ความชุกและรูปแบบการดื้อยาต้านจุลชีพของเชื้อ *Vibrio parahaemolyticus* และ *Vibrio vulnificus* ที่แยกได้จากหอยตะโกรมในประเทศไทย

นางสาวรุ่งอรุณ คันโธ

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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PREVALENCE AND ANTIMICROBIAL RESISTANT PATTERNS OF *VIBRIO PARAHAEMOLYTICUS* AND *VIBRIO VULNIFICUS* ISOLATED FROM PACIFIC OYSTERS (*CRASSOSTREA BELCHERI*) IN THAILAND

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A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title PREVALENCE AND ANTIMICROBIAL RESISTANT PATTERNS OF *VIBRIO PARAHAEMOLYTICUS* AND *VIBRIO VULNIFICUS* ISOLATED FROM PACIFIC OYSTERS (*CRASSOSTREA BELCHERI*) IN THAILAND

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รุ่งอรุณ คันโธ : ความชุกและรูปแบบการดื้อยาต้านจุลชีพของเชื้อ *Vibrio parahaemolyticus* และ *Vibrio vulnificus* ที่แยกได้จากหอยตะโกรมในประเทศไทย (PREVALENCE AND ANTIMICROBIAL RESISTANT PATTERNS OF *VIBRIO PARAHAEMOLYTICUS* AND *VIBRIO VULNIFICUS* ISOLATED FROM PACIFIC OYSTER (*CRASSOSTREA BELCHERI*) IN THAILAND, อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.น.สพ.ดร. ธงชัย เฉลิมชัยกิจ, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ. นพ.อนันต์ จงเถลิง, 129 หน้า

V. parahaemolyticus และ *V. vulnificus* เป็นสาเหตุสำคัญของโรคอาหารเป็นพิษอันเนื่องมาจากการบริโภคอาหารทะเล ซึ่งนับว่าเป็นปัญหาสำคัญทางด้านสาธารณสุขในหลายๆ ประเทศ เชื้อ *V. parahaemolyticus* ทำให้เกิดโรคกระเพาะอาหารและลำไส้อักเสบ ส่วนเชื้อ *V. vulnificus* มักเป็นสาเหตุของการติดเชื้อในกระแสโลหิตซึ่งมีอัตราการตายที่สูง สำหรับหอยนางรมหรือหอยตะโกรมนับว่าเป็นอาหารทะเลที่นิยมบริโภคดิบ หากหอยมีการปนเปื้อนเชื้อเหล่านี้ อาจทำให้ผู้บริโภคเสี่ยงต่อการได้รับเชื้อเข้าสู่ร่างกายได้ ดังนั้นการศึกษาในด้านความชุก ปริมาณ และการดื้อต่อสารต้านจุลชีพของเชื้อ *V. parahaemolyticus* และ *V. vulnificus* ในหอยตะโกรมอาจใช้เป็นข้อมูลทางด้านคุณภาพของหอยตะโกรมสำหรับการบริโภค การศึกษานี้ได้ทำการเก็บตัวอย่างหอยตะโกรมจากอ่าวบ้านดอน จังหวัดสุราษฎร์ธานีซึ่งเป็นแหล่งผลิตหอยตะโกรมที่สำคัญของประเทศไทย จำนวน 360 ตัวอย่างจาก 5 บริเวณคือ ไชยา เฉงอะ กระแดะ ท่าทอง และกระแดะแจะ ในช่วงเดือนเมษายน 2549 ถึงมีนาคม 2550 เพื่อนำมาศึกษาหาปริมาณเชื้อ *V. parahaemolyticus* และ *V. vulnificus* ด้วยวิธี MPN (FDA-BAM, 2004) และทดสอบความไวรับของเชื้อต่อยา Doxycycline, Norfloxacine, Ciprofloxacine และ Cefotaxime ด้วยวิธี agar dilution (CLSI, 2008) การศึกษาหาปริมาณเชื้อด้วยวิธี MPN มีการทดสอบยืนยันสปีชีส์โดยวิธี PCR โดยใช้ *vvhA* gene สำหรับยืนยันสปีชีส์เชื้อ *V. vulnificus* และวิธี multiplex PCR (*tlh*, *tdh*, *trh* gene) สำหรับเชื้อ *V. parahaemolyticus* โดย *tlh* gene ซึ่งเป็นยีนที่จำเพาะต่อสปีชีส์ของเชื้อ *V. parahaemolyticus* ส่วน *tdh* เป็นยีนที่เกี่ยวข้องกับการสร้าง thermal direct hemolysin (TDH) และ *trh* gene เกี่ยวข้องกับ TDH-relate hemolysin สำหรับยืนยันการเป็นเชื้อก่อโรค ซึ่ง Kanagawa phenomenon เป็นการทดสอบการสร้าง TDH บนอาหารเลี้ยงเชื้อ การสร้างเอนไซม์ urease เป็นการบ่งชี้การมียีน *trh* ของเชื้อ สำหรับการทดสอบยืนยันสปีชีส์ด้วยวิธี PCR สามารถแยกเชื้อ *V. parahaemolyticus* ได้ 1,457 isolates แต่ไม่พบ *tdh* และ/หรือ *trh* gene สอดคล้องกับการแสดงผลลบของ Kanagawa phenomenon และการสร้าง urease โดยเชื้อ *V. vulnificus* 609 isolates พบ *vvhA* gene การทดสอบการสร้างเอนไซม์ protease, gelatinase, DNase, lipase, lecithinase, mucinase และ elastase อาจจะเป็นปัจจัยที่เกี่ยวข้องกับความรุนแรงของเชื้อในการทำให้เกิดโรค ผลการศึกษาตัวอย่างหอยตะโกรมจำนวน 360 ตัวอย่าง พบเชื้อ *V. parahaemolyticus* จำนวน 172 ตัวอย่าง (47.78%) พบเชื้อ *V.vulnificus* จำนวน 15 ตัวอย่าง (4.17%) และพบทั้ง *V. parahaemolyticus* และ *V. vulnificus* จำนวน 84 ตัวอย่าง (23.3%) ปริมาณเชื้อ *V. parahaemolyticus* พบสูงสุดในเดือนพฤษภาคม ในตัวอย่างหอยตะโกรมจากไชยา เฉงอะ และกระแดะ โดยพบมากกว่า 10^7 MPN/g สำหรับเชื้อ *V. vulnificus* พบว่ามีปริมาณต่ำกว่า 3.0 MPN/g ในเดือนสิงหาคม กุมภาพันธ์ และมีนาคม แต่ในเดือนธันวาคมและมกราคม พบว่าปริมาณเชื้อสูงมากกว่า 10^4 MPN/g ปริมาณ *V. parahaemolyticus* จะมากกว่า *V. vulnificus* ในทุกตัวอย่างที่เก็บจากบริเวณเดียวกัน ยกเว้นในเดือนธันวาคมและมกราคมที่ *V. vulnificus* มีปริมาณมากกว่า ปริมาณเฉลี่ยของเชื้อ *V. parahaemolyticus* และ *V. vulnificus* ในช่วงที่ศึกษาคือ 3.2±2.5 log MPN/g และ 1.5±2.2 log MPN/g ตามลำดับ โดยค่าเฉลี่ยของปริมาณเชื้อ *V. parahaemolyticus* และ *V. vulnificus* ในตัวอย่างแต่ละบริเวณมีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) ในเดือนสิงหาคม กุมภาพันธ์และมีนาคมพบว่าปริมาณเฉลี่ยของเชื้อ *V. parahaemolyticus* ต่ำกว่าค่าที่ FDA/WHO (2001) กำหนด <10,000 MPN/g และไม่พบเชื้อ *V.vulnificus* ในตัวอย่างหอยตะโกรม ดังนั้นช่วงดังกล่าวอาจเหมาะสมต่อการบริโภคหอยตะโกรม เมื่อสุ่มเลือกเชื้อ *V. vulnificus* และ *V. parahaemolyticus* จำนวน 3 isolates ต่อตัวอย่างที่พบเชื้อเพื่อทดสอบการสร้างเอนไซม์และความไวรับของเชื้อต่อยาต้านจุลชีพ พบว่าเชื้อ *V. vulnificus* จำนวน 297 isolates และ *V. parahaemolyticus* 768 isolates มีการสร้างเอนไซม์ gelatinase และ protease ได้ทั้งหมด ซึ่ง *V. vulnificus* สามารถสร้างเอนไซม์ DNase และ lipase (100%) hemolysin (14.8%) และ lecithinase (52.4%) แต่ไม่พบการสร้างเอนไซม์ mucinase และ elastinase ทั้งนี้เชื้อ *V. parahaemolyticus* มีความไวรับต่อยา doxycycline (98.4%) norfloxacin (96.1%) ciprofloxacin (76.6%) และ cefotaxime (70.7%) ส่วนเชื้อ *V. vulnificus* ทุก isolates มีความไวรับต่อ doxycycline, norfloxacin, ciprofloxacine (100%) และ 84.5% ต่อ cefotaxime ดังนั้น จากการศึกษานี้พบว่า *V. parahaemolyticus* มีความชุกค่อนข้างสูงในหอยตะโกรม ซึ่งต่างจากเชื้อ *V. vulnificus* และเชื้อทั้งสองสปีชีส์ยังคงมีการดื้อต่อยาต้านจุลชีพต่ำ

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RUNGAROON KHANTHO: PREVALENCE AND ANTIMICROBIAL RESISTANT PATTERNS OF *VIBRIO PARAHAEMOLYTICUS* AND *VIBRIO VULNIFICUS* ISOLATED FROM PACIFIC OYSTER (*CRASSOSTREA BELCHERI*) IN THAILAND. THESIS ADVISOR: ASSOC. PROF. THONGCHAI CHALERMCHAIKIT, PH.D., THESIS CO-ADVISORS: ASSIS. ANAN CHONGTHALEONG, M.D., 129 pp.

V. parahaemolyticus and *V. vulnificus* are major causes of foodborne diseases from seafood consumption. Both bacteria are significant public healthy problem in many countries. *V. parahaemolyticus* is cause of human gastroenteritis while *V. vulnificus* can cause septicemia with high mortality rate. Since, raw pacific oyster (*Crassostrea Balcheri*) is popular seafood menu. The contamination of these microorganisms on pacific oyster will be risk of foodborne infection. This study covered prevalence, densities and antimicrobial resistance patterns of *V. parahaemolyticus* and *V. vulnificus* in the pacific oyster. During April 2007 to March 2008, 360 samples of pacific oysters were collected from 5 different locations of the coast area of Bandon bay, Suratthani province, where the most oysters were cultured in 5 locations; Chaiya, Cha-ngor, Kadae, Tatong and Kadae-gae. Those samples were examined carefully to get the information and the data of the prevalence of two significant species of harmful bacteria, *V. parahaemolyticus* and *V. vulnificus*. The occurrence of antimicrobial resistant strains was also studied. The densities of both microorganism were determined by using a modification of the most probable number (MPN) technique described in the FDA-BAM (2004). Agar dilution method used for doxycycline, norfloxacine, ciprofloxacine and cefotaxime susceptibility test (CLSI, 2008). The biochemical method was used for the identification of the isolates. The *vvhA* gene was applied to confirm *V. vulnificus* strains by PCR method. Multiplex PCR method was used to identification of *V. parahaemolyticus* strains, the *tlh* gene was used to confirm species specific, *tdh* and *trh* gene determine the presence of the thermostable direct hemolysin gene and TDH-related hemolysin gene, respectively which associated with pathogenicity of *V. parahaemolyticus*. The Kanagawa phenomenon was assayed as phenotypic marker of thermostable direct hemolysin (TDH) toxin, the urease activity was assayed as phenotypic marker of *trh* gene; the protease and gelatinase activity of individual strains were examined to identify other potential virulence factors of *V. parahaemolyticus*. The *tlh*+ positive of 1,457 isolates was found but none *V. parahaemolyticus* strains carried out of the *tdh* and *trh* genes that also showed the negative for TDH and urease activity tests. *V. vulnificus* of 609 isolates were *vvhA* positive. From the study, prevalence of pathogenic strains of *V. parahaemolyticus* were 172 (47.78%), *V. vulnificus* were 15 (4.17%) and 84 (23.33%) were contaminated both organisms from 360 oyster. Quantitative study of *V. parahaemolyticus* in oysters from all harvested regions found the highest densities in May. The highest densities were observed in oysters harvested from Chaiya, Cha-ngor and Kadae which mostly exceeded 10^7 MPN/g. While *V. vulnificus* found from the oysters of all locations were below the detectable level 3.0 MPN/g in August, February and March. In December and January, *V. vulnificus* densities of all location showed exceeding of 10^4 MPN/g. *V. parahaemolyticus* densities were greater than those of *V. vulnificus* in lots from these same areas, except in December and January. In this study period, the total average densities of *V. parahaemolyticus* and *V. vulnificus* were 3.2±2.5 log MPN/g and 1.5±2.2 log MPN/g, respectively. There were some evidences showing that the mean log MPN values were not same among these five locations ($p < 0.05$). The densities of *V. parahaemolyticus* lower than FDA/WHO (2001) concerned value of <10,000 MPN/g and none *of V. vulnificus* was found in August, February and March which probable to appropriate for raw oyster consumption. The 297 isolates of *V. vulnificus* and 768 of *V. parahaemolyticus* isolates produced of the gelatinase and protease. All of *V. vulnificus* strains were positive for DNase and lipase (100%), hemolysin (14.8%), lecithinase (52.4%). All strains of *V. vulnificus* exhibited negative results either mucinase nor elastinase activity. *V. parahaemolyticus* strains were susceptible to doxycycline (98.4%), norfloxacin (96.1%), ciprofloxacin (76.6%) and cefotaxime (70.7%). While *V. vulnificus* strains found from Bandon bay were susceptible to doxycycline, norfloxacin, cipofloxacine (100%) and cefotaxime (84.5%). This study revealed that the pacific oyster at Bandon bay found high prevalence and densities *V. parahaemolyticus* than *V. vulnificus*. However, both bacteria were susceptible to tested antimicrobial agents.

Field of study : Medical Microbiology

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สถาบันวิทยบริการ
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ABBREVIATIONS

AGS	arginine-glucose slant
ATCC	American Type Culture Collection
BHA	brain heart infusion agar
CC	colistin-cellobiose agar
CFU	colony forming units
CIP	ciprofloxacin
CLSI	Clinical Laboratory Standards Institute
CTX	cefotaxime
cm	centimeter
°C	degree Celsius
DNA	deoxyribonucleic acid
DOX	doxycycline
DW	distilled water
EDTA	ethylenediaminetetraacetic acid
et.al.	et alli
EY	Egg yolk agar
g	gram
HCl	hydrochloric acid
I	intermediate resistance
KA	Slant alkaline /Butt acidic
Ka	Slant alkaline/ Butt slightly acidic
KK	Slant alkaline / Butt alkaline
KP	Kanagawa Phenomenon
l	liter
M	molar
mM	millimolar
mg	milligram (s)
ml	milliliter (s)
mm	millimeter (s)
min	minute (s)

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

NA	nutrient agar
NaCl	sodium chloride
NaOH	sodium hydroxide
NB	nutrient broth
NCCLS	National Committee for Clinical Laboratory Standards
No.	number
NOR	norfloxacin
PCR	polymerase chain reaction
R	resistant
rpm	round per minute
s	second
S	susceptible
TCBS	thiosulfate citrate bile salt sucrose
TSA	tryticase soy agar
TSB	trypticase soy broth
U	unit
UV	ultraviolet
μg	microgram
μl	microliter
V	volt
WA	Wagatsuma agar
WHO	World Health Organization
%	percent

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER I

INTRODUCTION

Vibrio spp. are bacterial flora in the aquatic ecosystem and excite to 13 of 34 species are associated with infections in humans and aquatic animals. Human infection follows either direct contact with aquatic environment or indirectly via contaminated food and water. *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* are major Vibrio spp. that cause of food-born disease. *V. cholerae* serogroups O1 and O139 are of the important serogroups that lead to cholera. Cholera are pandemics and epidemics mainly due to poor drinking water and poor personal hygiene. The other importance species are *V.parahaemolyticus* and *V. vulnificus* which are being the seafood-borne halophilic Vibrio. *V. parahaemolyticus* and *V. vulnificus* result gastroenteritis, wound infection and septicemia. *V. parahaemolyticus* has been implicated as a major species for global for foodborne illness especially resulting of seafood disease. There are several concerning reports food-born disease cause by V. parahaemolyticus around the world eg. in America (1-8), Europe (9, 10), Oceania (11-13), Africa (14, 15) and Asia (16-29). *V. vulnificus* is also an invasive species, that product the original effects of local wound infection, gastroenteritis, and septicemia (30-32). *V. vulnificus* can lead to the development of necrotizing fasciitis and primary septicemia, which occurs mostly in immunocompromised host-associated diseases such as hepatic disease, diabetes mellitus, chronic renal insufficiency, and adrenal insufficiency (33), 50% mortality rate among individuals with liver disease and elevated serum iron levels (34). *V. parahaemolyticus* and *V. vulnificus* illness associated with raw oyster consumption (31, 32, 35-38). In Thailand, raw oyster is a popular seafood that enhance the risk for these pathogen microorganism. Bandon Bay, Surathani province is the major area of the oyster production in Thailand. This study the prevalence and drug susceptibility of *V. parahaemolyticus* and *V. vulnificus* may approach indication of seafood for safety consumption and/or improving of the disease free seafood production. Evidence of drug resistant value may be used for developing of clinical treatment.

CHAPTER II

OBJECTIVES

1. To study prevalence of *V. parahaemolyticus* and *V. vulnificus* in Pacific Oyster (*Crassostrea belcheri*) in Thailand
2. To study microbial resistance of *V. parahaemolyticus* and *V. vulnificus* in Pacific Oyster (*Crassostrea belcheri*) in Thailand

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CHAPTER III

LITERATURE REVIEW

Vibrio spp. are of the aquatic marine microorganisms and often reports with the pathogen in human and aquatic animals (39). Vibrio spp. associate to human diseases by direct contact with sea water and sea animals or indirectly contaminate with food or water. There are 13 out of 34 Vibrio spp. are reports caused the human infection, wound infectio, septicemia and gastroenteritis. *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio cholerae* are the main species resulting disease while *V. parahaemolyticus* and *V. cholerae* result the gastroenteritis, *Vibrio vulnificus* is an important species for septicemia. The relation of pathogenic between environment isolated strain and clinical isolation is still unclear. Vibrio parahaemolyticus is found along the coast, seawater, plankton and sea animal.

Cholera reports in the developing countries than developed countries. *Vibrio cholerae* O1 and O139 are the serogroup that cause the cholera both pandemic and epidemic spread. Main reason of cholera related with drinking poor quality or contaminated water and individual hygiene (40). *V. cholera*e 01, biovar Eltor, serovar Inaba, non-cholera and non-cholera Vibrio (*V. cholerae* non-01; *V. fluvialis*; *V. furnissii*, *V. parahaemolyticus* and Vibrio spp.) were reported during the cholera epidemic in Brasil (41). *Vibrio cholerae*-O1, V*ibrio. cholerae* non-O1 and *Vibrio parahaemolyticus* were the main problem of epidemic occurred in Nigeria (42).

Vibrio parahaemolyticus is an important species for foodborne disease which was discovered in 1953 for pandemic spread and affected to public health, which concerned with outbreaks of acute gastroenteritis, and also serious wound infection, it is an uncommon event for septicemia.

Vibrio parahaemolyticus

1. Occurrence of *Vibrio parahaemolyticus*

Distribution of *V. parahaemolyticus* in marine environments.

V. parahaemolyticus is an estuarine bacteria which usually found contaminating with marine products. *V. parahaemolyticus* was found predominantly in hemolymph of blue crabs up to 21% hemolymph samples between May and November in Maryland (43) *Vibrio parahaemolyticus* was present in Brazil in which 5% and 19% of fish and shell fish were contaminated respectively (7). The Mussels (*Perna perna*), which was harvested in three different stations on the coast of Ubatuba over a 1 year period showed the ranges of most probable number (MPN 100 g^{-1}) were *Vibrio alginolyticus* (<3-24,000), *V. parahaemolyticus* (<3-24,000), *V. fluvialis* (<3-1100), *V. cholerae* non-O1 (<3-23), *V. furnissii* (<3-30), *V. mimicus* (<3-9) and *V. vulnificus* (<3-3). The highest incidence was observed of *V. alginolyticus* (92-100%), followed by *V. parahaemolyticus* (67-92%), *V. fluvialis* (34-67%), *V. vulnificus* (8-17%), *V. furnissii* (0-17%), *V. mimicus* (0-17%) and *V. cholerae* non-O1 (0-8%). Positive results in the Kanagawa was also found 0.51% of *V. parahaemolyticus* strains (44). *V. parahaemolyticus* is also opportunistic agent of pound disease in *Penaeus merguiensis* as the opportunistic infections (45). Prevalence of vibrio spp. isolated from the environments, occurrened 2–13% of *Vibrio parahaemolyticus* and 1–4% of *V. vulnificus* in water, sediment and shrimp samples from multiple shrimp farm along the east and west coast of India. Only 2 out of 47 isolates were *tdh* positive and one contained the *trh* gene. However, since *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* species are recognized as the major species that cause the seafood illness, it is the necessity to pay attention to post-harvest handling and adequate cooking (46). *V. parahaemolyticus* occurred 11.6% of the in natural and precooked mussels, all strains tested were urease-positive and 28.5% were Kanagawa-positive, which suggests that they have potential pathogenic for humans and O10:K52 serotype was predominance. The results also show the epidemiological relation of *V. parahaemolyticus* in cases of gastroenteritis in human who consumed the mussel without sufficient cooking (100 °C/15 min) (47). The densities and the

occurrence of pathogenic strains in shellfish had been after the outbreaks of *V. parahaemolyticus* in the water environmental Washington, Texas, and New York in 1997-1998, by developing the nonradioactive DNA probes. The oyster samples from Washington and Texas showed the total densities of this bacteria exceeding 10,000/g. Pathogenic strains, defined as those hybridizing with *tdh* and/or *trh* probes, were detected in a few samples, mostly were Puget Sound oysters, and at low densities (usually <10 g^{-1}). Oppressive sampling in Galveston Bay associated constant water temperature (27.8 to 31.7°C) and *V. parahaemolyticus* levels (100 to 1,000 g^{-1}) during the summer. Salinity varied from 14.9 to 29.3 ppt. (48).

Prevalence of *V. parahaemolyticus* in seafood.

V. parahaemolyticus is a common problem of bacterial food poisoning in Singapore that related preference of the local population whom consume raw or partially cooked seafood. Seventy eight percents of a variety which imported seafood were found to be contaminated with *V. parahaemolyticus* (7). In Taiwan, the examination of 686 samples of seafood imported from Hong Kong, Indonesia, Thailand and Vietnam for *V. parahaemolyticus* and discovered these organism 315 (45.9%) samples. The incidence of *V. parahaemolyticus* in the products imported from Hong Kong and Thailand was markedly higher than the products from Indonesia and Vietnam. Such a rates found from shrimp, crab, snail, lobster, sand crab, fish and crawfish were 75.8, 73.3, 44.3, 44.1, 32.5, 29.3 and 21.1%, respectively (49). In Japan, *tdh* gene positive *V. parahaemolyticus* was detected in 33 out of 329 seafood samples (10.0%). The number of *tdh*-positive *V. parahaemolyticus* ranged from <3 to 93/10 g**.** TDH-producing strains of *V. parahaemolyticus* disovered in short-necked clam, hen clam, and rock oyster samples. Representative strains of *V. parahaemolyticus* in seafood by molecular method indicated that most O3:K6 *tdh*-positive strains belonged to the pandemic O3:K6 clone and suggested that serovariation took place in the Japanese environment (50). *V. parahaemolyticus* contaminated in prepacked portions of fresh marine fish in Italy and carrying a DNA fragment specific for *V. parahaemolyticus*, but resulted negative to PCR amplification of the virulence-related tdh gene (51). In 2006, New York City, New York state, Oregon, and Washington health

departments reported *V. parahaemolyticus* O4:K12 infected in 177 cases who related consumption the cluster or shellfish in the restaurant (52). In United States, 1998 to July 1999, 370 lots of oysters were sampled (restaurants, retail seafood markets, wholesale seafood markets). Densities of *V. vulnificus* and *V. parahaemolyticus* used MPN-BAM, DNA probes and enzyme immunoassay. The densities of both *V. vulnifcus* and *V. parahaemolyticus* in market oysters from all harvest regions followed a seasonal distribution, with highest densities in the summer, especially the samples collected from the Gulf Coast, exceeded 10,000 MPN/g. The majority lots 78% harvested in the North Atlantic, Pacific, and Canadian Coasts contain *V. vulnificus* densities below then detectable level, 0.2 MPN/g; none exceeded 100 MPN/g. *V. parahaemolyticus* densities were greater than those of *V. vulnificus* in lots from these same areas, with some lots exceeded 1,000 MPN/g. Some lots from the Mid-Atlantic state exceeded 10,000 MPN/g for both *V. vulnificus* and *V. parahaemolyicus*. There was a significant correlation between *V. vulificus* and *V. parahaemolyticus* densities ($r = 0.72$, $n = 202$, $P < 0.0001$), but neither density correlated with salinity. 9 of 3,429 (0.3%) *V. parahaemolyticus* cultures positive for *tdh* and in 8/198 (4.0%) lots of oysters (53). The most probable number-polymerase chain reaction (MPN-PCR) method using for targeting the species-specific thermolabile hemolysin gene (*tlh*) and *tdh* for virulence strain. The incidence of *V. parahaemolyticus* in retailed seafood market was found 29/30 (96.7%) of mollusc samples, 4/30 (13.3%) of fish samples and 11/20 (55.0%) of crustacean samples. Levels of *V. parahaemolyticus* were below 10^4 MPN/100 g in all fish and crustacean samples tested. However, they were above 10^4 MPN/100 g in 11/30 (36.7%) of the mollusc samples. In all types of seafood for raw consumption, the level of *tdh*-positive organisms was below the limit of detection (< 30 MPN/100 g). In seafood for cooking, *V. parahaemolyticus* was found in 20/20 (100%) of mollusc samples, 15/20 (75.0%) of fish samples and 9/20 (45.0%) of crustacean sample, 18/20 (90.0%) of mollusc samples were above 10^4 MPN/100 g. In 7/20 (35.0%) of the mollusc samples, *tdh*-positive organisms were found and their levels ranged from 3.6x10 to 1.1 x 10^3 MPN/100 g (54). In Japan, between 2002 and 2004, TDH gene (*tdh*) and TRH gene (*trh*)-positive *V. parahaemolyticus* in sea water was 11%, in sediment 16%, and in shellfish 26% (55). In Brazil, during June 1998 to March 1999, the Oysters and estuarine water

samples were collected monthly. *Aeromonas* spp., *Plesiomonas shigelloides*, *Vibrio cholerae* O1, *Vibrio parahaemolyticus*, and *Vibrio vulnificus* were counted in oyster by MPN method. All the water samples found *V. parahaemolyticus* and 10% for *V. vulnificus,* which *V. parahaemolyticus* Kanagawa-negative was detected in all oyster samples (3.6 to $\geq$ 2.4 x 10^3 MPN/g). About 80% of the untreated oyster found *V. vulnificus* (<3 - 11.0 MPN/g). The fecal indicators did not correlate with Vibrio presence (p>0.05), and no correlation between temperature, salinity, and bacteria (p > 0.05) (56).

