

**THE STUDY OF SEROTYPES AND VIRULENCE GENES OF
VIBRIO PARAHAEMOLYTICUS IN HEALTHY CARRIERS
AT FROZEN SEAFOOD PLANTS**

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CAPT. THANUNCHA ATHAJARIYA

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FROZEN SEAFOOD PLANTS**



Thanunch *Athajariya*
.....
Capt.Thanuncha Athajariya,
Candidate

Orasa Suthienkul
.....
Prof.Orasa Suthienkul,
Ph.D.(Medical Science)
Major-Advisor

K. Siripanichg
.....
Assoc.Prof.Kanokrat Siripanichgon,
B.Sc.(PT),M.D., M.P.H.
Co-Advisor

Fuangfa Utrarachkij
.....
Assist.Prof.Fuangfa Utrarachkij,
M.Sc.(Public Health)
Co-Advisor

Rassmidara Hoonsawat
.....
Assoc.Prof.Rassmidara Hoonsawat,
Ph.D.
Dean
Faculty of Graduate Studies

Pakpimol Mahannop
.....
Assoc.Prof.Pakpimol Mahannop,
Ph.D.(Trop. Med.)
Chair
Master of Science (Public Health)
Major in Infectious Disease and
Epidemiology
Faculty of Public Health

Thesis
entitled

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was submitted to the Faculty of Graduate Studies, Mahidol University
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on
May 20 , 2004

Thanunch Athajariya

Capt.Thanuncha Athajariya,
Candidate

Orasa Suthienkul

Prof.Orasa Suthienkul,
Ph.D.(Medical Science)
Chair

K. Siripanichgon

Assoc.Prof.Kanokrat Siripanichgon,
B.Sc.(PT),M.D.,M.P.H.
Member

Usanee Suthisarnsun

Assist.Prof.Usanee Suthisarnsun
M.D.,DM.T&H.,Dip. American board
of Pediatrics
Member

Rudiwilai Samakose

Assoc.Prof.Rudiwilai Samakoses,
M.D.,D.M.T&H.
Member

Rassmidara Hoonsawat

Assoc.Prof.Rassmidara Hoonsawat,
Ph.D.
Dean
Faculty of Graduate Studies
Mahidol University

Chalermchai Chaikittiporn

Assoc.Prof.Chalermchai Chaikittiporn,
Dr.P.H.(Epidemiology)
Dean
Faculty of Public Health
Mahidol University

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Capt. Thanuncha Athajariya

THE STUDY OF SEROTYPES AND VIRULENCE GENES OF *VIBRIO PARAHAEMOLYTICUS* IN HEALTHY CARRIERS AT FROZEN SEAFOOD PLANTS

CAPT. THANUNCHA ATHAJARIYA 4436073 PPH/M

M.Sc.(PUBLIC HEALTH) MAJOR IN INFECTIOUS DISEASES AND EPIDEMIOLOGY

THESIS ADVISORS : ORASA SUTHIENKUL, Ph.D.(MEDICAL SCIENCE),KANOKRAT SIRIPANICHGON, B.Sc.(PT),M.D.,M.P.H., FUANGFA UTRARACHKIJ, M.Sc.(PUBLIC HEALTH)

