, 85–89.

Shekhar, M.S., Azad, I.S., Ravichandran, P., 2006. Comparison of dot blot and PCR diagnosis techniques for detection of white spot syndrome virus in different tissues of *Penaeus monodon*. Aquaculture 261, 1122–1127.

Shih, H.H., 2004. Neutralization of white spot syndrome virus by monoclonal antibodies against viral envelope proteins. Taiwania 49, 159–165.

Sithigorngul, P., Chauychuwong, P., Sithigorngul, W., Longyant, S., Chaivisuthangkura, P., Menasveta, P., 2000. Development of a monoclonal antibody specific to yellow head virus (YHV) from *Penaeus monodon*. Dis. Aquat. Org. 42, 27–34.

Sithigorngul, P., Rukpratanporn, S., Longyant, S., Chaivisuthangkura, P., Sithigorngul, W., Menasveta, P., 2002. Monoclonal antibodies specific to yellow-head virus (YHV) of *Penaeus monodon*. Dis. Aquat. Org. 49, 71–76.

Sithigorngul, W., Rukpratanporn, S., Pecharaburanin, N., Longyant, S., Chaivisuthangkura, P., Sithigorngul, P., 2006. A simple and rapid immunochromatographic test strip for detection of white spot syndrome virus (WSSV) of shrimp. Dis. Aquat. Org. 72, 101–106.

Sithigorngul, W., Rukpratanporn, S., Sittidilokratna, N., Pecharaburanin, N., Longyant, S., Chaivisuthangkura, P., Sithigorngul, P., 2007. A convenient immunochromatographic test strip for rapid diagnosis of yellow head virus infection in shrimp. J. Virol. Methods 140, 193–199.

Takahashi, Y., Itami, T., Maeda, M., Suzuki, N., Kasornchandra, J., Supamattaya, K., Khongpradit, R., Boonyaratpalin, S., Kondo, M., Kawai, K., Kusuda, R., Hirono, I., Aoki, T., 1996. Polymerase chain reaction (PCR) amplification of bacilliform virus (RV-PJ) DNA in *Penaeus japonicus* Bate and systemic ectodermal and mesodermal baculovirus (SEMBV) DNA in *Penaeus monodon* Fabricius. J. Fish Diseases 19, 399–403.

Tsai, J.M., Wang, H.C., Leu, J.H., Hsiao, H.H., Wang, A.H., Kou, G.H., Lo, C.F., 2004. Genomic and proteomic analysis of thirty-nine structural proteins of shrimp white spot syndrome virus. J. Virol. 78, 11,360–11,370.

van Hulten, M.C.W., Westenberg, M., Goodall, S.D., Vlak, J.M., 2000. Identification of two major virion protein genes of White Spot Syndrome virus of shrimp. Virology 266, 227–236.

Wongteerasupaya, C., Thongcheua, W., Boonsaeng, V., Panyim, S., Tassanakajon, A., Withyachumnarnkul, B., Flegel, T.W., 1997. Detection of yellow-head virus (YHV) of *Penaeus monodon* by RT-PCR amplification. Dis. Aquat. Org. 31, 181–186.

Xie, X., Xu, L.M., Yang, F., 2006. Proteomic analysis of the major envelope and nucleocapsid proteins of white spot syndrome virus. J. Virol. 80, 10,615–10,623.

You, Z., Nadala Jr., E.C.B., Yang, J., van Hulten, M.C.W., Loh, P.C., 2002. Production of polyclonal antiserum specific to the 27.5 kDa envelope protein of white spot syndrome virus. Dis. Aquat. Org. 51, 77–80.

Zhang, X., Huang, C., Tang, X., Zhuang, Y., Hew, C.L., 2004. Identification of structural proteins from shrimp white spot syndrome virus (WSSV) by 2DE-MS. Proteins 55, 229–235.

Zhou, Q., Xu, L., Li, H., Qi, Y.P., Yang, F., 2009. Four major envelope proteins of white spot syndrome virus bind to form a complex. J. Virol. 83, 4709–4712.