***V. parahaemolyticus* and Oyster**

V. parahaemolyticus, the leading cause of seafood-associated gastroenteritis in the world, typically is associated with the consumption of raw oysters. The US Food and Drug Administration conducted a microbial risk assessment to characterize the risk of contracting *V. parahaemolyticus* infections from consuming raw oysters, CDC reported the outbreak of *V. parahaemolyticus* infection associated with eating raw oysters (1, 2, 35, 57, 58). Outbreak of *V. parahaemolyticus* in British Columbia related to also raw oysters (4, 59). Pathogenic *V. parahaemolyticus* in oysters harvested in Europe caused of food born disease in Spain. Outbreak of *V. parahaemolyticus* infection associated with the consumption of oyster from Alaska (35), Long Island, New Jersey and New York (60). In 1981, six cases of gastroenteritis and one wound infection associated with *V. parahaemolyticus* were reported to public health agencies in Washington and Oregon. An exploration display that all of the gastroenteric illnesses were related with eating raw oysters these are the suggestions for the revision of *V. parahaemolyticus* risk assessment associated with consumption of raw live oysters (61-63). The incidence of diarrhea associated with infection by Vibrio species was inquired at the Interscience Conference on Antimicrobial Agents and Chemotherapy held in New Orleans in 1986. The risk of diarrhea was significantly higher in those who ate raw or cooked oysters in which the five Vibrio species identified from stool specimens, *V. parahaemolyticus* was most common and was most strongly associated with diarrhea (64). In 1997, *V. parahaemolyticus* infections resulted the largest outbreak in North America. 209 cases was related with eating raw oysters harvested from California, Oregon, and Washington in the

United States and from British Columbia in Canada but only one patient died from septicemia (59). The report confirmed relation of *V. parahaemolyticus* isolated from the majority of patients tested and from environmental samples of oysters examined by the pulsed-field gel electrophoresis were highly related across clinical and oyster isolates (35).

2. Incidence of *V. parahaemolyticus in* food poisoning

V. parahaemolyticus was important marine bacteria which related with gastroenteritis outbreaks around the world. *V. parahaemolyticus* had various serotypes that associate food-born disease, cause of illness and died in the worldwide.

2.1 Incidence in Asia

V. parahaemolyticus is the most frequent cause of food-born disease in Japan (16, 17). Three hundreds and seventy one isolates *V. parahaemolyticus* collected from patients related in foodborne illness outbreaks in Taiwan during 1992 and 1995. Serovars O5:K15 (18.5%), O4:K8 (16.2%), O3:K29 (12.5%), O1:K56 (8.3%), O2:K3 (6.5%), and O4:K12 (6.0%) were the most serovars frequently isolated from the patients. Most of the isolates were susceptible to cephalothin, gentamicin, nalidixic acid, tobramycin and tetracycline. About 10% of the isolates were resistanted to seven or more antibiotics, 92.4% showed beta-hemolysis on Wagatsuma blood agar plate and approximately 62.1% of these exhibited detectable amounts of thermostable direct hemolysin (65). Taiwan reports case of liver cirrhosis with septicemia caused by this organism died 12 hours after admission (18-21). All rectal swabs for culture were collected from the CDC of the Department of Health in Taiwan. During 1995 and 2001, 1,171 outbreaks of food-borne illness, related 109,884 cases, were reported in northern Taiwan, of which 735 (62.8%) were caused by bacterial infection, particularly *V. parahaemolyticus* (86.0%) (21). In March 1974, eight infected patients in Chandpur, Bangladesh had the symptom of illness were severe abdominal cramps, nausea, vomiting and bloody diarrhea with onset 20 min to 9 h after eating fish at the restaurant. *V. parahaemolyticus* serotype O3K5 was isolated from rectal

cultures of all eight exhibited Kanagawa-positive. The short incubation, severity of abdominal cramps and grossly bloody stools distinguish this illness from that usually associated with *V. parahaemolyticus* infection in the United States (66). Southeast Asia, the data of foodborne disease occurred in China showed *V. parahaemolyticus* accounted the largest number of outbreaks and cases (23-25), Vietnam, Singapore including Thailand (26, 27), (28, 29). Serovar O3:K6, O1:K25, O1:KUT of pandemic *V. parahaemolyticus* isolates were identified as a major cause of diarrhoea in Thailand and a new *V. parahaemolyticus* isolate, serovar O3:K46 were closely related and clearly distinct from the non-pandemic isolates (67). The Department of Medical Science, Ministry of public Health in Thailand recenty reported 1,000-2,000 gastroenteritis patients who consumed raw or undercooked seafood contaminated V. parahaemolyticus.

2.2 Incidence in Europe

In Europe, pandemic *V. parahaemolyticus* O3:K6 strains have been detected in clinical samples in many countries. The first evidence for the presence in France and suggest the presence and persistence in French coastal areas of this pandemic O3:K6 serovar, which is indistinguishable from the O3:K6 clone isolated in Bangladesh in 1996 (9). In 1989, *V. parahaemolyticus* was the major type of bacteria that resulted the acute gastroenteritis in Spain (68) and also being the result food born disease in 64 cases during August and September 1999, by the consumption of live oysters from a typical outdoor street market in Galicia, (Spain). The strains isolates were Kanagawa phenomenon positive and produced thermostable direct hemolysin, which is related to pathogenic occurence in humans (61, 62). Pandemic strain *V. parahaemolyticus* O3:K6 was also isolated from stool samples of diarrhea patient in Italy (69).

2.3 Incidence in America and the United State

The outbreak of *V. parahaemolyticus* related to raw oysters consumptions in British Columbia (4), The *V. parahaemolyticus* infections caused by the pandemic clone emerged in the coasts of Peru had the connection with the 1997 El Nino episode. The epidemic dissemination of this clone matched the expansion and dynamics of the poleward propagation and the receding of the El Nino waters (70). In the United States, during 1988 and 1997, foodborne outbreaks and sporadic infections from Vibrio species in 4 Gulf Coast states were reported

continuously to the Centers for Disease Control and Prevention (CDC). Three hundred and forty four sporadic *V. parahaemolyticus* infections were reported; 59% were gastroenteritis, 34% wound infections, 5% septicemia and 2% were from other exposures. 45% of patients were hospitalized for their infections, and 88% of persons with acute gastroenteritis were reported eatening raw oysters during a week before their illness occurred. Between 1973 and 1998, 40 outbreaks of *V. parahaemolyticus* infections were reported to the CDC, and these outbreaks included 11,000 cases. Most of these outbreaks occurred during the warmer months and were distributed among seafood, particularly shell-fish (1-3, 71). In August 1971, three food-borne disease outbreaks in Maryland caused *V. parahaemolyticus*, steamed crabs were the vehicle of two outbreaks infection, and the third, crab salad prepared from canned crabmeat. About 57.0% illned persons of estimated 745 at risk involved in the outbreaks, but no deaths occurred (72). *V. parahaemolyticus* serotype 04:K11 was the importance cause of the outbreaks in Maryland, 1971. It was recovered from patients in each outbreak and gave a positive Kanagawa reaction, an indication of enteropathogenicity. Other patient isolates included types 03:K30, 03:K33, and an untypable isolate, all of which were Kanagawa negative. Food isolates included serotypes 03:K30, 02:K28, and two untypable isolates, all of which were also Kanagawa negative (73). In August 1972, *V. parahaemolyticus* caused of an outbreak in Covington, the estimated 600 persons involved acute gastroenteritis, diarrhea and abdominal pain were major symptoms. Shrimp was incriminated as the vehicle of infection. Multiple serotypes of *V. parahaemolyticus* were cultured from stools of sick individuals and from leftover shrimp. This outbreak was qualify to the failure of the shrimp boiling process to destroy *V. parahaemolyticus* in which the fresh shrimp were naturally colonized at the time of delivery to the processing plant (74). During May and June 1998, *V. parahaemolyticus* infections increased in Texas. Four hundreds and sixteen persons in 13 states had gastroenteritis after eating oysters harvested from Galveston Bay. *V. parahaemolyticus* serotype O3:K6 isolated from 28 available stool specimens. These were the first reported outbreak of *V. parahaemolyticus* serotype O3:K6 infection in the United States. The emergence of a virulent serotype and elevated seawater temperatures and salinity levels may have contributed to this large multistate outbreak of *V. parahaemolyticus* (75). During

May and July, 2006, New York City, New York state, Oregon, and Washington health departments reported a total of 177 cases of *V. parahaemolyticus* infection that related consumption raw shellfish (52). Incidence of *V. parahaemolyticus* not associated food born had report in August 2005, which was the cause of wound infection in Hurricane Katrina that made landfall, with major impact on the U.S. Gulf Coast (76).

3. Virulence factors of *V. parahaemolyticus*

The multiplex polymerase chain reaction method used for relation of *V. parahaemolyticus* isolated from patients belong to serogroups O1 and O4, whereas, the isolates from oysters belong to serogroups O1, O4 and O5. The thermolabile haemolysin gene, *tl* used species specific confirming V*. parahaemolyticus*. The presence of a *V. parahaemolyticus* serogroup O1 and O4 contained *tl*, either the thermostable direct haemolysin gene, *tdh,* or the thermostable direct haemolysin-related gene, *trh*, or both, were also positive for urease. The presence of the haemolysin genes, serogroup profiles and urease production in *V. parahaemolyticus* isolated from human patients correlated with the oysters collected during the outbreaks (57). *Vibrio parahaemolyticus* which is islated from cases associated with oyster consumption mostly were obtained from outbreaks in Washington, Texas, and New York. The oyster samples were collected from environments, and food sources on the Pacific, Atlantic, and Gulf Coasts of the United States and from clinical sources in Asia. The food and environmental isolates were defined as being pathogenic by using DNA probes to detect the presence of the thermostable direct hemolysin (*tdh*) gene. The clinical isolates from the United States were used multiplex PCR to confirm the species identification and the presence of *tdh* and to test for the *tdh*-related hemolysin *trh*. Most of the environmental, food, and clinical isolates from the United States were positive for *tdh, trh*, and urease production. Outbreak-associated isolates from Texas, New York, and Asia were predominantly serotype O3:K6 and possessed only *tdh*. The combination of serotyping and ribotyping showed that the Pacific Coast *V. parahaemolyticus* population appeared to be distinct from that of either

the Atlantic Coast or Gulf Coast. That certain serotypes or ribotypes are more essential for human disease (77).

4. Drug susceptibility pattern

About 63% of *V. parahaemolyticus* isolated from shrimps, *Penaeus monodon* collected from the region of the Deltaic Sundarbans (West Bengal, India), showed the resistance to ampicillin, cephalexin, and kanamycin. However, all of these strains were sensitive to norfloxacin, nitrofurantoin, nalidixic acid and tetracycline (78). Three hundreds and seventy one isolates of *V. parahaemolyticus* collected from patients involved with the foodborne illness outbreaks in Taiwan from 1992 to 1995 were characterized. The most frequently isolated serovars were O5:K15, O4:K8, O3:K29, O1:K56, O2:K3 and O4:K12. Most of these isolates were susceptible to gentamicin, nalidixic acid, tobramycin, tetracycline and cephalotin. About 10% of the isolates were resistant to seven or more antibiotics (65). The pathogenic halophilic vibrios isolated from seafood were studied in susceptibility patterns to 27 antimicrobial agents and beta-lactamase production. All isolates were uniformly sensitive to meropenem, choramphenicol, imipenem but resistant to lincomycin. All were highly sensitive to doxycycline, cefotaxime, ciprofloxacin, oxolinic acid, trimethoprim-sulphamethoxazole, flumequine and nalidixic acid. Some strains of *V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus* apparently had mechanisms of resistance to several beta-lactam antibiotics (79). All 204 *V. parahaemolyticus* strains isolated from diarrhea patients admitted to hospitals in several provinces in Indonesia were analyzed antimicrobial susceptibility patterns. The result showed increase resistance to ampicillin and cephalotin. About 3-15% of isolated strains were resistance to tetracycline and chloramphenicol, but were susceptibility to trimethoprim-sulfamethoxazole, ceftriaxone, norfloxacin and ciprofloxacin (80). The total of 95 *V. parahaemolyticus* strains isolated from Thai adults during 2001 and 2002 in Thailand, showed that 52% of isolates were resistant to ampicillin and sulfisoxazole, but all isolates were susceptible to ciprofloxacin and trimethoprim-sulfamethoxazole, two antibiotics commonly used to treat traveller's diarrhea (67).

5. Method of analysis for detection of *Vibrio parahaemolyticus*

Vibrio parahaemolyticus is a marine bacterium with a worldwide distribution and is frequently associated with human outbreaks of infection. Detection and isolation of *V. parahaemolyticus* from natural sources is often problematical because of limitations in the analytical procedures.

Duplicate samples of shellstock oysters were collected monthly (September 1997 to May 1998) from the same four restaurants and three wholesale seafood markets in the Gainesville, where several methodologies were tried and was showed the interesting result in details. The standard MPN method (BAM-MPN) and a new direct plating procedure (direct-VPAP) analyzed for total *V. parahaemolyticus* densities. Both methods employed an alkaline phosphatase-labeled DNA probe (VPAP) targeting the species-specific thermolabile hemolysin (*tlh*) gene to confirm suspect colonies as *V. parahaemolyticus*. The highest monthly geometric mean *V. parahaemolyticus* density was observed in October of 1997 (approximately 3,000/g) with similarly high values during September and November of 1997. From December 1997 to May 1998 mean densities were generally less than 100/g, falling to approximately 10/g in February and March. A strong correlation (r = 0.78) between the direct-VPAP and BAM-MPN methods for determining *V. parahaemolyticus* densities in market-level oysters was observed. The direct-VPAP method was more rapid and precise while the BAM-MPN was more sensitive and may better recover stressed cells. The utilization of the VPAP probe for identification of *V. parahaemolyticus* sharply reduced the labor for either method compared to biochemical identification techniques used in earlier *V. parahaemolyticus* surveys (81). *V. parahaemolyticus* densities and the occurrence of pathogenic strains isolated from seawater and organic material in the cool weather season, from a coast of the Seto-Inland Sea, Japan. Occurrence of *tdh* and/or *trh* genes by the polymerase chain reaction (PCR), using isolated DNA from enrichment culture of the samples. The difference in detection between the MPN-culture and the MPN-PCR techniques appeared to be significant. About 95% of the samples were positive for *V. parahaemolyticus* (with densities of 3 to >1400 cells per 100 ml water or 10 g organic samples) by the most-probable-number (MPN)-PCR technique with species-specific *toxR* primers, but only 40%

were positive by the conventional MPN-culture technique (with densities ranging from 3 to 240 cells per 100 ml water or 10 g organics). Furthermore, the *tdh* and *trh* genes were positive in 55% and 20% of samples, respectively, by the MPN-PCR technique. No *tdh* and *trh* gene-positive strains were isolated by the conventional MPN-culture procedure (82).

For the detection and enumeration of *V. parahaemolyticus* in seafood, especially for samples that show many colonies other than *V. parahaemolyticus* on TCBS agar, the MPN-PCR method may be more convenient and reliable than the MPN-TCBS method. The studies compared *V. parahaemolyticus* densities in spiked and naturally contaminated seafood samples by the MPN method combined with a PCR procedure (MPN-PCR method) targeting the species-specific thermolabile hemolysin gene (*tlh)*, and by the MPN method using subcultivation of alkaline-peptone-water (APW) enrichment culture on thiosulfate-citrate-bile-sucrose (TCBS) agar (MPN-TCBS method). In naturally contaminated seafood samples, the numbers of *V. parahaemolyticus* enumerated by the MPN-PCR method were higher than those by the MPN-TCBS method. In the case of the MPN-TCBS method, isolation of *V. parahaemolyticus* from some APW cultures was difficult because of the overgrowth of many colonies other than *V. parahaemolyticus* on TCBS agar. In contrast, the PCR technique could detect *tlh* from APW culture without isolation of *V. parahaemolyticus*, so the possibility of failing to obtain a positive result in APW culture by the MPN-PCR method was considered to be lower than that by the MPN-TCBS method. However, the PCR technique reduces the time and labor more the MPN-TCBS method (83).

The incidence of *V. parahaemolyticus* in an aquatic environment depends upon many ecological factor, sea water and organic material were collected during the warm weather season from a coast of the Seto Inland Sea, Japan. *V. parahaemolyticus* densities and the occurrence of pathogenic strains were analyzed by polymerase chain reaction (PCR), using isolated DNA from enrichment culture of the samples. About 99% of samples were positive for *V. parahaemolyticus* with densities of 3 to> 1400 cells per 100 ml of water or 10 g of organic samples by the most-probable-number (MPN)-PCR technique, but only 76.6% were positive by the conventional MPN culture technique, with densities ranging from 3 to> 1400 cells per 100 ml of water or 10 g of organics. For the

MPN-PCR technique exhibited 41.5% positive *tdh* and 8.5% *trh* genes of the samples. No *tdh* and *trh* gene-positive strains were isolated by the conventional MPN culture procedure (84). The sensitivity of MPN-PCR was 100 times higher than that of direct PCR. The MPN-PCR test could be completed in less than 16 h from the time of sample preparation. The Two hundreds and twenty five seafood samples from Qingdao, 165 were positive for the presence of *V. parahaemolyticus*, with an MPN value of >719 per gram, and about 41.5% of samples were positive for *tdh* gene-possessing cells. That showed MPN-PCR method was rapid, sensitive, and reliable for comprehensive detection and quick quantitative determination of V*. parahaemolyticus* in seafood and it revealed the potential risk of illness associated with their consumption (85). The combination of conventional and molecular protocols used for the investigation of *V. parahaemolyticus* was the best procedures to detection of this organism in environmental matrixes. The direct-PCR after the sample enriched in APW broth was the most successful method for the detection of *V. parahaemolyticus* with the A/P procedure and enumeration by MPN. Better detection was obtained with MPN than with the A/P protocol. Conversely, the plate culture procedure showed better results with the two-step enrichment protocol in which CHROM agar Vibrio was used as the selective agar (86).

In 1995, *V. parahaemolyticus* O3:K6 strains isolated from Southeast Asian travelers and from cases of diarrhea in Calcutta, India in 1996 were exhibited to belong to a unique clone characterized by possession of the *tdh* gene but not the *trh* gene and by unique arbitrarily primed PCR (AP-PCR) profiles. *V. parahaemolyticus* isolated in a hospital in Bangladesh between 1977 and 1998, only 22 of 227 strains positive to the new O3:K6 clone. The O3:K6 strains isolated from clinical sources in Taiwan, Laos, Japan, Thailand, Korea, and the United States between 1997 and 1998 were also shown the belonging of the new O3:K6 clone. The clonality of the new O3:K6 strain was also confirmed by analysis of the *toxRS* sequence. The *toxRS* sequences of the representative strains of the new O3:K6 clone differed from those of the O3:K6 strains isolated before 1995 at least at 7 base positions within a 1,346-bp region. The *toxRS* sequences were 100% identical to that of the new O3:K6 clone (87). The open reading frame 8 (ORF8) DNA-specific oligonucleotide primers tested in PCR method used for detection

pandemic *V. parahaemolyticus* O3:K6 strain, these strain in pure cultures and seeded waters from the Gulf of Mexico were detected for efficiency this method. The minimum level of detection by the PCR method was 1 pg of purified genomic DNA or 10^2 ORF8-positive *V. parahaemolyticus* O3:K6 cells in 100 ml of water. The effectiveness of this method for the detection of ORF8-positive isolates in environmental samples was tested in gulf water seeded with 10-fold serial dilutions of this pathogen. A detection level of 10^3 cells per 100 ml of gulf water was achieved (88).

Vibrio vulnificus

The *Vibrio vulnificus* includes three biotypes that have been defined on the basis of differences in biochemical and serology properties. General habitat of Biotype 1 was the sea water and environmental coast that reaults seafood disease after consuming the raw shellfish, and being the cause of wound infection.

1. Occurrence of *Vibrio vulnificus*

Vibrio vulnificus, a normal bacterial inhabitant of estuaries, is of concern because it can be a potent human pathogen, causing septicemia, wound infections, and gastrointestinal disease in susceptible hosts. This species seldom cause of gastroenteritis, but often being a main cause to wound infection in individuals who associated with marine environments (31). In waterborne disease and outbreak report, 2003-2004, 142 cases reported Vibrio illnesses were associated with recreational water exposure, the most commonly reported species were *Vibrio vulnificus*, *V. alginolyticus*, and *V. parahaemolyticus*. *V. vulnificus* illnesses associated with recreational water exposure were the highest Vibrio illness hospitalization (87.2%) and mortality rates (12.8%). During 2005-2006, 189 cases reported Vibrio illnesses, which *V. vulnificus* illnesses associated with recreational water exposure was the highest Vibrio illness hospitalization (77.6%) and 22.4% mortality rates (89, 90). The first reported case of peritonitis associated

with sepsis and gastroenteritis from *V. vulnificus*, which this organism was isolated from the patient's blood and peritoneal fluid from the same batch of oysters at the restaurant where the patient had visited (91).

1.1 Distribution of *V. vulnificus* in marine environments

V. vulnificus is an estuarine bacterium which is the causative agent of both food-borne disease and wound infection. Although *V. vulnificus* is commonly found in molluscan shellfish at high numbers and the occurrence of *V. vulnificus* in plankton confirms the role of plankton as a potential reservoir for this pathogen (92). *V. vulnificus* was isolated from oysters and water of the Squamscott, Piscataqua, and Oyster Rivers in United States during May 1989 through December 1990. there was correlation between temperature, salinity, and the presence of *V. vulnificus* in water and oysters (93). In Brazil, Mussels (*Perna perna*) harvested in three different stations on the coast of Ubatuba, *V. vulnificus* occurred <3.3 MPN 100 g-1, the incidence was observed for *V. vulnificus* (8-17%) (44). Seventy-seven strains of *V. vulnificus* were isolated from 11 environmental samples in Taiwan and seventy two isolates (91%) were indole-positive, a characteristic of biotype 1. The remaining five strains although indole-negative, a characteristic previously found exclusively in biotype 2 strains, were all ornithine decarboxylase- and mannitol-positive, which has never been reported for biotype 2 strains. Fifty-seven ribotypes were identified among these isolates, indicating the great genetic divergence in this species. Seventeen of the 30 environmental isolates (56.7%) exhibited virulence comparable to the clinical isolates in the mouse, *V. vulnificus* strains in the marine environments might be pathogenic to humans (94). During June 2004 and May 2005, ninety samples in seven shellfish-growing areas of the German Wadden Sea were analysed. The analysis included the compulsory microbiological parameters *Escherichia coli*, Salmonella spp. and Vibrio spp. The result showed that Vibrio spp. was detected in 74.4% of 90 samples, *Vibrio alginolyticus* (51.2%), *Vibrio parahaemolyticus* (39.5%). *Vibrio vulnificus* (3.5%). *V. parahaemolyticus* and *V. vulnificus* were not found in the samples collected at low water temperatures and also *E. c*oli is not a reliable indicator for the contamination with Vibrio spp. (95).

1.2 *Vibrio vulnificus* and Oyster

Since the early 1970s, the global consumption of shellfish has considerably increased while the outbreaks of shellfish-associated infection occurred in several part of the world. The majority of outbreaks have been linked to oysters, followed by clams and mussels. For outbreaks of *Vibrio vulnificus* infection, which results a mortality rate up to 50% in immunodeficiency person (96). The raw or undercooked seafood, commonly raw oysters, can lead to a primary septicaemia with a fatality rate of 50-60%. An unusual symptom, occurring in 69% of 274 cases, the development of secondary lesions, typically on the extremities, which are generally severe (often a necrotizing fasciitis) and require tissue debridement or amputation (31). Forty oyster samples (*Crassostrea rhizophorae*) served raw in 15 restaurants in the city of Rio de Janeiro were analyzed and subjected to enrichment in APW with the addition of 1 and 3% NaCl and incubated at 37°C for 24 hours. The cultures were seeded onto thiosulfate citrate bile sucrose agar (TCBS) and the suspected colonies were subjected to biochemical characterization. *Vibrio parahaemolyticus*, *Vibrio carchariae*, *Vibrio alginolyticus* and *Vibrio vulnificus* were the main species (>60%) in the results (97).

2. Incidence of *Vibrio vulnificus* in food poisoning and wound infection

Vibrio vulnificus is found usually in marine coastal waters. Infection with this organism associated with seafood poisoning disease, which expected to raw seafood consumption. Beside, *V. vulnific*us caused the wound infection when the open wound exposed with contaminated sea water. The most reports of *V. vulnificus* patients related with hepatic disease and immunodeficiency syndrome (98). Outbreaks of shellfish-associated infection have been reported for last several decades, since the early 1970s when the shellfish consumption in the world has increased. Most than half of the reports have originated from the United States, but Europe, Asia and Australia have been less information. The majority of outbreaks have been linked to oysters, followed by clams and mussels. Hepatitis A virus caused the largest ever shellfish-associated outbreak, *V. vulnificus* infected patients showed the mortality rate up to 50% (96).

2.1 Incidence in Asia

V. vulnificus caused septicemia in Japan, an outbreak of *V. vulnificus* infection was confirmed in the Yatsushiro area, Kumamoto prefecture, during late June and early July in 2001. Six septicemia and one wound infection of all 7 cases associated with existing hepatic disorders such as hepatitis or hepatic cirrhosis caused by hepatitis C virus or long-term alcohol abuse (99-102). The number of reported cases of infection attributable to *V. vulnificus* in Taiwan has increased since the first case was reported in 1985. There were an analyze of molecular epidemiologic features of this pathogens isolated from 84 clinical-case, during 1995 and 2000 (103). *V. vulnificus* infection related with either primary septicemia or wound infection, fourteen patients presented with primary septicemia, and four with wound infection in Taiwan, 1994. Thirteen patients had alcoholism or chronic liver disease, two had peptic ulcer disease, one was steroids abuser, and one patient had thalassemia and chronic liver disease. Chronic liver diseases and liver cirrhosis are common disease in Taiwan, the Taiwanese take a high risk for *Vibrio vulnificus* infection, which the overall mortality was 55.6% (104). In Thailand, there were reports of *V. vulnificus* infection in King Chulalongkorn Memorial Hospital, eight septicemia patients infected with *V. vulnificus*, all of the patients were immunocompromised hosts. Four patients had cirrhosis of the liver, three patients were heavy alcohol drinkers and one had systemic lupus erythematosis. After that, six of these got renal failure, four patients died shortly after admission. Two survived with clinical course of tubular necrosis (105). During 2001 and 2006, DMSC of Thailand reported fifty six septicemia patients caused by *V. vulnificus* infection. Thirty four cases were the patients who lived in the central part of the country, 9 cases in the northeastern and also 9 cases in the south. The number of patients was highest in June and December. These all infections were related with the consumption of raw seafood especially oysters.

2.2 Incidence of *V.vulnificus* in Europe

The *V. vulnificus* infection is rare in Europe, A 59-year-old female patient with a history of malignant lymphoma presented with symptoms of septicaemia. The symptom showed skin of the extremities of bullous, necrotizing plaques and

Vibrio vulnificus was detected from blood culture. The infection was most likely acquired while swimming in the unusually warm Baltic Sea through inadvertent swallowing of sea water (106). Infections caused by *V. vulnificus* were unusual in Spain, the patient whose abraded skin on the leg and contact with seawater. The patient died suddenly, probably due to septicemia or bacteriamia caused by this organism (107). During the unusually warm summer in Denmark in 1994, eleven patients of *V. vulnificus* infection were reported (108).