ABSTRACT

Many studies show that *Vibrio parahaemolyticus* can be detected from healthy contacts of diarrheal patients, villagers living near the sea, and healthy employees from seafood processed plants without showing any symptom of diarrhea. The carrier state of these healthy carriers who harbour *V. parahaemolyticus* has not been characterized. In this research, serotypes and virulence genes of *V. parahaemolyticus* were studied in 10,214 rectal swab samples from healthy workers of the frozen seafood plant and frozen processed seafood plant at Samut Prakan province during July 2002 to August 2003. *V. parahaemolyticus* was found in 147 samples (1.4%) from 139 healthy workers throughout the year. Heiberg's type VII and type V of *V. parahaemolyticus* were found in 64% and 18% of the samples, respectively. The isolates from another 18% of the samples tested positive for both Heiberg types. A total of 64 O:K serotypes were identified among 999 isolates of *V. parahaemolyticus* from 147 positive samples, with O1:KUT (untypable) which was the most dominant serotype (11.9%), followed by O5:KUT (11.5%), O10:KUT (8.0%) and O11:KUT (7.1%). Only 1.7% of 999 isolates produced both thermostable direct hemolysin (TDH) and urease enzyme (TRH), whereas 32.8% of isolates produced only TDH and 8% produced only urease enzyme. Furthermore, 269 of 999 isolates were selected on the basis of their different Heiberg types and serotypes from all positive samples to determine their virulence genes (*tdh* and *trh*) by using multiplex PCR and antimicrobial susceptibility pattern. Both *tdh* and *trh* genes were positive in 4.8% of the isolates, 25.3% contained only *tdh*⁺, 4.8% only *trh*⁺ and 65.1% only *tdh*⁻*trh*⁻. All isolates with *trh*⁺ produced urease enzyme and 7.4% of *tdh*⁺ isolates did not produce TDH. Antimicrobial susceptibility of the 269 isolates were tested with 8 antimicrobial agents, and more than 96% of them were susceptible to norfloxacin, nalidixic acid, nitrofurantoin, tetracycline, cotrimoxazole and gentamicin. Of the isolates, 27.9% and 17.8% were resistant to ampicillin and colistin, respectively. Multidrug resistant patterns were also found in 7.8% of 269 isolates, in which 5.9% showed resistance to ampicillin and colistin. Five transient healthy carriers from the frozen processed seafood plant were *V. parahaemolyticus* positive two to three times during the study period. However, all isolates from each transient healthy carrier were of different serotypes and strains. TDH with *tdh*⁺ isolate and TRH with *trh*⁺ isolate were identified in two carriers. This study has shown the significant findings and characteristics of *V. parahaemolyticus* among healthy carriers in the frozen seafood plants. Further research is suggested on the roles of carriers in transmission, pathogenic mechanism and virulence gene expression.

KEY WORDS: *VIBRIO PARAHAEMOLYTICUS* / HEALTHY CARRIER / VIRULENCE GENES / SEROTYPES / MULTIPLEX PCR

การศึกษาซีโรไทป์และยีนที่ควบคุมการก่อโรคของ วibriโอ พาราฮีโมลิติกัส ในพาหะในโรงงานอาหารทะเลแช่แข็ง (THE STUDY OF SEROTYPES AND VIRULENCE GENES OF *VIBRIO PARAHAEMOLYTICUS* IN HEALTHY CARRIERS AT FROZEN SEAFOOD PLANTS)

ร.อ.หญิง ธัญญา อัครจริยา 4436073 PHPH/M

วท.ม. (สาขารณสุขศาสตร์) สาขาวิชาเอกโรคติดเชื้อและวิทยาการระบาด

คณะกรรมการควบคุมวิทยานิพนธ์ : อรษา สุตเชิษฐกุล, Ph.D. (Medical science), กนกรัตน์ ศิริพานิชกร, B.Sc. (PT), พ.บ., M.P.H., เพ็ญฟ้า อุดรารัตติกิจ, วท.ม. (สาขารณสุขศาสตร์)

