การพัฒนาชุดตรวจวินิจฉัยโรคติดเชื้อเพื่อการผลิตเชิงพาณิชย์

Development of Immuno Test Kit for Commercial Production

(การผลิตโมโนโคลนอลแอนติบอดีต่อ *Vibrio parahaemolyticus* และ *V. vulnificus*)

(Monoclonal Antibody production against *Vibrio parahaemolyticus* and *V. vulnificus*)

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กิตติกรรมประกาศ

งานวิจัยครั้งนี้ได้รับทุนสนับสนุนจากงบประมาณแผ่นดินประจำปี 2548 จากฝ่ายวิจัย มหาวิทยาศรีนครินทรวิโรฒ คณะผู้วิจัยขอขอบคุณสถาบันต่างๆ ที่กรุณามอบแบคทีเรียสายพันธุ์ต่างๆ ที่ใช้ในการศึกษาครั้งนี้

CENTEX = CENTEX Shrimp, Mahidol University, Thailand.
CPF = Charoen Phokphan Foods Public Co. Ltd. Thailand.
DASB = Department of Aquatic Science, Burapa University, Thailand.
DMSC = Department of Microbiology, Chulalongkorn University, Thailand.
DMSM = Department of Microbiology, Mahidol University, Thailand.
DMST = Department of Medical Sciences, Ministry of Public Health, Thailand.
VMARC = Veterinary Medical Aquatic Research Center, Chulalongkorn University, Thailand.

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การพิสูจน์ทราบและการจำแนก *Vibrio parahaemolyticus* 3 isolate ด้วยโมโนโคลนอลแอนติบอดี

บทคัดย่อ

โมโนโคลนอลแอนติบอดี (MAb) 6 กลุ่มผลิตจากการฉีดหนูด้วยเชื้อ *Vibrio parahaemolyticus* 3 isolates (VPV, VPB, VPC) โดย MAb 2กลุ่มแรกมีความจำเพาะต่อ VPV เท่านั้น โดยจับกับแอนติบอดีที่น้ำหนักโมเลกุล 40 kDa และ 10-15 kDa MAb กลุ่มที่ 3 จำเพาะต่อ VPB เท่านั้น และจับกับแอนติเจนที่มีน้ำหนักโมเลกุล 20 kDa และแถบขนาด 10-15 kDa MAb กลุ่มที่ 4 และ 5 จำเพาะต่อ VPC โดย MAb ในกลุ่มที่ 4 จับกับแอนติเจนเหมือนกับ MAb ในกลุ่มที่ 3 (20, 10-15 kDa) ส่วน MAb ในกลุ่มที่ 5 จับกับแอนติเจนที่มีขนาดประมาณ 10 kDa คล้าย MAb ในกลุ่มที่ 2 สำหรับ MAb กลุ่มที่ 6 สามารถจับกับ *V. parahaemolyticus* ทั้ง 3 isolates แต่มีปฏิกิริยาข้ามกับ Vibrio ชนิดต่างๆ ได้แก่ *V. alginolyticus*, *V. harveyi*, *V. vulnificus*, และ *V. fluvalis* และจับอ่อนๆ กับ *V. mimicus* แต่ไม่จับกับ *V. odalii* และ *V. penaeicida* MAb ในกลุ่มนี้จับกับแอนติเจนที่มีน้ำหนักโมเลกุลประมาณ 40 kDa MAb ส่วนใหญ่ (ยกเว้น MAb ในกลุ่ม 6) สามารถใช้ตรวจสอบการติดเชื้อ *V. parahaemolyticus* ในเนื้อเยื่อโดยวิธี immunohistochemistry MAb ในกลุ่ม 1-3 สามารถใช้แยก *V. parahaemolyticus* ที่แสดง serotype เดียวกัน (O5:K33) ออกจากกันได้ ความไวในการตรวจแบคทีเรียโดยวิธี dot blotting ประมาณ 10^5-10^7 CFU/มล. จากการใช้ MAb ในการตรวจสอบการติดเชื้อ Vibrio ในกุ้งขาวและปลากระพงขาว พบว่ามีการติดเชื้อ Vibrio โดย MAb กลุ่มที่ 5 ซึ่งชี้ให้เห็นว่าสามารถใช้ MAb ในการตรวจการติดเชื้อได้โดยตรงถ้าเชื้อมีสายพันธุ์ใกล้เคียงกับเชื้อที่ใช้ในการทดลองครั้งนี้ ซึ่งสามารถทำได้รวดเร็วโดยวิธี dot blotting และยืนยันการติดเชื้อในสัตว์โดยวิธี immunohistochemistry

Simple identification and differentiation of three *Vibrio parahaemolyticus* isolates with monoclonal antibodies

Abstract

Six groups of monoclonal antibodies (MAbs) specific to *Vibrio parahaemolyticus* were generated using whole cell of three isolates of *V. parahaemolyticus* (VPV, VPB, VPC) for immunization. The first and the second groups of monoclonal antibodies demonstrated specificity only to the first isolate of the *V. parahaemolyticus* (VPV), and bound to antigens at molecular masses around 40 and a smear of 10-15 kDa, respectively. The third group of MAbs were specific to the second isolate of *V. parahaemolyticus* (VPB) only and recognized antigens at molecular masses of 20 kDa and a smear band at 60-90 kDa. The fourth and the fifth groups of MAbs recognized the third isolate of *V. parahaemolyticus* (VPC). The fourth group of MAbs bound to the antigens as recognized by MAbs in the third group. The fifth group of MAbs bound to an antigen at molecular mass of 10 kDa. The sixth group of MAbs recognized all three isolates of *V. parahaemolyticus* but demonstrated strong cross-reactivity to other five *Vibrio* spp.; *V. alginolyticus*, *V. harveyi*, *V. vulnificus*, and *V. fluvialis*,. These MAbs also bound to *V. mimicus* and did not bind to *V. odalii* and *V. penaeicida*. The MAbs in this group bound to an antigen at molecular mass of 40 kDa. All of the MAbs except for the sixth group MAbs in the sixth group can recognize the bacterial infection in tissues from experimentally infected in shrimp by means of immunohistochemistry. The MAbs in group 1, 2 and 3 can be used to differentiate two *V. parahaemolyticus* (VPV and VPB), even though they demonstrated the same serotype (O5:K33). The sensitivity of bacterial detection by dot-blotting was 10^5 to 10^7 CFU/ml depending on the groups of MAbs. The survey of naturally bacterial infection in diseased white shrimps *Litopenaeus vannamei* and sea bass *Lates calcarieer* by immunohistochemistry using all groups of MAbs revealed that the shrimps were positive with MAbs in group 5 only. This evidence indicated that all of these MAbs can be used for direct identification of Vibriosis in various aquatic animals, infected by similar strains of *V. parahaemolyticus* used in this study. Therefore, quick and simple identification of *V. parahaemolyticus* can be performed by dot blotting and the Vibriosis in animals could be verified by immunohistochemistry using these MAbs.

การตรวจการติดเชื้อ *Vibrio vulnificus* ในกุ้งด้วยโมโนโคลนอลแอนติบอดี

บทคัดย่อ

โมโนโคลนอลแอนติบอดี (MAb) 7 โคลนผลิตจากการฉีด *Vibrio vulnificus* 3 isolates (VVC, VVB, VVBS) ซึ่งสามารถแบ่งออกเป็น 5 กลุ่มคือ MAb กลุ่มที่ 1 จำเพาะต่อ VVC โดยไม่มีปฏิกิริยาข้ามกับ *V. vulnificus* อีก 2 isolates โดยจับกับแอนติเจนที่มีขนาดประมาณ 65 kDa MAb กลุ่มที่ 2 และ 3 จำเพาะต่อ VVB โดยจับกับแอนติเจนขนาด 6 kDa และ 35 kDa ตามลำดับ MAb กลุ่มที่ 4 จำเพาะต่อ VVBS ซึ่งไม่สามารถจับกับแอนติเจนในสภาพเสียสภาพใน Western blotting ได้ MAb กลุ่มที่ 5 มีความจำเพาะกว้างสามารถจับกับ *Vibrio vulnificus* ทั้ง 3 isolates และจับกับ Vibrio ชนิดต่างๆ ได้เช่น *V. parahaemolyticus*, *V. mimicus*, *V. harveyi*, *V. fluvialis*, *V. cholerae* และ *V. alginolyticus* แต่ไม่สามารถจับกับ *V. penaeicida* และ *V. odalii* โดย MAb นี้จับกับแอนติเจนขนากประมาณ 5 kDa MAb ในกลุ่ม 1 และกลุ่ม 2 สามารถใช้ตรวจสอบการติดเชื้อ *Vibrio vulnificus* ในเนื้อเยื่อโดยวิธี immuno-histochemistry MAb ทั้งหมดสามารถใช้ตรวจสอบ *Vibrio vulnificus* โดยวิธีง่ายๆ เช่น dot blotting ซึ่งมีความไวอยู่ระหว่าง 10^5-10^6 CFU/มล.

Detection of *Vibrio vulnificus* infection in shrimp with monoclonal antibodies

Abstract

Seven monoclonal antibodies (MAbs) were generated against 3 isolates of *V. vulnificus* (VVC, VVB, VVBs) using whole cell as antigens. The monoclonal antibodies can be divided into five groups according to their specificities. MAbs in groups 1 were specific to the first isolate of *V. vulnificus* (VVC only) without any cross-reaction to the other two isolates of *V. vulnificus* (VVB, VVBs) and other bacterial species. The MAbs in group 1 bound to a band of antigen at molecular mass of 65 kDa. The MAbs in group 2 and 3 were specific to the second isolate of *V. vulnificus* (VVB) and bound to the antigens at 5 and 35 kDa, respectively. The MAb in group 4 was specific to the third isolate of *V. vulnificus* (VVBs only) and it bound to the native protein only since it did not recognized any protein in Western blot assay. The MAb in group 5 was broadly specific. It recognized all the isolates of *V. vulnificus*, and almost all *Vibrio* spp. tested; *V. parahaemolyticus*, *V. mimicus*, *V. harveyi*, *V. fluvialis*, *V. cholerae* and *V. alginolyticus* but did not recognize *V. penaeicida*. This MAb in group 5 recognized an antigen at 5 kDa. The antibodies in group 1 and 2 can be used to detect the bacteria in the tissues by immunohistochemistry. All antibodies can be easily used to detect the bacteria by dot blotting with sensitivity range from 10^5 to 10^6 CFU/ml.

2.1

Simple identification and differentiation of three *Vibrio parahaemolyticus* by monoclonal antibodies.

Simple identification and differentiation of three *Vibrio parahaemolyticus* by monoclonal antibodies.

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Abbreviation Title: Monoclonal antibodies specific to *Vibrio parahaemolyticus*.

Key words: *Vibrio parahaemolyticus*, dot blotting, immunohistochemistry, detection, Monoclonal antibody, Western blotting.

Abstract

Six groups of monoclonal antibodies (MAbs) specific to *Vibrio parahaemolyticus* were generated using whole cell of three isolates of *V. parahaemolyticus* (VPV, VPB, VPC) for immunization. The first and the second groups of monoclonal antibodies demonstrated specificity only to the first isolate of the *V. parahaemolyticus* (VPV), and bound to antigens at molecular masses around 40 and a smear of 10-15 kDa, respectively. The third group of MAbs were specific to the second isolate of *V. parahaemolyticus* (VPB) only and recognized antigens at molecular masses of 20 kDa and a smear band at 60-90 kDa. The fourth and the fifth groups of MAbs recognized the third isolate of *V. parahaemolyticus* (VPC). The fourth group of MAbs bound to the antigens as recognized by MAbs in the third group. The fifth group of MAbs bound to an antigen at molecular mass of 10 kDa. The sixth group of MAbs recognized all three isolates of *V. parahaemolyticus* but demonstrated strong cross-reactivity to other five *Vibrio* spp.; *V. alginolyticus*, *V. harveyi*, *V. vulnificus*, and *V. fluvialis*,. These MAbs also bound to *V. mimicus* and did not bind to *V. odalii* and *V. penaeicida*. The MAbs in this group bound to an antigen at molecular mass of 40 kDa. All of the MAbs except for the sixth group MAbs in the sixth group can recognize the bacterial infection in tissues from experimentally infected in shrimp by means of immunohistochemistry. The MAbs in group 1, 2 and 3 can be used to differentiate two *V. parahaemolyticus* (VPV and VPB), even though they demonstrated the same serotype (O5:K33). The sensitivity of bacterial detection by dot-blotting was 10^5 to 10^7 CFU/ml depending on the groups of MAbs. The survey of naturally bacterial infection in diseased white shrimps *Litopenaeus vannamei* and sea bass *Lates calcarieer* by immunohistochemistry using all groups of MAbs revealed that the shrimps were positive with MAbs in group 5 only. This evidence indicated that all of these MAbs can be used for direct identification of Vibriosis in various aquatic animals, infected by similar strains of *V. parahaemolyticus* used in this study. Therefore, quick and simple identification of *V. parahaemolyticus* can be performed by dot blotting and the Vibriosis in animals could be verified by immunohistochemistry using these MAbs.

Introduction

Vibrio parahaemolyticus is a Gram-negative, motile, oxidase-positive, straight or curved rod shape, facultative anaerobic bacteria. *V. parahaemolyticus* and other *Vibrio* spp. are ubiquitous in aquatic habitats and can be readily isolated from water sediments and seafoods such as oysters, mussels and fish (Vuddhakul et al., 2000; Baffone et al., 2005).

Contaminated raw or improperly cooked sea foods are often regarded as important vehicles of diarrhea transmission in human (Chan et al., 1989; Kaufman et al., 2002; Zhang and Austin 2005). Vibriosis is one of the most prevalent on high mortality not only in larval cultures but also in shrimp production. *V. parahaemolyticus* have been isolated from diseased shrimp along with other *Vibrio* spp. (Liu et al, 1996, Sung et al., 2001). Recently, pathogenic *V. parahaemolyticus*, has been isolated from diseased abalones with withering syndrome (Lui et al., 2000). In the artificial infection experiment using two strains of *V. parahaemolyticus* isolated from stool of acute gastroenteritis patient and haemolymph of diseased abalone, both strains were lethal to abalone (Lee et al., 2003). Diagnosis of Vibriosis is usually performed by bacterial culture, isolation and identification. The culture process employs enrichment of the sample in liquid medium and subsequent isolation of colonies on selective plating media. Unfortunately, numbers of *Vibrio* spp. are not only taxonomically similar to *V. parahaemolyticus* but also appear similar in colonial morphology on the selective media. Therefore, additional biochemical tests or other reliable identification methods are required for *V. parahaemolyticus* recognition (Gopal et al., 2005; Yam et al., 1999). These microbilogical processes are laborious and time consuming and has low sensitivity and reliability. However, the routine biochemical test such as API20E system revealed that clinical isolates of *V. parahaemolyticus* showed significant differences in up to five biochemical tests which may lead to misidentification of *V. parahaemolyticus* (Martinez-Urtaza et al., 2006). Several methods for *V. parahaemolyticus* detection such as ELISA of *V. parahaemolyticus* toxins (Honda et al, 1995), polymerase chain reaction of *tox*R, *tdh*, *trh* genes (Kim et al., 1999; Kaufman et al., 2002; Gopal 2005) have been developed. The use of these methods are still limited due to the availability of the antiserum against these toxins and high cost of molecular techniques; moreover, they can not be used for detection of most environmental strains since the *tdh* and/or *trh* genes are usually presence in the clinical isolates (Kaufman et al., 2002; Osawa et al., 2002). This report was a preliminary study of production of monoclonal antibodies specific to *V. parahaemolyticus* in order to provide specific immunological tools for rapid and simple identification of *V. parahaemolyticus* infection in shrimp and aquatic animals.

Materials and Methods

Bacterial culture and antigen preparation

Vibrio parahaemolyticus (VPV) serotype O5:K33, isolated from diseased aquatic animals was kindly provide by Veterinary Medical Aquatic Research Center, Chulalongkorn University, Thailand. The second isolate of *V. parahaemolyticus* (VPB) serotype O5:K33 isolated from diseased *P. monodon*. The third isolate of *V. parahaemolyticus* (VPC) serotype O10:KUT was isolated from patient stool at Phramongkutklao hospital, Bangkok, Thailand. The sources of other bacteria used for determination of cross-reactivity were indicated in Table 1. The bacteria were grown to log phase with agitation at 37°C in tryptic soy broth (TSB; Becton and Dickenson Company) supplemented with 2% (w/v) NaCl. The bacteria were harvested by centrifugation at 3,500 X *g* for 20 min at 4 °C. Bacterial pellets were washed twice with sterile 0.15 M phosphate buffered saline (PBS) pH7.2, re-suspended in PBS, heat killed at 60 °C for 30 min, and adjusted to the OD of 1 at 610 nm corresponded to the concentration of the bacteria about 1-2 X 10^8 cells/ml as determined by direct cell counting using haematocytometer. The bacterial suspension was divided into small aliquots and stored at -70°C until use.