2.3. Incidence in America and United State

V. vulnificus wound infections was serious infection underlying hepatic disease in Mexico caused of severe illnesses (109). The *V. vulnificus* transmission by raw oyster consumption 3 days before hospitalization resulted the patients to septic shock and *V. vulnificus* was isolated from blood culture (110). Infections caused by *Vibrio vulnificus* were first reported in 1979 by Blake et al. of the U.S. Centers for Disease Control. Sixty-two patients with *V. vulnificus* infection were reported to the Florida Department of Health and Rehabilitative Services during 1981 and 1987. The three clinical syndromes found were 38 cases of primary septicemia, 17 cases of wound infections, and 7 cases of gastrointestinal illness without septicemia or wound infections. 55% mortality rate for patients with primary septicemia and 24% for wound infections, no deaths occurred in those with gastrointestinal illness. The main cause of primary septicemia and gastrointestinal illness was raw oyster consumption, liver disease was caused of primary septicemia, and wound or sustaining a wound in contact with seawater for wound infections (111). The *V. vulnificus* has emerged as the most virulent foodborne pathogen in the United States with a hospitalization rate of 0.910 and a case-fatality rate of 0.390. Infections following ingestion of raw or undercooked seafood, commonly raw oysters, can lead to a primary septicaemia with a fatality rate of 50-60%. The most cases occur in persons with certain underlying diseases which are either immunocompromising or which lead to elevated serum iron levels (e.g. liver cirrhosis, chronic hepatitis, haemochromatosis) (31).

3. Virulence factors

V. vulnificus utilizes a variety of virulence factors, the colonial morphology, enzymatic activity and animal assays were for characteristics related to their infectivity used for investigated in twenty strains of *V. vulnificus* isolated from the environment. All *V. vulnificus* strains were presence of DNase, chitinase, amylase, lecithinase and gelatinase, but haemolytic activity was absent, and variable results were obtained in elastase, collagenase and chondroitinase. 70% of the strains were lethal to adult mice, while 45% caused fluid accumulation in suckling mice. Although all strains had opaque colonies, only 3 of the 20 had the three enzymes elastase, collagenase and gelatinase, and only one of these was virulent in animal assays (112). The virulence factors of *V. vulnificus* are not yet well understood. The hydrolytic enzymes have been implicated in the pathogenesis of this micro-organism. One hundred and thirty three V. vulnificus strains isolated from 45 seafood samples showed that 100% of these strains were positive for the production of lecithinase and lipase (Tween-80), 99.2% for caseinolytic protease, 96.9% for DNase, 96% for haemolytic against sheep blood cells, 65.4% for mucinase and 46.6% for elastase. None of the strains was positive for the production of collagenase. For morphologic colonies test on BHI and NA, 59.4% of strains showed opaque morphology on BHI agar and 57.9% on nutrient agar, 10.5% presented translucent morphology on both agars and 30.1 and 31.6% of strains showed a mixture of opaque and translucent morphology on BHI agar and nutrient agar, respectively. None of the translucent colonies was virulent to mice. Twenty nine (64.4%) presented opaque strains could lethal to adult mice (113). The capsular polysaccharide (CPS) is a major virulence factor in *Vibrio vulnificus*, and encapsulated strains have an opaque, smooth (OpS) colony morphology, while nonencapsulated strains have a translucent and smooth (TrS) colony morphology. The OpR and TrR strains both produce three-dimensional biofilm structures that are indicative of rugose extracellular polysaccharide (rEPS) production, that OpR strains retain expression of CPS and are virulent in an iron-supplemented mouse model, while TrR strains lack CPS and are avirulent (114). Phospholipase A (PLA) was importance enzyme in *V. vulnificus*-induced cytotoxicity and lethality that related the pathogenesis of *V. vulnificus* infection

(115). The metalloprotease (vEP) was extracellular enzyme which a broad-specificity protease that could function as a prothrombin activator and a fibrinolytic enzyme to interfere with blood homeostasis as part of the mechanism associated with the pathogenicity of *V. vulnificus* in humans and thereby facilitate the development of systemic infection. The purified and characterized an extracellular metalloprotease (vEP) could cleave various blood clotting-associated proteins such as prothrombin, plasminogen, fibrinogen, and factor Xa. The cleavage of prothrombin produced active thrombin capable of converting fibrinogen to fibrin that associated with skin lesions and serious hemorrhagic complications (116). *V. vulnificus* protease (VVP) was the major toxic factor causing skin damage in the patients having septicemia. VVP is a metalloprotease and degrades important proteins including elastin, fibrinogen, and plasma proteinase inhibitors of complement components. VVP causes skin damages through activation of the Factor XII-plasma kallikrein-kinin cascade and/or exocytotic histamine release from mast cells, and a haemorrhagic lesion through digestion of the vascular basement membrane (117). Fifty six kilodaltons of cytolysin has been suggested as a possible virulence factor in *Vibrio vulnificus* infections. The DNA encoding cytolytic activity was sequenced and found that the sequence contained two open reading frames, *vvhA* and *vvhB*. *vvhA* encoded the structural gene for the cytolysin and contained the N-terminal amino acid sequence for the protein. Regions of the *vvhA* gene showed homology to the structural gene for the *Vibrio cholerae* E1 Tor hemolysin (118). The metalloprotease secreted from *V. vulnificus* caused of edematous skin lesions (119). The environmental *V. vulnificus* strains were phenotypically indistinguishable from clinical isolates. There were no differences between environmental and clinical strains on the basis of biochemical characteristics or antimicrobial susceptibility patterns and that approximately 90% of the environmental strains tested produced in vitro virulence factors and in vivo pathogenicity for mice comparable to those produced by clinical *V. vulnificus* isolates (120).

4. Drug susceptibility

Vibrio vulnificus is a serious opportunistic human pathogen commonly found in subtropical coastal waters, and is the leading cause of seafood-borne mortality. There were few studies had verified the effectiveness of commonly prescribed antibiotics in *V. vulnificus* treatment. There were no differences between environmental and clinical strains on the antimicrobial susceptibility patterns. The thirty strains isolated from environment sensitive to amikacin, ampicillin, carbennicillin, cephalothin, chloramphenicol, erythromycin, gentamicin,novobiocin, trimethoprim-sulfamethoxazole and tetracycline but resistant to colistin (120). *Vibrio vulnificus* was detected in both wild-caught South Carolina (SC) and farm-raised imported shrimp samples showed only the isolate from farm-raised shrimp was resistant to nalidixic acid and trimethoprim (121). *V. vulnificus* isolated diseased fresh water prawn showed different degrees of sensitivity to different antimicrobial agents. It was highly sensitive to each of the antibiotics rifadin, virbamycin, oflaxcin, garamycin, flummox and trimethoprim/sulfamethoxzole and resistant to amoxicillin, nalidixic acid, unasyn, velosef, negram and claforan. The minimal inhibitory concentration of trimethoprin/sulfamethoxzole for the studied pathogen, *V. vulnificus* was 0.31/5.93 μg (122). *V. parahaemolyticus* 168 strains and 151 *V. vulnificus* isolated from Louisiana Gulf and retail oysters in 2005 and 2006. The susceptibility pattern showed both microorganisms remained susceptible to the majority of antimicrobials tested; reduced susceptibility was detected only in *V. parahaemolyticus* for ampicillin (81%; MIC $\geq$16 microg/ml). Additionally, *V. parahaemolyticus* displayed significantly higher MICs for cefotaxime, ciprofloxacin, and tetracycline than *V. vulnificus* (123).

5. Method of analysis for detection of *V. vulnificus*

The culture method and a real-time polymerase chain reaction (PCR) assay was used for detection of the growth of *V. vulnificus* in an enriched culture of seawater in Japan. In the analysis of the bacterial populations in enrichment culture after 4h of incubation, the growth of *V. vulnificus* on agar media was

inhibited by the rapid growth of *V. parahaemolyticus* and the 100 times larger initial populations of bacteria other than *V. vulnificus* and *V. parahaemolyticus*, the molecular methods were effective and detect *V. vulnificus* accurately (124). The PCR assay was used to confirm isolates and to directly detect *V. vulnificus* in environmental concentrated samples from the Mediteranean coastal. The specific primers of cytotoxin/hemolysin gene and the variable regions of 16S rRNA species-specific for *V. vulnificus* were used to assay. Direct detection in marine samples was more frequently carried out than isolation of culturable forms. The 16S rRNA primers were the most sensitive molecular tool as they allowed detection of *V. vulnificus* in 79.1% of samples, which the low incidence of *V. vulnificus* detection required a molecular approach (92). The TaqMan assay, a quantitative real-time polymerase chain reaction (PCR) used for detecting of the number of *V. vulnificus* from environmental samples, this method was developed to target the *toxR* gene of *Vibrio vulnificus*, which rapidly, effectively and quantitatively for monitoring *V. vulnificus* contamination in seawater and seafoods such as oysters (125).

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER IV

MATERIALS AND METHODS

Methodology scheme

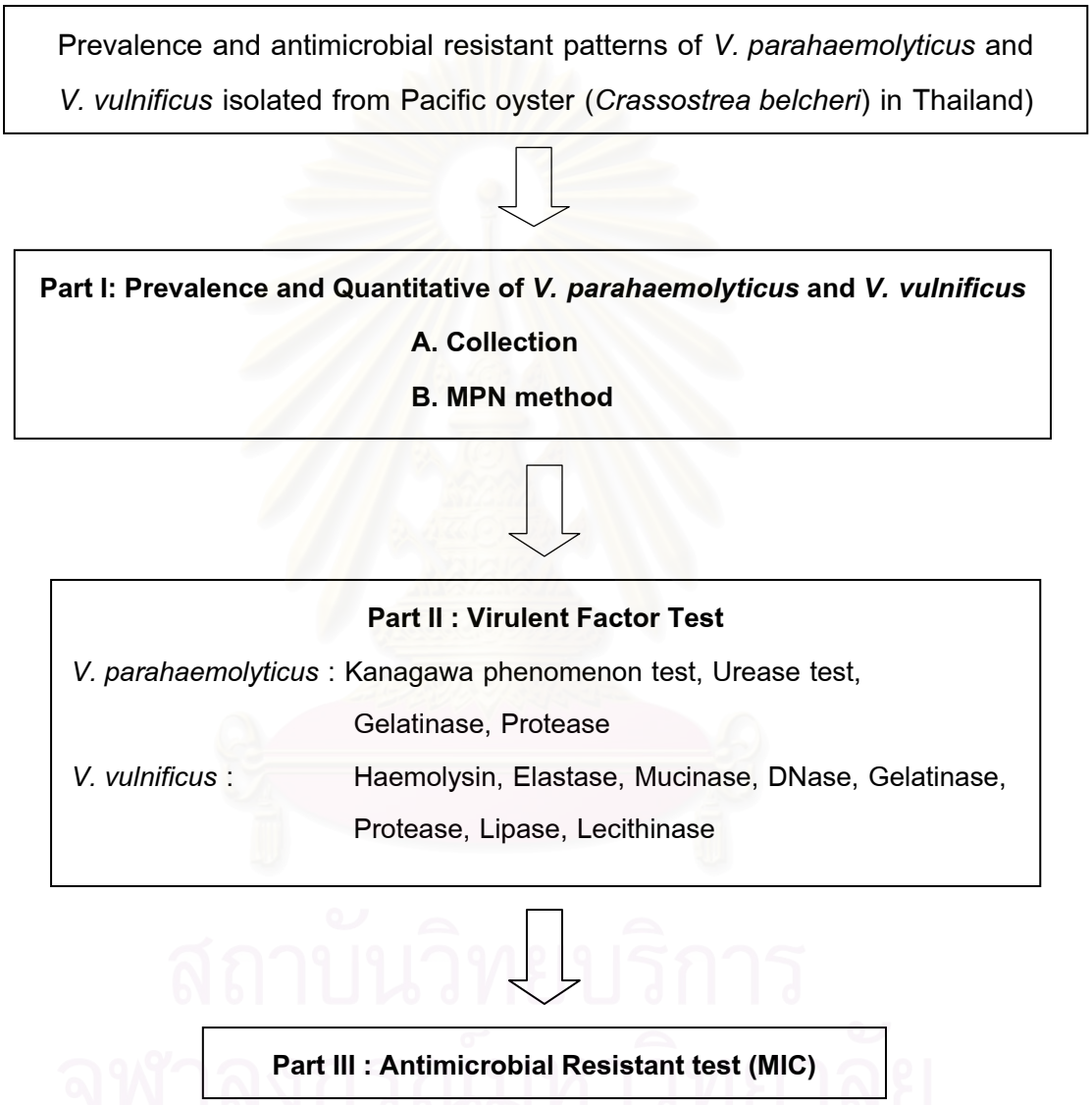

Figure 1 Methodology Scheme

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Part I : Prevalence and Quantitative of *V. parahaemolyticus* and *V. vulnificus*

A. Collection : Each month, 30 living oysters were collected from Bandon Bay

B. MPN Method :

Sample preparation : 25 grams sample of oyster in 225 ml PBS

↓

Enrichment: Inoculate APW (three tubes MPN)

Incubate

↓ 37 ºC, 18 h

Streak turbidity MPN tubes

Isolation : to CC and TCBS agar

37 ºC, 24 h

↓ ↓

Yellow colonies on CC agar — Bluish-green colonies on TCBS agar

Purification: Three on average typical colonies/plate were selected and purified by streak onto T1N1 for biochemical screening test.

Screening : preliminary biochemical test

- TSI, AGS, Oxidase
- Arginine hydrolase, Ornithine decarboxylase, Lysine decarboxylase
- Motillity, Salt tolerance (0, 3, 6, 8, 10 %)
- Acid from arabinose, D-cellobiose, glucose, lactose and sucrose
- ONPG

Confirmation : PCR method

Figure 2 Methodology Scheme

Table 1 Biochemical characteristics of human pathogenic Vibrionaceae commonly encountered in seafood (126)

		V. alginolyticus	*V. cholerae*	*V. fluvialis*	*V. furnissii*	*V. hollisae*	*V. metschnikovii*	*V. mimicus*	*V. parahaemolyticus*	*V. vulnificus*	*A. hydrophilia**	*P. shigelloides**
TCBS agar		Y	Y	Y	Y	NG	Y	G	G	G	Y	G
mCPC agar		NG	P	NG	NG	NG	NG	NG	NG	Y	NG	NG
CC agar		NG	P	NG	NG	NG	NG	NG	NG	Y	NG	NG
AGS		KA	Ka	KK	KK	Ka	KK	KA	KA	KA	KK	nd
Oxidase		+	+	+	+	+	-	+	+	+	+	+
Arginine dihydrolase		-	-	+	+	-	+	-	-	-	+	+
Ornithine decarboxylase		+	+	-	-	-	-	+	+	+	-	+
Lysine decarboxylase		+	+	-	-	-	+	+	+	+	V	+
Growth in (w/v):	0% NaCl	-	+	-	-	-	-	+	-	-	+	+
	3% NaCl	+	+	+	+	+	+	+	+	+	+	+
	6% NaCl	+	-	+	+	+	+	-	+	+	+	-
	8% NaCl	+	-	V	+	-	V	-	+	-	-	-
	10% NaCl	+	-	-	-	-	-	-	-	-	-	-
Growth at 42°C		+	+	V	-	nd	V	+	+	+	V	+
Acid from:	Sucrose	+	+	+	+	-	+	-	-	-	V	-
	D-Cellobiose	-	-	+	-	-	-	-	V	+	+	-
	Lactose	-	-	-	-	-	-	-	-	+	V	-
	Arabinose	-	-	+	+	+	-	-	+	-	V	-
	D-Mannose	+	+	+	+	+	+	+	+	+	V	-
	D-Mannitol	+	+	+	+	-	+	+	+	V	+	-
ONPG		-	+	+	+	-	+	+	-	+	+	-
Voges-Proskauer		+	V	-	-	-	+	-	-	-	+	-
Sensitivity to:	10 µg O/129	R	S	R	R	nd	S	S	R	S	R	S
	150 µg O/129	S	S	S	S	nd	S	S	S	S	R	S
Gelatinase		+	+	+	+	-	+	+	+	+	+	-
Urease		-	-	-	-	-	-	-	V	-	-	-

* *Aeromonas hydrophila, Plesiomonas shigelloides.* Abbreviations: TCBS, thiosulfate-citrate-bile salts-sucrose; mCPC, modified cellobiose-polymyxin B-colistin; AGS, arginine-glucose slant; Y = yellow, NG = no or poor growth, S = susceptible, nd = not done, G = green, V = variable among strains, R = resistant, P = purple, V = variable, KK = Slant alkaline / Butt alkaline, KA = Slant alkaline /Butt acidic, Ka = Slant alkaline/ Butt slightly acidic

A. Collection

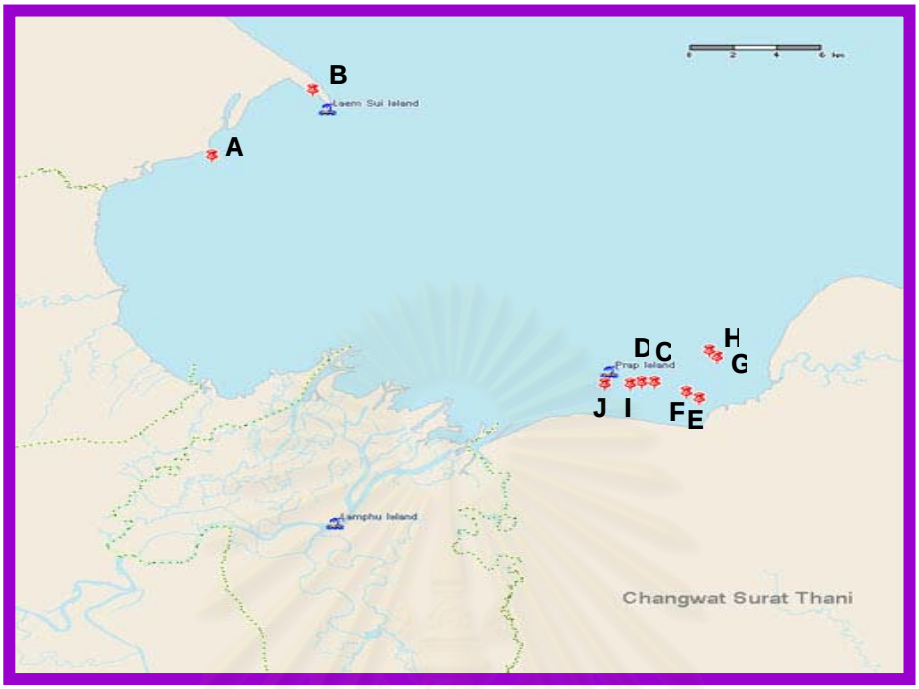

Figure 3 Sampling sites in Bandon Bay

Table 2 Locations of the Pacific oyster samplings in Bandon Bay, Suratthani Province

Location	Farm	Latitude	Longitude
Chaiya	Chaiya 1 (A)	09º 21’ 27.6”	099º 15’ 46.6”
	Chaiya 2 (B)	09º 23’ 48.6”	099º 18’ 21.9”
Cha-ngor	Cha-ngor 1 (C)	09º 13’ 17.9”	099º 27’ 03.0”
	Cha-ngor 2 (D)	09º 13’ 19.1”	099º 26’ 43.1”
Ka-dae	Ka-dae 1 (E)	09º 12’ 42.8”	099º 28’ 10.9”
	Ka-dae 2 (F)	09º 12’ 57.2”	099º 27’ 51.2”
Ta-tong	Ta-tong 1 (G)	09º 14’12.6”	099º 28’ 39.1”
	Ta-tong 2 (H)	09º 14’ 26.4”	099º 28’ 25.5”
Kadae-gae	Kadae-gae 1 (I)	09º 13’ 15.8”	099º 26’ 24.8”
	Kadae-gae 2 (J)	09º 13’ 15.1”	099º 25 46.5”

Three hundred and sixty of Pacific oyster samples had been collected during April 2007 to March 2008. Each month, thirty samples of Pacific Oysters were collected from ten farms in Bandon Bay, Suratthani province, each farm for 3 living oyster samples. The Oyster samples were collected and were brought to laboratory for analysis within 24 hour.

B. MPN method (FDA, BAM 2004)

1. Sample preparation

A Pacific oyster was one sample. Then the oysters arrived to the laboratory, were cleaning and shucking by aseptic technique.

2. Enrichments

The samples were added PBS buffer to 1:10 dilution and homogenized by stomacher (Figure 4). Each homogenized samples were diluted in decimal dilution PBS for 10^{-1}-10^{-9} dilution. One ml of each dilution was added into 9 ml of APW for MPN three-tubes method in enrichment strep, the MPN procedure as described in the standard procedure (126).

Figure 4 Stomatcher apparatus

3. Isolation

The pacific oyster samples were enriched in akaline peptone water (APW) broth and incubated at 37°C for 18 h, each APW tube that turbid after incubation was streaked onto a tetrathiosulfate citrate bile salt sucrose (TCBS) agar plate for screening *V. parahaemolyticus* and a cellobiose-collistin (CC) agar plate for screening *V. vulnificus* and incubated at 37°C for 24 h. Three or more typical colonies V. parahaemolyticus colonies (round, 2-3 mm diameter green or blue green) were selected from each TCBS plates and one or more typical colonies if essential. Three or more typical *V. vulnificus* colonies (flat yellow with opaque centers and translucent peripheries, about 2 mm diameter) were selected from CC agar plates and one or more typical colonies if essential.

4. Screening and Identification

These colonies were streaked on tryptic soy agar (TSA agar) add 2% NaCl and incubated at 37°C for 24 h. After incubating, the colonies were subjects to be primarily identified by Gram staining, oxidase, TSI, AGS, motility and their ability of growing in 0%, 3%, 6%, 8% and 10% NaCl broth. Colonies those were gram-negative rod shape, pleomorphic organism, oxidase positivtive, motile and could growth in 3%, 6% and 8% NaCl broth for *V. parahaemolyticus*, 3% and 6% NaCl broth positive for *V. vulnificus*. Those positive colonies had been subcultures on TSA+2% NaCl agar and identifying by their property to fermentation sugar, amino acid decarboxylase and specific ONPG test for *V. vulnificus.*

4.1 Gram staining procedure

The organisms were smeared on a clean slide and allowed air drying. The slide was heated fix by pass a flame. Gram crystal violet was dropped on the smear for 1 minute, the slide was washed with water and drained. The gram iodine solution was dropped on the smear, and washed with water after 1 minute. Next, the smear was decolorized with 95% ethanol and then washed with water. Gram safanin solution was dropped on the smear for 30 seconds. The smear was allowed to dry and then examined by microscopy under 100x objective lens.

4.2 Biochemical Characteristic test

4.2.1 Oxidase test

Drop oxidase reagent on filter paper and using sterilized toothpicks pick up colony on filter paper.

Positive control is *Vibrio parahaemolyticus* ATCC 17802

Interpretation criteria

The positive result was shown as dark-blue colors on filter paper.

The negative result was not shown as dark-blue colors on filter paper.

4.2.2 TSI (Triple sugars iron)

Pure colony culture on tryptic soy agar (added 2% NaCl) was stabed into the butt and streaked on the slant agar. Incubate at 37°C for 18 h.

Positive controls are *Vibrio parahaemolyticus* ATCC 17802

Vibrio vulnificus ATCC 27562

Negative control is *Escherichia coli* ATCC 25922

Interpretation criteria

The positive result was shown as acid butt (yellow) and alkaline slant (purple) or acid slant but no gas or H_2S.

The negative result was not shown as acid butt and alkaline slant colors.

4.2.3 AGS (Arginine glucose slant)

Pure colony culture on tryptic soy agar (added 2% NaCl) was stabed into the butt and streaked on the slant agar. Incubate at 37°C for 18 h.

Positive controls are *Vibrio parahaemolyticus* ATCC 17802 and

Vibrio vulnificus ATCC 27562

Negative control is *Escherichia coli* ATCC 25922

Interpretation criteria

The positive result was shown as acid butt (yellow) and alkaline slant (purple) but no gas or H_2S.

The negative result was not shown as acid butt and alkaline slant colors.

4.2.4 Motility test

Pure colony culture on tryptic soy agar (added 2% NaCl) was stabed into the motile medium to depth approximately 2 cm. Incubate at 37°C for 24 h.

Interpretation criteria

The positive result was shown as growth extending into the agar from the stab line.

The negative result growth was only on stab line.

4.2.5 Salinity tolerant test

Pure colony culture on tryptic soy agar (added 2% NaCl) was adjusted a density equivalent to approximately 10^8 CFU/ml. Inoculate bacterial suspension 100 μl into 1% tryptone broth with 0%, 3%, 6%, 8% and 10% NaCl 3 ml and mix well. Incubate at 37°C for 18 h.

Positive controls are *Vibrio parahaemolyticus* ATCC 17802 and *Vibrio vulnificus* ATCC 27562

Negative control is *Escherichia coli* ATCC 25922

Interpretation criteria

The positive result was turbid broth when compare with negative control.

The negative result was not turbid broth which the same to negative control.

4.2.6 Sugar fermentation

Pure colony culture on TSA (added 2% NaCl) was adjusted a density equivalent to approximately 10^8 CFU/ml. Inoculate bacterial suspension 100 μl into 1% concentration of sugar broth 3 ml and mix well. Incubate at 37°C for 24 h.

Interpretation criteria

The positive result was change color of media from green to yellow.

The negative result was not change color of media.

4.2.7 Arginine dihydrolysis

Pure colony culture on TSA (added 2% NaCl) was adjusted a density equivalent to approximately 10^8 CFU/ml. Inoculate bacterial suspension

100 μl into 1% concentration of amino broth 3 ml, mix well and covered with paraffin oil. Incubate at 37°C for 24 h.

Interpretation criteria

The positive result was not change color of media.

The negative result was change color of media from purple to yellow.

4.2.8 Ornithine decarboxylase

Pure colony culture on TSA (added 2% NaCl) was adjust a density equivalent to approximately 10^8 CFU/ml. Inoculate bacterial suspension 100 μl into 1% concentration of amino broth 3 ml, mix well and covered with paraffin oil. Incubate at 37°C for 24 h.

Interpretation criteria

The positive result was not change color of media (purple).

The negative result was change color of media from purple to yellow.

4.2.9 Lysine decarboxylase

Pure colony culture on TSA (added 2% NaCl) was adjusted a density equivalent to approximately 10^8 CFU/ml. Inoculate bacterial suspension 100 μl into 1% concentration of sugar broth 3 ml and mix well and covered with paraffin oil. Incubate at 37°C for 24 h.

Interpretation criteria

The positive result was not change color of media (purple).

The negative result was change color of media from purple to yellow.

4.2.10 ONPG test

Pure colony culture on TSA (added 2% NaCl) was adjusted a density equivalent to approximately 10^8 CFU/ml. Inoculate bacterial suspension 100 μl into PBS 0.5 ml, pick up a ONPG disc into the culture tube. Incubate at 37°C for 24 h.

Interpretation criteria

The positive result was yellow broth.

The negative result was not yellow broth.