บทคัดย่อ

ภาวะพาหะของ วibriโอ พาราฮีโมลิติกัส นั้นไม่สามารถให้คำจำกัดความที่ชัดเจนได้ แต่มีหลายรายงานที่พบว่าสามารถตรวจพบ วibriโอ พาราฮีโมลิติกัส ได้ในคนปกติโดยที่ไม่มีอาการและอาการแสดงของอาการระว่ง โดยเฉพาะผู้ที่สัมผัสกับผู้ป่วยหรือผู้พาหะระว่ง, ผู้ที่อาศัยอยู่ในหมู่บ้านที่ติดทะเล และในพนักงานที่ทำงานในโรงงานอาหารทะเลแปรรูปแช่แข็ง จากการศึกษาซีโรไทป์และยีนที่ควบคุมการก่อโรคของ วibriโอ พาราฮีโมลิติกัส จาก 10,214 ตัวอย่างอาการระว่งของพนักงานในโรงงานอาหารทะเลแช่แข็งและโรงงานอาหารทะเลแปรรูปแช่แข็งในจังหวัดสมุทรปราการ ระหว่างเดือนกรกฎาคม 2545 ถึง สิงหาคม 2546 สามารถตรวจพบเชื้อ วibriโอ พาราฮีโมลิติกัส ได้ตลอดทั้งปี จำนวนทั้งสิ้น 147 ตัวอย่าง (ร้อยละ 1.4) จากพนักงาน 139 คนโดยไม่มีอาการและอาการแสดงของโรคอาการระว่ง จากการศึกษาเชื้อ วibriโอ พาราฮีโมลิติกัส ที่แยกจาก 139 ตัวอย่างพบว่าร้อยละ 64 และ 18 ของเชื้อ เป็น Heiberg's type VII และ type V อีกร้อยละ 18 ของตัวอย่างตรวจพบทั้ง 2 ไทป์ ซีโรไทป์ของ วibriโอ พาราฮีโมลิติกัส ที่ตรวจพบทั้งหมดมี 64 ซีโรไทป์โดยพบ O1:KUT มากที่สุด ร้อยละ 11.9 รองลงมาคือ O5:KUT ร้อยละ 11.5, O10:KUT ร้อยละ 8.0 และ O11:KUT ร้อยละ 7.1 การศึกษาปัจจัยก่อโรคโดยการตรวจฮีโมลิติน (TDH) และเอนไซม์ซูริเอส (TRH) พบว่ามีเพียงร้อยละ 1.7 จาก 999 ไอโซเลตที่พบปัจจัยการก่อโรคทั้ง 2 ชนิด, ร้อยละ 32.8 พบให้ผลบวกต่อ TDH (K⁺) และร้อยละ 8 ผลิตเอนไซม์ซูริเอส (TRH⁺) การตรวจยีนที่ควบคุมการก่อโรค (*tdh*, *trh*) ด้วยวิธี multiplex PCR โดยเลือกจากผลของปฏิกิริยาทางชีวเคมีและซีโรไทป์ที่แตกต่างกัน 269 จาก 999 ไอโซเลต พบว่ามีเพียงร้อยละ 4.8 เท่านั้นที่มียีนควบคุมการเกิดโรคทั้ง 2 ชนิด (*tdh*⁺, *trh*⁺) ร้อยละ 25.3 มีเพียง *tdh*⁺ อย่างเดียว และ วibriโอ พาราฮีโมลิติกัส ร้อยละ 65.1 ไม่พบยีน (*tdh*, *trh*) ทุกไอโซเลตที่มี *trh*⁺ สามารถผลิตเอนไซม์ซูริเอสได้ การศึกษาความไวของ วibriโอ พาราฮีโมลิติกัส ต่อยาต้านจุลชีพ 8 ชนิด พบว่ามากกว่าร้อยละ 96 ของเชื้อมีความไวต่อยาต้านจุลชีพ 6 ชนิดคือ norfloxacin, nalidixic acid, nitrofurantoin, tetracycline, cotrimoxazole and gentamicin และร้อยละ 27.9 คือ คอติยา ampicillin ร้อยละ 17.8 คือ คอติยา colistin การคือต่อยามากกว่า 1 ชนิดพบได้ร้อยละ 7.8 จากทั้งหมด 269 ไอโซเลต ร้อยละ 5.9 คือ คอติยา ampicillin และ colistin จากการศึกษาตามพาหะของ วibriโอ พาราฮีโมลิติกัส พบว่ามีเพียง 5 คนจากโรงงานอาหารทะเลแปรรูปแช่แข็งที่ตรวจพบเชื้อนี้ได้ซ้ำ 2-3 ครั้งในคนเดียวก้นตลอดระยะเวลาในการติดตาม โดยไม่มีอาการและอาการแสดงของอาการระว่ง อย่างไรก็ตามทุกไอโซเลตของพาหะทั้ง 5 คนนี้ มีซีโรไทป์และสายพันธุ์ที่ต่างกัน ในจำนวนพาหะทั้ง 5 คนนี้มีเพียง 2 คนเท่านั้น ที่มีปัจจัยและยีนที่ก่อโรคคือ มี TDH⁺ และ *tdh*⁺ gene และ TRH⁺ และ *trh*⁺ gene การศึกษานี้ทำให้ได้ข้อมูลที่สคัญและลักษณะของ วibriโอ พาราฮีโมลิติกัส ที่ตรวจพบในคนที่เป็พาหะในโรงงานอาหารทะเล ซึ่งจะนำไปสู่การศึกษาต่อไปถึงบทบาทของพาหะ ในด้านการแพร่กระจายเชื้อ การก่อโรค และการแสดงออกของยีนที่ก่อโรค