In this study all three isolates of *V. parahaemolyticus* were used for immunization in two forms, heat killed and denatured forms. The denatured form was prepared by mixing the bacteria with treatment buffer (4% SDS, 10 % mercaptoethanol) at a ratio of 1:1 (v/v) and boiled for 1 min, then dialyzed against three changes of PBS at 12 hr intervals.

Immunization

Two sets of four Swiss mice were injected intraperitoneally with 50 µl of two differently bacterial antigen preparations treated by heat killed and SDS-mercaptoethanol. The first bacterial antigen preparation was a mixture of treated VPV (10^8 CFU/ml) mixed with complete Freund's adjuvant at a ratio of 1:1. The second bacterial antigen preparation was a mixture of treated VPB and VPC (1:1 ratio, each with 10^8 CFU/ml), mixed with complete Freund's adjuvant at a ratio of 1:1. Each set of mice were subsequently injected 3 more times with the same set of antigen preparation mixed with incomplete Freund's adjuvant at two week intervals. One week after the fourth injection, mouse antisera were collected, preabsorbed with excess *V. harveyi* and tested with all three isolates *V. parahaemolyticus* by Western blotting. The best performing mouse was later boosted 3 days before hybridoma production.

Hybridoma production.

A cell fusion protocol was adapted from the method developed by Kohler & Milestein (1975) with modifications described by Mosmann et al. (1979). A P3X myeloma cell line was used as the fusion partner. Fusion products from 1 mouse were plated on 30 microculture plates (96 well/plate). After identification of the positive cultures by screening methods; dot blotting, Western blotting and immunohistochemistry as described below, cells were cloned by the limiting dilution method and stored in liquid nitrogen.

Dot-blotting

Cell suspensions of various heat-killed of bacteria approximately 10^8 CFU/ml were used for screening. Heat killed bacterial samples (1 µl/spot) were applied to nitrocellulose membrane, baked at 60°C for 10 min, and incubated in hybridoma conditioned medium diluted to 1:20 dilution in 5% blotto (5% nonfat drymilk, 0.1% Triton X-100 in PBS) for 4 h. After extensive washing in 0.5% Blotto, 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 0.5%Blotto 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). Hybridoma cultures that displayed immunoreactivity against *V. parahaemolyticus* were confirmed for bacterial specificity by Western blotting and immuno-histochemistry .

SDS-PAGE and Western blot analysis

V. parahaemolyticus and other bacterial lysates were separated by 15% SDS-PAGE according to the method described by Laemmli (1970). Samples were electrophoresed for 6 h at 30 V and gels were stained using Coomassie brilliant blue R-250. For Western blotting, samples resolved by SDS-PAGE were electro-blotted onto nitrocellulose membrane using Transblot apparatus (Bio-Rad). The nitrocellulose membrane was incubated in 5% Blotto for 10 min, treated with 1:200 hybridoma conditioned-medium for 4 h and then processed as described above in the dot blotting section. Molecular weight markers (Bio-Rad) were used as standards.

Immunohistochemistry.

Artificial infection:

The Pacific white shrimp *L. vannamei* with 15-20g weight was artificially infected with each isolate of *V. parahaemolyticus* (VPV, VPB or VPC) by injection via the second walking leg. When the shrimp exhibited pathogenic symptoms such as slow moving and loss of balance, they were killed in the cold water and the cephalothoraces were cut and fixed in Davidson fixative for 24 h, then processed for paraffin sectioning.

Natural infection:

Natural vibriosis samples of *L. vannamei* (5-10 g body weight) were obtained from a farm in Patoomthani Province, Thailand. Shrimps exhibited several black lines on the lateral carapace epithelium. They were fixed and processed for paraffin section as described above.

Formalin fixed of natural vibriosis sea bass *Lates calcarieer* (20-30 g body weight) was kindly provided by a hatchery in Chachoengsoa province. The fish exhibited haemorrhage and ulceration of skin on body and abdomen. The visceral mass was dissected out and processed for paraffin section as above.

Serial sections (8 μm thickness) were prepared and processed for indirect immuno-peroxidase staining using various MAbs and GAM-HRP diluted 1:1000 with 10% calf serum in PBS. Peroxidase activity was revealed by incubation with 0.03% DAB and 0.006% hydrogen peroxide in PBS. Preparations were counter-stained with haematoxylin and eosin Y (H&E), dehydrated in graded ethanol series, clear in xylene and mounted in permount (Sithigorngul et al., 2000, 2002). Positive immunoreactivity was visualized as brown coloration against the pink and purple colors of H&E.

Class and subclass determination.

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

Sensitivity of MAb for detection of *V. parahaemolyticus* determined by dot-blotting.

Serial dilution of *V. parahaemolyticus* (beginning with 10^8 CFU/ml) in PBS was performed and 1 μl of each dilution was spotted onto a nitrocellulose membrane before fixing in 10% formalin for 10 min and processing for dot blotting using various MAbs as described above. The lowest bacterial dilution that showed distinct and clear immunoreactivity was determined.

Results and Discussion

After the fourth immunization, the antisera from each mouse at dilution of 1:20,000 were preabsorbed with lysate of *Vibrio harveyi* to remove non-specific binding activity against common epitopes of *Vibrio* spp., before determination of the specificity by Western blotting. The antisera from all mice demonstrated some distinct immunoreactive bands of *V. parahaemolyticus* antigens with light cross-reactivity to *V. harveyi* antigens. Therefore, the best response mouse was used as spleen donor for hybridoma production.

From two fusions, the cells from each fusion were laid in 30 microculture plates and about half (1500 wells/fusion) contained hybridoma colonies from which approximately 200 wells gave positive reaction on the first screening by dot blotting against *V. parahaemolyticus* isolates used for immunization. After the second screening with dot blotting against three isolates of *V. parahaemolyticus* and various bacteria, Western blotting and immuno-histochemistry were performed. Strongly positive immunoreactivity antibody-producing clones with high specificity and some limited cross-reactivity to the closely related *Vibrio* spp. were selected and cloned. Only 9 hybridoma clones were selected and established as high stability cell lines which can be divided into 6 groups according to antibody specificity (Table 2, Fig.1 and 2).

Group one consisted of two MAbs that bound to the first isolate of *V. parahaemolyticus* (VPV) only and did not show any cross-reactivity to the other two isolates of *V. parahaemolyticus* (VPB and VPC), and other bacteria by dot blotting (Fig.1A). They bound to a broad smear band of the antigen with molecular mass around 40 kDa (Fig. 2B).

Group two consisted of one MAb that showed similar specificity and characteristic as MAbs in group one (Fig. 1B). However, this MAb bound to a smear of the antigen with molecular mass approximately of 10-15 kDa (Fig. 2B).

Group three consisted of one MAb that bound to the second isolates *V. parahaemolyticus* (VPB) on ly (Fig. 1C). It bound to antigens with molecular mass approximately 20 kDa and smear around 60-90 kDa (Fig. 2C).

Group four consisted of one MAb that bound to the antigens with the same molecular masses as recognized by MAbs in group three (20 and 60-90 kDa; Fig. 2D) but exhibited different specificity. They recognized the third isolate of *V. parahaemolyticus* (VPC) only (Fig. 1D).

Group five consisted of two MAbs that recognized VPC only (as MAb in group 4did; Fig 1E) but bound to the antigen at approximately 10-15 kDa similar to similar molecular masses of antigens recognized by MAbs in group two (Fig. 2D).

Group six consisted of one MAb, that recognized all three isolates of *V parahaemolyticus* with different affinity and also demonstrated cross-reactivity to other *Vibrio* spp. including *V. alginolyticus*, *V. harveyi*, *V. vulnificus*, *V. fluvialis* and *V. cholerae*, and lightly cross-reacted to *V. mimicus* but did not bind to *V. ordalii* and *V. penaeicida* (Fig. 1F). This MAb bound to an antigen at molecular mass approximately 40 kDa (Fig. 2C).

All MAbs (except for MAb in group six) were also able to recognize *V. parahaemolyticus* infection in the tissues of artificially infected *L. vannamei* injected with each isolates (Fig. 3). Most of the MAbs are IgM class except for one antibody in group 1 which is IgG2b (Table 2).

The sensitivity testing for detection of *V. parahaemolyticus* with dot blot assay was range from 10^5 to 10^7 CFU/ml depending on the group of MAbs (Table 2). Since the sample volume for dot blot assay is small (1 µl), the development of other type of enzyme immuno-assay such as sandwich ELISA would improve the sensitivity due to the increased of volume of bacterial sample.

Two isolates of *V. parahaemolyticus* (VPV and VPB) were isolated from aquatic animals and identified as the same serotype (O5:K33). However, they are different in protein profiles as demonstrated in SDS-PAGE (Fig. 2A lane 1 and 2) and can be differentiated clearly by MAbs in group 1, 2 and 3.

In naturally infected *L. vannamei*, strongly positive result was found in hepatopancreas with MAbs in group 5 (VPC 701: Fig. 3E), specific to VPC isolated from patient stool but did not recognized by MAb in group 4, also specific to the VPC. The positions of infection in hepatopancreas were different between artificially and naturally infected *L. vannamei*. In artificial infection, the immunoreactivity was found in the interstitial tissue (Fig. 3D) while in the natural infection the immunoreactivity was found in the tubular cells (Fig 3E).

In naturally infected sea bass *Lates calcarieer* the positive immunoreactivity was also found with MAbs in group 5 (VPC 701; Fig 4F) but did not recognized by MAb in group 4 similar to that found in the naturally infected *L. vannamei*.

This evidence indicated that the bacteria in naturally infected *L. vannamei* and sea bass are different from the *V. parahaemolyticus* (VPC) used for immunization. Moreover the infection by various isolates of *V. parahaemolyticus* was not host specific since in previous

observation reported that the strains from diarrhea patients were also caused lethal in Abalone (Lee et al., 2003).

The nature of the antigens recognized by these MAbs were not defined in this study. However, MAbs in group 1 to 5 recognized the antigens as smear broad bands, suspected to be polysaccharide. All of MAbs demonstrated high specificity to each isolate of *V. parahaemolyticus* used for immunization only without showing any cross reactivity among the three isolates of *V. parahaemolyticus* and other bacteria.

The MAb specific to common antigens among *V. parahaemolyticus* was not obtained in this study. However, MAb in group six could bind to all three isolates of *V. parahaemolyticus* with different intensity in reactivity and could bind to the closely related *Vibrio* species as well. Therefore, this group of MAbs can be used for general identification of several *Vibrio* spp. related to *V. parahaemolyticus*.

In conclusion, MAbs specific to each isolate of *V. parahaemolyticus* and MAbs specific to *Vibrio* spp. related to *V. parahaemolyticus* were generated. These MAbs can be used for simple detection of the bacteria from culture by dot blotting and for detection of bacterial infection in shrimp tissues by immunohistochemistry. It is promising for production of MAbs specific to other isolates and to common epitopes among various *V. parahaemolyticus* isolates using combination of different isolates for immunization and careful screening of the desired hybridoma as successfully done in *V. harveyi* (Phianphak et al., 2005) and *V. alginolyticus* (Sithigorngul et al., 2006).

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Table 1. List of bacterial isolates and sources used in this study.

Bacteria	Sources	Remarks
Vibrio parahaemolyticus 1 (VPV) O5:K33	VMARC	Isolated from Aquatic animals
V. parahaemolyticus 2 (VPB) O5:K33	DASB	Isolated from *P. monodon*
V. parahaemolyticus 3 (VPC) O10:KUT	DMSC	Isolated from patient's stool
Vibrio alginolyticus 22082	DMST	Isolated from stool
V. vulnificus	DASB	Isolated from sea bass
V. mimicus 22089	DMST	Isolated from food
V. harveyi 1526	CENTEX	Isolated from *P. monodon*
V. fluvialis 22086	DMST	Isolated from stool
V. chloerae	DMSC	
V. penaeicida	DMSC	
Vibrio ordalii	DASB	
Photobacterium damselae damselae	DASB	Isolated from sea bass
Aeromonas hydrophila 22097	DMST	Isolated from carp's kidney
A. sobria 22099	DMST	Isolated from stool
A. caviae 22106	DMST	Isolated from stool
Plesiomonas shigelloides 22107	DMST	Isolated from rectal swab
Escherichia coli ATCC25922	CPF	
Salmonella Typhi	DMSM	
Salmonella Typhimurium ATCC1408	CPF	
Salmonella Enteriditis 7108	DMST	
Shigella flexneri	DMSM	
Morganella morganii	DMSM	
Enterobacter cloacae	DMSM	
Klebsiella pneumoniae	DMSM	
Proteus vulgaris	DMSM	
Pseudomonas aeruginosa	DMSM	
Enterococcus faecalis	DMSC	

CENTEX = CENTEX Shrimp, Mahidol University, Thailand.
CPF = Charoen Phokphan Foods Public Co. Ltd. Thailand.
DASB = Department of Aquatic Science, Burapa University, Thailand.
DMSC = Department of Microbiology, Chulalongkorn University, Thailand.
DMSM = Department of Microbiology, Mahidol University, Thailand.
DMST = Department of Medical Sciences, Ministry of Public Health, Thailand.
VMARC = Veterinary Medical Aquatic Research Center, Chulalongkorn University, Thailand.

Table 2. Specificity of monoclonal antibodies. The binding of antibodies to various bacteria was determined by dot blot using bacteria at approximately 10^8 CFU/ml: VPV = *V. parahaemolyticus* isolate 1, VPB = *V. parahaemolyticus* isolate 2, VPC = *V. parahaemolyticus* isolate 3, VV = *V. vulnificus*, VF = *V. fluvialis*, VC = *V. cholera*, VA = *V. alginolyticus*. The intensity of staining was arbitrary scored as +++ = very intense staining, + = light staining, - = not staining. IHC = immunohistochemistry

Group	MAb (isotype)	Sensitivity Dot Blot (CFU/ml)	Antigen Western blot (kDa)	IHC	Bacterial immunoreactivity (Dot blot)
1.	VPV 22(M), VPV 54 (G2b)	2.5 X 10^5	40	++	VPV(+++)
2.	VPV 767 (M),	5 X 10^5	10-15	+	VPV (++)
3.	VPB25, 897 (M)	1 X 10^6	20, 60-90	++	VPB (+++)
4.	VPC 381 (M)	1 X 10^6	20, 60-90	++	VPC (+++)
5.	VPC701, 841 (M)	1 X 10^7	10-15	++	VPC (+++)
6.	VPV486 (M)	1 X 10^7	40	-	VPV, VPB, VPC, VA,VH, VV, VF VC (++)VM(+)