5. PCR Confirmation

Confirmation for species specific gene and Pathogenesis gene by PCR method for *V. vulnificus* and multiplex PCR method for *V. parahaemolyticus*

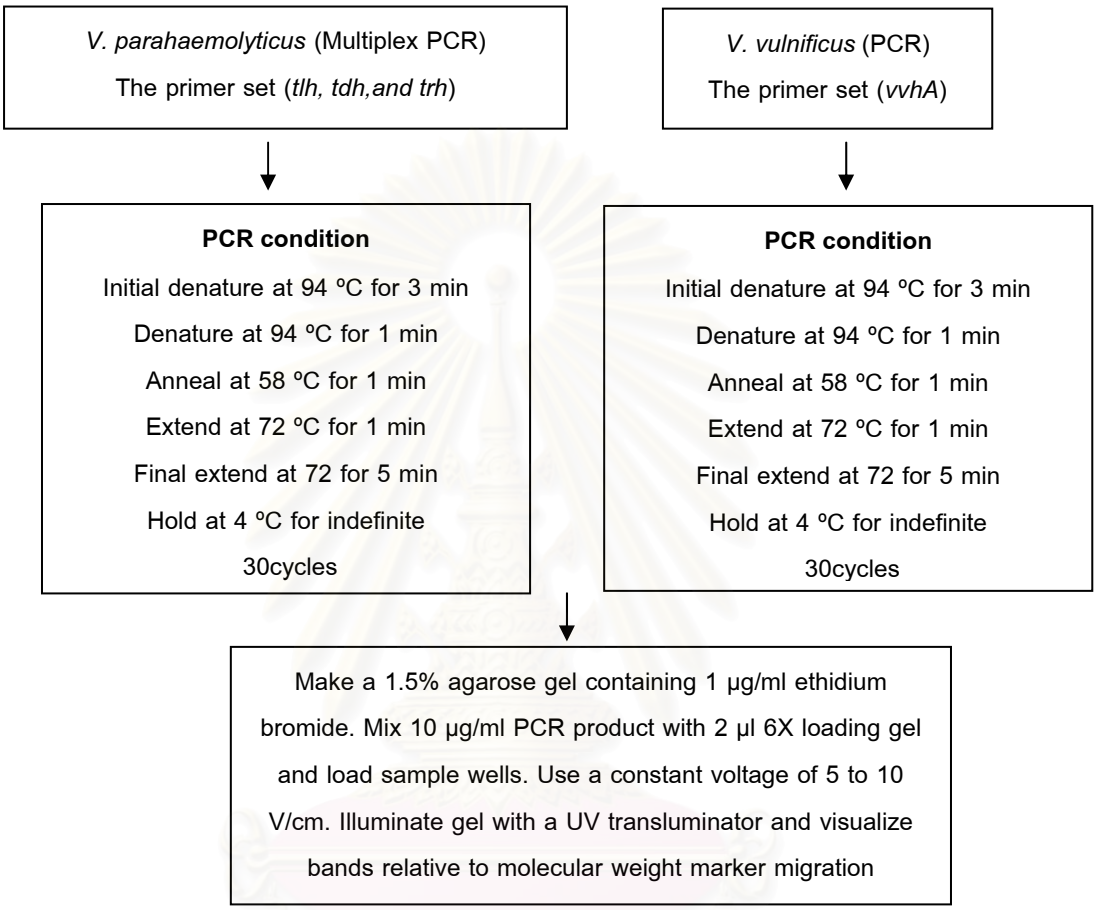

Figure 5 PCR Methodology Scheme

DNA Extraction

Each of the selected colonies of *V. parahaemolyticus* and *V. vulnificus* were inoculated into nutrient broth (NB) medium containing 1% NaCl and incubated overnight at 37 ºC. One milliliter of the overnight culture was examined by centrifuge at 3 minutes, 15,000Xg and washed the pellet twice with physiological

saline. Suspend the pellet in 1 ml distilled water and heated for 10 minutes at 100 °C and centrifuge at 5 minutes, 13000Xg.

Primer (84)

The three primer sets of *V. parahaemolyticus* followed by Bej et.al (127), that show in Table 3 were added to the reaction mixtures for 1.25 μl each the primers. The *vvhA* primer of *V. vulnificus* was added to the reaction tubes for confirmed *V. vulnificus* that followed by Lien-I Hor et.al (94, 126, 128) (Table 4).

Table 3 Oligodeoxynucleotide primers for *Vibrio parahaemolyticus (127)*

Target	Size of PCR product (bp)	Primer sequence (5' to 3')
tlh	450	Forward - AAAGCGGATTATGCAGAAGCACTG
		Reverse – GCTACTTTCTAGCATTTTCTCTGC
tdh	270	Forward - GTAAAGGTCTCTGACTTTTGGAC
		Reverse – TGGAATAGAACCTTCATCTTCACC
trh	500	Forward - TTGGCTTCGATATTTTCAGTATCT
		Reverse - CATAACAAACATATGCCCATTTCCG

Table 4 Oligodeoxynucleotide primers for *Vibrio vulnificus (94, 126, 128)*

Target	Size of PCR product (bp)	Primer sequence (5' to 3')
vvhA	519	Forward - CCGCGGTACAGGTTGGCGCA
		Reverse - CGCCACCCACTTTCGGGCC

Multiplex PCR assay

The multiplex PCR assay for *V. parahaemolyticus* followed by Bej et.al. (127), was performed in a total volume of 25 μl containing PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP, 2 μM concentration each primer (*tlh, tdh* and *trh*) and 0.625 U of *Taq* DNA polymerase. DNA amplification was carried out with the following thermal cycling profile: initial denaturation at 94°C for 3 min, 30 cycles of amplification (denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min) and a final extension at 72°C for 5 min in a thermal cycle Thermal cycler TC-312. PCR products were analyzed on a 1.5% agarose with 0.5X Tris-borate-EDTA (TBE) buffer. A 100 bp DNA ladder was used as the molecular size marker. The gel were stained with ethidium bromide and photographed under UV light. The PCR assay for *V. vulnificus* was developed from Lien-I Hor(129), 1995. In the total volume 25 μl containing PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), 2 μM of *vvhA* primer and 0.625 U of *Taq* DNA polymerase. DNA amplification was carried out with the following thermal cycling profiles same as *V. parahaemolyticus* analysis.

Analysis of PCR products

Amplification of *tlh*, *tdh* and *trh V. parahaemolyticus* specific targets produced distinct bands corresponding to their respective molecular sizes that were easily recognizable by Bej *et.al.*, 1999 (Figure 6). The *vvhA* PCR product of *V. vulnificus* exhibited 519 bp, which showed in Figure 7.

Quality control

Each multiplex PCR assay was carried out with a negative control containing all of the reagents without a DNA template. A *tlh, tdh* and *trh* strain (*V. parahaemolyticus* DMST 22013), was used as quality control strains. For tested *vvhA* of *V. vulnificus* used *V. vulnificus* ATCC 27562 for quality control strain.

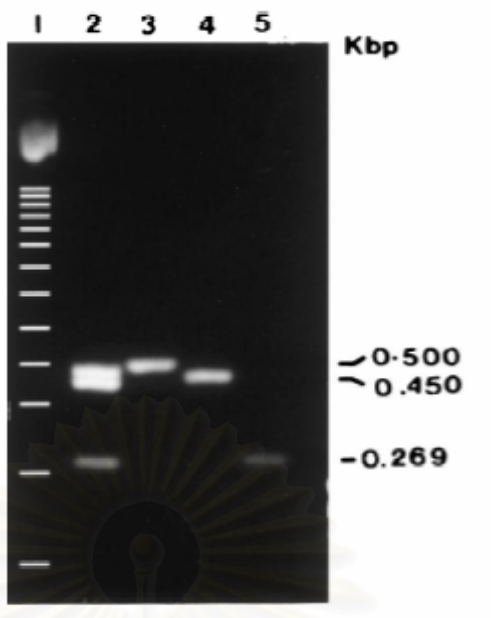

Figure 6 PCR product reference bands of *tlh*, *tdh* and *trh* gene (127)

Agarose gel electrophoresis showing the results from PCR amplification of genomic DNA from *V.parahaemolyticus* F113A. Lane 1, 123-bp DNA ladder (Gibco) as size marker enhanced by PRORFLP (Nashville, TN, USA) computer software; lane 2, multiplex PCR DNA ProScan amplification using oligonucleotide primers specific for the *tl*, *tdh* and the *trh* genes showing three bands of DNA with expected molecular weights of 0.450 kbp, 0.269 kbp, and 0.5 kbp, respectively; lane 3, PCR amplification using oligonucleotide primers specific for the *trh* gene; lane 4, PCR amplification using oligonucleotide primers specific for the *tlh* gene; lane 5, PCR amplification using oligonucleotide primers specific for the *tdh* gene.

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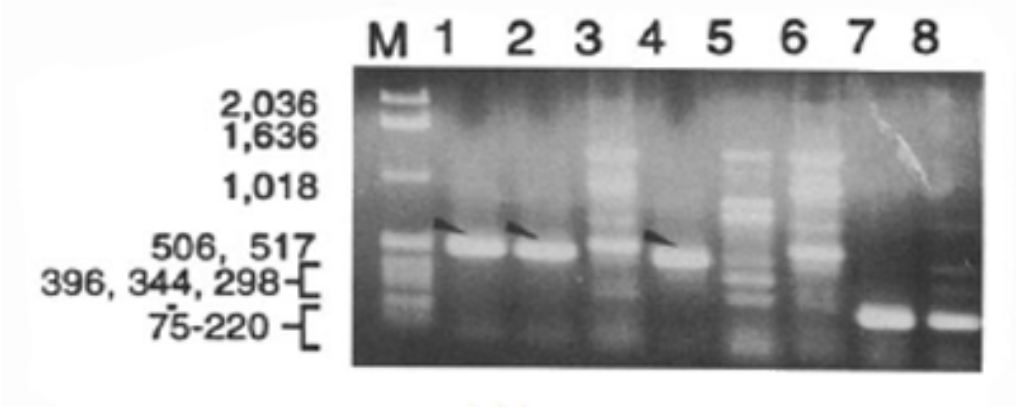

Figure 7 PCR product reference bands by Lien-I Hor et al., 1995 (94)

Isolation and Characterization of *Vibrio vulnificus* Inhabiting the Marine Environment of the Southwestern Area of Taiwan. Agarose gel electrophoresis of PCR products, a Photograph of the gel stained with ethidium bromide. The arrows indicate the amplified DNA fragment (519 bp) from the *vvhA* gene of V. *vulnificus.*

Prevalence and Quantitative of *V. parahaemolyticus* and *V. vulnificus*

The colonies were finally identified polymerase chain reaction test as *V. parahaemolyticus* and *V. vulnificus* least one of the two colonies was positive for each category, the tube was positive considered and apply the 3-tube–MPN tables for final enumeration of the organism.

The pacific oyster samples that contaminated *V. parahaemolyticus* and *V. vulnificus* were relevance to prevalence of *V. parahaemolyticus* and *V. vulnificus*.

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Table 5 For 3 tubes each at 0.1, 0.01, and 0.001 g inoculate, the MPN per gram and 95 percent confidence intervals (126)

Pos. tubes			MPN/	Conf. lim.		Pos. tubes			MPN/g	Conf. lim.	
0.10	0.01	0.001	g	Low	High	0.10	0.01	0.001		Low	High
0	0	0	<3.0	--	9.5	2	2	0	21	4.5	42
0	0	1	3.0	0.15	9.6	2	2	1	28	8.7	94
0	1	0	3.0	0.15	11	2	2	2	35	8.7	94
0	1	1	6.1	1.2	18	2	3	0	29	8.7	94
0	2	0	6.2	1.2	18	2	3	1	36	8.7	94
0	3	0	9.4	3.6	38	3	0	0	23	4.6	94
1	0	0	3.6	0.17	18	3	0	1	38	8.7	110
1	0	1	7.2	1.3	18	3	0	2	64	17	180
1	0	2	11	3.6	38	3	1	0	43	9	180
1	1	0	7.4	1.3	20	3	1	1	75	17	200
1	1	1	11	3.6	38	3	1	2	120	37	420
1	2	0	11	3.6	42	3	1	3	160	40	420
1	2	1	15	4.5	42	3	2	0	93	18	420
1	3	0	16	4.5	42	3	2	1	150	37	420
2	0	0	9.2	1.4	38	3	2	2	210	40	430
2	0	1	14	3.6	42	3	2	3	290	90	1,000
2	0	2	20	4.5	42	3	3	0	240	42	1,000
2	1	0	15	3.7	42	3	3	1	460	90	2,000
2	1	1	20	4.5	42	3	3	2	1100	180	4,100
2	1	2	27	8.7	94	3	3	3	>1100	420	--

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Part II Virulent Factors Tests

1. Virulent factors of *V. parahaemolyticus*

Kanagawa Phenomenon Test

V. parahaemolyticus growth in TSA (added 2% NaCl) and incubated over night at 37 ^{0}C. The pure colonies on TSA were spot onto the Wagatsuma agar and incubated 24 h at 37 ^{0}C.

Interpretation criteria

The Kanagawa Phenomenon positive result was clear zone around colony on Wagatsuma agar

The negative result was not clear zone around colony

Urease test

Pure colonies of *V. parahaemolyticus* on TSA (added 2% NaCl) were streaked on Christensen's urea agar supplemented with NaCl, 2% final concentration and incubated at 37°C for 24 h.

Interpretation criteria

The positive result was pink color of media.

The negative result was not change color of media.

Gelatinase

Pure colonies of *V. parahaemolyticus* on TSA (added 2% NaCl) were streaked on gelatin salt agar and incubated at 37°C for 24 h.

Interpretation criteria

The positive result was clear zone around streaked line after poured with 1% $HgCl_2$

The negative result was not change around streaked line.

Protease

Pure colonies of *V parahaemolyticus* on TSA (added 2% NaCl) were streaked on skim milk agar and incubated at 37°C for 24 h.

Interpretation criteria

The positive result was clear zone around streaked line.

The negative result was not change around streaked line.

2. Virulent factors of *V. vulnificus*

Hemolysin

V. vulnificus growth in TSA (added 2% NaCl) were streaked on blood agar that added 2% NaCl and incubated 24 h at 37 ^{0}C.

Interpretation criteria

The positive result was clear zone around colony.

The negative result was not clear zone around colony.

DNase test

V. vulnificus growth in TSA (added 2% NaCl) were streaked on DNase agar added 2% NaCl and incubated 24 h at 37 ^{0}C.

Interpretation criteria

The positive result was clear zone around streaked line then pour with 1N HCl

The negative result was not clear zone around streaked line.

Protease test

V. vulnificus growth in TSA (added 2% NaCl) were streaked on skim milk agar added 2% NaCl, incubated 24 h at 37 ^{0}C.

Interpretation criteria

The positive result was clear zone around streaked line.

The negative result was not clear zone around streaked line.

Lipase test

V. vulnificus growth in TSA (added 2% NaCl) were streaked on egg yolk agar and Tween-80 added 2% NaCl, incubated 24 h at 37 ^{0}C.

Interpretation criteria

The positive result was turbidity crystal around streaked line on tween-80 or glister color on egg yolk agar.

The negative result was neither turbidity crystal around streaked line or nor glister color.

Lecithinase test

V. vulnificus growth in TSA (added 2% NaCl) were streaked on egg yolk agar added 2% NaCl, incubated 24 h at 37 ^{0}C.

Interpretation criteria

The positive result was turbidity around streaked line on egg yolk agar.

The negative result was not turbidity around streaked line.

Mucinase test

V. vulnificus growth in TSA (added 2% NaCl) were streaked on Mucin agar added 2% NaCl, incubated 72 h at 37 ^{0}C.

Interpretation criteria

The positive result was clear zone around streaked line on Mucin agar.

The negative result was not clear zone around streaked line.

Elastase test

V. vulnificus growth in TSA (added 2% NaCl) were streaked on Elastin agar added 2% NaCl, incubate 72 h at 37 ^{0}C.

Interpretation criteria

The positive result was clear zone around streaked line on Elastin agar.

The negative result was not clear zone around streaked line.

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PART III : Antimicrobial resistant test (130)

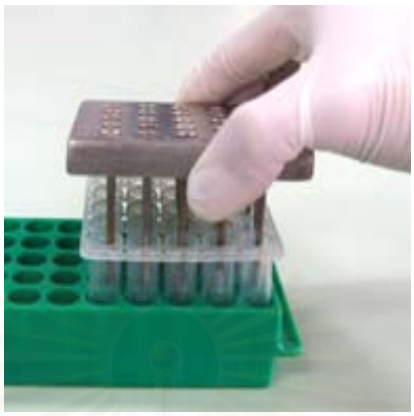

Figure 8 Show replicator pins in MIC method

1. Agar dilution test

1.1 Media and antimicrobial agents

1.1.1 Media

Mueller-Hinton (MH) agar supplement with 1% NaCl which meets the requirements of the CLSI standard is considered the reference medium.

1.1.2 Antimicrobial agents

To determine minimum inhibitory concentrations (MICs) of forth antibacterial agents, ciprofloxacin, doxycycline, norfloxacin and cefotaxime were used in this study.

1.2 Preparation of stock solutions

1.2.1 To calculate the stock solutions following formula :

$$\text{Weight of powder (mg)} = \frac{\text{Volume (ml)} \times \text{Concentration } (\mu g/ml)}{\text{Potency of powder } (\mu g/mg)}$$

1.2.2 Antibacterial agents were dissolved in solvents are listed in Table 6 and were diluted in diluents, as sterile distilled water.

1.2.3 To store stock solutions frozen in aliquots at -20°C or below until used.

1.3 Preparation of working solutions

1.3.1 Use two fold dilution series of agar dilutions MICs.

1.3.2 Diluting a 5,120 mg/l stock solution, the range of concentrations tested each antibacterial agents follow by Table 7.

1.3.3 Dilution schemes are given in Table 8, the schemes involve adding 18 ml volumes of MH agar to 2 ml volumes of each an antimicrobial solution. This study is diluting a 5,120 mg/l stock solution.

1.4 Preparation of plates

The sterilized MH agar supplement with 1% NaCl to cool at 50°C in a water bath. Prepare a dilution series of antimicrobial agents, as above, in 50 ml containers. Include a drug-free control. Add 2 ml of antimicrobial solution each a concentration to each MH agar containers, mix thoroughly, and pour the MH agar into sterile petri dishes on a level surface. Allow the plates to set at room temperature and dry the plates so that no drops of moisture remain on the surface of the agar. Do not over dry plates. Plates should not be stored unless the agents have been shown to be stable on storage.

1.5 Preparation of inoculum

Standardize the density of inoculum to give 10^4 colony-forming units (CFU) per spot on the agar. Use four or five colonies of a pure culture to avoid selecting and atypical variant. At 0.5 Mcfarland may be used for visual compatision to adjust the suspension to a density equivalent to approximately 10^8 CFU/ml. Dilute the suspensions of organisms in 0.85% to 10^7 CFU/ml. Plates must be inoculated within 30 min of standardizing the inoculum, to avoid changes in inoculum density.

1.6 Inoculation plates

Mark the plates so that the orientation is obvious. Transfer diluted bacterial suspensions to the wells of an inoculum replicating apparatus (Figure 8). Use the apparatus to transfer the inocula to the series of plates, including a control plate without antimicrobial agent. Replicator pins 2.5 mm in diameter will transfer about 1 μl, i.e. an inoculum of 10^4 CFU/spot. Allow the inoculum spots to dry at room temperature before inverting the plates for incubation.

1.7 Incubation of plates

Incubate plates at 37°C in air for 18 hours. In order to avoid uneven heating, do not stock more than five high.

1.8 Interpretation of result

The MIC is the lowest concentration of the agent that completely inhibits visible growth as judged by the naked eyes, disregarding a single colony or a thin haze within the area of the inoculated spot. Interprete follow in Table 9 and analyzed susceptibility test data by WHONET 5 program (1999).

1.9 Quality Control

Reference strains of *Escherichia coli* ATCC 25922, *Pseudomonas aeroginosa* ATCC 27853 were used for quality control in the agar dilution test (Table 10).

Table 6 Solvents and diluents for dissolving antibacterial agents (130)

Antimicrobial agents	Solvents	Diluents
Doxycycline	Water	Water
Norfloxacin	0.1N NaOH	Water
Ceprofloxacin	Water	Water
Cefotaxime	Water	Water

Table 7 The range of concentrations tested each an antibacterial agents (130)

Antimicrobial agents	Range of concentration tested μg/ml)
Doxycycline	1 , 2 , 4 , 8 , 16 , 32 , 64
Norfloxacin	1 , 2 , 4 , 8 , 16 , 32
Ceprofloxacin	0.25 , 0.5 , 1 , 2 , 4 , 8
Cefotaxime	2, 4 , 8 , 16 , 32 , 64 , 128, 256

Table 8 The dilution schemes of antimicrobial for use in agar dilution (130)

Step	Concentration (μg/ml)	Source	Volume use (131)	Add distilled water (131)	Intermediate Conc. (μg/ml)	1:10 Dilution in agar	Log 2
1	5,120	Stock	-	-	5,120	512	9
2	5,120	Step 1	1	1	2,560	256	8
3	5,120	Step 1	1	3	1,280	128	7
4	1,280	Step 3	1	1	640	64	6
5	1,280	Step 3	1	3	320	32	5
6	1,280	Step 3	1	7	160	16	4
7	160	Step 6	1	1	80	8	3
8	160	Step 6	1	3	40	4	2
9	160	Step 6	1	7	20	2	1
10	20	Step 9	1	1	10	1	0
11	20	Step 9	1	3	5	0.5	-1
12	20	Step 9	1	7	2.5	0.25	-2
13	2.5	Step 12	1	1	1.25	0.125	-3

Table 9 MIC standard range and their interpretation for the antimicrobial agents for *Vibrio* spp. (130)

Antimicrobial agent	MIC breakpoint		
	Resistant	Intermediate	Susceptible
Doxycycline	$\geq$ 16	8	$\leq$4
Norfloxacin	$\geq$ 16	8	$\leq$4
Ceprofloxacin	$\geq$ 4	2	$\leq$1
Cefotaxime	$\geq$64	16-32	$\leq$8

Table 10 MIC of reference control for MIC determination (μg/ml) (130)

Antimicrobial Agents	MIC determination (μg/ml)	
	Pseudomonas aerginosa ATCC 27853	*Escherichia coli* ATCC 25922
Doxycycline	-	0.5-2
Norfloxacin	1-4	0.03-0.12
Ceprofloxacin	0.25-1	0.004-0.016
Cefotaxime	8-32	0.03-0.12

PART IV : REFERENCE BACTERIAL STRAINS

1. For biochemical characteristic test

Vibrio parahaemolyticus ATCC 17802 and *Vibrio vulnificus* ATCC 27562 were used for positive control of biochemical test. *Escherichia coli* ATCC 25922 was used for negative control of biochemical test.

2. For susceptibility test (agar dilution test)

Reference strains of *Escherichia coli* ATCC 25922 and *Pseudomonas aeroginosa* ATCC 27853 were used for quality control in the agar dilution test.

3. For PCR

3.1 *Vibrio parahaemolyticus* strain DMST 22013 carrying *tlh*, *tdh and trh* gene was used for positive control in PCR amplification step, free template solution was used for negative control strains in PCR amplification.

3.2 *Vibrio vulnificus* strain ATCC 27562 carrying *vvhA* gene was used for positive control in PCR amplification, free template solution was used for negative control strains in PCR amplification.

4. For virulence factor test

Vibrio parahaemolyticus strain DMST 22013 for Wagatsuma phenomenon test, *Bacillus cereus* was positive reference strain for haemolysin, proteinase, lecithinase, lipase, DNase. *Bacillus subtilis* was positive reference strain for elastase. *Bacillus polymyxa was* positive reference strain for mucinase and *E. coli* ATCC 25922 is negative control.

PART V : CULTURE PRESERVATION

1. Media for culture preservation

   Motile medium add 1% NaCl

2. Preservation method

   Use pure single colony stab into the motile medium and drop paraffin upper agar and store at room temperature.

PART VI : STATISTICAL ANALYSIS

The MPN value that were indetermine (< 0.3 or < 3.0) as a result of no positive tubes in any of the series were assigned a v of *V. parahaemolyticus* and *V. vulnifcus* were transformed alue halfway between the maximum value and zero (i.e., 0.15 or 1.5, respectively). The MPN counts were converted to base 10 logarithms before being subjected to analysis. Variance due to the three-tubes MPN method was estimated by five sets of ten farms measurements, each ten farms set was performed on the same three oysters (131).

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CHAPTER V

Results

Part I : Prevalence and Quatitative of *Vibrio parahaemolyticus* and *Vibrio vulnificus*

A total of 360 living oysters samples were collected from Bandon bay in April 2007 through March 2008. Chaiya, Cha-ngor, Ka-dae, Ta-tong and Kadae-gae. All of the samples were analysed within 24 hours after collection.

The turbid MPN tubes were streaked onto TCBS agar for selected *V. parahaemolyticus* and CC agar for selected *V. vulnificus*. The green or blue green colonies on TCBS agars and 2-3 mm. of yellow, fried egg colonies on CC agars were selected for gram strain, oxidize test, TSI, AGS, motility, salt tolerance, amino hydrolysis, sugar utilization and ONPG test.

Then *V. parahaemolyticus* and *V. vulnificus* were isolated and identified with conventional biochemical method, the PCR method was used to confirm. The *tlh* was species specific gene, confirmed for *V. parahaemoylticus* species. The virulence gene were *tdh* and/or *trh* gene, *tdh* gene produce thermostable direct hemolysin (TDH) that is associated with the Kanagawa phenomenon, *trh* gene code for TDH-related hemolysin (TRH) related with urease activity for pathogenic strains of *V. parahaemolyticus.* The *vvhA* gene associated with virulence *V. vulnificus* strain.

The *tlh* gene positive of *V. parahaemolyticus* strains exhibited 1,457 of *V. parahaemolyticus* isolates from 256/360 oyster samples but none of *V. parahaemolyticus* isolates positive for *tdh* and/or *trh* gene, which related with Kanagawa phenomenon and urease activity negative tests (Table 11 and Figure 9, 10). The PCR conformed of *V. vulnificus* isolates were 609 of 99/360 in the oyster samples positive for *vvhA* gene (Figure 11 and 12).

Table 11 Confirmation for species specific gene and pathogenesis gene of *V. parahaemolyticus* isolated from Bandon bay

Target genes			Kanagawa phenomenon	Urease production
tlh	***tdh***	***trh***		
1,457	0	0	0	0

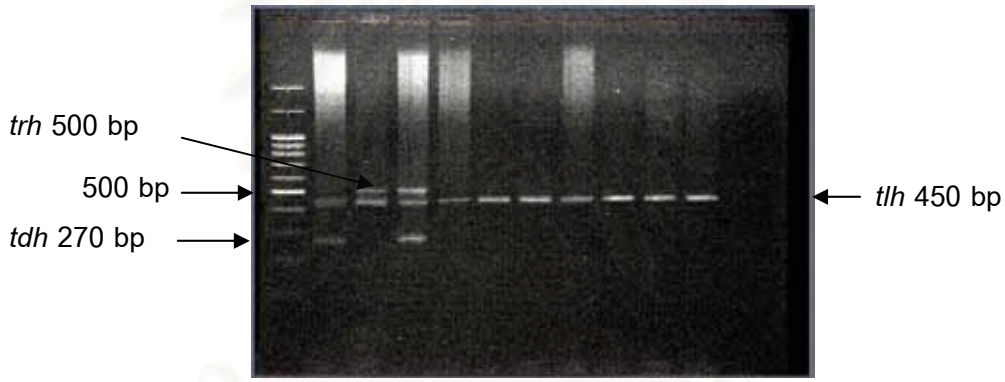

Figure 9 Agarose gel electrophoresis of V. parahaemolyticus PCR products. Lane 1, lamda ladder for molecular weight standard; lane 2, *tlh* and *tdh* of *V. parahaemolyticus* DMST 22013; lane 3, *tlh* and *trh* of *V. parahaemolyticus* DMST 22013; lane 4, *tlh, tdh* and *trh* of *V. parahaemolyticus* DMST 22013; lane 5-11, *V. parahaemolyticus* isolated from pacific oysters; lane 12, negative control

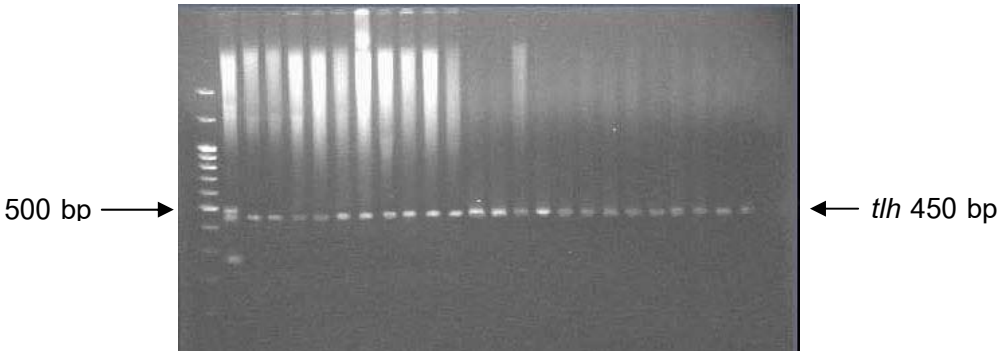

Figure 10 Agarose gel electrophoresis of PCR products.