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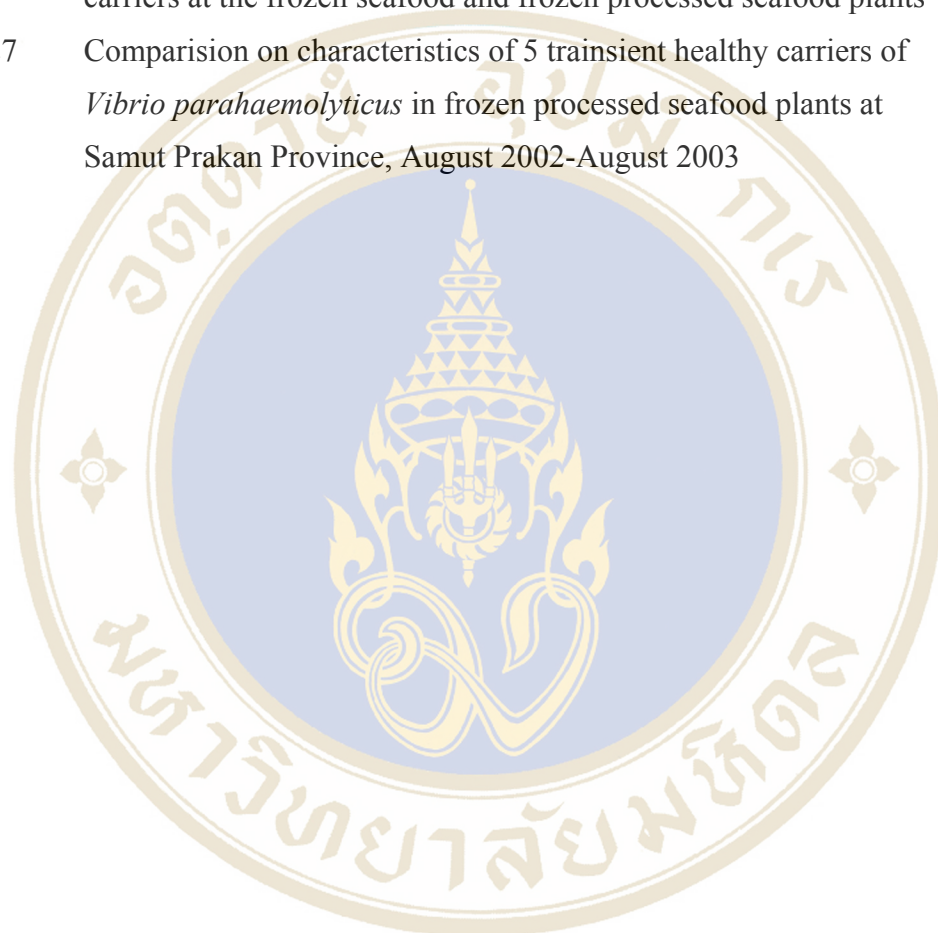
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LIST OF ABBREVIATIONS

APW	=	Alkaline peptone water
bp	=	Base pair
°C	=	Degree celcius
DDW	=	Deionized distilled water
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxynucleoside triphosphate
EDTA	=	Ethylenediaminetetraacetic acid
g	=	Gram
h	=	Hour
HCl	=	Hydrochloric acid
l	=	Litre
LB	=	Luria-Bertani medium
MC	=	MacConkey agar
mg	=	Milligram
ml	=	Millilitre
mm	=	Millimeter
mM	=	Millimolar
min	=	Minute
µg	=	Microgram
µl	=	Microlitre
NaCl	=	Sodium chloride
OD	=	Optical density
PCR	=	Polymerase chain reaction
rpm	=	Revolution per minute
TBE	=	Tris-Borate buffer
TCBS	=	Thiosulfate citrate bile salt sucrose agar

LIST OF ABBREVIATIONS (cont.)

TE	=	Tris-EDTA buffer
Tris-HCl	=	Tris hydrochloride buffer
U	=	Unit
UV	=	Ultraviolet



CHAPTER 1

INTRODUCTION

Statement of problem

Vibrio parahaemolyticus is the most important enteropathogen that causes acute gastroenteritis, bacterial food poisoning, and traveller's diarrhea (1, 2). It is responsible for approximately 40 to 60 percent of foodborne outbreaks in Japan, especially during the summer. In many countries, especially in Southeast Asia, the isolation of *V. parahaemolyticus* varies from 2 to 11 percent from seafood (2, 3). It has also been identified as the cause of food poisoning outbreaks in other countries such as in India 11%, Vietnam 8.5-15%, Indonesia 2.6-3.7%, and Korea 1.5% (4-6). In Thailand, the isolation rate of *V. parahaemolyticus* gastroenteritis during 1983 to 1996 was ranged from 4.5% to 7.6% (7). This organism has been a major cause of gastroenteritis when compared to other organisms isolated from diarrheal patients in Bamrasnaradura Infectious Disease Hospital in 1992 to 1998 (8, 9). *V. parahaemolyticus* has also been a major cause of foodborne outbreak ranking the first in Thailand since 1992 to 2003 (9). The spectrum of disease varies from a mild gastroenteritis to a dysenteric syndrome. Diarrhea and abdominal cramps are the most frequent and earliest symptoms, accompanied by nausea, vomiting, and headache (2, 7, 10-12).