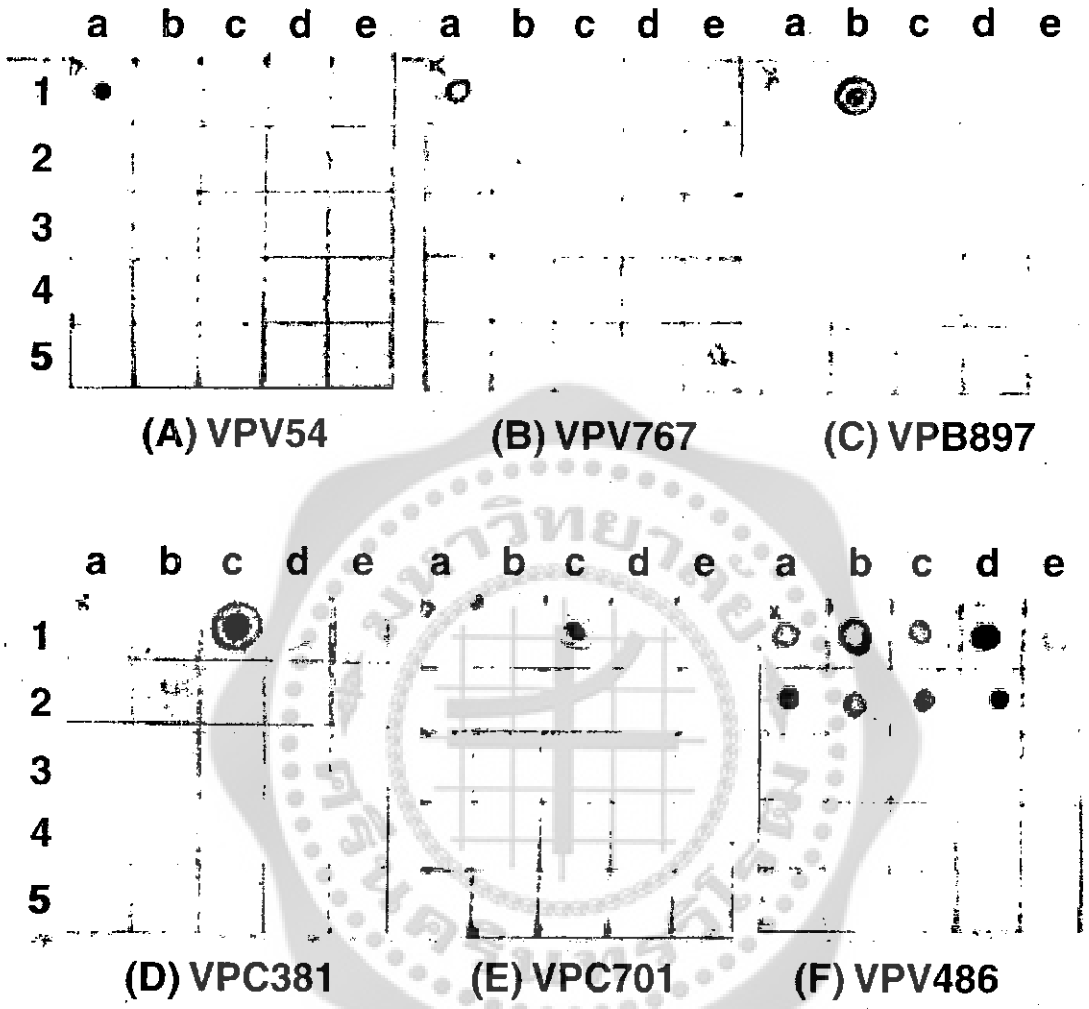
Figure Legends

Figure 1. Cross-reactivity testing of MAbs by dot blotting. Heat killed bacteria (10^8 CFU/ml) were spot on nitrocellulose membrane (1ul/spot) and treated with various MAbs from each group (only one of representative for each group were demonstrated) (A) VPV54, (B) VPV767, (C) VPB897, (D) VPC381, (E) VPC701 and (F) VPV486.
Row 1. a. *V. parahaemolyticus* (VPV), b. *V. parahaemolyticus* (VPB), c. *V. parahaemolyticus* (VPC), d. *V. alginolyticus*, e. *V. mimicus*.
Row 2. a. *V. harveyi*, b. *V. fluvialis*, c. *V. cholerae*, d. *V. vulnificus*, e. *V. penaeicida*.
Row 3. a. *V. ordalii*, b. *Photobacterium damselae*, c. *Aeromonas hydrophila*, d. *A. sobriae*, e. *A. caviae*, e. *Escherichia coli*.
Row 4. a. *Plesiomonas shigelloides*, b. *Escherichia coli*, c. *Samonella* Typhi. d. *Shigella flexneri*, e. *Morganella morganii*.
Row 5. a. *Enterobacter cloacae*, b. *Klebsiella pneumoniae*, c. *Proteus vulgaris*, d. *Pseudomonas aeruginosa*, e. *Enterococcus faecalis*.

Figure 2. SDS-PAGE and Western blot analysis. (A) Homogenates from three isolates of *V. parahaemolyticus* 1. VPV, 2. VPB and 3. VPC were separated by 12% SDS-PAGE and stained with Coomassie blue or transferred to nitrocellulose membrane and treated with MAbs: (B) VPV54 and VPV767, (C) VPB897 and VPV 486, (D) VPC 381 and VPC701. S = low range (left) and high range (right) protein standard. Separated Western blot analyses with each MAb were performed before combination of two MAbs were used on the same blot.

Figure 3. Immunohistochemistry. Indirect immunoperoxidase staining of tissues from (A to D) experimentally infected *L. vannamei*, (E) naturally infected *L. vannamei* and (F) naturally infected *Lates calcarieer* using MAbs (A) VPV54 (gill), (B) VPV767 (muscle), (C) VPB 897 (muscle), (D) VPC 381 (hepatopancreas), (E) VPC701 (hepatopancreas), (F) VPC701 (intestine). The tissues were counter stained with Eosin Y only.

Figure 1


a b c d e a b c d e a b c d e
1
2
3
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(A) VPV54
(B) VPV767
(C) VPB897
a b c d e a b c d e a b c d e
1
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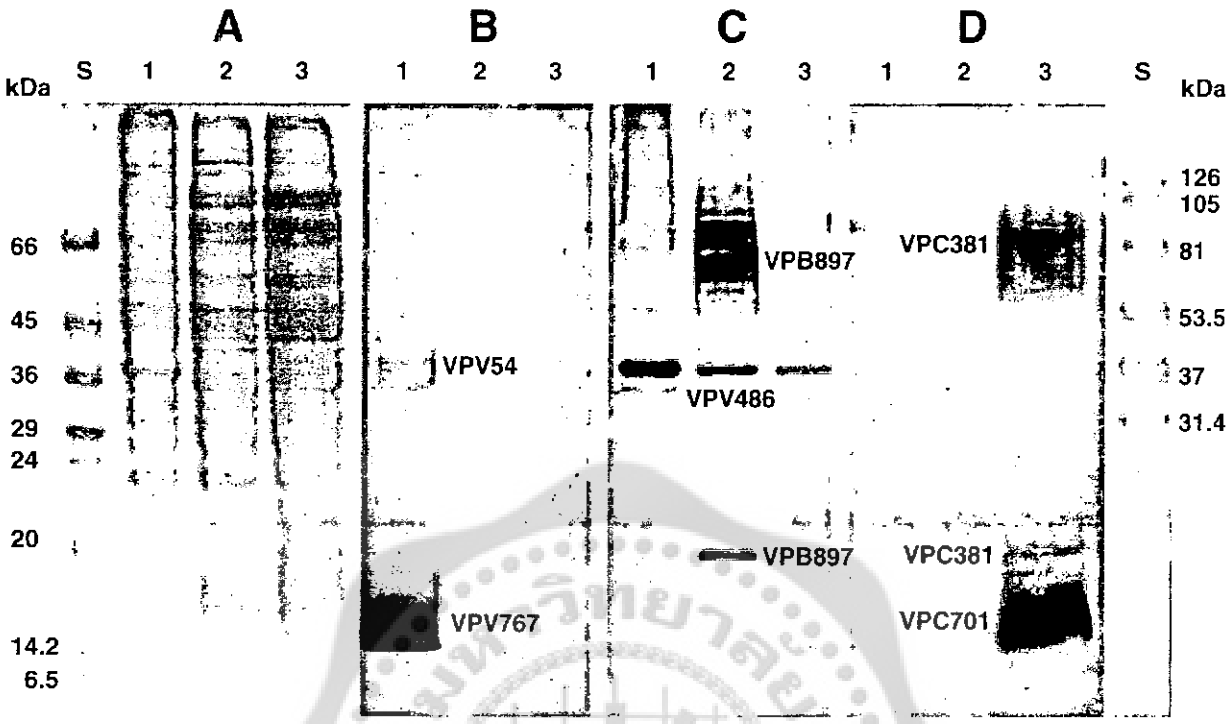
(D) VPC381
(E) VPC701
(F) VPV486

Figure 2


A
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VPV54
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126
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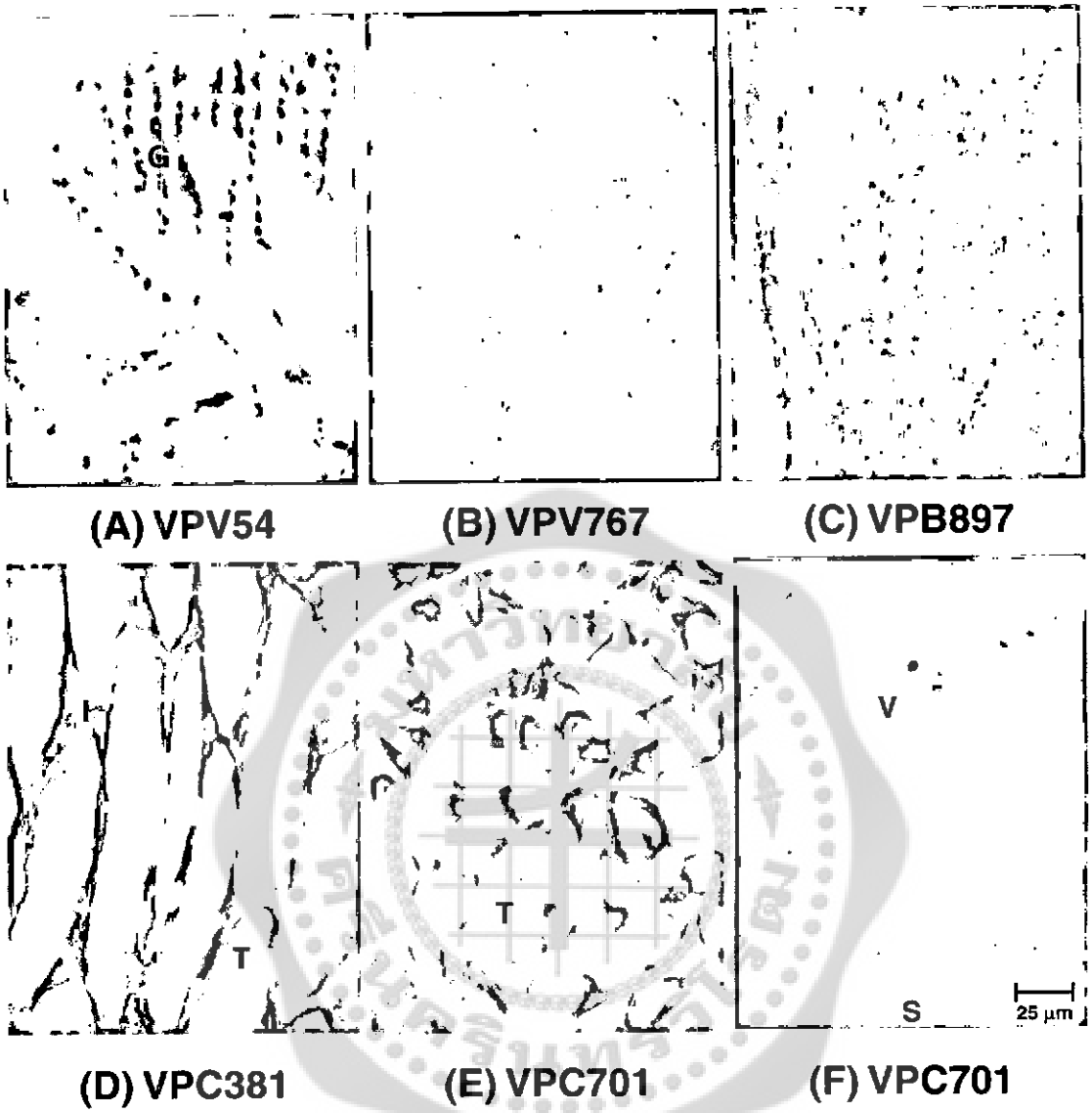
Figure 3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(A) VPV54
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(C) VPB897
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(D) VPC381
(E) VPC701
(F) VPC701

2.2

Detection of *Vibrio vulnificus* infection in shrimp with monoclonal antibodies

Detection of *Vibrio vulnificus* infection in shrimp with monoclonal antibodies

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2 Tables
3 Figures

Abbreviation Title: Monoclonal antibody specific to *Vibrio vulnificus*.

Key word: *Vibrio vulnificus*, dot blotting, immunohistochemistry, Lipopolysaccharide (LPS), sea bass *Lates calcarieer*, *Penaeus monodon*.

Abstract

Seven monoclonal antibodies (MAbs) were generated against 3 isolates of *V. vulnificus* (VVC, VVB, VVBs) using whole cell as antigens. The monoclonal antibodies can be divided into five groups according to their specificities. MAbs in groups 1 were specific to the first isolate of *V. vulnificus* (VVC only) without any cross-reaction to the other two isolates of *V. vulnificus* (VVB, VVBs) and other bacterial species. The MAbs in group 1 bound to a band of antigen at molecular mass of 65 kDa. The MAbs in group 2 and 3 were specific to the second isolate of *V. vulnificus* (VVB) and bound to the antigens at 5 and 35 kDa, respectively. The MAb in group 4 was specific to the third isolate of *V. vulnificus* (VVBs only) and it bound to the native protein only since it did not recognized any protein in Western blot assay. The MAb in group 5 was broadly specific. It recognized all the isolates of *V. vulnificus*, and almost all *Vibrio* spp. tested; *V. parahaemolyticus*, *V. mimicus*, *V. harveyi*, *V. fluvialis*, *V. cholerae* and *V. alginolyticus* but did not recognize *V. penaeicida*. This MAb in group 5 recognized an antigen at 5 kDa. The antibodies in group 1 and 2 can be used to detect the bacteria in the tissues by immunohistochemistry. All antibodies can be easily used to detect the bacteria by dot blotting with sensitivity range from 10^5 to 10^6 CFU/ml.

Introduction

Vibrio vulnificus, a Gram negative marine bacterium has been the focus of attention due to its roles as pathogen in fish (Esteve-Gassent et al., 2003), shrimp (Sung and Song, 1996), and human. This species causes two kinds of clinical manifestations; fatal septicemia after consumption of contaminated seafood (Bender and Romig, 2004; Summer and Ross, 2002), or severe wound infections from exposure to the seawater or handling of fish or shellfish (Petel et al., 2002; Midturi et al., 2005). Three biotypes designated 1, 2 and 3 have been established based upon characteristics, such as indole production, host specificity (Linkous and Oliver, 1999), serotype (Biosca et al., 1996) and genetic subtyping (Ripabell1 et al., 2003). Those in biotype1 are pathogenic to human, as opposed to biotype 2 that are commonly infected eel while biotype 3 appeared to be pathogen of both (Amaro and Biosca, 1996: Biosca et al., 1997a). Biochemical identification and application of selective media specific for *V. vulnificus* are time consuming and still required immunological and molecular identification for confirmation of the organism (Cerda'-Cue'llar et al., 2000; Biosca et al., 1996). Biotype 2 is virulent to eels, negative for indole reaction, and serological homogeneous, whereas strains of biotype1 are a virulent, indole positive, and serological

heterogenous (Biosca et al., 1997b). Five monoclonal antibodies generated against LPS of 17 clinical isolates of *V. vulnificus* were specifically used to group these isolates into 6 serovars without cross reaction among these groups while the antisera could not differentiate these serovars from each other (Martin and Siebeling, 1991). Therefore monoclonal antibody seem to be more appropriate for identification of the bacteria due to its monospecific property. Recently, two isolates of *V. vulnificus* (biotype1 and biotype 2) were isolated from infected sea bass (*Lates calcarieer*) from a hatchery in Chachoengsoa province, Thailand according to their differences in colony forming and indole reaction. In order to provide a convenient and accurate tool for diagnosis of *V. vulnificus* infection, monoclonal antibodies against these two isolates of *V. vulnificus* from sea bass and one isolate from Chulalongkorn University, Thailand were produced and preliminary used for identification of *V. vulnificus* infection in fish and shrimp occurred naturally.

Materials and Methods

Bacterial cultured and antigen preparation

Two isolates of *Vibrio vulnificus*; biotype 1 (VVBs = a slow growing colony) and biotype 2 (VVB = a fast growing colony) were isolated from naturally infected seabass (*Lates calcarieer*) in a hatchery at Chachoengsoa province, Thailand. Another isolate of *V. vulnificus* (VVC) was kindly provided by Department of Microbiology, Chulalongkorn University. The sources of various bacteria used for cross-reactivity testing were indicated in Table 1. The bacteria were grown with agitation at 37°C in tryptic soy broth (TSB; Merck) to log phase. In case of *Vibrio* spp, the TSB was supplemented with 2% (w/v) NaCl. The bacteria were harvested by centrifugation at 3500 X *g* for 20 min at 4 °C. Bacterial pellets were washed twice with sterile PBS (0.15 M phosphate buffered saline, pH 7.2), resuspended in PBS, heat killed at 60°C for 30 min, and adjusted to the OD of 1 at 610 nm (approximately 10^8 CFU/ml). The bacterial suspension was divided into small aliquots and stored at -70°C until use.