Lane 1, lamda ladder for molecular weight standard; lane 2, *tlh, tdh and trh* of *V. parahaemolyticus* DMST 22013; lane 3-25, *V. parahaemolyticus* isolated from pacific oysters; lane 26, negative control

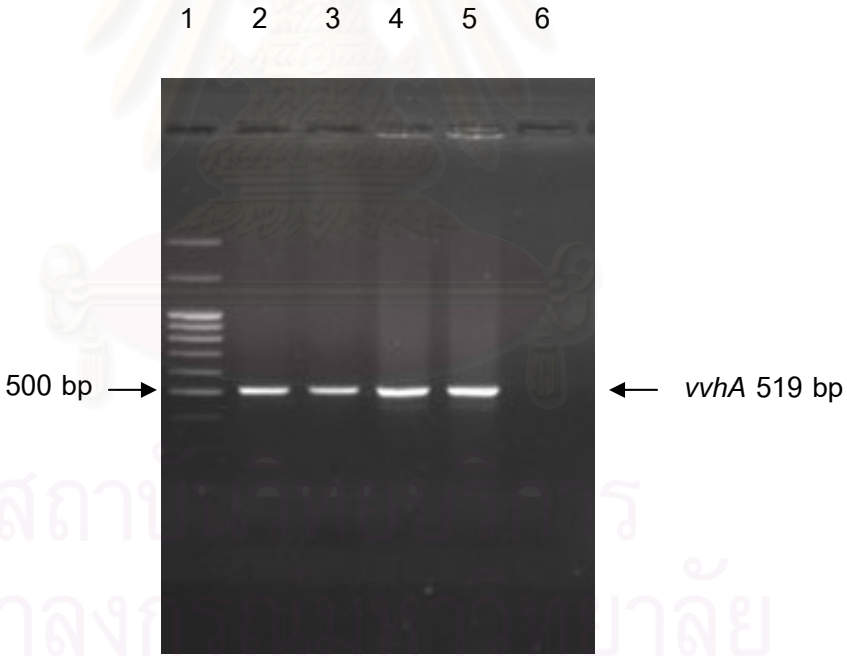

Figure 11 Agarose gel electrophoresis of *vvhA* PCR products.

Lane 1, lamda ladder for molecular weight standard; lane 2, 3 *V. vulnificus* ATCC 27562; lane 4, 5 *V. vulnificus* DMSC 5852; lane 6, negative control

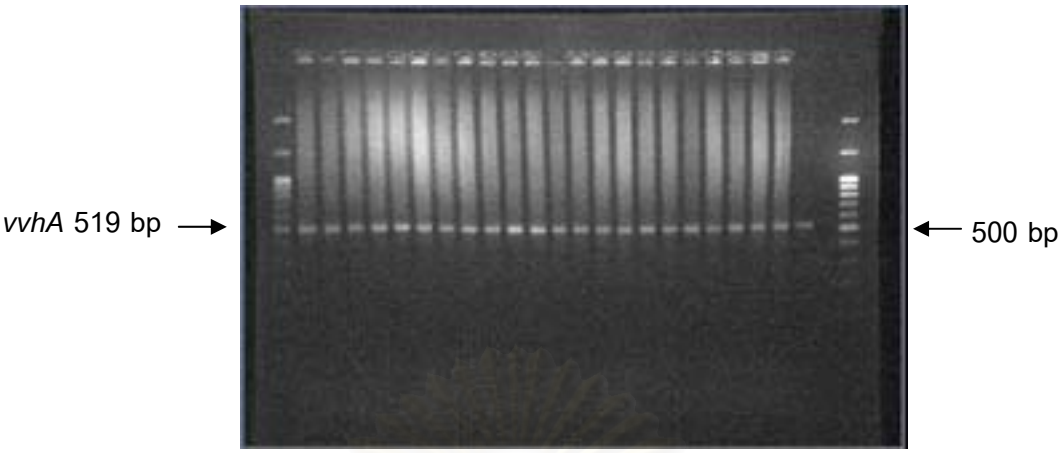

Figure 12 Agarose gel electrophoresis of *vvhA* PCR products.

Lane 1,26 lamda ladder for molecular weight standard; lane 2 *V. vulnificus* ATCC 27562; lane 3-24, *V. vulnificus* isolated from pacific oysters; lane 25, negative control

Prevalence of *V. parahaemolyticus* and *V. vulnificus*

The prevalence of *V. parahaemolyticus* and *V.vulnificus* in the pacific oyster samples from Bandon Bay during April 2007-March 2008 provide in Table 12 and Graph 1. Prevalence of *V. parahaemolyticus* was 172 (47.78 %), *V. vulnificus* was 15 (4.17%), *V. parahaemolyticus* and *V. vulnificus* were 84 (23.33%), which these pacific oyster samples were contaminated *V. parahaemolyticus* and/or *V. vulnificus* were 271 (75.28%) of 360 pacific oyster samples. During December 2007 to January 2008 found high prevalence of both, especially in January (93.3%). All of oyster samples positive for *V. parahaemolyticus* in February and March 2008, but none *V. vulnificus*. *V. vulnificus* exhibited high prevalence during December 2007 and January 2008, which all of the oyster samples in January contaminated for *V. vulnificus.*

Table 12 Prevalence of *V.parahaemolyticus* and *V.vulnificus* in Pacific oyster samples isolated from Bandon Bay (April, 2007-March, 2008)

Month	Prevalence			Total
	V.parahaemolyticus Number (%)	*V.vulnificus* Number (%)	*V.parahaemolyticus* & *V.vulnificus* (%)	N = 30/month (%)
April,07	6 (20)	0 (0)	6 (20)	12 (40)
May,07	15 (50)	0 (0)	9 (30)	24 (80)
June,07	18 (60)	0 (0)	6 (20)	24 (80)
July,07	15 (50)	0 (0)	6 (20)	21 (70)
August,07	15 (50)	0 (0)	0 (0)	15 (50)
September,07	12 (40)	1 (10)	2 (6.7)	15 (50)
October,07	19 (63.3)	0 (0)	6 (20)	25 (83.3)
November,07	12 (40)	6 (20)	0 (0)	18 (60)
December,07	0 (0)	6 (0)	21 (70)	27 (90)
January,08	0 (0)	2 (0)	28 (93.3)	30 (100)
February,08	30 (100)	0 (0)	0 (0)	30 (100)
March,08	30 (100)	0 (0)	0 (0)	30 (100)
Total (n= 360) /12 month	172 (47.78)	15 (4.17)	84 (23.33)	271 (75.28)

During April 2007 to March 2008, prevalence of *V. parahaemolyticus* in pacific oysters was regularly contamination, which occurrence all study period but *V. vulnificus* less occurred in oyster samples, that none appeared of *V. vulnificus* in August 2007, February and March 2008.

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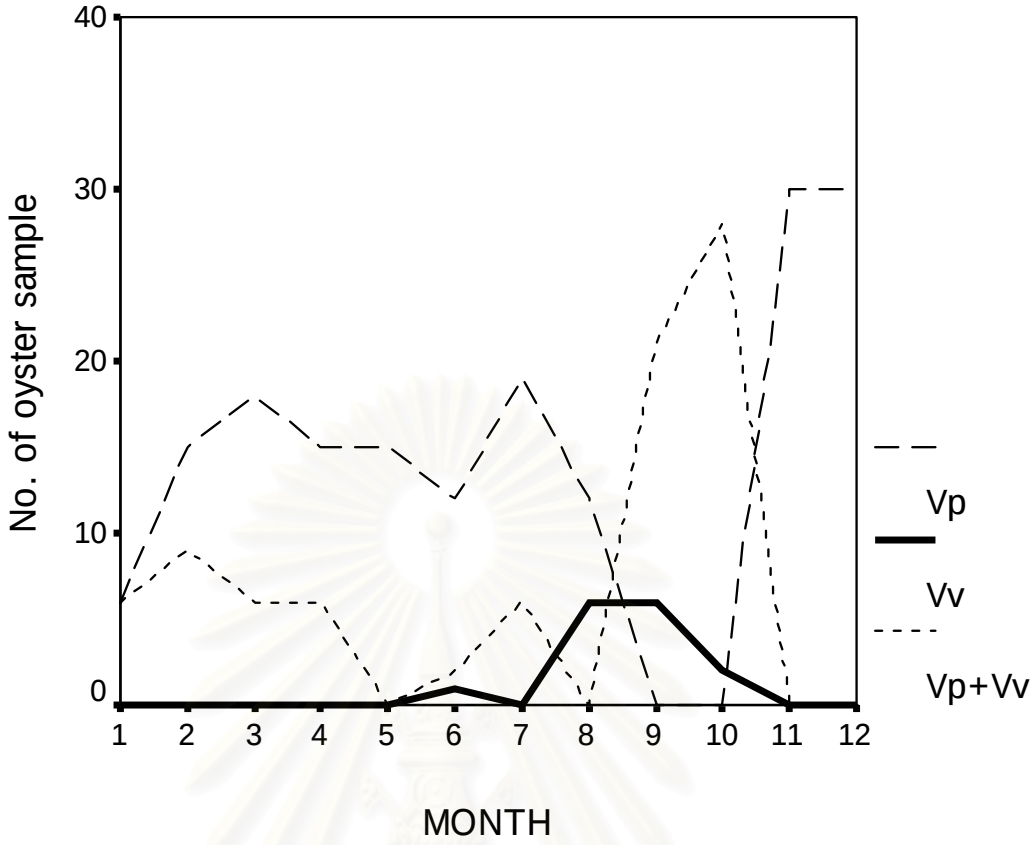

Graph 1 Monthly number the pacific oyster samples from the Bandon bay that show positive of *V. parahaemolyticus* and *V. vulnificus.* (n=30 per month)

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The densities of *V. parahaemolyticus* contaminated in the pacific oysters collected from Bandon bay during April, 2007 to March, 2008 exhibited in Table 13. In April, May, July, August, October, November, December and January found average densities of *V. parahaemolyticus* over 4 log MPN/g, especialy highest in May (6.3±2.2 log MPN/g) but less than in June (3.6±0.9 log MPN/g), September (3.7±1.1 log MPN/g), Febuary (3.6±0.8 log MPN/g) and March (2.4±0.7 log MPN/g). The average densities of *V. parahaemolyticus* in the 256 pacific oysters were 4.5±1.7 log MPN/g. The minimal density of *V. parahaemolyticus* was 1.9 log MPN/g and the maximal density was 8.4 log MPN/g.

Table 13 Quantitative of *V. parahaemolyticus* contaminated in the pacific oysters from Bandon Bay, Surat Thani Province (April, 2007-March, 2008)

Month	Number of oyster samples contaminated Vp	Mean log MPN/g ± SD	Mean log MPN/g	
			Min.	Max.
April,07	12	5.7±1.2^{abdeh}	4.2	7.6
May,07	24	6.3±2.2^{ab}	2.2	8.4
June,07	24	3.6±0.9^{cf}	2.2	5.6
July,07	21	5.4±1.7^{adeghi}	3.0	7.6
August,07	15	5.8±1.1^{ade}	3.6	7.0
September,07	14	3.7±1.1^{cfgj}	2.6	5.2
October,07	25	5.7±0.8^{ade}	4.6	8.0
November,07	12	4.5±0.6^{dfghij}	3.6	5.2
December,07	21	4.7±1.5^{adghij}	2.2	7.3
January,08	28	4.5±1.5^{dghij}	2.4	7.3
February,08	30	3.6±0.8^{cf}	2.6	5.2
March,08	30	2.4±0.7	1.9	5.0
Total	256	4.5±1.7^{fghij}	1.9	8.4

MPN = most probable number, SD = standard deviation of mean

Values followed by the same letter within a column are not significantly different by Nonparametric method, Mann-Whitney U-test, ($p<0.05$).

The densities of *V. vulnificus* contaminated in the 99 pacific oysters collected from Bandon bay during April, 2007 to March, 2008 exhibited in Table 14. The average densities of *V. vulnificus* low in April (2.7±0.5 log MPN/g) and July (2.1±0.2 log MPN/g), but hight in May, June, September, October, November, December and January especialy highest in December (5.9±1.0 log MPN/g). The average densities of *V. vulnificus* in the 99 pacific oysters were 4.9±1.3 log MPN/g. The minimal density of *V. vulnificus* was 1.9 log MPN/g and the maximal density was 8.7 log MPN/g.

Table 14 Quantitative of *V. vulnificus* contaminated in the pacific oysters from Bandon Bay, Surat Thani Province (April, 2007-March, 2008)

Month	Number of oyster samples contaminated Vv	Mean log MPN/g ± SD	Mean log MPN/g	
			Min.	Max.
April,07	6	2.7±0.5	2.2	3.4
May,07	9	4.4±0.5^{acd}	3.6	5.2
June,07	6	5.3±0.2^{bce}	5.0	5.6
July,07	6	2.1±0.2	1.9	2.3
September,07	3	4.9±0.3^{abce}	4.6	5.2
October,07	6	5.3±0.3^{bce}	5.0	5.9
November,07	6	4.2±0.2^{ad}	3.9	4.3
December,07	27	5.9±1.0^{bce}	4.2	8.7
January,08	30	5.0±0.7^{abc}	4.0	7.0
Total	99	4.9±1.3^{abc}	1.9	8.7

MPN = most probable number, SD = standard deviation of mean

Values followed by the same letter within a column are not significantly different by Nonparametric method, Mann-Whitney U-test, ($p<0.05$).

Quantitative of *V. parahaeolyticus* and *V. vulnificus* in the pacific oysters from Bandon Bay, Surat Thani Province (April,2007-March, 2008)

The *V. parahaemolyticus* and *V. vulnificus* isolated and identification by conventional biochemical method and confirm by PCR method, least one or two colonies of these organisms in each plate of MPN tube dilution show positive result for PCR test that the MPN tube was considered positive. The number of MPN tube positive was calculated MPN value per gram of sample.

The densities of *V. parahaemolyticus* in Chaiya during April, 2007 to March, 2008, were over 4 log MPN/g, especialy in May (7.5±0.4 log MPN/g), but less in Febuary (3.5±0.7 log MPN/g) and March (3.5±1.0 log MPN/g)

The densities of *V. vulnificus* were highest in December (4.7±0.4 log MPN/g), January (4.4±0.3 log MPN/g), April (2.7±0.5 log MPN/g), and July (2.1±0.2 log MPN/g), respectively.

During study period time, the average densities of *V. patrahaemolyticus* in Chaiya were more than *V. vulnificus*, which 5.3±1.4 log MPN/g and 1.3±1.7 log MPN/g, respectively (Table 15 and Graph 2).

The densities of *V. parahaemolyticus* in Cha-ngor during April 2007 to March 2008, were over 4 log MPN/g, especialy in May (7.4±0.1 log MPN/g), but less in June (3.3±0.4 log MPN/g), September (2.8±0.2 log MPN/g), November (3.9±0.2 log MPN/g) and March (2.3±0.3 log MPN/g).

The densities of *V. vulnificus* were highest in December (5.6±0.9 log MPN/g), June (5.3±0.2 log MPN/g), January (4.5±0.3 log MPN/g) and May (4.3±0.6 log MPN/g), respectively.

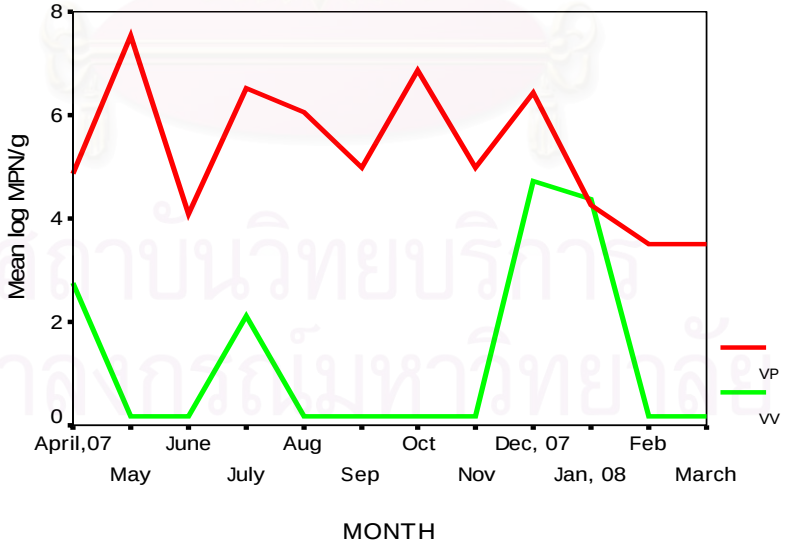
During study period time, the average densities of *V. patrahaemolyticus* in Cha-ngor were more than *V. vulnificus*, except in June, December and January. The total density of *V. parahaemolyticus* in the pacific oyster collected from Cha-ngor was 4.8±1.8 log MPN/g and 1.8±2.3 log MPN/g for *V. vulnificus* (Table 16 and Graph 3).

Table 15 Monthly counts of *V. parahaemolyticus* and *V. vulnificus* in the pacific oyster samples from the Chaiya, Ban don Bay.

Month	Average Vp (log MPN± SD)	
	V. parahaemolyticus	*V. vulnificus*
April, 07	4.9±0.6^{agh}	2.7±0.5
May, 07	7.5±0.4^{bf}	0.2±0.0^{a}
June, 07	4.1±0.3^{cg}	0.2±0.0^{a}
July, 07	6.5±0.7^{def}	2.1±0.2
August, 07	6.1±0.5^{defh}	0.2±0.0^{a}
September, 07	5.0±0.2^{agh}	0.2±0.0^{a}
October, 07	6.9±0.8^{bdef}	0.2±0.0^{a}
November, 07	5.0±0.1^{agh}	0.2±0.0^{a}
December, 07	6.4±0.6^{def}	4.7±0.4^{b}
January, 08	4.3±0.8^{acgh}	4.4±0.3^{b}
February, 08	3.5±0.7^{cg}	0.2±0.0^{a}
March, 08	3.5±1.0^{cg}	0.2±0.0^{a}
Total	5.3±1.4^{aegh}	1.3±1.7^{a}

MPN = most probable number, SD = standard deviation of mean

Values followed by the same letter within a column are not significantly different by Nonparametric method, Mann-Whitney U-test, ($p<0.05$).



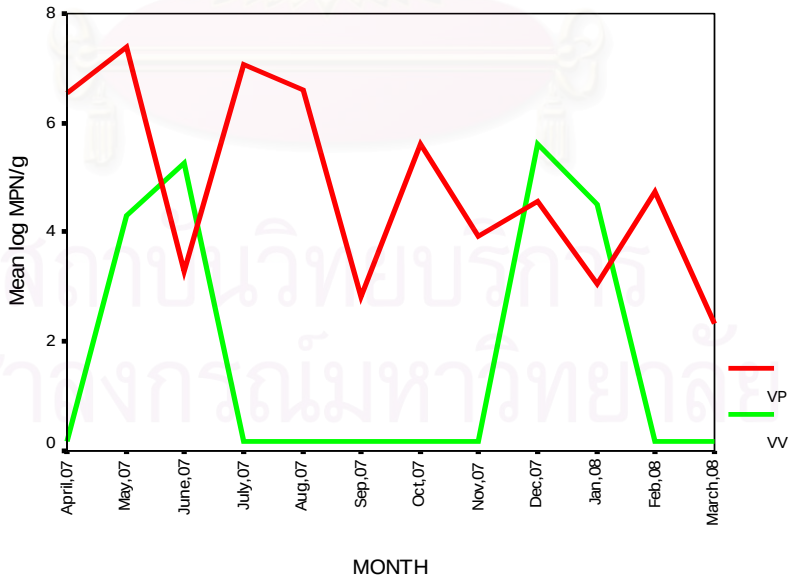
Graph 2 Monthly Densities of *V. parahaemolyticus* and *V. vulnificus* in the pacific oyster from the Chaiya during a 12-month period. Each point represents the geometric mean (n=6) of the log MPN of bacteria per gram of the pacific oyster meat.

Table 16 Monthly counts of *V. parahaemolyticus* and *V. vulnificus* in the pacific oyster samples from the Cha-ngor, Ban don Bay.

Month	Average Vp (log MPN± SD)	
	V. parahaemolyticus	*V. vulnificus*
April, 07	6.8±1.1 ^{abdeg}	0.2±0.0 ^{a}
May, 07	7.4±0.1 ^{abd}	4.3±0.6
June, 07	3.3±0.4 ^{cjk}	5.3±0.2
July, 07	7.1±0.6 ^{abe}	0.2±0.0 ^{a}
August, 07	6.6±0.4 ^{adek}	0.2±0.0 ^{a}
September, 07	2.8±0.2 ^{fj}	0.2±0.0 ^{a}
October, 07	5.6±0.5 ^{adgk}	0.2±0.0 ^{a}
November, 07	3.9±0.2 ^{hk}	0.2±0.0 ^{a}
December, 07	4.6±0.3 ^{ik}	5.6±0.9
January, 08	3.1±0.3 ^{cfj}	4.5±0.3
February, 08	4.7±0.3 ^{ik}	0.2±0.0 ^{a}
March, 08	2.3±0.3	0.2±0.0 ^{a}
Total	4.8±1.8 ^{ceghik}	1.8±2.3 ^{a}

MPN = most probable number, SD = standard deviation of mean

Values followed by the same letter within a column are not significantly different by Nonparametric method, Mann-Whitney U-test, ($p<0.05$).



Graph 3 Monthly Densities of *V. parahaemolyticus* and *V. vulnificus* in the pacific oyster from the Cha-ngor during a 12-month period. Each point represents the geometric mean (n=6) of the log MPN of bacteria per gram of the pacific oyster meat.

The densities of *V. parahaemolyticus* in Kadae during April 2007 to March 2008, were over 4 log MPN/g, especialy in May (7.5±0.6 log MPN/g), but less in July (3.8±0.8 log MPN/g), December (4.1±1.6 log MPN/g), January (3.9±1.2 log MPN/g), Febuary (2.9±0.3 log MPN/g), March (2.0±0.2 log MPN/g), which April, August, September and November were 0.2±0.0 log MPN/g.

The densities of *V. vulnificus* were highest in December (6.1±0.4 log MPN/g), June (5.3±0.2 log MPN/g), January (5.0±0.7 log MPN/g), and November (4.2±0.2 log MPN/g), respectively. During study period time, the average densities of *V. patrahaemolyticus* in Kadae were more than *V. vulnificus*, except in November, December and January. The total density of *V. parahaemolyticus* in the pacific oyster collected from Kadae was 2.9±2.4 log MPN/g and 1.4±2.3 log MPN/g for *V. vulnificus* (Table 17 and Graph 4).

The densities of *V. parahaemolyticus* in Tatong during April 2007 to March 2008, were lower 4 log MPN/g, except in January (6.6±0.6 log MPN/g).

The densities of *V. vulnificus* were highest in December (6.1±0.5 log MPN/g), January (5.1±0.4 log MPN/g), and May (2.4±2.5 log MPN/g), respectively.

During study period time, the average densities of *V. patrahaemolyticus* in Tatong were more than *V. vulnificus*, except in December. The total density of *V. parahaemolyticus* in the pacific oyster collected from Kadae was 1.7±2.0 log MPN/g and 1.3±2.2 log MPN/g for *V. vulnificus* (Table 18 and Graph 5).

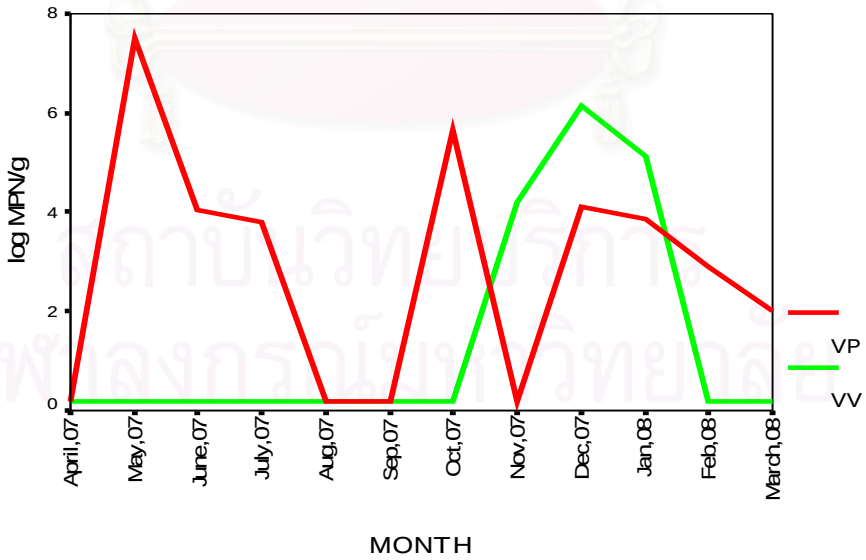
The densities of *V. parahaemolyticus* in Kadae-gae during April 2007 to March 2008, were lower 4 log MPN/g, except in October (4.9±0.3 log MPN/g), but less in June (1.7±1.6 log MPN/g), August (2.0±2.0 log MPN/g), September (1.0±1.3 log MPN/g), January (3.4±2.6 log MPN/g), Febuary (3.3±0.6 log MPN/g), March (2.0±0.2 log MPN/g), which April, May, July, November and December were 0.2±0.0 log MPN/g. The densities of *V. vulnificus* were highest in January (5.9±0.8 log MPN/g), October (5.3±0.3 log MPN/g), December (4.0±4.2 log MPN/g), September (2.6±2.6 log MPN/g) and the other had 0.2±0.0 log MPN/g. During study period time, the average densities of *V. patrahaemolyticus* in Kadae-gae more than *V. vulnificus*, except in September, October, December and January. The total density of *V. parahaemolyticus* in the pacific oyster collected from Kadae-gae was 1.6±1.9 log MPN/g and 1.6±2.5 log MPN/g for *V. vulnificus* (Table 19 and Graph 6).

Table 17 Monthly counts of *V. parahaemolyticus* and *V. vulnificus* in the pacific oyster samples from the Kadae, Ban don Bay.

Month	Average Vp (log MPN± SD)	
	V. parahaemolyticus	*V. vulnificus*
April, 07	0.2±0.0 [a]	0.2±0.0 [a]
May, 07	7.5±0.6	0.2±0.0 [a]
June, 07	4.1±1.3 [bcefg]	0.2±0.0 [a]
July, 07	3.8±0.3 [bcef]	0.2±0.0 [a]
August, 07	0.2±0.0 [a]	0.2±0.0 [a]
September, 07	0.2±0.0 [a]	0.2±0.0 [a]
October, 07	5.6±0.2 [de]	0.2±0.0 [a]
November, 07	0.2±0.0 [a]	4.2±0.2
December, 07	4.1±1.6 [bcdefg]	6.1±0.4
January, 08	3.9±1.2 [bcefg]	5.0±0.7
February, 08	2.9±0.3 [befg]	0.2±0.0 [a]
March, 08	2.0±0.2 [h]	0.2±0.0 [a]
Total	2.9±2.4 [bcefgh]	1.4±2.3 [a]

MPN = most probable number, SD = standard deviation of mean

Values followed by the same letter within a column are not significantly different by Nonparametric method, Mann-Whitney U-test, ($p<0.05$).