The pathogenic mechanism of *V. parahaemolyticus* have not been defined or elucidated. At present, two hemolysins are important virulence factors in the pathogenesis of *V. parahaemolyticus*, thermostable direct hemolysin (TDH) and thermostable direct hemolysin related hemolysin (TRH). Hemolysis on Wagatsuma's medium (special blood agar) has been routinely used to identify pathogenic strains. A clear hemolytic zone is called Kanagawa phenomenon (KP⁺), which thermostable

direct hemolysin (TDH) causes hemolysis of red blood cells. Kanagawa phenomenon is thought to be closely associated with the enteropathogenicity of the organism. Eighty-eight to 96 % of human isolates produce TDH or KP⁺. One to 2% of environmental strains produce TDH or KP⁺ while 99% are TDH⁻ or KP⁻. Some environmental isolates produced TRH in small percentage (13-17). The rapid detection of *V. parahaemolyticus* hemolysins and its genes has been reported such as immunodiagnosis methods, modified Elek test, immuno halo test (18), enzyme-linked immunosorbent assay (ELISA) (19), and DNA colony hybridization test (DCH) (20).

V. parahaemolyticus was considered as giving negative result for urea hydrolysis. However, urease positive strains were first reported by Huq *et al.*(21) isolated from case of gastroenteritis. In 1995, Suthienkul *et al.*(16) indicated that urease production by *V. parahaemolyticus* strains strongly correlates with the possession of the *trh* gene. Thus, the urease-positive phenotype can be considered an useful marker and indication of virulent *V. parahaemolyticus* in clinical diagnosis. Recently, the several studies have applied PCR technique to detect hemolysin genes both *tdh* and *trh* of *V. parahaemolyticus* in clinical and environmental samples resulted in increasing the sensitivity and specificity of the detection (16, 22-25).

The most frequent source of *V. parahaemolyticus* food poisoning resulted from ingestion of seafood, that has been inadequately cooked or raw and then allowed to remain at ambient temperature for several hours. Sometimes they cross-contaminated with kitchen utensils such as chopping boards, knives, and dishes (10, 26-30). The enteropathogenicity of *V. parahaemolyticus* by feeding broth cultures of isolates to human volunteers and found that administration of relatively large numbers of KP⁻ cells ($1-2 \times 10^{10}$) did not cause any symptoms of gastroenteritis, but abdominal discomfort and diarrhea were observed after administration of $2 \times 10^5 - 3 \times 10^7$ cells of KP⁺ strains (31). Previously, *V. parahaemolyticus* has rarely been cultured from asymptomatic people, and no carrier state has been identified. There is unknown mammalian reservoir of the infection that has been reported (10). However, the organism was isolated from 0.3% of 2,000 Japanese healthy carriers, and 2.5% of healthy sushi chef (4, 32). Study of *V. parahaemolyticus* gastroenteritis in Culcutta

was showed that about 15% of the healthy contact of these patients were detected to be carriers (33). In Thailand, cross-sectional study on the epidemiology of *V. parahaemolyticus* in two villages of Samut Sakhon province was demonstrated that 87% to 97% of the positive cases did not have diarrhea and apparently healthy during the time of the investigation (34). Recently, Bangtrakulnonth *et al.*(35) reported about 2.0% of frozen seafood and 3.7% of frozen processed seafood plants employees harboured this vibrio without showing any symptom of diarrhea.

However, the carrier state of healthy carriers have not been clearly elucidated. In this study, frozen seafood plants employees were examined for their *V. parahaemolyticus* carrier status. The isolated *V. parahaemolyticus* strains were determined the virulence factors, serotypes and drug resistant patterns.

Objectives

General objective

To characterize of *V. parahaemolyticus* isolated from healthy carriers in the frozen seafood and frozen processed seafood plants employees.

Specific objectives

1. To determine detection rate of *V. parahaemolyticus* among rectal swab samples collected from healthy workers of the two frozen seafood plants.
2. To study the difference of biochemical reactions among 10 isolates/sample of *V. parahaemolyticus* from individual healthy carriers.
3. To study the serotype distribution of *V. parahaemolyticus* isolated from the healthy carriers.
4. To determine the virulence genes; *tdh* and *trh* genes by multiplex PCR and hemolysin production (TDH and TRH) of *V. parahaemolyticus* strains isolated from rectal swab samples of the healthy carriers.