Two forms of antigen from three isolates of *V. vulnificus* (VVC, VVB, VBBs) including heat killed and denatured forms, were used for immunization. The denatured form was prepared by mixing the bacteria with treatment buffer (4% SDS and 10% mercaptoethanol) at a ratio of 1:1 and boiled for 1 min, then dialyzed against three changes of PBS at 12 hr intervals.

Immunization

The mixture of heat-killed and denatured form (with a ratio of 1:1) from of 3 isolates of *V. vulnificus* (10^8 CFU/ml) was prepared. Four Swiss mice were injected intraperitoneally with 50 µl of the prepared mixture mixed with complete Freund's adjuvant at a ratio of 1:1. Mice were subsequently injected three more times with the same antigen mixed with incomplete Freund's adjuvant at two weeks intervals. One week after the fourth injection, mouse antisera were collected, prabsorbed with excess *Vibrio harveyi* to remove non-specific binding to common epitopes of *Vibrio* spp., and tested against *V. vulnificus*, *V. harveyi* and *Escherichia coli* by Western blotting. The best performing mouse that its antiserum demonstrated strongly distinct bands on *V. vulnificus* was later boosted three days before hybridoma production.

Hybridoma production

A cell fusion protocol was adapted from the method developed by Kohler & 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 was plated on 30 micro-culture plates (96 wells/plate). After identification of the positive cultures by screening methods; including dot blotting, Western blotting and immunohistochemistry as described below, cells were cloned by limiting dilution method and stored in liquid nitrogen.

Dot blotting

Cell suspension of various heat-killed bacteria approximately 10^8CFU/ml was used for screening. Heat-killed bacterial samples (1 µl/spot) were applied to nitrocellulose membrane, baked at 60°C for 10 min, and incubated in hybridoma conditioned medium diluted to 1:20 with 5% Blotto (5% nonfat dry milk, 0.1% Triton X-100 in PBS) for 4 hr. After extensive washing in 0.5% Blotto, 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 hr. The membrane was then washed for 5 min in 0.5% Blotto and developed for 5 min in substrate mixture containing 0.006% hydrogen peroxide, 0.03% diaminobenzidine (DAB) and 0.05% cobalt chloride in PBS (Sithigorngul et al., 2000). Hybridoma cultures that displayed immunoreactivity against *V. vulnificus*, were confirmed for bacterial specificity by dot blotting against various bacteria, Western blotting and immunohistochemistry before cloning and cryopreservation.

SDS-PAGE and Western blot analysis

V. vulnificus and other bacterial lysates were separated by 12% SDS-PAGE according to the method described by Laemmli (1970). Samples were electrophoresed for 6 hr at 30 V and part of the gel was stained using Coomassie brilliant blue R-250. For Western blotting, sample resolved by SDS-PAGE in another part of the gel were electro-blotted onto nitrocellulose membrane using Transblot apparatus (Bio-Rad). The nitrocellulose membrane was incubated in 5% blotto for 10 min, each lane of sample was separately treated with 1:200 hybridoma conditioned medium from each clone, and then processed as described above in dot blotting section. Low molecular weight markers (Sigma) were used as a standard.

Immunohistochemistry

The black tiger shrimp *Penaeus monodon* (weight 10-15 g) was artificially infected with *V. vulnificus* (VVB) (25 µl of 10^7 CFU/ml) via the fourth walking leg. When the shrimps displayed unbalanced and slow movement, the cephalothoraces were cut and fixed in Davidson fixative for overnight before processing for paraffin sectioning.

Serial sections (8 µm thickness) were prepared and processed for indirect immuno-peroxidase staining using various MAbs (at 1:100 dilution) as primary antibodies and GAM-HRP (at 1:1000) as secondary antibody. Immunoreactivity was revealed by incubation with 0.006% hydrogen peroxide, 0.03% DAB in PBS for 5 min. Preparations were counter-stained with haematoxylin and eosin Y (H&E), dehydrated in graded ethanol series, cleared in xylene and mounted in Permount (Sithigorngul et al., 2000; 2002). Positive immunoreactivity was visualized as brown coloration against the pink and purple colors of H&E.

Class and subclass determination

Class and subclass of mouse immunoglobulins produced by the hybridomas was determined by sandwich ELISA using Zymed's MouseAB ID kit.

Sensitivity of MAb for detection of *V. vulnificus* determined by dot blotting.

Serial dilution (beginning with 10^8 CFU/ml) in PBS was performed and 1 µl of each dilution of *V. vulnificus* was spotted onto nitrocellulose membrane before fixing in 10% formalin for 10 min and processing for dot blotting using various MAbs as described above. The lowest bacterial dilution that showed distinct and clear immunoreactivity was determined.

Results and Discussion

After the fourth immunization with *V. vulnificus*, the antisera from four mice at dilution of 1:20,000 were preabsorbed with lysate of *Vibrio harveyi* before determination of the specificity by Western blotting. All antisera demonstrated several immunoreactive bands on *V. vulnicus* lysate without cross-reactivity to *V. harveyi* and *E. coli*. They also displayed distinct immunoreactive bands against antigens among three isolates of *V. vulnificus* isolated in Thailand (VVB, VVBs, VVC); therefore, one mouse with the strongest immunoreactivity was used as spleen donor for hybridoma production.

From one fusion, the cell mixture was laid in 30 microculture plates and about half of the well (1500 wells) contained hybridoma colonies. About 100 wells gave positive reaction for the first screening against combination of three isolates of *V. vulnificus*. After the second screening with Western blotting, strongly positive immunoreactivity-producing clones with high specificity and some limited cross-reactivity to the related bacteria were selected and cloned. Only 7 hybridoma clones were selected and established as high stability cell lines which can divided into 5 groups according to their specificities (Table 2).

MAbs in group 1 consisted of the antibodies that bound specifically to only one isolate of *V. vulnificus* (VVC) (Fig. 1A). These antibodies bound a band of antigen at molecular mass around 65 kDa (Fig. 2B). The antibodies can be used to detect bacterial infection in tissues by means of immunohistochemistry (Fig 3A and B).

MAb in group 2 consisted of one MAb that bound only to the second isolate of *V. vulnificus* (VVB) (Fig. 1B). The antibody recognized a smear of antigen at molecular mass around 5 kDa (Fig. 2C). This MAb can be used to detect the bacteria by means of immunohistochemistry as well (Fig. 3C and D).

MAbs in group 3 demonstrated specificity to VVB only, the same as MAb in group 2 did. (Fig. 1C). However, MAbs in group 3 bound to different antigen at the molecular mass around 35 kDa (Fig 2D).

Mab in group 4 was specific to the third isolate of *V. vulnificus* (VVBs) only (Fig. 1D). The molecular mass of this antigen was unknown since the antibody did not bind to the denatured antigen in Western blotting.

MAb in group 5 bound to all 3 isolates of *V. vulnificus*, and cross-reacted strongly to most of *Vibrio* spp. tested such as *V. parahaemolyticus, V. alginolyticus, V. fluvialis, V. mimicus, V. cholerae* and lightly cross-reacted to *V. harveyi*, but did not cross-reacted to *V. penaeicida* and other bacteria (Fig. 1E). The antibody bound to an antigen at molecular mass

around 5 kDa (Fig. 2E). However, this MAb can not detect the bacteria in tissues by immunohistochemistry.

The sensitivity for detection of *V. vulnificus* by dot blotting ranged from 5 X 10^4 to 5 X 10^6 CFU/ml depending on the group of antibodies (Table 2). The sensitivity of detection by MAbs in group 1 was close to that of the antiserum against *V. vulnificus* in previous report in which the antiserum can be used to detect bacteria around 10^4-10^5 cells/well (100 ul) by ELISA (Biosca et al., 1997b). However, this technique required highly pure culture for the assay which is not required by dot blotting. To improve the efficiency of the detection, pre-enrichment of the sample in alkaline peptone water (APW) or supplemented with polymyxin B (APWP) had been used to improve the detection of *V. vulnificus* from seawater and sediment (Hoi et al., 1998).

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Table1. List of bacterial isolates and sources used in this study.

Bacteria	Sources	Remarks
Vibrio vulnificus biotype1 (VVBs)	DABU	isolated from seabass
V. vulnificus biotype 2 (VVB)	DABU	isolated from seabass
V. vulnificus (VVC)	DMSC	
V. parahaemolyticus 3 (VPC) O10:KUT	DMSC	Isolated from patient's stool
Vibrio alginolyticus 22082	DMST	Isolated from stool
V. mimicus 22089	DMST	Isolated from food
V. harveyi 1526	CENTEX	Isolated from *P. monodon*
V. fluvialis 22086	DMST	Isolated from stool
V. cholerae	DMSC	
V. penaeicida	DMSC	
Vibrio ordalii	DASB	
Photobacterium damselae damselae	DASB	Isolated from sea bass
Aeromonas hydrophila 22097	DMST	Isolated from carp's kidney
A. sobria 22099	DMST	Isolated from stool
A. caviae 22106	DMST	Isolated from stool
Plesiomonas shigelloides 22107	DMST	Isolated from rectal swab
Escherichia coli ATCC25922	CPF	
Salmonella Typhi	DMSM	
Salmonella Typhimurium ATCC1408	CPF	
Salmonella Enteriditis 7108	DMST	
Shigella flexneri	DMSM	
Morganella morganii	DMSM	
Enterobacter cloacae	DMSM	
Klebsiella pneumoniae	DMSM	
Proteus vulgaris	DMSM	
Pseudomonas aeruginosa	DMSM	
Enterococcus faecalis	DMSC	

CENTEX = CENTEX Shrimp, Mahidol University, Thailand.
CPF = Charoen Phokphan Foods Public Co. Ltd. Thailand.
DABU = Department of Aquatic Science, Burapa University, Thailand.
DMSC = Department of Microbiology, Chulalongkorn University, Thailand.
DMSM = Department of Microbiology, Mahidol University, Thailand.
DMST = Department of Medical Sciences, Ministry of Public Health, Thailand.

Table 2. Specificity of MAbs. The binding of antibodies to various bacteria was determined by dot blotting using bacteria with approximately 10^8 CFU/ml: VVC = *V. vulnificus* from DMSC , VVB and VVBs = *V. vulnificus* isolated from sea bass, VA = *V. alginolyticus*; VC = *V. cholerae*; VF = *V. fluvialis*; VH = *V. harveyi*; VM = *V. mimicus*; VP = *V. parahaemolyticus*. The intensity of staining was arbitary scored as +++ = very intense staining, ++ = moderate staining, + = light staining, - not staining, IHC = Immunohistochemistry.

Group MAb (isotype)	Sensitivity testing By dot blotting (CFU/ml)	Detected antigen by Western blotting (kDa)	IHC	Bacterial immunoreactivity (dot blotting)
1. VVC42 (G2a) VVC23 (G1)	5 X 10^6	65	+	VVC (+++)
2. VVB158 (G2b)	5 X 10^4	5	+	VVB (+++)
3. VVB 12, 48 (G1)	5 X 10^6	35	-	VVB (+++)
4. VVBs66	5 X 10^6	-	-	VVBs (+++)
5. VVB100	5 X 10^5	5	-	VV, VP, VA, VM, VF, VC +++) VH (++)

Figure Legends

Figure 1. Cross-reactivity of MAbs assayed by dot blotting. Heat killed bacteria (~10^8 CFU/ml) were spotted onto nitrocellulose membrane (1µl/spot) and treated with various MAbs (only one representative for each group were demonstrated)
Row 1: a) *Vibrio vulnificus* (VVC); b) *V. vulnificus* (VVB); c) *V. vulnificus* (VVBs); d) *V. parahaemolyticus*; e) *V.alginolyticus*.
Row 2: a) *V. mimicus*; b) *V. harveyi* 639; c) *V. fluvialis*; d) *V. cholerae*; e)*V. penaeicida*.
Row 3: a) *Aeromonas hydrophila* 1234; b) *A. sobria* 12446; c) *A. caviae* NICMB13016; d) *Plesiomonas shigelloides*; e) *Escherichia coli* ATCC 25922.
Row 4: a) *Salmonella* Typhi; b) *Salmonella* Typhimurium; c) *Salmonella* Enteritidis; d) *Shigella flexneri*; e)*Morganella morganii*.
Row 5: a) *Enterobacter cloacae*; b) *Klebsiella pneumoniae*; c) *Proteus vulgaris*; d) *Pseudomonas aeruginosa*; e) *Enterococcus faecalis*.

Figure 2. SDS-PAGE and Western blot analysis. (A) lysates of *V. vulnificus*; VVC and VVB were electrophoresed and stained with Coomassie blue or transferred onto nitrocellulose membrane and treated with representative MAb from each group (B) VVC42, (C) VVB158, (D) VVB48, (E) VVB100. S = standard marker proteins.

Figure 3. Immunohistochemistry of *V. vulnificus* infection. Tissues from artificially infected tiger prawn *Penaeus monodon* were treated with MAbs; VVC42 (A and B) and VVB158 (C and D). (A) hepatopancreas, (B) muscle, (C) mouth appendage (D) subcuticular gland.

Figure 1

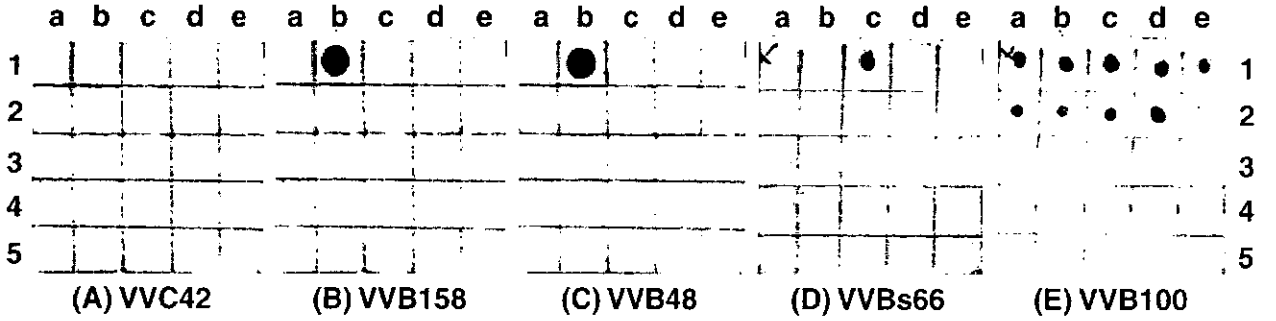

Figure 2

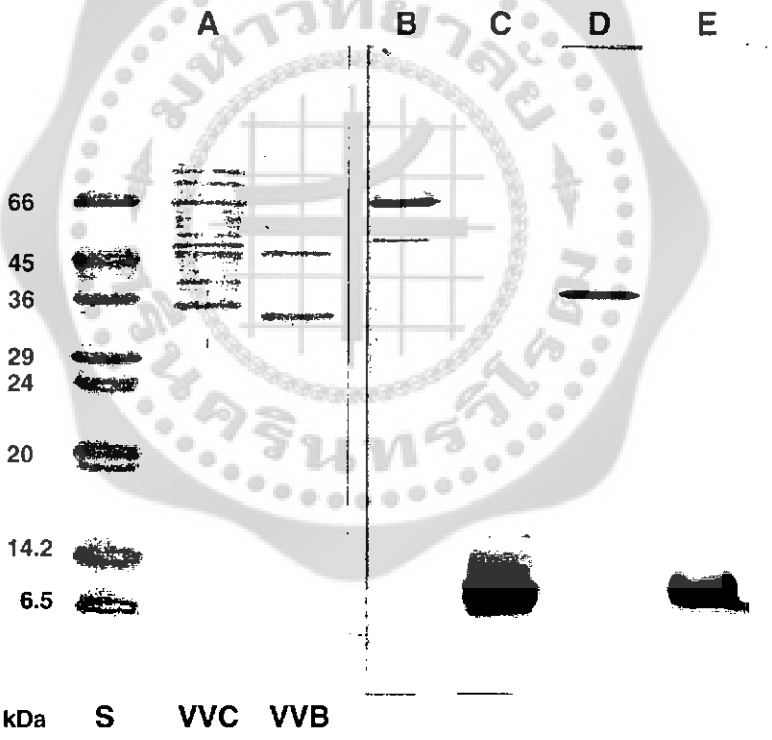

Figure 3

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   ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ

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บทคัดย่อ
ABSTRACTS

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เทคโนธานี มหาวิทยาลัยเทคโนโลยีสุรนารี
TECHNOPOLIS, SURANAREE UNIVERSITY OF TECHNOLOGY

Abstract: Four groups of monoclonal antibodies (MAbs) against *Vibrio alginolyticus* 14800 were generated using whole cell of bacteria for immunization. The first group of monoclonal antibodies demonstrated high specificity that bound to only the *V. alginolyticus* 14800 but did not bind to the other two *V. alginolyticus* isolates, they bound to a smear band of antigen at molecular mass range from 12 to 23 kDa. The second group of antibodies recognized all three *V. alginolyticus* isolates tested, they bound to 2 major antigens at molecular mass of 10 and 18 kDa. The third group of MAbs recognize all 3 isolates of *V. alginolyticus* but bound to a very high molecular weight antigen. The fourth group of MAbs recognized several species of Vibrio tested with different affinities; *V. parahaemolyticus*, *V. harveyi*, *V. fluvialis* and *V. vulnificus* but did not recognize *V. mimicus*, *V. cholera* and *V. penaeicida*. It bound to a protein at 26 kDa. The MAbs in group 1, 2 and 3 can recognized the bacteria in tissues by means of immunohistochemistry. This evidence demonstrate the heterogeneity among various isolates of *V. alginolyticus*: therefore, production MAbs against different isolates is required to develop a specific tool for serotyping of *V. alginolyticus*.