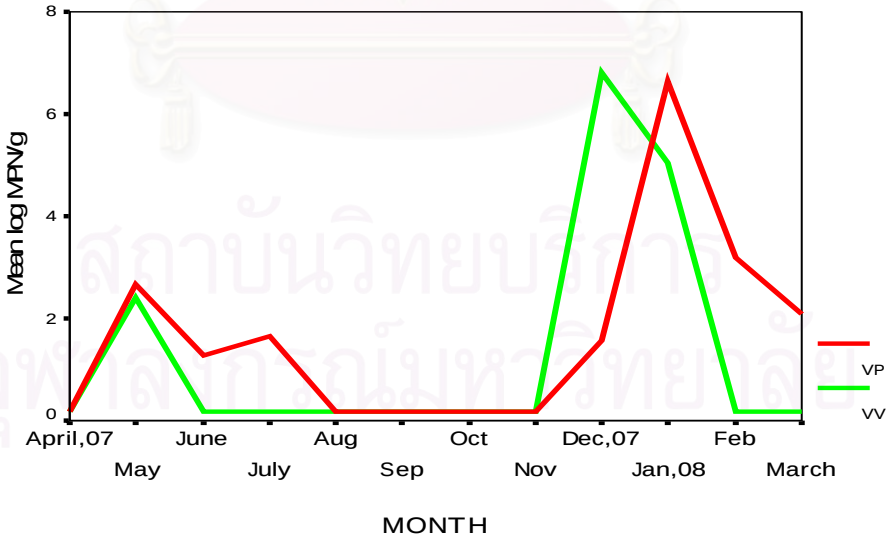
Graph 4 Monthly Densities of *V. parahaemolyticus* and *V. vulnificus* in the pacific oyster from the Kadae during a 12-month period. Each point represents the geometric mean (n=6) of the log MPN of bacteria per gram of the pacific oyster meat.

Table 18 Monthly counts of *V. parahaemolyticus* and *V. vulnificus* in the pacific oyster samples from the Tatong, Ban don Bay.

Month	Average Vp (log MPN± SD)	
	V. parahaemolyticus	*V. vulnificus*
April, 07	0.2±0.0 [acd]	0.2±0.0 [a]
May, 07	2.7±0.3 [bdeg]	2.4±2.5 [b]
June, 07	1.3±1.3 [acdfg]	0.2±0.0 [a]
July, 07	1.7±1.6 [abcdefg]	0.2±0.0 [a]
August, 07	0.2±0.0 [acd]	0.2±0.0 [a]
September, 07	0.2±0.0 [acd]	0.2±0.0 [a]
October, 07	1.0±2.0 [acdg]	0.2±0.0 [a]
November, 07	0.2±0.0 [acd]	0.2±0.0 [a]
December, 07	1.6±1.5 [abcdefg]	6.1±0.5
January, 08	6.6±0.6	5.1±0.4
February, 08	3.3±0.6 [bd]	0.2±0.0 [a]
March, 08	2.1±0.2 [cdfg]	0.2±0.0 [a]
Total	1.7±2.0 [bcdfg]	1.3±2.2 [ab]

MPN = most probable number, SD = standard deviation of mean

Values followed by the same letter within a column are not significantly different by Nonparametric method, Mann-Whitney U-test, ($p<0.05$).



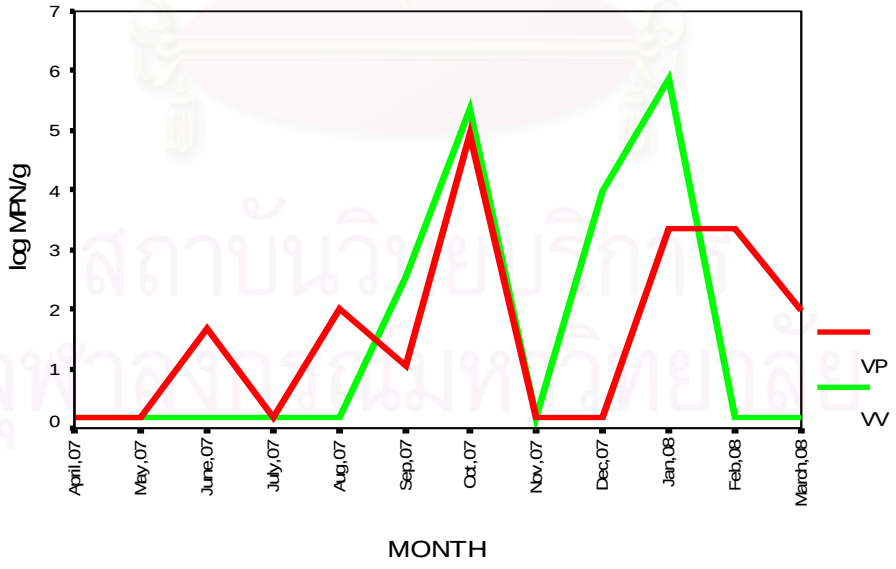
Graph 5 Monthly Densities of *V. parahaemolyticus* and *V. vulnificus* in the pacific oyster from the Tatong during a 12-month period. Each point represents the geometric mean (n=6) of the log MPN of bacteria per gram of the pacific oyster meat.

Table 19 Monthly counts of *V. parahaemolyticus* and *V. vulnificus* in the pacific oyster samples from the Kadae-gae, Ban don Bay.

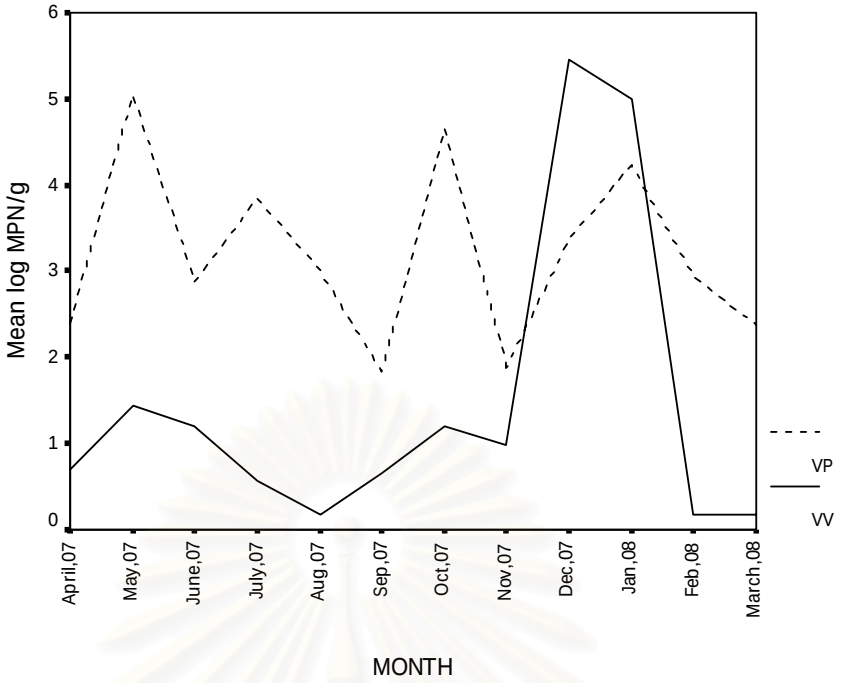
Month	Average Vp (log MPN± SD)	
	V. parahaemolyticus	*V. vulnificus*
April, 07	0.2±0.0 abc	0.2±0.0 a
May, 07	0.2±0.0 abc	0.2±0.0 a
June, 07	1.7±1.6 abcefg	0.2±0.0 a
July, 07	0.2±0.0 abc	0.2±0.0 a
August, 07	2.0±2.0 abcefg	0.2±0.0 a
September, 07	1.0±1.3 abceg	2.6±2.6 bd
October, 07	4.9±0.3 de	5.3±0.3 cd
November, 07	0.2±0.0 abc	0.2±0.0 a
December, 07	0.2±0.0 abc	4.0±4.2 bcd
January, 08	3.4±2.6 bcdefg	5.9±0.8 cd
February, 08	3.3±0.6 befg	0.2±0.0 a
March, 08	2.0±0.2 bcefg	0.2±0.0 a
Total	1.6±1.9 abceg	1.6±2.5 ab

MPN = most probable number, SD = standard deviation of mean

Values followed by the same letter within a column are not significantly different by Nonparametric method, Mann-Whitney U-test, ($p<0.05$).



Graph 6 Monthly Densities of *V. parahaemolyticus* and *V. vulnificus* in the pacific oyster from the Kadae-gae during a 12-month period. Each point represents the geometric mean (n=6) of the log MPN of bacteria per gram of the pacific oyster meat.



Graph 7 Total quantitative of *V. parahaemolyticus* and *V. vulnificus* in the Pacific oysters from Bandon bay in April, 2007 – March, 2008.

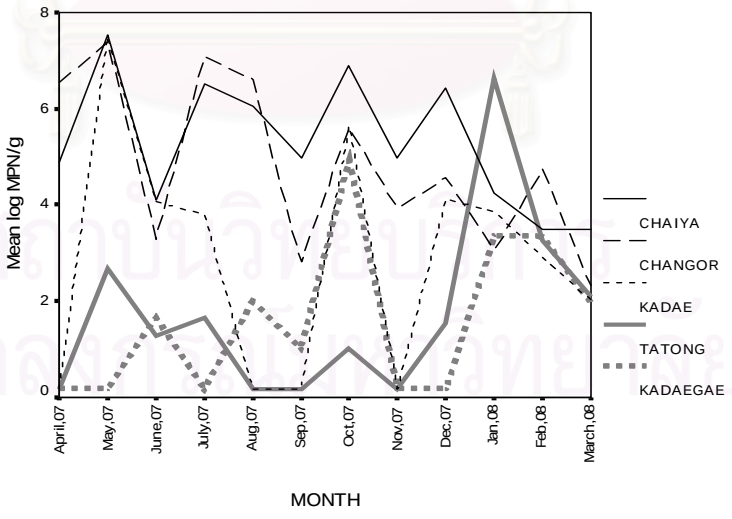
The occurrence of *V. parahaemolyticus* in 256 (71.1%) of 360 the oyster samples isolates from Bandon bay during April 2007 to March 2008. Densities varied considerably (0.2 to 7.5 log MPN/g) in the oyster samples from each location sampling (Table 20). All *V. parahaemolyticus* densities over 2 log MPN g^{-1} (10^2 MPN g^{-1}) between January to March 2008 (Graph 8), high densities of *V. parahaemolyticus* isolated from oyster samples in Chai-ya, Cha-ngor and Kadae in May 2007 were over 7 log MPN g^{-1} (10^7 MPN g^{-1}), which densities of *V. parahaemolyticus* in the oyster samples from Chaiya and Cha-ngor over 4 log MPN g^{-1} in studied period except lower in February and March 2008 but these densities over 2 log MPN g^{-1}. The oyster samples collected from Ta-tong and Kadae-gae had low densities of *V. parahaemolyticus* but over 4 log MPN g^{-1} in Octuber and January of Kadae-gae and Ta-tong, respectively. The total densities of *V. parahaemolyticus* isolated from the pacific oyster samples in Bandon Bay, were 3.2±2.5 log MPN g^{-1}. Differences among the five locate with respects to log MPN data were analyzed by nonparametric method. There are evidence that the mean log MPN values are difference at the five site ($p < 0.05$).

Table 20 Total counts of *V. parahaemolyticus* in the pacific oyster samples from the Ban don Bay.

Month	Average Vp (log MPN± SD)					
	Chaiya	Cha-Ngor	Kadae	Ta-tong	Kadae-jae	Total
April, 07	4.9±0.6^{agh}	6.8±1.1^{abdeg}	0.2±0.0^{a}	0.2±0.0^{acd}	0.2±0.0^{abc}	2.4±2.9^{acdefhikl}
May, 07	7.5±0.4^{bf}	7.4±0.1^{abd}	7.5±0.6	2.7±0.3^{bdeg}	0.2±0.0^{abc}	5.1±3.1^{bgj}
June, 07	4.1±0.3^{cg}	3.3±0.4^{cjk}	4.1±1.3^{bcefg}	1.3±1.3^{acdfg}	1.7±1.6^{abcefg}	2.9±1.6^{acdehikl}
July, 07	6.5±0.7^{def}	7.1±0.6^{abe}	3.8±0.3^{bcef}	1.7±1.6^{abcdefg}	0.2±0.0^{abc}	3.8±2.8^{acdegikl}
August, 07	6.1±0.5^{defh}	6.6±0.4^{adek}	0.2±0.0^{a}	0.2±0.0^{acd}	2.0±2.0^{abcefg}	3.0±3.0^{acdefghijkl}
September, 07	5.0±0.2^{agh}	2.8±0.2^{fj}	0.2±0.0^{a}	0.2±0.0^{acd}	1.0±1.3^{abceg}	1.8±2.0^{aefhj}
October, 07	6.9±0.8^{bdef}	5.6±0.5^{adgk}	5.6±0.2^{de}	1.0±2.0^{acdg}	4.9±0.3^{de}	4.7±2.4^{bdeg}
November, 07	5.0±0.1^{agh}	3.9±0.2^{hk}	0.2±0.0^{a}	0.2±0.0^{acd}	0.2±0.0^{abc}	1.9±2.2^{acefhjk}
December, 07	6.4±0.6^{def}	4.6±0.3^{ik}	4.1±1.6^{bcdefg}	1.6±1.5^{abcdefg}	0.2±0.0^{abc}	3.4±2.5^{acdeikl}
January, 08	4.3±0.8^{acgh}	3.1±0.3^{cfj}	3.9±1.2^{bcefg}	6.6±0.6	3.4±2.6^{bcdefg}	4.2±1.8^{bdegij}
February, 08	3.5±0.7^{cg}	4.7±0.3^{ik}	2.9±0.3^{befg}	3.3±0.6^{bd}	3.3±0.6^{befg}	2.9±1.6^{acdehijkl}
March, 08	3.5±1.0^{cg}	2.3±0.3	2.0±0.2^{h}	2.1±0.2^{cdfg}	2.0±0.2^{bcefg}	2.4±0.7^{acefh}
Total	5.3±1.4^{aegh}	4.8±1.8^{ceghik}	2.9±2.4^{bcefgh}	1.7±2.0^{bcdfg}	1.6±1.9^{abceg}	3.2±2.5^{adeikl}

MPN = most probable number, SD = standard deviation of mean

Values followed by the same letter within a column are not significantly different by Nonparametric method, Mann-Whitney U-test, ($p<0.05$).



Graph 8 Total densities of *V. parahaemolyticus* in the oyster from the Ban don Bay during a 12-month period. Each point represents the geometric mean (n=6) of the log MPN of bacteria per gram of the pacific the oyster meat.

The occurrence of *V. vulnificus* in 99 (27.5%) of 360 the oyster samples isolates from Bandon bay during April 2007 to March 2008. Densities varied considerably (0.2 to 5.9 log MPN g^{-1}) in the oyster samples from each location sampling (Table 21). All of *V. vulnificus* densities over 4 log MPN g^{-1} (10^4 MPNg^{-1}) in December 2007 and January 2008 (Graph 9), high densities of *V. vulnificus* isolated from oyster samples in Ka-dae, Ta-tong and Kadae-jae nearly 6 log MPN g^{-1} in December 2007 and January 2008. The densities of *V. vulnificus* were lower 3 MPN g^{-1} in all oyster sample in Bandon bay in August 2007 and February, March 2008. In April 2007, the densities of *V. vulnificus* were lower 3 MPN g^{-1}, but except the pacific oyster collected from Chaiya (2.7±0.5 log MPN g^{-1}). In May, the densities of *V. vulnificus* could detect in oyster samples from Cha-ngor (4.3±0.6 MPN g^{-1}) and Ta-tong (2.4±2.5 MPN g^{-1}). In June and July, *V. vulnificus* densities could detected in oyster samples from Cha-ngor (5.3±0.2 MPN g^{-1}) and Chaiya (2.1±0.2 MPN g^{-1}), respectively. In September and August, V. vulnificus was detected in only the oyter samples collected from Kadae-gae. MPN counts were below the lower limit of sensitivity for the method (3 per g) in 66.7% of Chaiya and Cha-ngor, 75% of Kadae and Kadae-gae, 79.2% of Ta-tong. The total densities of *V. vulnificus* isolated from the pacific oyster samples in Bandon Bay, were 1.5±2.2 log MPN g^{-1}. Differences among the five locate with respects to log MPN data were analyzed by nonparametric method. There are evidence that the mean log MPN values were not same at the five site ($p < 0.05$).

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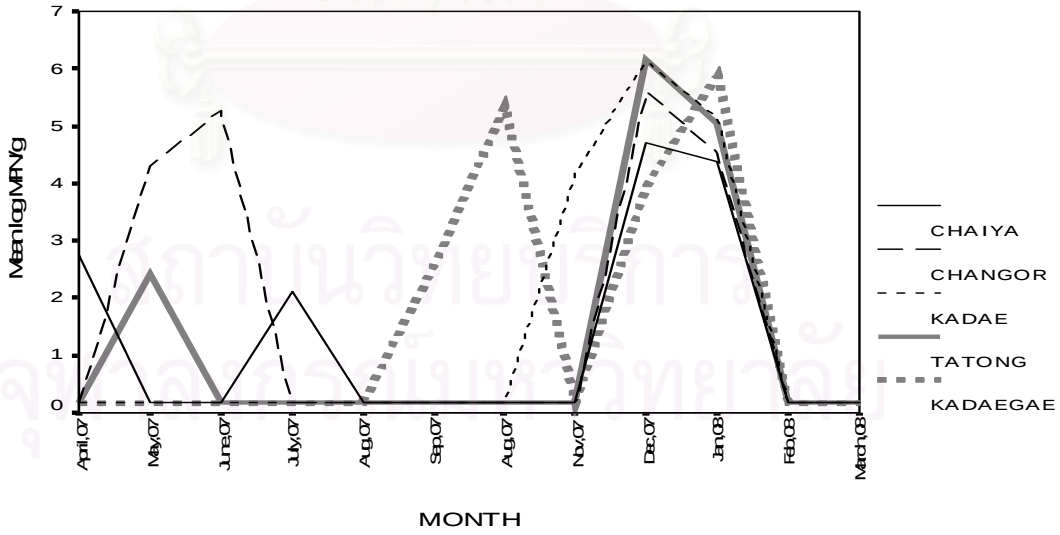
Table 21 Toatal counts of *V. vulnificus* in the pacific oyster samples from the Ban don Bay

Month	Average Vv (log MPN± SD)					
	Chaiya	Cha-Ngor	Kadae	Ta-tong	Kadae-gae	Total
April, 07	2.7±0.5	0.2±0.0[a]	0.2±0.0[a]	0.2±0.0[a]	0.2±0.0[a]	0.7±1.1[ac]
May, 07	0.2±0.0[a]	4.3±0.6	0.2±0.0[a]	2.4±2.5[b]	0.2±0.0[a]	1.5±2.0[ac]
June, 07	0.2±0.0[a]	5.3±0.2	0.2±0.0[a]	0.2±0.0[a]	0.2±0.0[a]	1.2±2.1[ac]
July, 07	2.1±0.2	0.2±0.0[a]	0.2±0.0[a]	0.2±0.0[a]	0.2±0.0[a]	0.6±0.8[ac]
August, 07	0.2±0.0[a]	0.2±0.0[a]	0.2±0.0[a]	0.2±0.0[a]	0.2±0.0[a]	0.2±0.0[bcd]
September, 07	0.2±0.0[a]	0.2±0.0[a]	0.2±0.0[a]	0.2±0.0[a]	2.6±2.6[bd]	0.7±1.5[abcd]
October, 07	0.2±0.0[a]	0.2±0.0[a]	0.2±0.0[a]	0.2±0.0[a]	5.3±0.3[cd]	1.2±2.1[ac]
November, 07	0.2±0.0[a]	0.2±0.0[a]	4.2±0.2	0.2±0.0[a]	0.2±0.0[a]	1.0±1.6[ac]
December, 07	4.7±0.4[b]	5.6±0.9	6.1±0.4	6.1±0.5	4.0±4.2[bcd]	5.4±2.1
January, 08	4.4±0.3[b]	4.5±0.3	5.0±0.7	5.1±0.4	5.9±0.8[cd]	5.0±0.7
February, 08	0.2±0.0[a]	0.2±0.0[a]	0.2±0.0[a]	0.2±0.0[a]	0.2±0.0[a]	0.2±0.0[bcd]
March, 08	0.2±0.0[a]	0.2±0.0[a]	0.2±0.0[a]	0.2±0.0[a]	0.2±0.0[a]	0.2±0.0[bcd]
Total	1.3±1.7[a]	1.8±2.3[a]	1.4±2.3[a]	1.3±2.2[ab]	1.6±2.5[ab]	1.5±2.2[a]

MPN = most probable number, SD = standard deviation of mean

Values followed by the same letter within a column are not significantly different by Nonparametric method, Mann-Whitney U-test, ($p<0.05$).



Graph 9 Total densities of *V. vulnificus* in the pacific oyster from the Bandon Bay during a 12-month period. Each point represents the geometric mean (n=6) of the log MPN of bacteria per gram of the oyster meat.

Part II Virulence Factors Test

After PCR method was used for confirm *V. parahaemolyticus* and *V. vulnificus*, three isolates of each these organisms in positive oyster samples were selected by random sampling for virulence factor test and antimicrobial resistance test. Seven hundred and sixty eight of *V. parahaemolyticus* isolates were selected from 256 of oyster samples that carried *V. parahaemolyticus*, 297 of *V. vulnificus* isolates were selected from 99 of oyster samples which contaminated *V. vulnificus.*

Seven hundred and sixty eight of *V. parahaemolyticus* isolates were examined for Watgatsuma phenomenon test for TDH production, Urease activity related with TDH-related hemolysin (BAM online), protease and gelatinase examined for probable virulence strain.

Cultures were grown in brain heart infusion (BHI) add 2% NaCl , incubated at 37 ºC for 24 h and streaked on TSA add 2% NaCl , incubated at 37 ºC for 18 h. Pure colonies was streaked on Watgatsuma agar, urea agar ,skim milk agar for protease test and gelatine agar for gelatinase test.

After incubated at 37 ºC for 24 h, the enzyme activity showed in Table 22. None *V. parahaemolyticus* strains exhibited positive result for Wagatsuma agar and urease activity, which related with PCR method for *tdh* and *trh* gene, respectively (Figure 13 and 14).

All *V. parahaemolyticus* could be produced protease and gelatinase that showed 100% positive result of both enzyme activity tests.

Table 22 Number and percentage of positive strains for the virulence factors tests. Kanagawa phenomenon on Watgatsuma agar, urease test, Protease and Gelatinase test (768 strains of *Vibrio parahaemolyticus*)

Virulent Factors Test	No. of positive strains	%
WA	0	0
Urea test	0	0
Protease	768	100
Gelatinase	768	100

Two hundred and ninety seven of *V. vulnificus* isolates were examined for virulence factor; protease, gelatinase, DNase, lipase (egg yolk, Tween 80), lecithinase, haemolysin, mucinase and elastase were examined for probable virulence strain.

Cultures were grown in brain heart infusion (BHI) add 2% NaCl , incubated at 37 ºC for 24 h and streaked on TSA add 2% NaCl , incubated at 37 ºC for 18 h. Pure colonies was streak onto the plates assay media.

After incubated at 37 ºC for 24 h, the enzyme activity showed in Table 23. The enzymes proteas, gelatinase, DNase and Lipase were produced by almost all strains. Lecithinase was produced by 52.5 %, haemolysin by 14.8%. None of *V. vulnificus* isolates was positive for the production of mucinase and elastase (Figure15-19).

Table 23 Number and percentage of positive strains for the enzyme production of 297 *V. vulnificus* strains.

Enzyme	No. of positive strains	%
Protease	297	100
Gelatinase	297	100
DNase	297	100
Lipase (egg yolk)	297	100
Lipase (Tween 80)	291	98
Lecithinase	156	52.5
Haemolysin	44	14.8
Mucinase	0	0
Elastase	0	0

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Figure 13 Kanagawa phenomenon positive of *V. parahaemolyticus* DMST 22013 on Wagatsuma agar

Figure 14 Urease positive of *V. parahaemolyticus* DMST 22013 on Christensen's urea agar supplemented with 2% NaCl

Figure 15 **DNase positive of *V. vulnificus* on DNase agar supplemented with 2% NaCl**

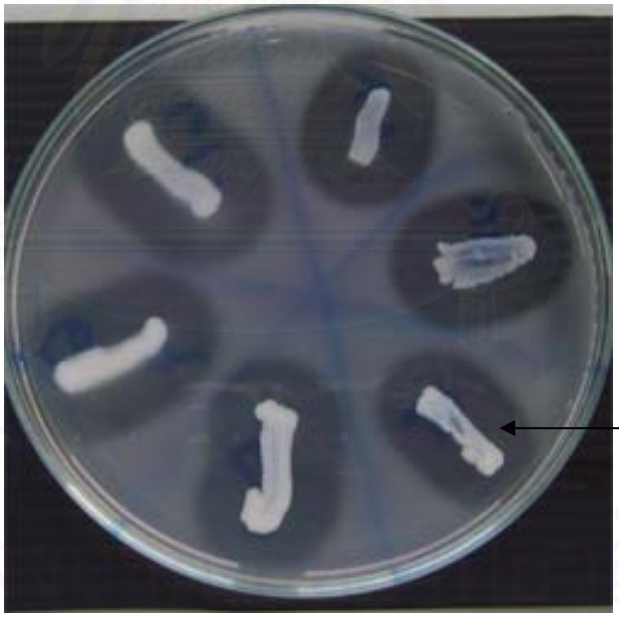


Figure 16 **Gelatinase positive of *V. vulnificus* on Gelatin agar supplemented with 2% NaCl**

Figure 17 **Protease positive of *V. vulnificus* on Skim milk agar supplemented with 2% NaCl**

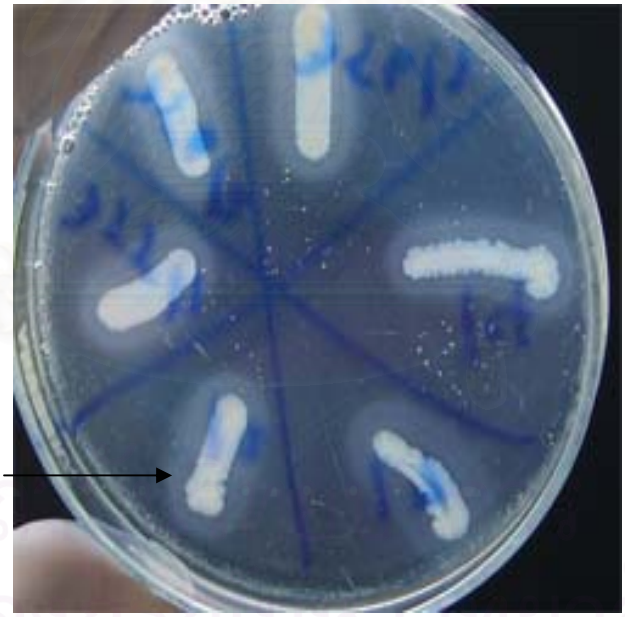


Figure 18 **Lipase positive of *V. vulnificus* on Tween 80 agar supplemented with 2% NaCl**

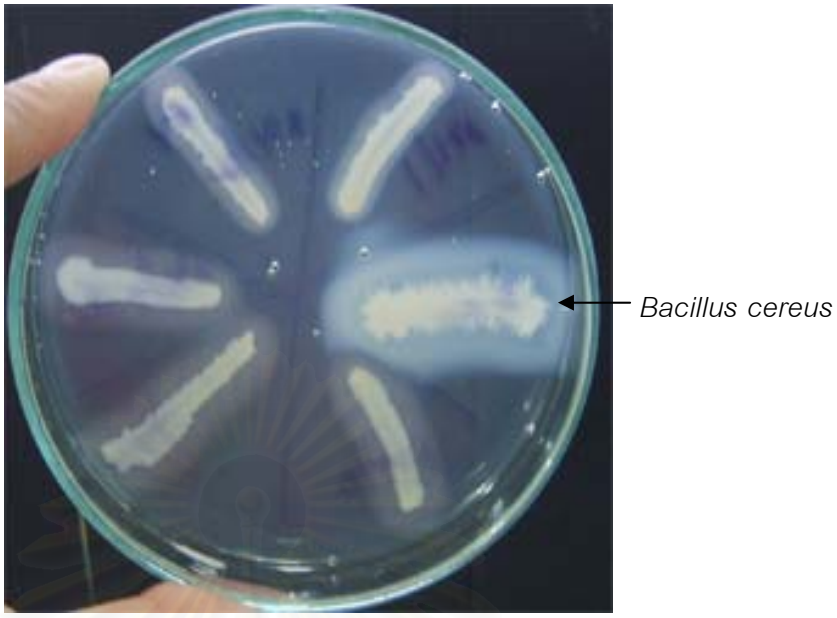


Figure 19 Lipase and Lecithinase positive of *V. vulnificus* on Egg Yolk agar supplemented with 2% NaCl

Part III Antimicrobial Resistant Pattern

After PCR method was used for confirm *V. parahaemolyticus* and *V. vulnificus*, three isolates of each these organisms in positive oyster samples were selected by random sampling for antimicrobial resistance test. Seven hundred and sixty eight of *V. parahaemolyticus* strains were selected from 256 of oyster samples that carried *V. parahaemolyticus*, 297 of *V. vulnificus* strains were selected from 99 oyster samples which contaminated *V. vulnificus.*

Susceptibility of four antibiotics performed by agar dilution method (Figure 20). *Escherichia coli* ATCC 25922 and *Pseudomonas aerginisa* ATCC 27853 were the recommended reference strains for agar dilution method by CLSI, 2008.