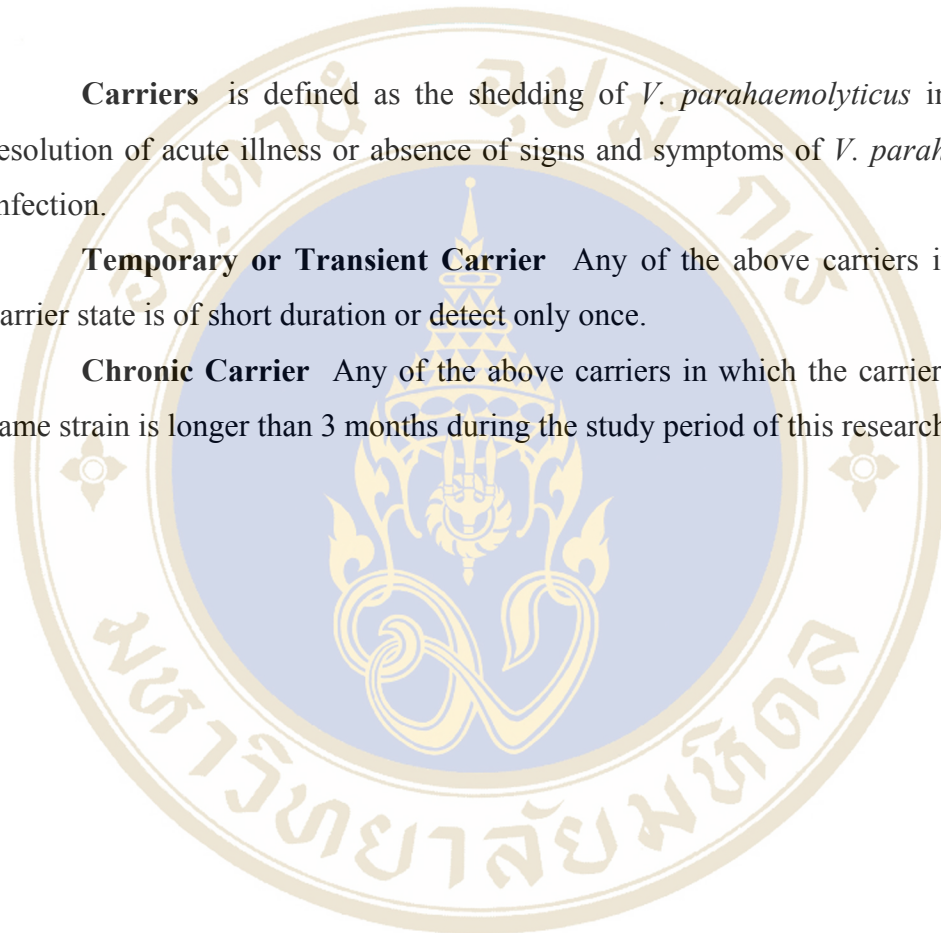
5. To study the antimicrobial susceptibility of *V. parahaemolyticus* isolated from healthy carriers.

Definition of terms

Carriers is defined as the shedding of *V. parahaemolyticus* in stool after resolution of acute illness or absence of signs and symptoms of *V. parahaemolyticus* infection.

Temporary or Transient Carrier Any of the above carriers in which the carrier state is of short duration or detect only once.

Chronic Carrier Any of the above carriers in which the carrier state of the same strain is longer than 3 months during the study period of this research.



CHAPTER 2

LITERATURE REVIEW

History

Vibrio parahaemolyticus is an important cause of seafood related gastroenteritis and traveller's diarrhea which was first isolated by Fujino *et al.*(1) in 1950 from a mass outbreak of food poisoning in Japan. "Shirasu" (a small, half-dried sardines) was named of suspected to be the cause of food poisoning. The outbreak involved 272 persons became ill and 20 (1%) died. In Thailand, the first outbreak of *V. parahaemolyticus* gastroenteritis was reported by the Southeast Asian Treaty Organization (SEATO) in 1970 (36). This organism was recovered to 25% in diarrheal patients. In 1974, Attasampunna (37) reported the isolation of *V. parahaemolyticus* during November 1970 to June 1973 from 7,930 diarrheal patients who admitted in the Infectious Disease Hospital near Bangkok and found that 10.7% in cool season and 22.5% in summer. Seafood was involved in over half of the cases studied. The isolation rate of *V. parahaemolyticus* gastroenteritis during 1983 to 1996 was ranged from 4.5% to 7.6% (7). In 1998, *V. parahaemolyticus* was found 22%, ranking the first organism caused gastroenteritis in Thailand when compared with other organisms (8).