B0037-DIFFERENTIATION OF TWO ISOLATES OF *Vibrio vulnificus* WITH MONOCLONAL ANTIBODY

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Abstract: Five groups of monoclonal antibodies (MAbs) against two isolates of *Vibrio vulnificus* were generated using whole cell of bacteria for immunization. The first group of monoclonal antibodies demonstrated high specificity to only one isolate of the *V. vulnificus* (VVC) but did not bind to the other two *V. vulnificus* isolates. They bound to a protein at molecular mass of 66 kDa. The second group of antibodies bound to the same protein as recognized by the first group of MAbs but demonstrated light cross reactivity to *V. fluvialis*. The third, fourth and fifth groups of MAbs recognized only the second isolate of *V. vulnificus* (VVB). The third group bound to only native protein and did not recognize denatured protein by Western blotting. The fourth and the fifth groups bound to proteins with molecular masses of 6.5 and 37 kDa, respectively. MAbs in group 1 and 4 can recognized the bacteria in tissues by means of immunohistochemistry. This evidence indicated the heterogeneity among *V. vulnificus* population that could be differentiated by MAbs. Additional MAbs specific to other isolates of *V. vulnificus* are required to provide specific tools for identification of this bacteria.

B0038-PRODUCTION OF MONOCLONAL ANTIBODIES SPECIFIC TO *Aeromonas hydrophila* USING WHOLE CELL ANTIGENS

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Abstract: Three groups of monoclonal antibodies (MAbs) against *Aeromonas hydrophila* 1234 were generated using whole cell of bacteria for immunization. The first group of monoclonal antibodies demonstrated high specificity only to the *A. hydrophila* 1234 but did not bind to the other two *A. hydrophila* isolates. They bound to a series of lipo-polysaccharide (LPS) with molecular masses range from 10 to 190 kDa. The second group of antibodies recognized two out of three *A. hydrophila* isolates, and bound to a series of LPS with molecular masses range from 5-190 kDa. The third group of MAbs recognized all 3 isolates of *A. hydrophila*, 2 isolates of *A. sobria*, and weakly bound to *A. caviae*. This group of MAbs bound to a 20 kDa protein. The MAbs in group 1 and 2 can recognize the bacteria in tissues by means of immunohistochemistry. Both groups of MAbs possibly bound to LPS at different sites in which the MAbs in group 2 bound to the main core of monomer while the MAbs in group 1 bound to the core at the polymerization site between monomers. The specific MAbs can be used to identify the

การแยกความแตกต่างของ *Vibrio vulnificus* 2 สายพันธุ์ด้วยโมโนโคลนอลแอนติบอดี

Differentiation of two isolates of ***Vibrio vulnificus*** with monoclonal antibody.

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บทคัดย่อ: โมโนโคลนอลแอนติบอดีต่อ *Vibrio vulnificus* 2 สายพันธุ์ ทั้ง 5 กลุ่ม ผลิตโดยใช้แบคทีเรียทั้งเซลล์ในการปลูกภูมิคุ้มกัน โมโนโคลนอลแอนติบอดีกลุ่มที่ 1 มีความจำเพาะต่อ *V. vulnificus* (VVC) แต่ไม่แสดงปฏิกิริยาข้ามกับ *V. vulnificus* อื่นอีก 2 สายพันธุ์ โดยกลุ่มนี้จับกับแถบโปรตีนที่มีน้ำหนักโมเลกุล 66 กิโลดาลตัน กลุ่มที่ 2 มีความจำเพาะกับแถบโปรตีนเดียวกันกับกลุ่มที่ 1 แต่แสดงปฏิกิริยาข้ามกับ *V. fluvialis* เล็กน้อย กลุ่มที่ 3, 4 และ 5 มีความจำเพาะต่อ *V. vulnificus* (VVB) เท่านั้น โดยกลุ่มที่ 3 จับกับโปรตีนที่คงสภาพและไม่จับกับแถบโปรตีนที่เสียสภาพของ Western blotting กลุ่มที่ 4 และ 5 จำเพาะกับแถบโปรตีนที่มีน้ำหนักโมเลกุล 6.5 และ 37 กิโลดาลตัน ตามลำดับ โมโนโคลนอลแอนติบอดีกลุ่มที่ 1 และ 4 สามารถตรวจการติดเชื้อ *V. vulnificus* ในเนื้อเยื่อด้วยวิธี immunohistochemistry ได้ งานวิจัยนี้บ่งชี้ความแตกต่างระหว่างสายพันธุ์ของ *V. vulnificus* ซึ่งสามารถจำแนกได้โดยใช้โมโนโคลนอลแอนติบอดี ด้วยเหตุนี้จึงจำเป็นต้องทำการผลิตโมโนโคลนอลแอนติบอดีที่จำเพาะต่อ *V. vulnificus* สายพันธุ์ต่าง ๆ ต่อไป เพื่อใช้เป็นเครื่องมือในการจำแนกแบคทีเรียชนิดนี้

Abstract: Five groups of monoclonal antibodies (MAbs) against two isolates of *Vibrio vulnificus* were generated using whole cell of bacteria for immunization. The first group of monoclonal antibodies demonstrated high specificity to only one isolate of the *V. vulnificus* (VVC) but did not bind to the other two *V. vulnificus* isolates. They bound to a protein at molecular mass of 66 kDa. The second group of antibodies bound to the same protein as recognized by the first group of MAbs but demonstrated light cross reactivity to *V. fluvialis*. The third, fourth and fifth groups of MAbs recognized only the second isolate of *V. vulnificus* (VVB). The third group bound to only native protein and did not recognize denatured protein by Western blotting. The fourth and the fifth groups bound to proteins with molecular masses of 6.5 and 37 kDa, respectively. MAbs in group 1 and 4 can recognized the bacteria in tissues by means of immunohistochemistry. This evidence indicated the heterogeneity among *V. vulnificus* population that could be differentiated by MAbs. Additional MAbs specific to other isolates of *V. vulnificus* are required to provide specific tools for identification of this bacteria.

Introduction

Vibrio vulnificus, a Gram negative bacterium is a natural inhabitant of estuarine waters, and possesses significant infections in fish (Esteve-Gassent et al., 2003), shrimp (Chanratchakool *et al*., 1995), and health threat to human from consumption raw seafood (Sumner and Ross 2002) or via wound infection (Midturi *et al*., 2005). Three biotypes designated 1,2 and 3 have been

established based upon characteristics, such as indole production, host specificity, serotype (Tison *et al.*, 1982) and genetic subtyping (Bisharat *et al.*, 1999). Those in biotype 1 are pathogenic to human, as opposed to biotype 2 that are commonly infected eel while biotype 3 appeared to be pathogen of both (Amaro and Biosca, 1996; Bisharat et al., 1999; Tison et al., 1982). Biochemical identification and application of selective-differential media specific for *V. vulnificus* are time consuming and still required immunological and molecular identification for confirmation of the identity of the organism (Harwood *et al.*, 2004). This study, is the attempt to develop the specific monoclonal antibody to *V. vulnificus* in order to provide tools for rapid, accurate and sensitive detection of *V. vulnificus*.

Methodology: Two out of three isolates of *V. vulnificus* (VVC, VVB) were used to immunize into four mice at two weeks intervals and only one of the best responded mouse was used as spleen donor for hybridoma production. The hybridoma clones producing antibodies specific to *V. vulnificus* were selected by dot blotting against three isolates of *V. vulnificus* and other Gram negative bacteria, and further screened by Western blotting and immunohistochemistry against *V. vulnificus* infected shrimp.

Results and Discussion

After the fourth immunization, the antisera from four mice at dilution of 1:20,000 were preabsorbed with the lysate of *V. harveyi* before determination of the specificity by Western blotting. All antisera demonstrated series of numerous immunoreactive bands without any cross-reactivity to *V. harveyi* and *Escherichia coli*. The serum from all mice demonstrated the distinct immunoreactive bands of proteins for each isolate of *V. vulnificus* used for immunization. Therefore, one mouse from each group was used as spleen donor for hybridoma production.

From each fusion, the cell mixed were laid in 30 microculture plates and about half (1500 wells) contained hybridoma colonies. Approximately 200 wells gave positive reaction on the first screening by dot blotting against *V. vulnificus* (VVC or VVB), after the second screening with Western blotting strongly positive immunoreactivity producing clones with high specificity and some limited crossreactivity to the related bacteria were selected and recloned. Only 22 hybridoma clones were selected, recloned and established as high stability cell lines which can be divided into 5 groups according to their specificities (Table1). The first and the second groups were from mice immunized with *V. vulnificus* VVC and the other 3 groups were from mice immunized with *V. vulnificus* VVB. **MAb group 1.** consists of nine MAbs, these MAbs bound only to one isolate of *V. vulnificus* (VVC) and did not show any crossreactivity to the other two isolates of *V. vulnificus* and other bacteria. They bound to a protein with molecular mass of 66 kDa. These MAbs also recognized *V. vulnificus* in the tissues of infected sea bass by means of immunohistochemistry. **MAb group 2.** consists of two MAbs that bound to only one isolate *V. vulnificus* (VVC) and the same protein as recognized by MAbs in group 1 but demonstrated light cross-reactivity to *V. fluvialis* and did not recognize the bacteria in tissue by immunohistochemistry. **MAb group 3.** consists of four MAbs, they recognized only one isolate of *V. vulnificus* (VVB). This group of MAbs did not recognize the denatured form of antigen and antigen in tissue, therefore, the molecular nature of the bound molecule by these antibodies was not further characterized. **MAb group 4.** consists of one MAb that bind to only one isolate of *V. vulnificus* (VVB) similar to the third group of MAb, but this antibody recognized a broad band of protein at molecular mass of 6.5 kDa. This antibody also bound lightly to the bacteria in the tissue. **MAb group 5.** consists of 6 MAbs that bound to only one isolate of *V. vulnificus* similar to MAbs in the third and the fourth groups but bound to a 37 kDa protein. These MAbs did not recognize the bacteria in tissues. The sensitivity testing for detection of *V. vulnificus* with dot blot assay was range from 5 $X10^4$ to 5 $X10^6$ CFU/ml depending on the group of MAbs (Table 1). Since the volume of dot blot assay is small (1 µl), the development of other type of

enzyme immuno-assay such as sandwich ELISA would improve the sensitivity of the test approximately by over 100 fold since the volume of the sample will be increased by a hundred fold. The *V. vulnificus* specific antibodies (group 1 and 4) can be used for detection of *V. vulnificus* in the tissues from infected prawn by immunohistochemistry. The MAbs in the group 1, 3, 4 and 5 were highly specific to only one isolate of *V. vulnificus* (VVC or VVB) with out any cross-reactivity to the other two *V. vulnificus* isolates. This evidence indicated the heterogeneity of *V. vulnificus* which could be differentiated by specific MAbs. Therefore, MAbs specific to other *V. vulnificus* isolates are required in order to provide specific tools for identification of various serotypes of *V. vulnificus*.

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Table 1. Specificities of monoclonal antibodies. The binding of antibodies to various bacteria was determined by dot blotting using bacteria with approximately 10^8 CFU/ml: VVC = *V. vulnificus* isolate 1, VVB = *V. vulnificus* isolate 2, VF = *V. fluvialis*. The intensity of staining was arbitrary scored as +++ = very intense staining, + = light staining, ± = very light staining, - = not staining. IHC = immunohistochemistry

Group	MAb (isotype)	Sensitivity of Dot Blotting (CFU/ml)	Detected antigen by Western blotting (kDa)	IHC	Bacterial immunoreactivity (Dot blotting)
1.	VVC 23, 38 (G1), 43,121,118 (G2a), 14,40,88,97 (G2b)	5×10^6	66	+	VVC (+++)
2.	VVC71, 90 (G2a),	5×10^6	66	-	VVC (+++), VF (++)
3.	VVB34, 202 (G2b), 3,152 (G3)	8×10^5	-	-	VVB (+++)
4.	VVB158 (G2b)	5×10^4	6.5	+	VVB (+++)
5.	VVB 4, 12, 47, 48, 110,174 (G1)	5×10^6	37	-	VVB (+++)

Abstract: The important glutinous rice variety Niaw Sanpahtawng was transformed by *Agrobacterium*-mediated transformation of embryogenic calli. Mature seeds were cultured on N6D medium for 4 weeks. The mature seed-derived embryogenic calli was transformed with pCAMBIA 1303 that contains *hptII* gene for resistance to hygromycin B and *gusA* gene as a reporter gene. Hygromycin resistant calli from several independent clones were obtained and showed GUS expression. The T_0 transgenic line was regenerated from hygromycin resistant calli. Genomic DNA of hygromycin-resistant calli and leaves from regenerated plant were isolated and subjected to PCR analysis using *hptII* and *gusA* genes specific primers. The results confirmed the integration of *hptII* and *gusA* genes in the genome of the transformed calli and plant. The transgenic plant is normally grown in the greenhouse. The efforts to improve transformation efficiency and regeneration system are currently performed by modification of various factors.

B0129-PRODUCTION OF MONOCLONAL ANTIBODIES SPECIFIC TO *Vibrio parahaemolyticus* USING WHOLE CELL ANTIGEN

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Abstract: Four groups of monoclonal antibodies (MAbs) against *Vibrio parahaemolyticuss* were generated using whole cell of bacteria for immunization. The first and the second groups of monoclonal antibodies demonstrated high specificity that bound to only one isolate of the *V. parahaemolyticus* (VPV) but did not bind to the other two *V. parahaemolyticus* isolates. The MAbs in the first and the second groups bound to proteins at molecular masses of 57 kDa and 32 kDa, respectively. The third group of MAbs also bound one isolate of *V. parahaemolyticus* (VPV) but demonstrated strong cross reactivity to *V. vulnificus, V. fluvialis, V. cholera* and *V. alginolyticus*. They bound to a protein of 55 kDa. The fourth group of MAb recognized all three isolates of *V. parahaemolyticus* and cross-reacted with other *Vibrio* spp. recognized by MAbs in the third group. All MAbs can recognize the bacteria in tissues by immunohistochemistry. This evidence indicated the heterogeneity among *V. parahaemolyticus* population that could be differentiated by the specific MAbs. Additional MAbs specific to other isolates of *V. parahaemolyticus* are required to provide specific tools for identification of *V. parahaemolyticus*.

B0130-INDUCED MUTATION IN *Adenium obesum* Balf. USING ETHYL METHANESULPHONATE

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Abstract: The mutant inductions in *Adenium obesum* Balf. using Ethyl Methanesulphonate (EMS) were investigated. Seeds were treated with EMS at concentration 0.3 % for 1, 2 and 3 hr., respectively. After treating the seeds were transferred to culture in soil until seedlings and transfered at 4 weeks to the same medium. Growth rate, morphological and biochemical changes of seedling were investigated. The result showed that phenotypic change caused by treat for 2 hr. Abnormal leaf shape with double vein of the mutant was observed. In order to determine genetic modification in the mutant genome, HAT-RAPD (High Annealing Temperature-RAPD) was chosen. Ten primers named OPAQ05, OPK12, OPAD01, OPAH03, OPAH12, OPAH16, OPAH18, OPAH19, OPAH20 and OPH20 were used to investigate genetic variation between the mutant and control. Primer OPAH03 was able to showed polymorphic band. These DNA fragments will be investigated for further analysis in gene involving in its phenotype change.