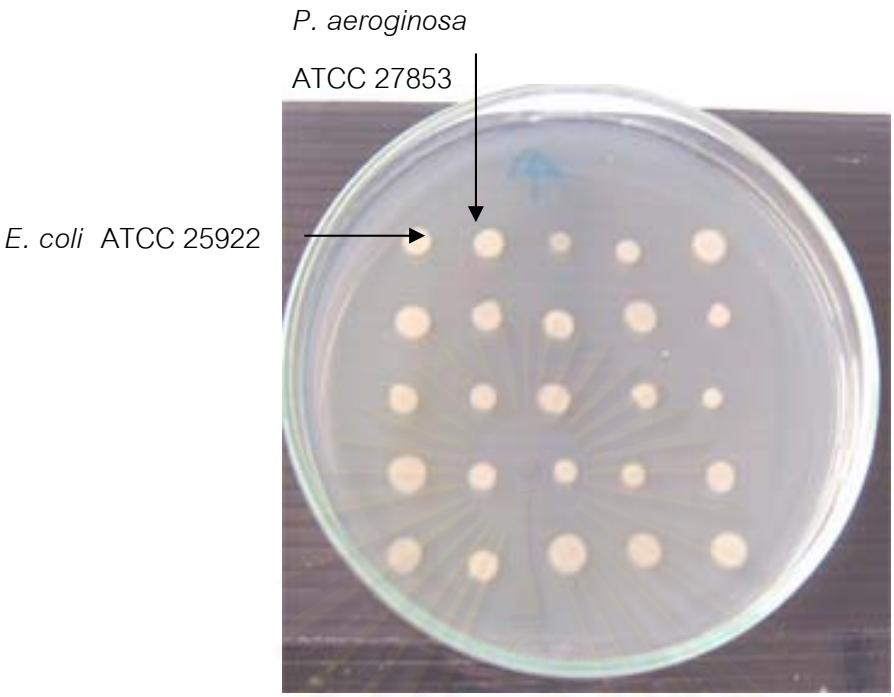


Figure 20 The area of the inoculated spot by agar dilution methods. Reference control: *Escherichia coli ATCC 25922 = E. coli, Pseudomonas aerginosa ATCC 27853 = P. aeroginosa*

Based on the CLSI-recommended breakpoints (130), the present the MIC distribution of *V. parahaemolyticus* isolated from Bandon bay, approximately 98.4% of the 768 isolates tested had doxycycline-susceptible (MICs of 4 μg/ml), 738 isolates (96.1%) had norfloxacin-susceptible (MICs of 4 μg/ml), 588 isolates (76.6%) had ciprofloxacin-susceptible (MICs of 1 μg/ml), 543 isolates (70.7%) had cefotaxime-susceptible (MICs 8 μg/ml). There were 12 (1.6%) doxycycline-intermediate of *V. parahaemolyticus*. The nonsusceptible isolates identified were 13 (1.7%) norfloxacine-resistant, 17 (2.2%) norfloxacin-intermediate, 44 (5.7%) cipofloxacine-resistant, 136 (17.7%) cipofloxacine-intermediate, 152 (9.5%) cifotaxime-resistant and 73 (19.8%) cifotaxime-intermediate of 768 *V. parahaemolyticus* isolates.

The MIC50 and MIC90 for doxycycline trended to be 1 μg/ml, the MIC50, MIC90 for norfloxacin trended to be 1 μg/ml and 4 μg/ml, respectively. The MIC50,

MIC90 for cipofloxacin trended to be 0.5 μg/ml and 2 μg/ml, respectively. The MIC50, MIC90 for cefotaxime trended to be 2 μg/ml and 32 μg/ml, respectively.

V.parahaemolyticus resisted to only norfloxacin 5 isolate (0.65%), norfloxacin and ciprofloxacin 8 isolates (1.05%), none norfloxacine and cefotaxime resistant strains. These organisms resisted to only cipofloxacine 30 isolates (3.9%), ciprofloxacin and cefotaxime 6 isolates (0.8%) and only cefotaxime 67 isolates (8.7%). None strains resisted to three/four antimicrobial agents (Table 24, 25 and Graph 10).

The MIC distribution of 297 *V. vulnificus* isolates exhibited in Table 26, 27 and Graph 11. All of *V. vulnificus* had suscept to doxycycline (MIC 4 μg/ml), norfloxacine (MIC 1 μg/ml), and cipofloxacine (MIC 1 μg/ml). There were 251 (84.5%) cefotaxime-susceptible, the only nonsusceptible isolates identified were 26 (8.8%) cefotaxime-resistant and 20 (6.7%) cefotaxime-intermediate.

.The MIC50 and MIC90 for doxycycline and Norfloxacine trended to be 1 μg/ml. The MIC50, MIC90 for cipofloxacin trended to be 0.25 μg/ml and 5 μg/ml, respectively. The MIC50, MIC90 for cefotaxime trended to be 2 μg/ml and 32 μg/ml, respectively.

Table 24 Antimicrobial susceptibility testing ranges, breakpoints for four antimicrobials tested and MIC distribution for 768 *V. parahaemolyticus* a isolates discovered from Bandon Bay in 2007 and 2008

Antimicrobial agent	Breakpoints ((μg/ml)*			MIC (μg/ml) distribution for					
	S	I	R	%S	%I	%R	MIC 50	MIC 90	MIC Range
Doxycycline	≤4	8	≥16	98.4	1.6	0	1	1	1-8
Norfloxacin	≤4	8	≥16	96.1	2.2	1.7	1	4	1-32
Cipofloxacin	≤1	2	≥4	76.6	17.7	5.7	0.5	2	0.25-8
Cefotaxime	≤8	16-32	≥64	70.7	19.8	9.5	2	32	2-64

*Breakpoints recommended by the CLSI, in M100-S18 (130). S, I and R stand for susceptible, intermediate and resistant susceptibility.

Table 25 Distribution of MICs and occurrence of resistance among *V. parahaemolyticus* (n=768) from Bandon bay.

Antibiotic	Distribution (%) of MICs										
	0.25	0.5	1	2	4	8	16	32	64	128	256
DOX			90.6	5.6	2.2	1.6					
NOR			78.1	11.3	6.6	2.2	1.2	0.5			
CIP	41.8	19	15.8	17.7	2.3	3					
CTX				56.1	5.6	9	12.9	6.9	9.5		

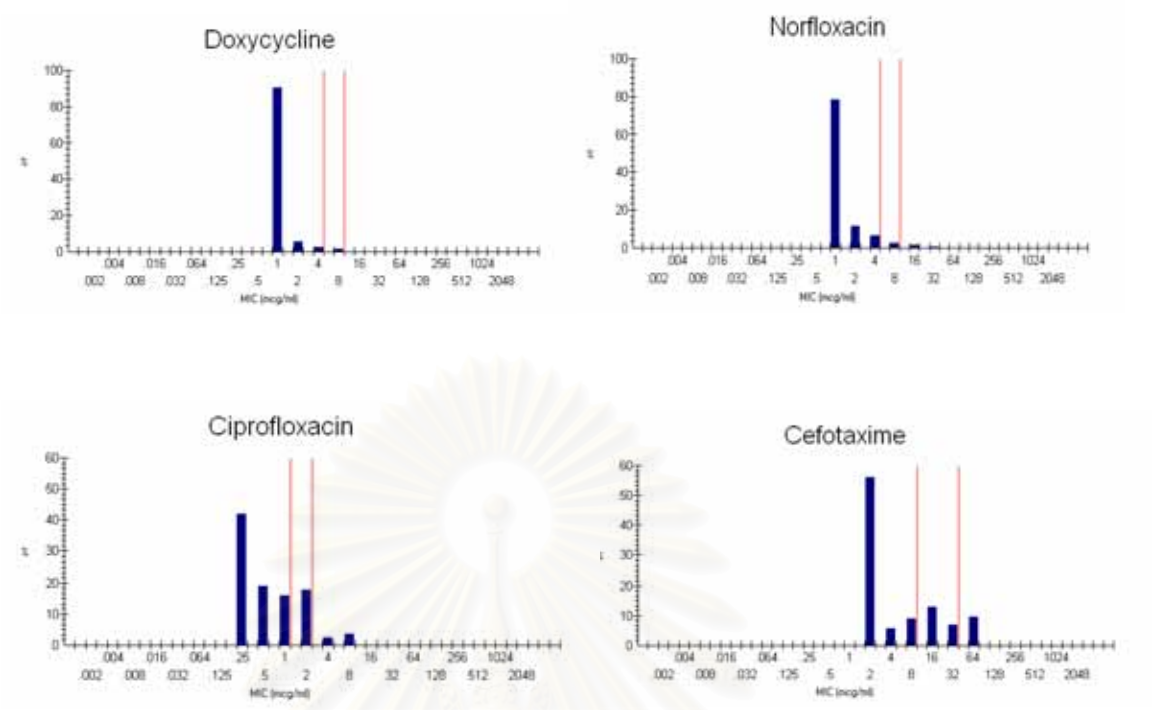
Table 26 Antimicrobial susceptibility testing ranges and breakpoints for four antimicrobials tested and MIC distribution for 297 *V. vulnificus* isolates discovered from Bandon Bay in 2007 and 2008

Antimicrobial agent	Breakpoints ((µg/ml)*			MIC (µg/ml) distribution for					
	S	I	R	%S	%I	%R	MIC 50	MIC 90	MIC Range
Doxycycline	≤4	8	≥16	100	0	0	1	1	1 – 4
Norfloxacin	≤4	8	≥16	100	0	0	1	1	1 – 1
Cipofloxacin	≤1	2	≥4	100	0	0	0.25	0.5	0.25 – 1
Cefotaxime	≤8	16-32	≥64	84.5	6.7	8.8	2	32	2 - 64

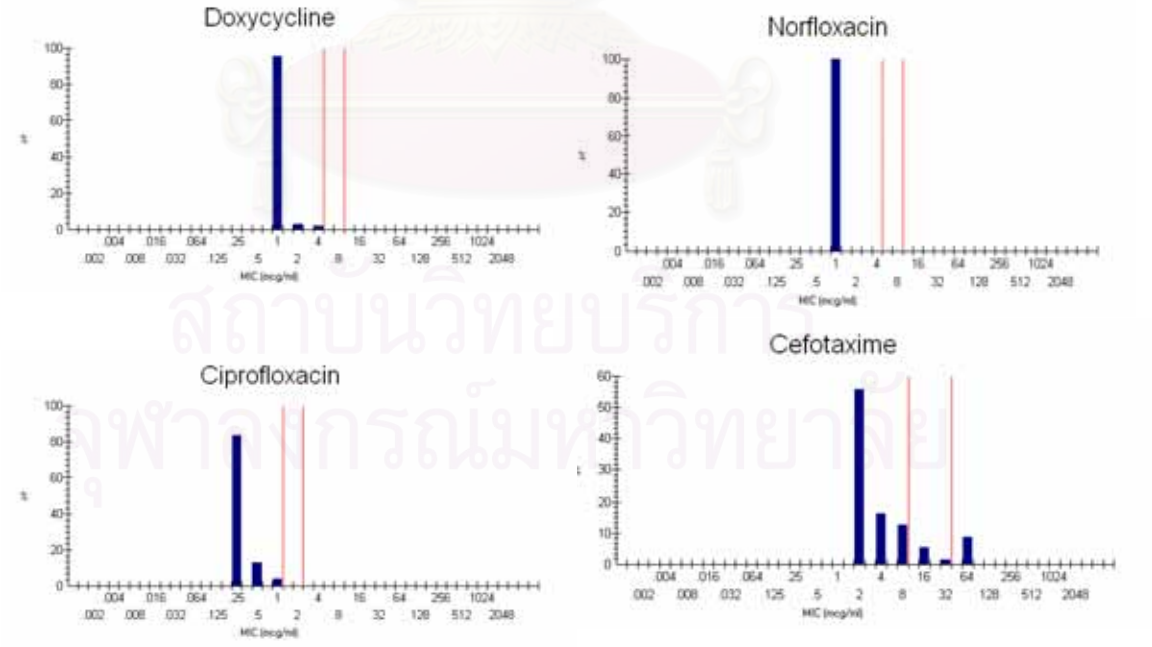
*Breakpoints recommended by the CLSI, in M100-S18 (130). S, I and R stand for susceptible, intermediate and resistant susceptibility.

Table 27 Distribution of MICs and occurrence of resistance among *V. vulnificus* (n=297) from Bandon bay.

Antibiotic	Distribution (%) of MICs										
	0.25	0.5	1	2	4	8	16	32	64	128	256
DOX			95.3	2.7	2						
NOR			100								
CIP	83.5	12.8	3.7								
CTX				55.9	16.2	12.5	5.4	1.3	8.8		



Graph 10 Antimicrobial susceptibility testing and breakpoints for Antimicrobial tested and MIC distribution. 768 isolates of *Vibrio parahaemolyticus* discovered from Bandon bay in 2007 and 2008



Graph 11 Antimicrobial susceptibility testing and breakpoints for Antimicrobials tested and MIC distribution. 297 isolates of *Vibrio vulnificus* discovered from Bandon Bay in 2007 and 2008

CHAPTER VI

DISCUSSION

V. parahaemolyticus and *V. vulnificus* are distributed in seawater environments and were associated with gastroenteritis, wound infections and septicaemia. There are frequency of infection by seafood consumption and direct contact with estuarine water (53).

In this study interested prevalence of *V. parahaemolyticus* and *V. vulnificus* contaminated in the oyster that was major produce in Bandon Bay, Suratthani. This is the most comprehensive survey of pathogenic vibrios from the farms to the markets. The trade of the oysters in the market, the oyster was collected from farms in Bandon bay and was transferred to the market for consumer by the car. Bangkok is the major market. This study resemble which refer to oyster in market, which the oyster samples were collected from Bandon bay in 10 farms, 3 oysters in farms each month for one year of study. The oyster samples were transfer to the laboratory of the Center for Antimicrobial Resistance Monitoring in Foodborne Pathogens (In collaborating with WHO), Department of Microbiology, Faculty of Veterinary Science, Chulalongkorn University and analysis within 24 h. The samples were detected densities by used three tubes MPN method, antimicrobial resistant pattern used agar dilution method and virulence factors concern to enzymes production in *V. parahaemolyticus* and *V. vulnificus*.

The MPN method used PCR method for confirmation these organisms, which were operated throughout this study (126, 127). However, selection media are known to have a decrease sensitivity of detection in natural populations. For a variety of reasons, include poor or no growth on selective media. Moreover, during subculture of enriched cultures on TCBS agar, target cells may not produce isolated colonies because of the background of other bacteria, including other Vibrio species. Such practical difficulties or limitations may also contribute to the lower recovery of *V. parahaemolyticus* by the MPN culture method (84). In this study, 360 samples of oyster from Bandon bay were examined in April 2007 to March 2008, which *V. parahaemolyticus* was discovered from 172 (47.78%)

samples, 15 (4.17%) samples of *V. vulnificus* and 84 (23.23%) samples contained both these organism.

In the many countries reported a 15-71% contamination rate of seafood by *V. parahaemolyticus* (29, 62). *V. parahaemolyticus* has been isolated and detected from seafoods, partially shellfish or bivalve mollusks from Thailand, Hongkong, Indonesia and Vietnam as well as other parts of the world (29, 49, 77). *V. parahaemolyticus* occurred in 62 of 100 cockles (*Anadara granosa*) samples at harvest from Kuala Selangor, Malaysia. The value great more than 1,100 MPN g^{-1} by MPN and PCR confirm method, that 2 sample were *tdh*+ and 11 samples were *trh*+ (132).

The current pathogenicity markers of *V. patahaemolyticus* are the thermostable direct hemolysin (*tdh*) and TDH-related hemolysin (*trh*) genes (82). This study obtained 1,457 isolates of *V. parahaemolyticus* were positive for *tlh* gene confirmation but none occurred *tdh* and/or *trh* gene in *V. parahaemolyticus* strains, which related with Kanagawa phenomenon and urease activity negative.

Pathogenic (*tdh* positive) *V. parahaemolyticus* was detected in 9 of 42 (21%) oyster samples at maximum exposure, in 5 of 19 (26%) sediment samples, but in 0 of 9 excreta samples (133). The prevalence of *trh* gene positive strains from environmental or seafood sample are very low, which 91 of 95 *V. parahaemolyticus* strains were isolated from case-control study of expatriates and Thai adults in Thailand, were positive for *tdh* gene and 5 isolates were positive for *trh* gene, all strains susceptible for ciprofloxacin an sulfa-trimethoprim (67).

FAD/CFSAN and WHO committee for safety seafood, which should contain less than 10,000 MPN/g of *V. parahaemolyticus*, which positive or negative Kanagawa phenomenon in seafood that ready to eat fishery products include raw oysters (134, 135).

The occurred of *V. parahaemolyticus* in 256/360 (71.11%) of the pacific oyster samples collected in Bandon bay between April 2007 to March 2008, exhibited in Table 20 and Graph 8. The densities varied considerably (0.2 to 7.5 log MPN g^{-1}, in Chaiya occurred high densities of *V. parahaemolyticus* all studied periods that over 4 log MPN g^{-1}, which exceeded the FDA/CFSAN and WHO level concern of 10,000 MPN/g, except in Febuary and May. Some pacific oyster

samples collected from Cha-ngor showed densities of *V. parahaemolyticus* over 4 log MPN g^{-1}, at this high level it could be harmful to consumers. The densities of *V. parahaemolyticus* in Kadae, Tatong and Kadae-gae showed rather low in the studied peroid. The average densities of *V. parahaemolyticus* were significantly different based on statistical analysis ($p \leq 0.05$). The total average density of *V. parahaemolyticus* isolated from the pacific oysters in Bandon Bay was 3.2±2.5 log MPN g^{-1}.

FAD/CFSAN and WHO/FAO committee for safety raw oyster consumption, which none found of *V. vulnificus* –presence of pathogenic organism. There was not adequate information to differentiate between virulent and avirulent strains of *V. vulnificus.* Therefore, all *V. vulnificus* strains were considered to be equally pathogenic (134-136).

The occurrence of *V. vulnificus* in 99 (27.5%) of 360 the oyster samples isolates from Bandon bay during April 2007 to March 2008. Densities varied considerably (0.2 to 6.1 log MPN g^{-1}) in the oyster samples from each location sampling (Table 21). The densities of all *V. vulnificus* over 4 log MPN g^{-1} in December 2007 and January 2008 (Graph 9), these mean difference is significant ($p \leq 0.05$). At this high level it could be harmful to consumers. The total average density of *V. vulnificus* isolated from the pacific oysters in Bandon bay was 1.5±2.2 log MPN g^{-1}.

This result exhibited prevalence (Table 12 and Graph 1) and densities of *V. parahaemolyticus* (Table 20 and Graph 8) and *V. vulnificus* (Table 21 and Graph 9) isolated from the pacific oyster in Bandon Bay. In August, Febuary and March, the average density of *V. parahaemolyticus* isolated from the pacific oysters in Bandon bay less than FDA level of concern at 4 log MPN g^{-1} and none *V. vulnificus* in these months, that possible for raw oyster safety to consumer.

However, CDC advises for preventing *V. vulnificus* infections, particularly among immunocompromised patients, including those with underlying liver disease to don't eat raw oysters or other raw shellfish, cook shellfish thoroughly, boiling or steam until the shells open and continue cooking for 5-9 minutes for shellfish in the shell, avoid cross-contamination of cooked seafood, eat shellfish promptly after cooking and refrigerate leftovers, avoid expose of open wounds or broken skin to

sea water or to raw shellfish and wear protective clothing when handing raw shellfish (137).

This study, the oyster were 8-12 cm. average size, which Suratthani Coastal Fisheries Research and Development Center reported the densities of Vibrio spp. not related with size of the oyster but associated with the quality of water in each season (138).

The virulence factors of *V. vulnificus* are not yet well understood. All of 768 strains *V. parahaemolyticus* and 297 *V. vulnificus* were produced protease and gelatinase (100%) (Table 22 and 23). These probable enzymes were important factors to the pathogenic infection by this organism because of the invasive potential. All 297 of *V. vulnificus* isolates positive for DNase, destruction of DNA and were hydrolysis lipase from egg yolk (100%), which 98% for tween 80. None *V. vulnificus* strains produced mucinase and elastase. These organisms showed 14.8% positive for hemolysin production in sheep red blood cells agar. Rodrigues reported the absence of haemolysin in all strains studies, these variance of virulence factors (112). *V. vulnificus* strains positive for lecithinase and lipase 100%, protease production 99.2% of 113 isolates, 65.4% for mucinase and 46.6% for elastase, 96% were haemolysis sheep blood agar but none collagenase production (113). The environmental *V. vulnificus* strains are phenotypically indistinguishable from clinical isolates and that approximately 90% of the environmental strains tested produced in vitro virulence factors and in vivo pathogenicity for mice comparable to those produced by clinical *V. vulnificus* isolates (120). Twenty strains of *V. vulnificus* isolated from the environment were investigated for characteristics related to their infectivity such as colonial morphology, enzymatic activity and animal assays. The presence of DNase, chitinase, amylase, lecithinase and gelatinase was observed in 100% of the strains, haemolytic activity was absent, and variable results were obtained in elastase, collagenase and chondroitinase. In the animal assays, 70% of the strains were lethal to adult mice, while 45% caused fluid accumulation in suckling mice. Although all strains had opaque colonies, only 3 of the 20 had the three enzymes elastase, collagenase and gelatinase, and only one of these was virulent in animal assays (64).

For antimicrobial therapy treated with doxycycline and a third generation cephalosporin e.g cefotaxime or a single regimen with a fluoroquinolone such as cipofloxacin. In children, doxycycline and fluoroquinolones are contraindicated, can be treated with trimethoprim-sulfamethoxazole plus a aminoglycoside (137). The first-line drugs including tetracycline, cefotaxime, ceftacidime and ciprofloxacin highly effective against *V. parahaemolyticus* and *V. vulnificus* isolated oyster from Louisiana Gulf Coast and retail outlets in 2005 to 2006 (123).

Based on the CLSI-recommended breakpoints (130), the present the MIC distribution of *V. parahaemolyticus* isolated from Bandon bay, approximately 98.4% of the 768 isolates tested had doxycycline-susceptible (MICs 4 μg/ml), 96.1% had norfloxacin-susceptible (MICs 4 μg/ml), 76.6% had ciprofloxacin-susceptible (MICs 1 μg/ml), 70.7% had cefotaxime-susceptible (MICs 8 μg/ml). There were 12 doxycycline-intermediate of *V. parahaemolyticus.* The nonsusceptible isolates identified were 13 norfloxacine-resistant, 17 norfloxacin-intermediate, 44 cipofloxacine-resistant, 136 cipofloxacine-intermediate, 152 cifotaxime-resistant and 73 cifotaxime-intermediate of 768 *V. parahaemolyticus* isolates.

The MIC50 and MIC90 for doxycycline trend to be 1 μg/ml. The MIC50, MIC90 for norfloxacin trended to be 1 μg/ml and 4 μg/ml, respectively, which both drugs fell into the susceptible range. The MIC50 for cipofloxacin and cefotaxime trended to be 0.5 μg/ml and 2 μg/ml, respectively, which fell into the susceptible range. The MIC90 for cipofloxacin trended to be 2 μg/ml and 32 μg/ml for cefotaxime, which fall into the intermediate range.

V.parahaemolyticus resisted to only norfloxacin 5 isolate (0.65%), norfloxacin and ciprofloxacin 8 isolates (1.05%), none norfloxacine and cefotaxime resistant strains. These organisms resisted to only cipofloxacine 30 isolates (3.9%), ciprofloxacin and cefotaxime 6 isolates (0.8%) and only cefotaxime 67 isolates (8.7%). None strains resisted to three/four antimicrobial agents.

All of 297 *V. vulnificus* had suscept to doxycycline (MIC 4 μg/ml), norfloxacine (MIC 1 μg/ml), and cipofloxacine (MIC 1 μg/ml). There were 251 (84.5%) cefotaxime-susceptible (MIC 8 μg/ml), the only nonsusceptible isolates identified were 26 (8.8%) cefotaxime-resistant (MIC 64 μg/ml), and 20 (6.7%) cefotaxime-intermediate (MIC 32 μg/ml).

The MIC50 and MIC90 for doxycycline and Norfloxacine trended to be 1 μg/ml. The MIC50, MIC90 for cipofloxacin trended to be 0.25 μg/ml and 5 μg/ml, respectively. The MIC50, MIC90 for cefotaxime trended to be 2 μg/ml and 32 μg/ml, respectively. The MIC50 and MIC90 for doxycycline, norfloxacine and cipofloxacine fell into the susceptible range, which MIC90 for cefotaxime fell into intermediate range. None resisted two/three antimicrobial agent strains.

Vibrios are aquatic bacteria, live in the coastal and estuarine waters, an open area particularly subject to environmental contaminations by agricultural run off or wastewater treatment plants, which may contain various levels of antimicrobials-resistant aquatic bacteria. Noticeable in the coastal inside area of Bandon Bay had many intensive marine shrimp farms (34,243 Rais) (139), the antimicrobial drugs used in these farms may be contaminated in run off water to seawater, cause of antimicrobial resistance bacteria.

Therefore, continued monitoring of both the prevalence and the antimicrobial susceptibility profiles of *V. parahaemolyticus* and *V. vulnificus* are of the importance to ensure the safety consumption of this oyster.