Characteristics

1. Morphology

V. parahaemolyticus is a halophilic, Gram-negative, 0.5 to 0.8 μm in diameter and 1.4 to 2.6 μm in length, straight to slightly curved rod bacterium with rounded ends that possess a polar flagellum (when grown in liquid medium) or peritrichous flagella (when grown in solid medium) (2, 38). It is a facultative anaerobe with both

respiratory and fermentative metabolism, with no gas produced during fermentation. It can grow on or in ordinary media containing 1 to 8% sodium chloride, but it grows best in the presence of 2 to 4% salt (2, 12).

2. Physiological and biochemical characteristics

V. parahaemolyticus is a slightly halophilic bacterium. The optimum NaCl concentration for the organism ranges from 2 to 4% and poor growth is exhibited in media below 0.5% NaCl. The upper and lower tolerance limits of NaCl for growth are 10% and 0.5%, respectively (2, 30, 39). The organism can grow over a pH ranging from 5.6 to 9.6, but the optimal pH for growth is between 7.6 and 8.6 (2, 30, 40). It can grow at 42°C, but growth is best at 30° to 35°C. The lowest recorded temperature of growth was in laboratory medium at 5°C (41). *V. parahaemolyticus* produces round, blue-green colonies on the widely *Vibrio*-selective thiosulfate citrate bile salts sucrose agar (TCBS). Typical colonies giving oxidase-positive reactions, nonfermenter of lactose. It is arginine-dihydrolase negative and ornithine-decarboxylase positive (2).

Epidemiology

1. Source of infection

The most frequent source of *V. parahaemolyticus* food poisoning resulted from ingestion of seafood that has been inadequately cooked or raw seafood and then allowed to remain at ambient temperatures for several hours. Sometimes they become contaminated with the vibrio from kitchen utensils such as chopping boards, knives, dishes (10, 26-30). Baker *et al.*(42) proposed three pathways by which seafood could become contaminated with sufficient numbers of *V. parahaemolyticus* to cause illness in humans. Each pathway is based on the condition that minimal number of organism is present initially and, therefore: (1) if food is allowed to remain unrefrigerated for a sufficient period of time before ingestion without cooking, or (2) is sufficiently cooked, or (3) is recontaminated after cooking, then illness can occur.

In Japan (1976), 91% (162/177) of shellfish and 87% (154/177) of immersed water obtained from Tokyo Central Wholesale Market were contaminated with *V. parahaemolyticus* (14).

In Thailand 1995, Limuthaitip (13) reported that the isolation of 200 raw seafood samples purchased from local markets in Bangkok from May to October 1994, found that 78% (155/200) of raw seafood samples were contaminated with *V. parahaemolyticus*. Mussel was found in highest isolation rates 100% (53/53), followed by cockle 96%, shrimp 68% and salted mangrove crab was contaminated in lowest 51%.

2. Outbreak

At first, food poisoning caused by *V. parahaemolyticus* was thought to be local problem in Japan, but it has now been recognized in many countries throughout the world. It has been found in coastal waters, estuarine waters, sediments, marine fishes, and shellfishes. Several investigators have reported the isolation of *V. parahaemolyticus* from over 15% of patients with diarrhea in India (Calcutta), Vietnam, Philippines, and Thailand. The isolation rates were greater than those of *Salmonella* and *Shigella* (4, 6, 36, 43). In 1999, WHO Surveillance Programme reported the food poisoning outbreak in Japan during 1996 to 1998. The outbreak of *V. parahaemolyticus* food poisoning is present in higher number during the summer month with a peak in August. There were 496 outbreaks during these three years. Only 6% of the outbreaks involving more than 500 cases occurred only twice. The most serovars caused food poisoning outbreaks since 1996 was O3:K6 (44).

The first outbreak of *V. parahaemolyticus* food poisoning outside of Japan was occurred in Maryland, U.S.A. in 1971. *V. parahaemolyticus* was recovered from the stool of patients and food samples. About 320 persons suffered from acute gastroenteritis (45, 46). In U.S.A. during 1969 to 1972, thirteen outbreaks of food poisoning due to *V. parahaemolyticus* (29).

In 1999, Wong *et al.*(47) studied on the characteristics of 308 clinical isolates of *V. parahaemolyticus* obtained during food poisoning outbreaks, mostly occurring from 1993 to 1995 in Taiwan and several environmental and clinical reference strains. The most frequent serovar isolated from these outbreaks were K15 (19.23%), followed by K8 (13.94%), K29 (12.98%), K56 (8.65%) and K12 (6.25%), respectively.