B0131-ISOLATION OF SPECIFIC BINDING PEPTIDES BY PHAGE DISPLAY TECHNOLOGY

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การผลิตโมโนโคลนอลแอนติบอดีที่จำเพาะต่อเชื้อ *Vibrio parahaemolyticus* โดยใช้เซลล์เป็นแอนติเจน

Production of monoclonal antibodies specific to *Vibrio parahaemolyticus* using whole cell antigen

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บทคัดย่อ: ในการทดลองนี้ได้ทำการผลิตโมโนโคลนอลแอนติบอดีต่อเชื้อ *V. parahaemolyticus* เป็นจำนวน 4 กลุ่ม โดยใช้เซลล์แบคทีเรียทั้งเซลล์ในการปลูกภูมิคุ้มกัน โมโนโคลนอลแอนติบอดีกลุ่มที่ 1 และ 2 แสดงความจำเพาะต่อ *V. parahaemolyticus* (VPV) เพียง 1 ไอโซเลทเท่านั้นโดยไม่ทำปฏิกิริยาต่อ *V. parahaemolyticus* อีก 2 ไอโซเลทที่เหลือ โดยโมโนโคลนอล 2 กลุ่มนี้จะแสดงความจำเพาะต่อโปรตีนที่มีน้ำหนักโมเลกุล 57 กิโลดาลตัน และ 32 กิโลดาลตัน ตามลำดับ โมโนโคลนอลแอนติบอดีกลุ่มที่ 3 สามารถทำปฏิกิริยากับ *V. parahaemolyticus* (VPV) เพียงไอโซเลทเดียวและสามารถแสดงปฏิกิริยาข้ามต่อ *V. vulnificus V. fluvialis V. cholera* และ *V. alginolyticus* โมโนโคลนอลแอนติบอดีกลุ่มที่ 4 สามารถทำปฏิกิริยากับ *V. parahaemolyticus* ทั้ง 3 ไอโซเลท และแสดงปฏิกิริยาข้ามต่อ *Vibrio* spp. อื่นๆ เช่นเดียวกับ โมโนโคลนอลแอนติบอดีกลุ่มที่ 3 โมโนโคลนอลแอนติบอดีทั้ง 4 กลุ่มนี้ สามารถตรวจติดตามการติดเชื้อ *V. parahaemolyticus* ในเนื้อเยื่อกุ้งกุลาดำด้วยวิธี immunohistochemistry จากผลการทดลองนี้แสดงให้เห็นถึงความหลากหลายที่เกิดขึ้นในประชากรของ *V. parahaemolyticus* ซึ่งแสดงให้เห็นได้โดยโมโนโคลนอลแอนติบอดีที่มีความจำเพาะเหล่านี้ อย่างไรก็ตามยังมีความต้องการโมโนโคลนอลแอนติบอดีที่มีความจำเพาะต่อ *V. parahaemolyticus* ไอโซเลทอื่นๆ เพื่อนำมาใช้สำหรับการตรวจติดตามการติดเชื้อแบคทีเรียชนิดนี้

Abstract: Four groups of monoclonal antibodies (MAbs) against *Vibrio parahaemolyticuss* were generated using whole cell of bacteria for immunization. The first and the second groups of monoclonal antibodies demonstrated high specificity that bound to only one isolate of the *V. parahaemolyticus* (VPV) but did not bind to the other two *V. parahaemolyticus* isolates. The MAbs in the first and the second groups bound to proteins at molecular masses of 57 kDa and 32 kDa, respectively. The third group of MAbs also bound one isolate of *V. parahaemolyticus* (VPV) but demonstrated strong cross reactivity to *V. vulnificus, V. fluvialis*, *V. cholera* and *V. alginolyticus*. They bound to a protein of 55 kDa. The fourth group of MAb recognized all three isolates of *V. parahaemolyticus* and cross-reacted with other *Vibrio* spp. recognized by MAbs in the third group. All MAbs can recognize the bacteria in tissues by immunohistochemistry. This evidence indicated the heterogeneity among *V. parahaemolyticus* population that could be differentiated by the specific MAbs. Additional MAbs specific to other isolates of *V. parahaemolyticus* are required to provide specific tools for identification of *V. parahaemolyticus*.

Introduction: *Vibrio parahaemolyticus* is a Gram-negative, motile, oxidase-positive, straight or curved rod-shaped, facultative anaerobe. They form part of the indigenous microflora of aquatic habitats of various salinity, and are the major causative agents for some of the most serious diseases in fish, shellfish and penaeid shrimp (Lightner, 1996). *Vibrio* spp. have also been closely associated with mass mortality of culture prawn in Taiwan (Liu *et al.*, 1996; Song *et al.*, 1993). The diagnosis of vibriosis is usually performed by bacterial culture, isolation and identification. The culture process employs enrichment of the sample in a liquid medium and the subsequent isolation of colonies on

selective plating media. Unfortunately, a number of other *Vibrio* spp. are not only taxonomically similar to *V. parahaemolyticus* but also appear similar in colonial morphology on the selective media. Thus, it has been necessary to utilize additional biochemical tests or other reliable identification methods for *V. parahaemolyticus* recognition. Moreover the bacteriological method has low sensitivity, laborious and time consuming. The use of immunodiagnosis to detect *V. parahaemolyticus* has been reported. Several methods for detection of thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) of *V. parahaemolyticus* which encoded by the *tdh* and *trh* genes, respectively, have been developed. These methods were ELISA, colonial DNA hybridization and polymerase chain reaction (PCR) (Honda *et al.*, 1980; Nishibuchi *et al.*, 1985; Yamamoto *et al.*, 1992; Suthienkul *et al.*, 1995; Karunasagar *et al.*, 1996). The *tdh* and/or *trh* genes of *V. parahaemolyticus* are usually present in clinical isolates of *V. parahaemolyticus* but *tdh* gene was not detected in most environmental strains of this organism. Thus, the above mentioned methods could detect most of clinical isolates of *V. parahaemolyticus* but are unfavorable to detect food or environmental isolates (Nishibuchi *et al.*, 1985; Yamamoto *et al.*, 1992; Kishishita *et al.*, 1992). In this study hybridomas secreting specific monoclonal antibodies to *V. parahaemolyticus* were developed for detection of *V. parahaemolyticus* infection in shrimp.

Methodology: *Vibrio parahaemolyticus* (VPV), kindly provided by Veterinary Medical Aquatic Research Center, Chulalongkorn University, Thailand was used to immunize into 4 Swiss mice for four times at two weeks intervals. The best response mouse was identified by Western blotting and used as spleen cell donor for hybridoma production. The hybridoma clones producing desired antibodies were identified by dot blotting against 3 isolates of *V. parahaemolyticus*, *Vibrio* spp. and other Gram negative bacteria, and then characterized by Western blotting and immunohistochemistry.

Results, Discussion and Conclusion: Eleven hybridoma clones were successfully isolated as established cell lines which can be divided into four groups according to their specificities of the MAbs produced (Table 1; Fig. 1): **MAbs group 1**. consists of seven MAbs and **MAb group 2** consists of one MAb. MAbs from both groups bound to only one isolate of *V. parahaemolyticus* (VPV) and did not show any crossreactivity to the other two *V. parahaemolyticus* isolates and other bacteria. The MAbs in the first and the second groups bound to proteins at molecular masses of 57 and 32 kDa respectively. These MAbs also recognized *V. parahaemolyticus* in the tissues from *V. parahaemolyticus* infected sea bass by immunohistochemistry. **MAbs group 3**. consists of two MAbs that bound to only one isolate of *V. parahaemolyticus* (VPV) but demonstrated cross-reactivity to *V. vulnificus*, *V. fluvialis*, *V. cholera* and *V. alginolyticus*. The MAbs bound to a protein of 55 kDa and recognized antigen in the tissues by immunohistochemistry. **MAb group 4**. consists of one MAb, that recognized all three isolates of *V. parahaemolyticus* and cross reacted to all *Vibrio* spp. recognized by MAbs in the third group. This group of MAbs did not recognize the denatured form of antigen but could bind to the antigen in the tissue.

The sensitivity testing for detection of *V. parahaemolyticus* with dot blot assay was range from 2.5 $X10^5$ to 10^7 CFU/ml depending on the group of MAbs (Table 1). Since the volume of dot blot assay is small (1 µl), the development of other types of enzyme immuno-assay such as sandwich ELISA would improve the sensitivity of the test by over 100 fold since the volume of the sample will be increased by a hundred fold. The *V. parahaemolyticus* specific antibodies (group 1 and 2) can be used for detection of *V. vulnificus* in the tissues from infected prawn by immunohistochemistry.

In conclusion, MAbs specific to one isolate of *V. parahaemolyticus* could be selected. Therefore, further production of monoclonal antibodies against different isolates of *V. parahaemolyticus* is need in order to provide immunological tools for identification of various serotypes of *V. parahaemolyticus*.

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Table 1. Specificities of monoclonal antibodies. The binding of antibodies to various bacteria was determined by dot blotting using bacteria at approximately 10^8 CFU/ml: VPV = *V. parahaemolyticus* isolate 1, VPB = *V. parahaemolyticus* isolate 2, VPM = *V. parahaemolyticus* isolate 3, VV = *V. vulnificus*, VF = *V. fluvialis*, VC = *V. cholera*, VA = *V. alginolyticus*. The intensity of staining was arbitrary scored as +++ = very intense staining, + = light staining, - = not staining. IHC = immunohistochemistry

Group	Mab (isotype)	sensitivity of dot blotting (CFU/ml)	detected antigen by Western blotting (kDa)	IHC	bacterial immunoreactivity (Dot blotting)
1	VPV 22, 353, 375, 376, 560, 665 (M) 481(G2b)	2.5×10^5	57	++	VPV(+++)
2	VPV 767(M)	5×10^5	32	+	VPV(+++)
3	VPV 486, 703 (G2b)	1×10^7	55	++	VPV, VV, VF, VC, VA (+++)
4	VPV 373(M)	5×10^5	-	+	VPV, VPB, VPM, VV, VF, VC, VA (+++)

PROCEEDINGS

The International Conference on Shrimp Biotechnology: New Challenges through Thai Shrimp Industry

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National Science and Technology Development Agency (NSTDA)
Ministry of Science and Technology (MOST)

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P-SHRIMP-20

Differentiation of Two Isolates of *Vibrio vulnificus* with Monoclonal Antibody

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Abstract

Five groups of monoclonal antibodies (MAbs) against two isolates of *Vibrio vulnificus* were generated using whole cell of bacteria for immunization. The first group of monoclonal antibodies demonstrated high specificity to only one isolate of the *V. vulnificus* (VVC) but did not bind to the other two *V. vulnificus* isolates. They bound to a protein at molecular mass of 66 kDa. The second group of antibodies bound to the same protein as recognized by the first group of MAbs but demonstrated light cross reactivity to *V. fluvialis*. The third, fourth and fifth groups of MAbs recognized only the second isolate of *V. vulnificus* (VVB). The third group bound to only native protein and did not recognize denatured protein by Western blotting. The fourth and the fifth groups bound to proteins with molecular masses of 6.5 and 37 kDa, respectively. MAbs in group 1 and 4 can recognized the bacteria in tissues by means of immunohistochemistry. This evidence indicated the heterogeneity among *V. vulnificus* population that could be differentiated by MAbs. Additional MAbs specific to other isolates of *V. vulnificus* are required to provide specific tools for identification of this bacteria.

Introduction

Vibrio vulnificus, a Gram negative bacterium is a natural inhabitant of estuarine waters, and possesses significant infections in fish (Esteve-Gassent et al., 2003), shrimp (Chanratchakool *et al.*, 1995), and health threat to human from consumption raw seafood (Sumner and Ross 2002) or via wound infection (Midturi *et al.*, 2005). Three biotypes designated 1,2 and 3 have been established based upon characteristics, such as indole production, host specificity, serotype (Tison *et al.*, 1982) and genetic subtyping (Bisharat *et al.*, 1999). Those in biotype 1 are pathogenic to human, as opposed to biotype 2 that are commonly infected eel while biotype 3 appeared to be pathogen of both (Amaro and Biosca, 1996; Bisharat et al., 1999; Tison et al., 1982). Biochemical identification and application of selective-differential media specific for *V. vulnificus* are time consuming and still required immunological and molecular identification for confirmation of the identity of the organism (Harwood *et al.*, 2004). This study, is the attempt to develop the specific monoclonal antibody to *V. vulnificus* in order to provide tools for rapid, accurate and sensitive detection of *V. vulnificus*.

Methodology

Two out of three isolates of *V. vulnificus* (VVC, VVB) were used to immunize into four mice at two weeks intervals and only one of the best responded mouse was used as spleen donor for hybridoma production. The hybridoma clones producing antibodies specific to *V. vulnificus* were selected by dot blotting against three isolates of *V. vulnificus* and other Gram negative bacteria, and further screened by Western blotting and immunohistochemistry against *V. vulnificus* infected shrimp.

Results and Discussion

After the fourth immunization, the antisera from four mice at dilution of 1:20,000 were preabsorbed with the lysate of *V. harveyi* before determination of the specificity by Western blotting. All antisera demonstrated series of numerous immunoreactive bands without any cross-reactivity to *V. harveyi* and *Escherichia coli*. The serum from all mice demonstrated the distinct immunoreactive bands of proteins for each isolate of *V. vulnificus* used for immunization. Therefore, one mouse from each group was used as spleen donor for hybridoma production.

From each fusion, the cell mixed were laid in 30 microculture plates and about half (1500 wells) contained hybridoma colonies. Approximately 200 wells gave positive reaction on the first screening by dot blotting against *V. vulnificus* (VVC or VVB), after the second screening with Western blotting strongly positive immunoreactivity producing clones with high specificity and some limited crossreactivity to the related bacteria were selected and recloned. Only 22 hybridoma clones were selected, recloned and established as high stability cell lines which can be divided into 5 groups according to their specificities (Table1). The first and the second groups were from mice immunized with *V. vulnificus* VVC and the other 3 groups were from mice immunized with *V. vulnificus* VVB. **MAb group 1.** consists of nine MAbs, these MAbs bound only to one isolate of *V. vulnificus* (VVC) and did not show any crossreactivity to the other two isolates of *V. vulnificus* and other bacteria. They bound to a protein with molecular mass of 66 kDa. These MAbs also recognized *V. vulnificus* in the tissues of infected sea bass by means of immunohistochemistry. **MAb group 2.** consists of two MAbs that bound to only one isolate *V. vulnificus* (VVC) and the same protein as recognized by MAbs in group 1 but demonstrated light cross-reactivity to *V. fluvialis* and did not recognize the bacteria in tissue by immunohistochemistry. **MAb group 3.** consists of four MAbs, they recognized only one isolate of *V. vulnificus* (VVB). This group of MAbs did not recognize the denatured form of antigen and antigen in tissue, therefore, the molecular nature of the bound molecule by these antibodies was not further characterized. **MAb group 4.** consists of one MAb that bind to only one isolate of *V. vulnificus* (VVB) similar to the third group of MAb, but this antibody recognized a broad band of protein at molecular mass of 6.5 kDa. This antibody also bound lightly to the bacteria in the tissue. **MAb group 5.** consists of 6 MAbs that bound to only one isolate of *V. vulnificus* similar to MAbs in the third and the fourth groups but bound to a 37 kDa protein. These MAbs did not recognize the bacteria in tissues. The sensitivity testing for detection of *V. vulnificus* with dot blot assay was range from 5 $X10^4$ to 5 $X10^6$ CFU/ml depending on the group of MAbs (Table 1). Since the volume of dot blot assay is small (1 µl), the development of other type of enzyme immuno-assay such as sandwich ELISA would improve the sensitivity of the test approximately by over 100 fold since the volume of the sample will be increased by a hundred fold. The *V. vulnificus* specific antibodies (group 1 and 4) can be used for detection of *V. vulnificus* in the tissues from infected prawn by immunohistochemistry. The MAbs in the group 1, 3, 4 and 5 were highly specific to only one isolate of *V. vulnificus* (VVC or VVB) with out any cross-reactivity to the other two *V. vulnificus* isolates. This evidence indicated the heterogeneity of *V. vulnificus* which could be differentiated by specific MAbs. Therefore, MAbs specific to other *V. vulnificus* isolates are required in order to provide specific tools for identification of various serotypes of *V. vulnificus*.