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CHAPTER VII

CONCLUSION

1. For PCR conformation; 1,457 of *V. parahaemolyticus* isolates positive to *tlh* gene, but none of *V. parahaemolyticus* strains was positive to *tdh* and *trh* gene, this related which Kanagawa phenomenon and urease activity negative. *V. vulnificus* isolates were positive to *vvhA* gene 609 isolates.
2. The prevalence of *V. parahaemolyticus* in the pacific oyster samples collected from Bandon bay were 172 (47.78%) of 360 oysters, 15 (4.17%) of 360 oysters positive to *V. vulnificus* and contained both organisms were 84 (23.33%) of 360 oysters. The pacific oysters contaminated *V. parahaemolyticus* and/or *V. vulnificus* were 271 (75.28%) of 360 oysters.
3. The densities of *V. parahaemolyticus* in Bandon bay between 0.2 log MPN g^{-1} to 7.5 log MPN g^{-1} and 0.2 log MPN g^{-1} to 5.9 log MPN g^{-1} of *V. vulnificus* , the mean difference are significant ($p \leq 0.05$).
4. Virulence factors test, the enzymes protease, gelatinase, DNase and Lipase were produced by almost all 297 strains of *V. vulnificus* strains, Lecithinase was produced by 52.5 %, haemolysin by 14.8% . None of *V. vulnificus* strains positive for the production of mucinase and elastase. All of 768 *V. parahaemolyticus* strains were positive to protease and gelatinase production.
5. Antimicrobial susceptibility pattern of 768 *V. parahaemolyticus* isolated from Bandon Bay were susceptible to doxycycline 756 isolates (98.4%), norfloxacin 738 isolates (96.1%), ciprofloxacin 588 isolates (76.6%) and cefotaxime 543 isolates (70.7%). *V. parahaemolyticus* resisted to only norfloxacin 5 isolate (0.65%), norfloxacin and ciprofloxacin 8 isolates (1.05%), none norfloxacine and cefotaxime resistant strains, resisted to only cipofloxacine 30 isolates (3.9%), ciprofloxacin and cefotaxime 6 isolates (0.8%) and only cefotaxime 67 isolates (8.7%). None strains resisted to three/four antimicrobial agents.

6. All 297 *V. vulnificus* strains in Bandon Bay were susceptible to doxycycline, norfloxacin, cipofloxacine (100%) and 251 isolates (84.5%) of *V. vulnificus* strains susceptible to cefotaxime. None resisted two/three antimicrobial agent strains.

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APPENDIX I

REAGENTS, MATERIALS and INSTRUMENTS

A. REAGENTS

Absolute ethanol	(Lab-scan, Ireland)
Agarose	(Research organics, Ohio)
Colistin sulfate	(Sigma, U.S.A.)
DNA ladder marker 100 bp	(SibEnzyme, U.S.A.)
dNTP set, 4x 25 μmol, 100 mM solution	(Amersham, U.S.A.)
Ethonediamine tetraacetic acid (EDTA)	(USB, U.S.A.)
Ethidium bromide	(USB, U.S.A.)
Oligodeoxynucleotides primers (200 nMole)	(GIBThai, Thailand)
Parafin liquid fisher 2.5 L	(Fisher Scientific, UK)
Sodium chloride	(Labscan, Ireland)
Sodium deoxycholate monohydrate	(Sigma, U.S.A.)
Taq DNA Polymerase (recombinant) 500U (with $MgCl_2$ and PCR buffer)	(QIAGEN, Germany)
Tris base	(USB, U.S.A.)

B. MATERIALS

Beaker	(Pyrex, U.S.A.)
Centrifuge tube	(Corning, Germany)
Cotton swabs	(HI-VAN, Thailand)
Cylinder	(Pyrex, U.S.A)
Eppendroff tube	(Axygen, U.S.A.)
Flask	(Pyrex, U.S.A.)
Glass bottle	(Pyrex, U.S.A.)
Glass screw tube	(Pyrex, U.S.A.)

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B. MATERIALS (continuous)

Glass tube	(Pyrex, U.S.A.)
Microcentrifuge tube	(Corning, Germany)
Multipoint inoculators	(KMIL, Thailand)
Petri dish	(Pyrex, U.S.A.)
Pipetman	(Gilson, France)
Pipet tip	(Greiner bio-one, Geramany)
Replicator pins	(KMIL, Thailand)
Screw cap tube	(Pyrex, U.S.A.)
Steri-loop	(Sterilin, UK)
Volumatic flask	(Witeg, Geramany)

C. INSTRUMENTS

Autoclave	(OMRON, Japan)
Dispenser 10 ml (Labmax)	(Witeg, Germany)
Freezer	(SHARP, Japan)
Gel Doc 2000	(Bio-Rad, U.S.A.)
Incubator	(Memmert, Germany)
Measurer	(Precisa, Swiss)
Microcentrifuge	(Witeg, Germany)
pH meter (Cyberscan 500)	(EUTECH, Singapore)
Power supply	(BRL, U.S.A.)
Refrigerator	(SANYO, Japan)
Thermal cycler TC-312	(TECHNE, U.S.A.)
Turbidometer	(Oxoid, England)
Vortex mixer (VM-300)	(Gemmy, U.S.A.)
Water bath	(Memmert, Germany)

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APPENDIX II

MEDIA PREPARATION

A. Medium

A1. Acid from carbohydrate

(Arabinose, D-Cellobiose, Glucose, Lactose, Sucrose, Salicin)

Peptone	10	g
Beef Extract	1	g
NaCl	20	g
0.2% Bromthymol blue	15	ml

pH, 6.8 ± 0.1.

Dissolve ingredient in 900 ml distilled water. Autoclave 15 minutes at 118°C. Cool to 50-55°C. Add 100 ml of 10% sugar to 1% final except salicin for 0.5% in final. Mix and dispense to sterile tubes.

A2. Alkaline Peptone Water

Peptone	10	g
NaCl	10	g

pH 8.5 ± 0.2

Dispense 10 ml portions into 20 x 150 screw-cap tubes. Sterile by autoclaving at 121 ºC for 10 minutes.

A3. Amino Decarboxylase Medium (Arginine, Lysine, Ornithine)

Peptone or gelysate	5	g
Yeast extract	3	g
Glucose	1	g
Bromcresol purple	0.02	g
NaCl	20	g

pH 6.5 ± 0.2

Add 5 g L-arginine, L-lysine, L-ornithine for Arginine Broth, Lysine Broth and Ornithine respectively. Broth Dispense 5 ml portions into 13 x 100 test tubes. Autoclave 10 min at 121°C. For control, use unsupplemented base.

A4. Arginine glucose slants (AGS)

Peptone	5	g
Yeast extract	3	g
Tryptone	10	g
NaCl	20	g
Glucose	1	g
L-Arginine (hydrochloride)	5	g
Ferric ammonium citrate	0.5	g
Sodium thiosulfate	0.3	g
Bromocresol purple	0.02	g
Agar	13.5	g

pH 6.8-7.0.

Suspend ingredients in 1 liter of distilled water and boil to dissolve. Dispense into tubes (for 13 x 100 mm tubes use 5 ml). Sterilize by autoclaving at 121°C 10-12 minutes. After sterilization, solidify as slants.

A5. 5% Blood Agar

Tryptic Soy Agar (Difco,U.S.A)

Pancreatic digest of casein	15	g
Enzymatic digest of soybean meal	5	g
NaCl	5	g
Agar	15	g

pH 7.3 ± 0.2.

Add NaCl to a final concentration of 1%. Dissolve 40 g in 1 liter of distilled water. Heat with agitation to dissolve agar. Sterilize by autoclaving at 121°C for15 minutes. Cool to 50°C. Add 5 ml sheep blood to 100 ml melted agar. Mix well before pouring.

A6. Cellobiose colistin (CC) agar

Solution 1

Peptone	10	g
Beef extract	5	g
NaCl	20	g
Agar	15	g
Distilled water	900	ml

pH 7.6.

Add 1000X Dye stock solution* 1 ml. Boil to dissolve agar. Cool to 48-55°C.

***1000X Dye stock solution**

Bromthymol blue	4	g
Cresol red	4	g

Dissolve dyes in 95% ethanol for 4% (w/v) stock solution. Using 1 ml of this solution per liter of CC agar

Solution 2

Cellobiose	10	g
Colistin	400,000	units

Dissolve cellobiose in distilled water by heating gently. Cool. Add antibiotics and filter sterilize. Add Solution 2 to cooled Solution 1, mix, and dispense into petri dishes. Final color, dark green to green-brown.

A7. Christensen's urea agar with added NaCl (2%)

Peptone	1	g
Dextrose	1	g
KH_2PO_4	2	g
Phenol red	0.012	g
NaCl	20	g
Agar	15	g

pH, 6.8 ± 0.1

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Heat with agitation to dissolve agar in 900 ml water (basal medium). Autoclave 15 minutes at 121°C. Cool to 50-55°C. Add 100 ml of 20% urea . Mix and dispense to sterile tubes. Slant tubes for 2 cm butt and 3 cm slant.

A8. DNase Test Agar (Difco, USA)

Tryptose	20	g
Deoxyribonucleic Acid	2.0	g
Agar	15	g
NaCl	5	g

pH 7.3 ± 0.2

Add NaCl to 20 g final and suspend 42 g of the powder in 1l of distilled water. Heat with agitation and boil 1-2 min to dissolve agar. Autoclave 15 min at 121°C. Cool to 45-50°C before pouring.

A9. Gelatin Salt Agar

Peptone	4	g
Yeast extract	1	g
Gelatin	15	g
NaCl	30	g
Agar	15	g

pH 7.2 ± 0.2

Suspend ingredients and boil to dissolve gelatin and agar. Autoclave 15 minutes at 121°C. Cool to 45-50°C. Pour plates.

A10. Motility test medium-2% NaCl

Beef extract	3	g
Peptone or gelysate	10	g
NaCl	20	g
Agar	4	g

pH 7.4 ± 0.2

Heat with agitation and boil 1-2 min to dissolve agar.. Dispense 8 ml portions into 16 x 150 screw-cap tubes. Autoclave 15 min at 121°C.

A11. MUELLER HINTON (MH) agar

MUELLER HINTON (MH) agar (Difco, 500 g)	38	g
NaCl	10	g

Adjust volume to 1000 ml with DDW and Sterilize by autoclaving

A12. Salt Tolerance Broth (0%, 3%, 6%, 8%, 10%)

Tryptone	10	g

Add NaCl to a final concentration of 0%,3%,6%,8%,10%. Dissolve in 1 liter of distilled water. Add in 16x130 test tubes. Sterilize by autoclaving at 121°C for15 minutes.

A13. Thiosulfate citrate bile salts sucrose (TCBS) agar (Eiken)

Yeast extract	5	g
Peptone	10	g
Sodium Citrate	10	g
Sodium Thiosulfate	7	g
Oxgall	5	g
Sodium Cholate	3	g
Saccharose	20	g
Sodium Chloride	10	g
Ferric citrate	1	g
Bromthymol blue	0.04	g
Thymol blue	0.04	g
Agar	15	g

pH 8.8

Dissolve 86 g in 1 liter of distilled water. Heat with agitation to dissolve agar. Do not autoclaving. Cool to 50°C before pouring.

A14. TSI (Difco, U.S.A.)

Beef extract	3	g
Yeast extract	3	g

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Peptone	15	g
Proteose peptone	5	g
Glucose	1	g
Lactose	10	g
Sucrose	10	g
$FeSO_4$	0.2	g
NaCl	5	g
$Na_2S_2O_3$	0.3	g
Phenol red	0.024	g
Agar	12	g

pH 7.4 ± 0.2

Add NaCl to 20 g final. Heat with agitation and boil 1-2 min to dissolve agar. Dispense 5 ml portions into 13 x 100 test tubes. Autoclave 15 min at 121°C. Before the media solidify, incline tubes to obtain 4-5 cm slant and 2-3 cm butt.

A15. TRYPTONE SOYA Broth (TSB)

TRYPTONE SOYA (TS) broth	30	g
NaCl	20	g

Adjust volume to 1000 ml with DDW and Sterilize by autoclaving

A16. Wagatsuma Agar (BAM,2004)

Yeast extract	3	g
Peptone	10	g
NaCl	70	g
K_2HPO_4	5	g
Mannitol	10	g
Crystal violet	0.001	g
Agar	15	g

pH 8.0 ± 0.2

Suspend ingredients in distilled water and boil to dissolve agar. Steam 30 min. Do not autoclave. Cool to 45-50°C. Add 50 ml of washed red blood cells to the cooled medium. Mix and pour into sterile Petri-dishes.

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REAGENT PREPARATION

REAGENT FOR DILUTION

1. Phosphate buffered saline (PBS)

NaCl	7.65	g
KH_2PO_4	0.210	g
Na_2HPO_4	0.724	g

Dissolve all ingredients in distilled water 1 litre. Autoclave 15 min at 121°C.

REAGENT FOR SUSCEPTIBILITY TEST

1. 0.85% NaCl

Sodium chloride	0.85	g

Adjust volume to 100 ml with DDW and Sterilize by autoclave 15 min at 121°C.

REAGENT FOR BIOCHEMICAL TEST

1. Oxidase Reagent

N,N,N',N'-Tetramethyl-p-phenylenediamine·2HCl	10

Dissolve reagent in 1 l of distilled water. Store in a dark glass bottle under refrigeration

REAGENT FOR DNA EXTRACTION AND PCR

1. 1.5% Agarose gel

Agarose	0.75	g
0.5x TBE buffer	50	ml

2. dNTP mixture, 300 µl (10mM)

dATP, 100 mM	30	µl
dCTP, 100 mM	30	µl

dGTP, 100 mM	30	µl
dGTP, 100 mM	30	µl
DDW	180	µl

3. DNA Ladder marker

DNA ladder marker	20	µl
DDW	40	µl

4. Ethidium bromide (10 mg/ml)

Ethidium bromide	1	g
DDW	100	ml

5. Loading dye

Bromphenol blue	0.25	g
Xylene cyanol	0.25	g
Ficoll 400	15	g
Sterilized water	100	ml

6. 10x Tris - borate - EDTA (TBE) buffer, 500 ml

Tris base	30.25	g
Boric acid	15.425	g
Na_2 EDTA.H_2O	18.86	g
Sterilized water	500	ml

Sterilize by autoclaving

5x TBE buffer, 500 ml

10x TBE buffer	25	ml
Sterilized water	475	ml

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APPENDIX III

Table 28 Antimicrobial susceptibility testing ranges and breakpoints for four antimicrobials tested and MIC distribution for 216 isolates of *Vibrio parahaemolyticus* discovered from Chai-ya district in 2007 and 2008

Antimicrobial agent	Breakpoints ((µg/ml)*			MIC (µg/ml) distribution for					
	S	I	R	%S	%I	%R	MIC 50	MIC 90	MIC Range
Doxycycline	4	8	16	96.8	3.2	0	1	2	1-8
Norfloxacin	4	8	16	97.7	2.3	0	1	4	1-8
Cipofloxacin	1	1-4	4	76.4	19.9	3.7	0.5	2	0.25-4
Cefotaxime	8	16-32	64	58.3	19	22.7	8	64	2-256

*Breakpoints recommended by the CLSI, in M100-S14 (130). S, I and R stand for susceptible, intermediate and resistant susceptibility.

Table 29 Antimicrobial susceptibility testing ranges and breakpoints for four antimicrobials tested and MIC distribution for 72 isolates of *Vibrio vulnificus* discovered from Chai-ya district in 2007 and 2008

Antimicrobial agent	Breakpoints ((µg/ml)*			MIC (µg/ml) distribution for					
	S	I	R	%S	%I	%R	MIC 50	MIC 90	MIC Range
Doxycycline	4	8	16	100	0	0	1	1	1-1
Norfloxacin	4	8	16	100	0	0	1	1	1-1
Cipofloxacin	1	1-4	4	100	0	0	0.25	0.25	0.25-1
Cefotaxime	8	16-32	64	95.8	0	4.2	2	4	2-128

*Breakpoints recommended by the CLSI, in M100-S14 (130). S, I and R stand for susceptible, intermediate and resistant susceptibility.

Table 30 Antimicrobial susceptibility testing ranges and breakpoints for four antimicrobials tested and MIC distribution for 552 isolates of *Vibrio parahaemolyticus* discovered from Kanchanadith district (Cha-ngor, Kadae, Tatong, Kadae, Kadae-gae) in 2007 and 2008

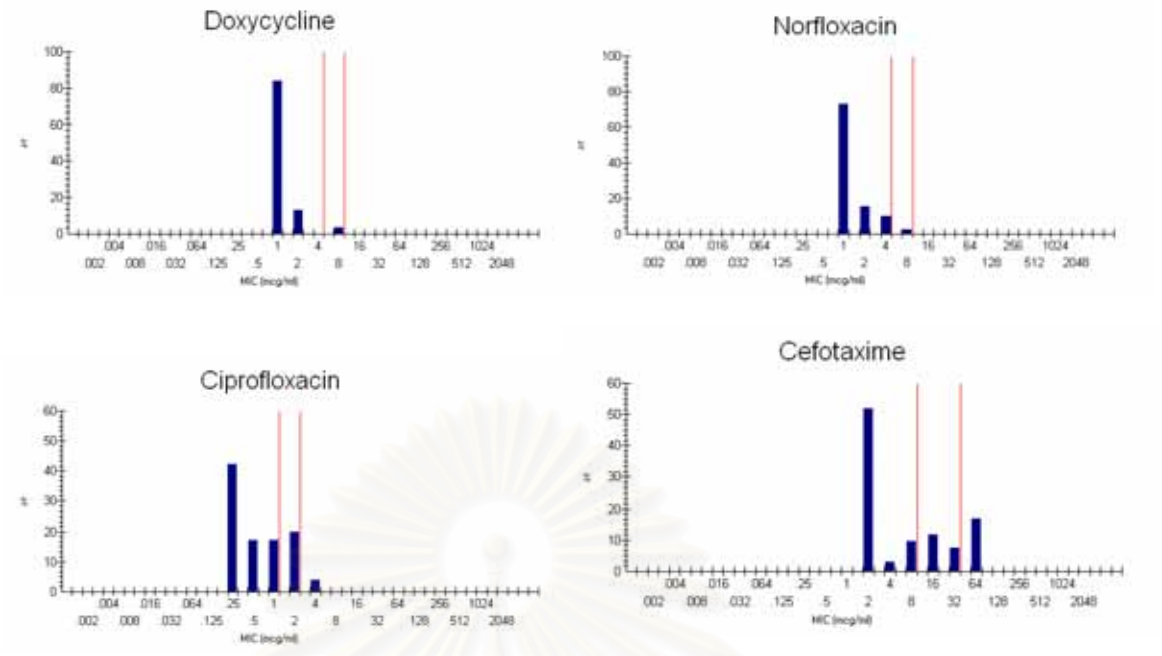
Antimicrobial agent	Breakpoints ((µg/ml)*			MIC (µg/ml) distribution for					
	S	I	R	%S	%I	%R	MIC 50	MIC 90	MIC Range
Doxycycline	4	8	16	99.5	0.5	0	1	1	1-8
Norfloxacin	4	8	16	95.5	2.2	2.4	1	2	1-32
Cipofloxacin	1	1-4	4	76.3	16.8	6.9	0.5	2	0.25-8
Cefotaxime	8	16-32	64	63.9	20.1	15.9	2	64	2-256

*Breakpoints recommended by the CLSI, in M100-S14 (130). S, I and R stand for susceptible, intermediate and resistant susceptibility.

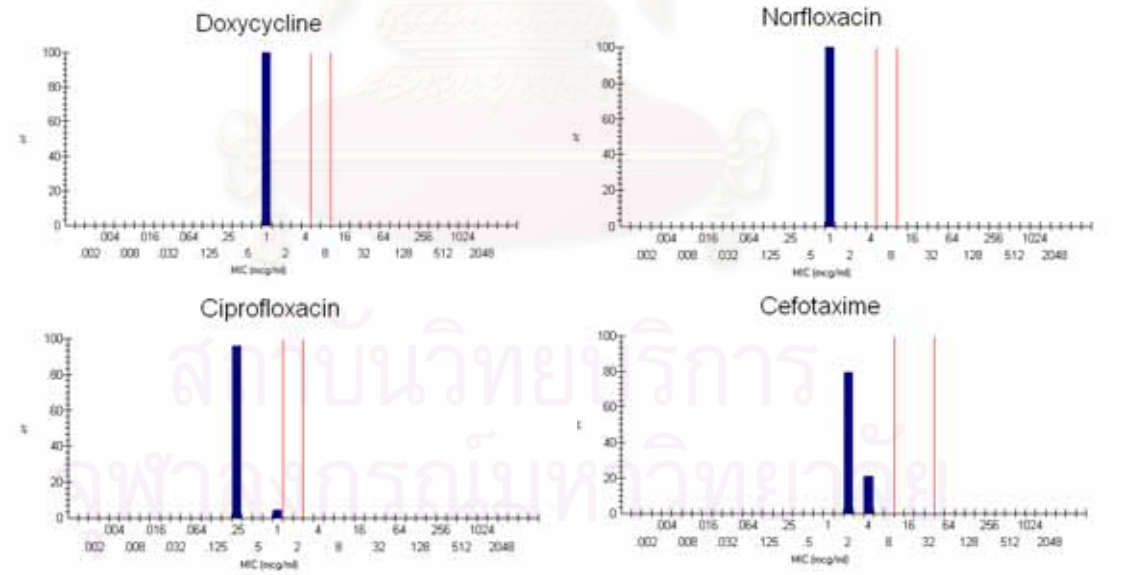
Table 31 Antimicrobial susceptibility testing ranges and breakpoints for four antimicrobials tested and MIC distribution for 225 isolates of *Vibrio vulnificus* discovered from Kanchanadith district (Cha-ngor, Kadae, Tatong, Kadae, Kadae-gae) in 2007 and 2008

Antimicrobial agent	Breakpoints ((µg/ml)*			MIC (µg/ml) distribution for					
	S	I	R	%S	%I	%R	MIC 50	MIC 90	MIC Range
Doxycycline	4	8	16	100	0	0	1	1	1-4
Norfloxacin	4	8	16	100	0	0	1	1	1-1
Cipofloxacin	1	1-4	4	100	0	0	0.25	0.5	0.25-1
Cefotaxime	8	16-32	64	72	9.3	18.7	4	64	2-128

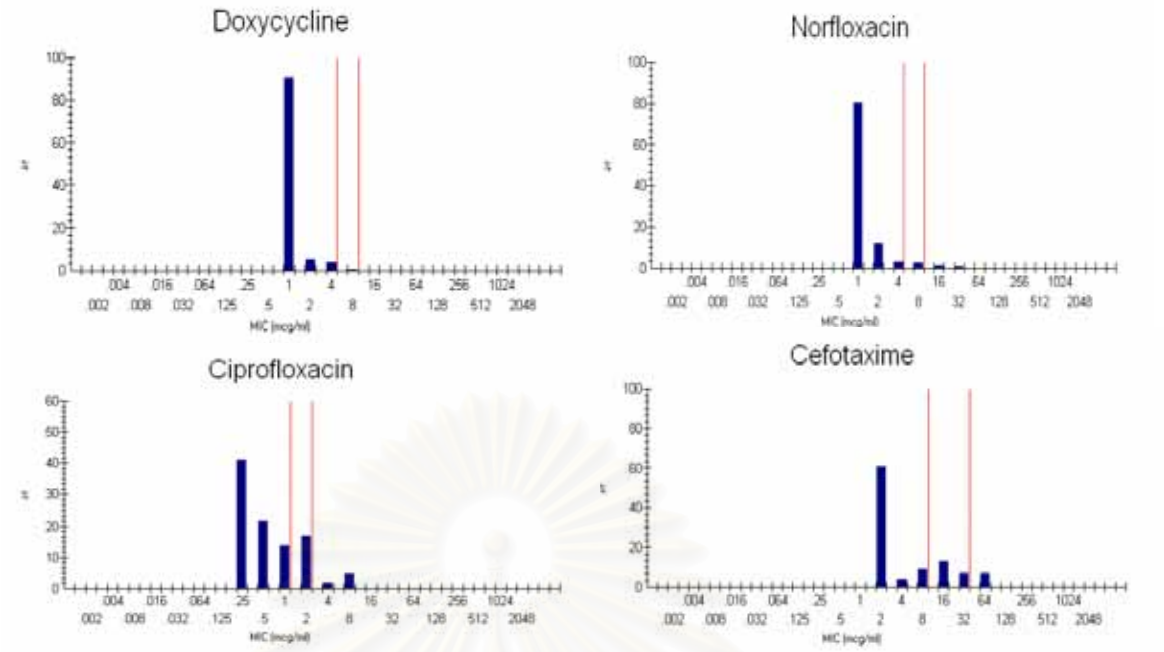
*Breakpoints recommended by the CLSI, in M100-S14 (130). S, I and R stand for susceptible, intermediate and resistant susceptibility.



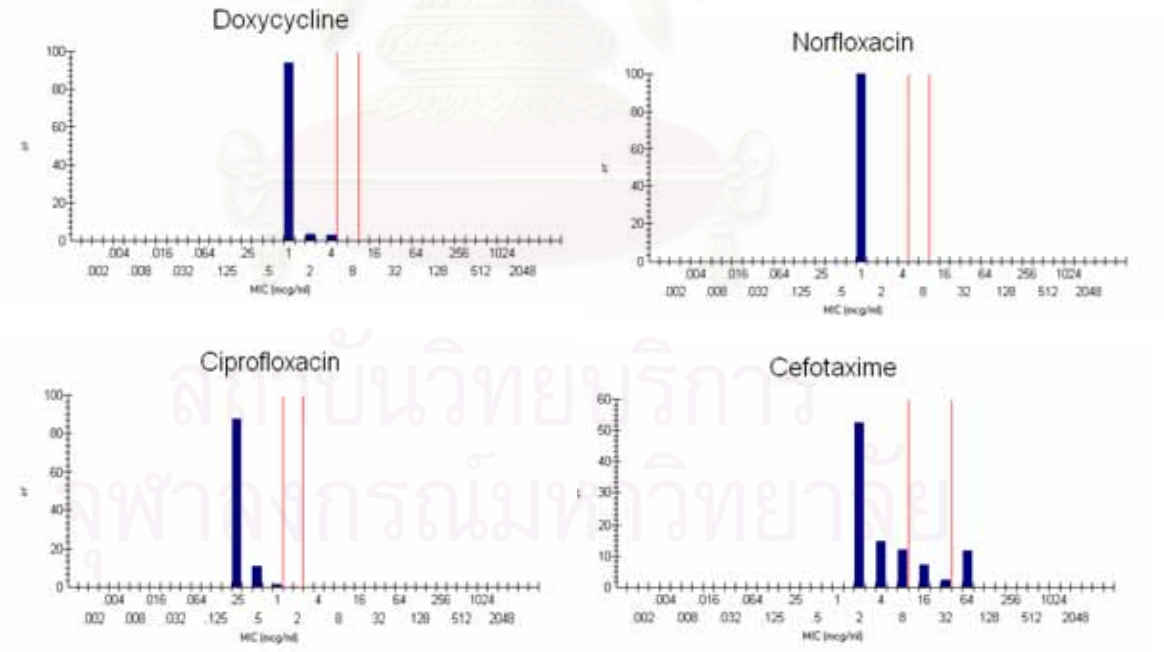
Graph 12 **Antimicrobial susceptibility testing and breakpoints for Antimicrobial tested and MIC distribution for 216 isolates of *Vibrio parahaemolyticus* discovered from Chai-ya district (Chaiya1 and Chaiya 2) in 2007 and 2008**



Graph 13 **Antimicrobial susceptibility testing and breakpoints for Antimicrobial tested and MIC distribution for 72 isolates of *Vibrio vulnificus* discovered from Chai-ya district (Chaiya1 and Chaiya 2) in 2007 and 2008**



Graph 14 **Antimicrobial susceptibility testing and breakpoints for Antimicrobial tested and MIC distribution for 552 isolates of *Vibrio parahaemolyticus* discovered from Kanchanadith district (Cha-ngor, Kadae, Tatong, Kadae, Kadae-gae) in 2007 and 2008**



Graph 15 **Antimicrobial susceptibility testing and breakpoints for Antimicrobial tested and MIC distribution for 225 isolates of *Vibrio vulnificus* discovered from Kanchanadith district (Cha-ngor, Kadae, Tatong, Kadae, Kadae-gae) in 2007 and 2008**

BIOGRAPHY

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