Table 1. List of bacterial isolates and sources used in this study. VMARC = Veterinary Medical Aquatic Research Center, Chulalongkorn University; AAHRI Aquatic Animal Health Research Institute, Department of Fisheries, Ministry of Agriculture; NICMB = Khonkaen University; DMSC = Department of Microbiology, Faculty of Science, Mahidol University; DMSC = Department of Microbiology, Faculty of Science, Chulalongkorn University, DABU = Department of Aquatic Science, Burapha University, DMST = Department of Medical Science, Ministry of Public Health ; BLP Co. Ltd.

Bacteria	Sources
Vibrio vulnificus (VVC)	DMSC
Vibrio vulnificus (VVB)	DABU
Vibrio vulnificus (VVBS)	DABU
Vibrio parahaemolyticus	DMSC
Vibrio mimicus DMST 15142	DMST
Vibrio harveyi 639	DMSM
Vibrio fluvialis	DMSC
Vibrio cholerae	DMSC
Vibrio alginolyticus DMST 14800	DMST
Vibrio penaeicida	DMSC

Aeromonas hydrophila 1234	VMARC
Aeromonas sobria DMST 12446	DMST
Aeromonas caviae 13016	NICMB
Plesiomonas shigelloides	AAHRI
Escherichia coli ATCC 25922	BLP Co. Ltd.
Salmonella Typhi	DMSM
Salmonella Typhimurium ATCC 1408	BLP Co. Ltd.
Salmonella Enteritidis DMST7108	DMST
Shigella flexneri	DMSM
Morganella morganii	DMSM
Enterobacter cloacae	DMSM
Klebsiella pneumoniae	DMSM
Proteus vulgaris	DMSM
Pseudomonas aeruginosa	DMSM
Enterococcus faecalis ATCC 7080	DMSC

Table 2. Specificities of monoclonal antibodies. The binding of antibodies to various bacteria was determined by dot blotting using bacteria with approximately 10^8 CFU/ml: VVC = *V. vulnificus* isolate 1, VVB = *V. vulnificus* isolate 2, VF = *V. fluvialis*. The intensity of staining was arbitrary scored as +++ = very intense staining, + = light staining, ± = very light staining, - = not staining. IHC = immunohistochemistry

Group	MAb (isotype)	Sensitivity of Dot Blotting (CFU/ml)	Detected antigen by Western blotting (kDa)	IHC	Bacterial immunoreactivity (Dot blotting)
1.	VVC 23, 38 (G1), 43,121,118 (G2a), 14,40,88,97 (G2b)	5×10^6	66	+	VVC (+++)
2.	VVC71, 90 (G2a),	5×10^6	66	-	VVC (+++), VF (++)
3.	VVB34, 202 (G2b), 3,152 (G3)	8×10^5	-	-	VVB (+++)
4.	VVB158 (G2b)	5×10^4	6.5	+	VVB (+++)
5.	VVB 4, 12, 47, 48, 110,174 (G1)	5×10^6	37	-	VVB (+++)

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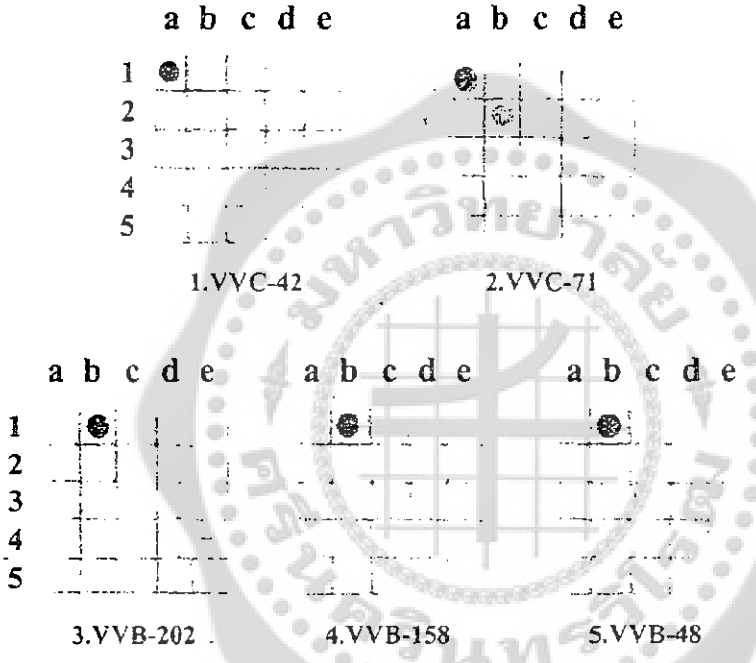
Figure Legends

Figure 1. Cross-reactivity of MAbs assayed by dot blot. Heated killed bacteria (10^8 CFU/ml) were spot on nitrocellulose membrane (1ul/spot) and treated with various MAbs (only one of representative for each group were demonstrated).



Row 1: (a) *Vibrio vulnificus* (VVC); (b) *Vibrio vulnificus* (VVB); (c) *Vibrio vulnificus* (VVBS); (d) *Vibrio parahemolyticus* ; (e) *Vibrio mimicus*
Row 2: (a) *Vibrio harveyi* 639 ; (b) *Vibrio fluvialis* ; (c) *Vibrio cholerae* ; (d) *Vibrio alginolyticus* ; (e) *Vibrio penaeicida* ;
Row 3:(a) *Aeromonas hydrophila* VMARC 1234 ; (b) *Aeromonas sobria* DMST 12446 ; (c) *Aeromonas caviae* NICMB 13016 ; (d) *Plesiomonas shigelloides* ; (e) *Escherichia coli* ATCC 25922
Row 4: (a) *Salmonella* Typhi ; (b) *Salmonella* Typhimurium ; (c) *Salmonella* Enteritidis ; (d) *Shigella flexneri* ; (e) *Morganella morganii*
Row 5: (a) *Enterobacter cloacae* ; (b) *Klebsiella pneumoniae* ;(c) *Proteus vulgaris* ; (d) *Pseudomonas aeruginosa* ; (e) *Enterococcus faecalis*

Figure 2. SDS-PAGE and Western blot analysis. *V. vulnificus* (VVC), *V. vulnificus* (VVB) were electrophoresed and stained with Coomassie blue or trsnsferred to nitrocellulose membrane and treated with MAb in group 1 (A), group 2 (B), group 4 (C), group 5 (D) as indicated in Fig. 1 except the MAb in group 3, low molecular weight standard proteins (S).

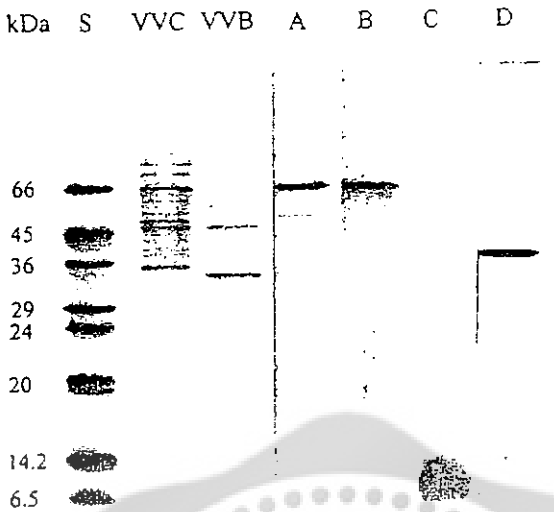
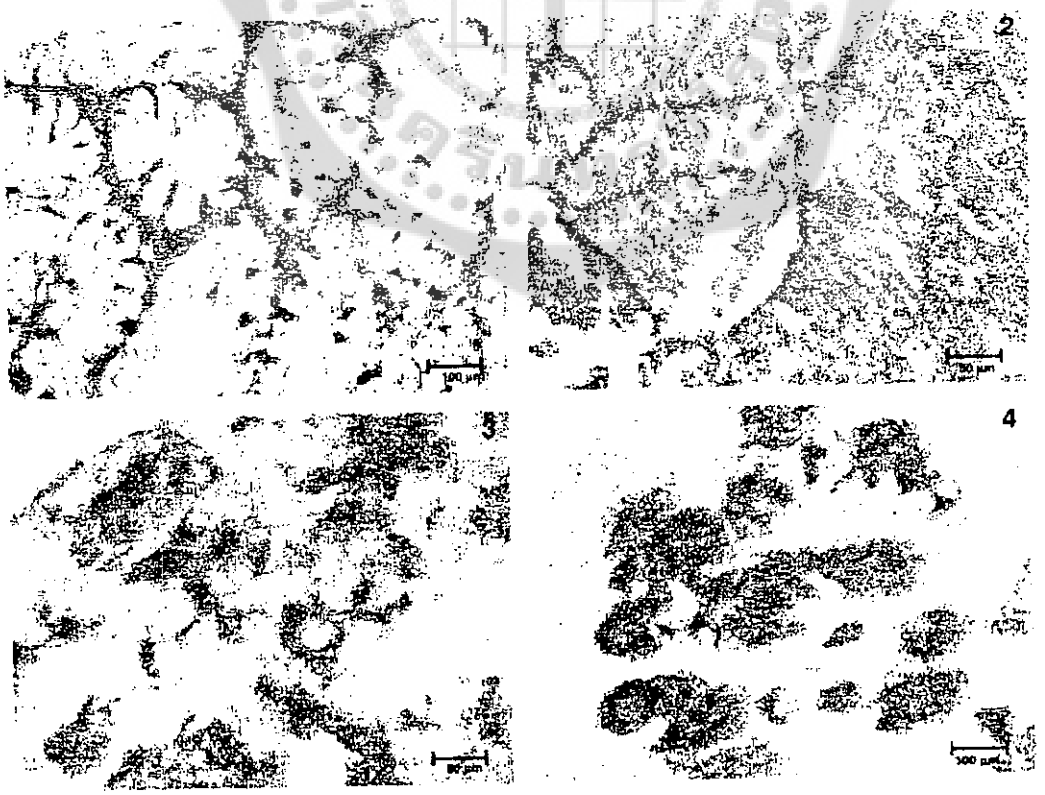


Figure 3. Immunohistochemistry of infected tissue from *P. monodon*: hepatopancreas (1), muscle (2), mouth appendage (3), subcuticular gland (4). 1 and 2 were from shrimp injected with *V. vulnificus* (VVC) and treated with VVC42 monoclonal antibody. 3 and 4 were from shrimp injected with *V. vulnificus* (VVB) and treated with VVB158 monoclonal antibody.



P-SHRIMP-21

Generation of Monoclonal Antibodies Specific to *Vibrio parahaemolyticus.*

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ur groups of monoclonal antibodies (MAbs) against *Vibrio parahaemolyticuss* were generated ing whole cell of bacteria for immunization. The first and the second groups of monoclonal tibodies demonstrated high specificity that bound to only one isolate of the *V. parahaemolyticus* PV) but did not bind to the other two *V. parahaemolyticus* isolates. The MAbs in the first and the cond groups bound to proteins at molecular masses of 57 kDa and 32 kDa, respectively. The third oup of MAbs also bound one isolate of *V. parahaemolyticus* (VPV) but demonstrated strong cross activity to *V. vulnificus, V. fluvialis, V. cholera* and *V. alginolyticus*. They bound to a protein of 55 Da. The fourth group of MAb recognized all three isolates of *V. parahaemolyticus* and cross-reacted th other *Vibrio* spp. recognized by MAbs in the third group. All MAbs can recognize the bacteria in sues by immunohistochemistry. This evidence indicated the heterogeneity among *V. rahaemolyticus* population that could be differentiated by the specific MAbs. Additional MAbs ecific to other isolates of *V. parahaemolyticus* are required to provide specific tools for entification of *V. parahaemolyticus*.

troduction

brio parahaemolyticus is a Gram-negative, motile, oxidase-positive, straight or curved rod-shaped, cultative anaerobe. They form part of the indigenous microflora of aquatic habitats of various linity (Colwell, 1984), and are the major causative agents for some of the most serious diseases in sh, shellfish and penaeid shrimp (Austin and Austin, 1987; de la Pena *et al.*, 1993; Egidius, 1987; ee *et al.*, 1996; Lightner, 1988; Muroga, 1995; Sugumar *et al.*, 1998). *Vibrio* spp. have also been osely associated with mass mortality of culture prawn in Taiwan (Liu *et al.*, 1996; Song *et al.*, 993). The diagnosis of vibriosis is usually performed by bacterial culture, isolation and entification. The culture process employs enrichment of the sample in a liquid medium and the bsequent isolation of colonies on selective plating media. Unfortunately, a number of other *Vibrio* sp. are not only taxonomically similar to *V. parahaemolyticus* but also appear similar in colonial orphology on the selective media. Thus, it has been necessary to utilize additional biochemical tests other reliable identification methods for *V. parahaemolyticus* recognition (Kaper *et al.*, 1980; Mclaughlin, 1995). Moreover the bacteriological method has low sensitivity and is laborious and me consuming. The use of immunodiagnosis to detect *V. parahaemolyticus* has been reported. everal methods for detection of thermostable direct hemolysin (TDH) and TDH-related hemolysin TRH) of *V. parahaemolyticus* which encoded by the *tdh* and *trh* genes, respectively, have been eveloped. These methods were ELISA, colonial DNA hybridization and polymerase chain reaction PCR) (Honda *et al.*, 1980; Nishibuchi *et al.*, 1985; Yamamoto *et al.*, 1992; Suthienkul *et al.*, 1995; Karunasagar *et al.*, 1996; Kowcachaporn *et al.*, 1997). The *tdh* and/or *trh* genes of *V. arahaemolyticus* are usually present in clinical isolates of *V. parahaemolyticus* but *tdh* gene was not etected in most environmental strains of this organism (Miyamoto *et al.*, 1969; Shirai *et al.*, 1990). Thus, the above mentioned methods could detect most of clinical isolates of *V. parahaemolyticus* but re unfavorable to detect food or environmental isolates (Nishibuchi *et al.*, 1985; Yamamoto *et al.*, 992; Kishishita *et al.*, 1992). In this study hybridomas secreting specific monoclonal antibodies to *V. arahaemolyticus* will be developed for detection of *V. parahaemolyticus* infection in shrimp.

Materials and Methods

Bacterial culture and antigen preparation

Vibrio parahaemolyticus (VPV) was isolated kindly provide by Veterinary Medical Aquatic Research Center, Chulalongkorn University, Thailand. The second isolate of *V. parahaemolyticus* (VPB) was kindly provided by Dr. Praparsiri Barnet, Department of Aquatic Science, Faculty of Science, Burapha University. The third isolate of *V. parahaemolyticus* (VPM) was from Department of Medical Sciences, Ministry of Public Health, Thailand. The sources of other bacteria used for determination of cross-reactivity were indicated in Table 1. The bacteria were grown with agitation at 37°C in tryptic soy broth (TSB; Merck) supplement with 2% (w/v) NaCl. to log phase. The bacteria were harvested by centrifugation at 3,500g for 20 min at 4 °C. Bacterial pellets were washed twice with sterile 0.15 phosphate buffered saline pH7.2 (PBS), re-suspended in PBS, heated kill at 60 °C for 30 min, and adjusted to the OD of 1 at 610 nm. The bacterial suspension was divided into small aliquots and stored at-70°C until used.

One isolates of *V. parahaemolyticus* (VPV) was prepared for immunization in two forms, heated kill and denatured form.. The denatured form was prepared by mixing the bacteria with treatment buffer (4 % SDS, 10%mercaptoethanol) at ratio of 1:1 (v/v) and boiled for 1 min, then dialyzed againist three changes of PBS at 12 hr intervals.

Immunization

Four Swiss mice were injected intraperitoneally with 50 ul of a mixture of heat killed, and SDS-mercapto-ethanol treated 10^8 CFU/ml of *V. parahaemolyticus* (VPV) at ratio of 1:1, mixed with complete Freund's adjuvant at a ratio of 1:1. Mice were subsequently injected 3 more times with the bacteria mixed with incomplete Freund's adjuvant at two wk intervals. One week after the fourth injection, mouse antisera were collected, preabsorbed with excess *V. parahaemolyticus* and tested with all three isolates *V. parahaemolyticus* by Western blot. The best performing mouse was later boosted 3 d before hybridoma production.

Hybridoma production.

A cell fusion protocol was adapted from the method developed by Kohler & Milestein (1975) with modifications described by Mosmann et al. (1979). A P3X myeloma cell line was used as the fusion partner. Fusion products from 1 mouse were plated on 30 microculture plates (96 well/ plate). After identification of the positive cultures by screening methods; dot blotting, Western blotting and immunohistochemistry as described below, cells were cloned by the limiting dilution method and stored in liquid nitrogen.

Dot-blotting

Cell suspension of heated killed of various bacteria approximately 10^8 CFU/ml were used for screening. Heat killed bacterial samples (1 ul/spot) were applied to nitrocellulose membrane, baked at 60°C for 10 min, and incubated in hybridoma conditioned medium from the culture diluted 1:20 dilution in 5% blotto (5% nonfat drymilk, 0.1% Triton X-100 in PBS) for 4 h. After extensive washing in 0.5% Blotto, 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 Blotto 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). Hybridoma cultures that displayed immunoreactivity against *V. parahaemolyticus* , were confirm for bacterial specificity by Western blot and immunohistochemistry before cloning and cryopreserving for further investigation

SDS-PAGE and Western blot analysis

V. parahaemolyticus and other bacterial lysates were separated by 15% SDS-PAGE according to the method described by Laemmli (1970). Samples were electrophoresed for 6 h at 30 V and gels were stained using Coomassie brilliant blue R-250. For Western blotting, samples resolved by SDS-PAGE

were electro-blotted onto nitrocellulose membrane using Transblot apparatus (Bio-Rad). The nitrocellulose membrane was incubated in 5% Blotto for 10 min, treated with 1:200 hybridoma conditioned-medium for 4 h and then processed as described above in the dot blotting section. Low molecular weight markers (Bio-Rad) were used as standards.

Immunohistochemistry.
The black tiger shrimp *Penaeus monodon* weight 15-20g was artificially infected with *V. parahaemolyticus* (VPV) by injection via the second walking leg. When the shrimp exhibit pathogenic symptoms such as slow moving and loss of balance, they were killed in the cold water and the head were cut and fixed in Davidson's fixative for 24 h and processed for paraffin sectioning.

Serial sections (8 um thickness) were prepared and processed for indirect immunoperoxidase staining using various MAbs and GAM-HRP diluted 1:1000 with 10% calf serum in PBS. Peroxidase activity was revealed by incubation with 0.03% DAB and 0.006% hydrogen peroxide in PBS. Preparations were counter-stained with haematoxylin and eosin Y (H&E), dehydrated in graded ethanol series, clear in xylene and mounted in permount (Sithigorngul et al., 2000, 2002). Positive immunoreactivity was visualized as brown coloration against the pink and purple of H&E.

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

Sensitivity of MAb for detection of V. parahaemolyticus determined by dot-blot.
Serial dilution of *V. parahaemolyticus* (begin from 10^8CFU/ml) in PBS was performed and 1 ul of each dilution was spotted onto a nitrocellulose membrane before fixing in 10% formalin for 10 min and processing for dot blotting using various MAbs as described above. The lowest bacterial dilution that showed distinct and clear immunoreactivity was determined.

Results and Discussion
After the fourth immunization, the antisera from four mice at dilution of 1:20,000 were preabsorbed with lysate of *Vibrio harveyi* before determination of the specificity by Western blot. All antisera demonstrated series of numerous bands without any cross-reactivity to *V. harveyi* and *Escherichia coli*. The serum from all mice demonstrated the distinct immunoreactive bands of protein for each isolate of *V. parahaemolyticus* used for immunization. Therefore, one mouse was used as spleen donor for hybridoma production.

From each fusion, the cell mixed were laid in 30 microculture plates and about half (1500 wells) contained hybridoma colonies from which approximately 200 wells gave positive reaction on the first screening by dot blot against *V. parahaemolyticus* (VPV), after the second screening with Western blot strong positive immunoreactivity antibody producing clones with high specificity and some limited crossreactivity to the related bacteria were selected and recloned. Only 11 hybridoma clone were selected, recloned and established high stability cell lines which can be divided into 4 groups according to their specificities (Table 2, Fig.1, 2, 3).

MAb group 1 consists of seventh MAbs and MAb group 2 consists of one MAb, MAbs from both group bound only to one isolate of *V. parahaemolyticus* (VPV) and did not show any crossreactivity to the other two isolates of *V. parahaemolyticus* and other bacteria (Fig.1). They bound to a protein molecular mass of 57 and 32 kD consecutively (Fig.2). These MAbs were also able to recognized *V. parahaemolyticus* in the tissues from infected sea bass by means of immunohistochemistry (Fig 3).

MAb group 3 consists of two MAbs that bound only one isolates *V. parahaemolyticus* (VPV) but demonstrated cross-react to *V. vulnificus, V. fluvialis, V. cholera* and *V. alginolyticus* (Fig. 1). The MAbs bound to a protein of 55 kD (Fig.2) and recognized antigen in the tissues by means of immunohistochemistry.

MAb group 4 consists of one MAbs, that recognized all three isolates of *V. parahaemolyticus* and cross reacted to all *Vibrio* spp. recognized by MAb in the third group.. This group of MAbs did not recognize the denatured form of antigen but could bind to the antigen in tissue.

The sensitivity test for detection of *V. parahaemolyticus* with dot blot assay was range from 2.5 $X10^5$ to 10^7 CFU/ml depending on the group of MAbs (Table 2). Since the volume of dot blot assay is small (1 µl), the development of other type of enzyme immuno-assay such as sandwich ELISA would improve the sensitivity of the by over 100 fold since the volume of the sample increases by hundred fold. The *V. parahaemolyticus* specific antibodies (group 1 and 2) can be used for detection of *V. vulnificus* in the tissues from infected prawn by means of immunohistochemistry.

Table 1. List of bacterial isolates and sources used in this study. VMARC = Veterinary Medical Aquatic Research Center, Chulalongkorn University; AAHRI Aquatic Animal Health Research Institute, Department of Fisheries, Ministry of Agriculture; NICMB = Khonkaen University; DMSM = Department of Microbiology, Faculty of Science, Mahidol University; DMSC = Department of Microbiology, Faculty of Science, Chulalongkorn University, DMST = Department of Medical Science, Ministry of Public Health; BLP Co. Ltd.

Bacteria	Sources
Aeromonas hydrophila 04082	AAHRI
A. sobria 1333	NICMB
A. caviae 1444	NICMB
Plesiomonas shigelloides	AAHRI
Vibrio parahaemolyticus (V)	VMARC
Vibrio parahaemolyticus(B)	DMSC
Vibrio parahaemolyticus (M)	DMSC
V. vulnificus	DMSC
V. fluvialis	DMSC
V.alginolyticus	DMSC
V.harveyi	DMSC
V.mimicus	DMSC
V.cholerae	DMSC
V.penaeicida	DMSC
Pseudomonas aeruginosa	DMSC
Enterococus faecalis ATCC7080	DMSC
Enterobacter cloacae	DMSM
Escherichia coli ATCC 25922	BLP Co. Ltd.
Klebsiella pneumoniae	DMSM
Morganella morganii	DMSM
Proteus vulgais	DMSM
Salmonella Enteritidis DMST710	DMST
Salmonella Typhi	DMSM
Salmonella Typhimurium ATCC 1408	BLP Co. Ltd.
Shigella flexneri	DMSM

Table 2. Specificity of monoclonal antibodies. The binding of antibodies to various bacteria was determined by dot blot using bacteria at approximately 10^8 CFU/ml: VPV = *V. parahaemolyticus* isolate 1, VPB = *V. parahaemolyticus* isolate 2, VPD = *V. parahaemolyticus* isolate 3, VV = *V. vulnificus*, VF = *V. fluvialis*, VC = V. cholera, VA = *V. alginolyticus*. The intensity of staining was arbitrary scored as +++ = very intense staining, + = light staining, - = not staining. IHC = immunohistochemistry

Group	Mab (isotype)	sensitivity of dot blotting (CFU/ml)	detected antigen by Western blotting (kDa)	IHC	bacterial immunoreactivity (Dot blotting)
1	VPV 22, 353, 375, 376, 560, 665 (M) 481(G2b)	2.5×10^5	57	++	VPV(+++)
2	VPV 767(M)	5×10^5	32	++	VPV(+++)
3	VPV 486, 703 (G2b)	1×10^7	55	+	VPV, VV, VF, VC, VA (+++)
4	VPV 373(M)	5×10^5	-	+	VPV, VPB, VPM, VV, VF, VC, VA (+++)

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Figure Legends

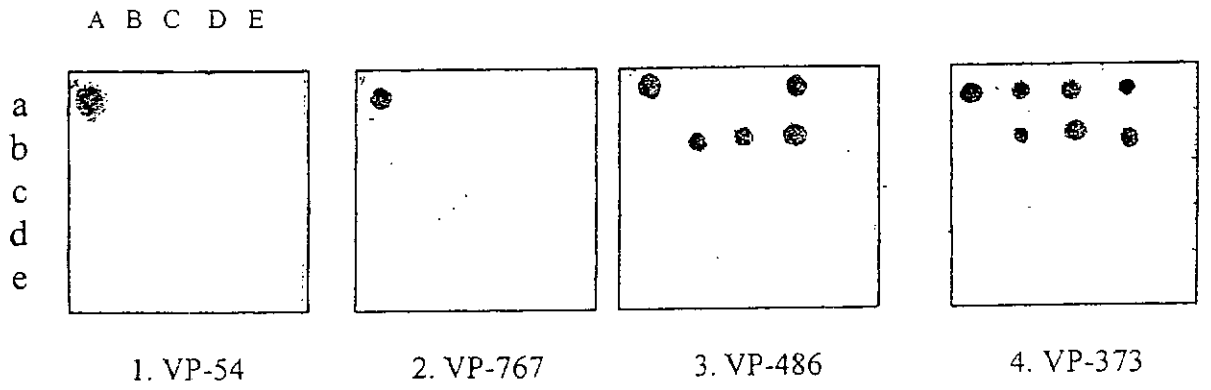

Figure 1. Cross-reactivity of MAbs assayed by dot blot. Heated killed bacteria (10^9 CFU/ml) were spot on nitrocellulose membrane (1ul/spot) and treated with various MAbs (only one of representative for each group were demonstrated).

Column A: Row (a) *V. parahaemolyticus* (V) (b) *V. parahaemolyticus* (B) (c) *V. parahaemolyticus* DMST5665 (M) *V. vulnificus* (e) *V. mimicus*
Column B: Row (a) *V. harveyi* (b) *V. fluvialis* (c) *V. cholera* (d) *V. alginolyticus* (e) *V.penaeicida*
Column C: Row (a) *Aeromonas hydrophila* (b) *A. sorbria* (c) *A. caviae* (d) *Plesiomonas shigelloides* (e) *Escherichia coli*
Column D: Row (a) *Salmonella Typhi* (b) *S. Typhimurium* (c) *S. Enteritidis* (d) *Shigella flexneri* (e) *Morganella morganii*
Column E: Row (a) *Enterobacter cloacae* (b) *Klebsiell. pneumoniae* (c) *Proteus vulgaris* (d) *Pseudomonas aeruginosa* (e) *Enterococus faecalis*

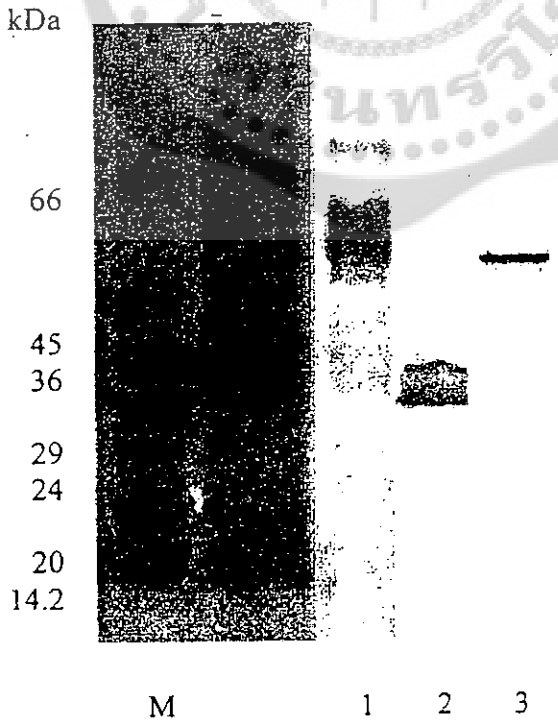

Figure 2. SDS-PAGE and Western blot analysis. Heated killed *V. parahaemolyticus* (VPV) homogenate was separated by SDS-PAGE and transferred to nitrocellulose membrane and treated with 4 MAbs as indicated in Fig. 1 except the MAb in group 4.

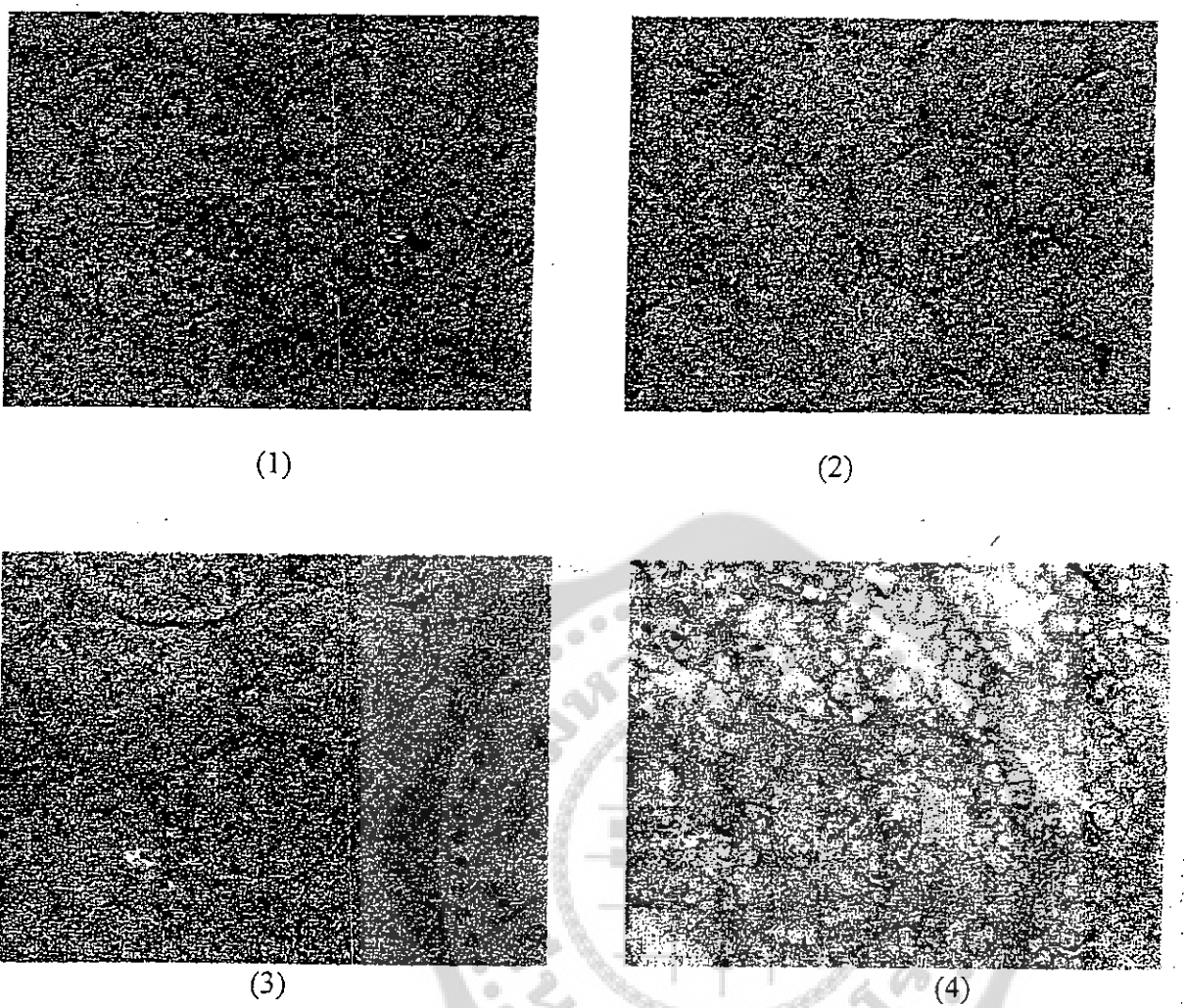

Figure 3. Immunohistochemistry Indirect immunoperoxidase staining of tissues from experimental infected *P. monodon*, using representative MAbs from each group 1. VP-54, 2. VP-767, 3. VP-486, 4. VP-373