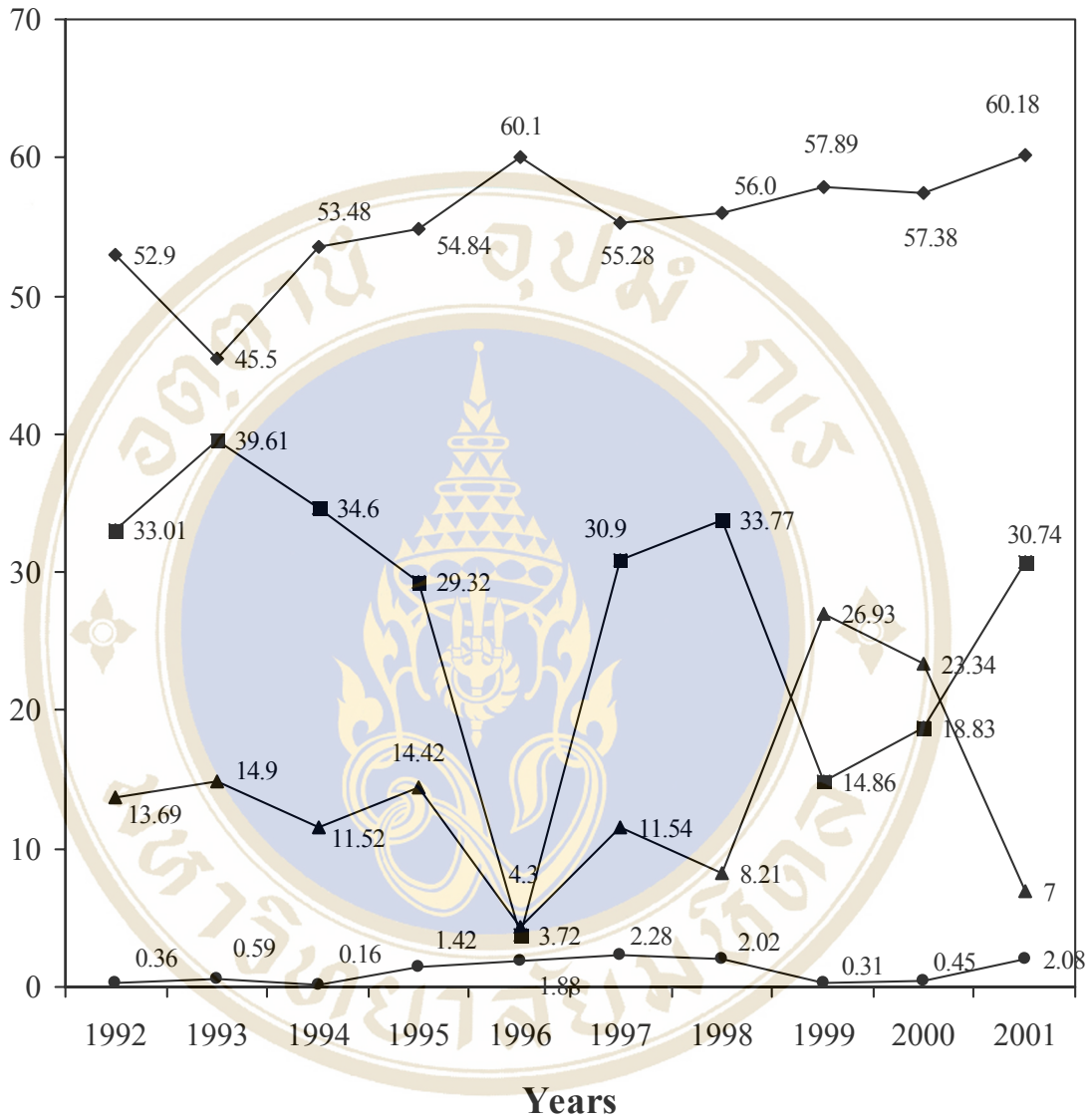
In Southeast Asia, Bonang *et al.*(5) examined rectal swab from 514 ill persons with acute gastroenteritis. *V. parahaemolyticus* was found in 3.7% (19/514). In 1972, Neumann *et al.*(43) examined the isolation rate of *V. parahaemolyticus* from patients with gastroenteritis in Vietnam was 8.5 to 15%.

In Thailand, the first outbreak of *V. parahaemolyticus* gastroenteritis was reported by SEATO in 1970 (36). This organism was recovered to 25% in diarrheal patients. In 1974, Attasampunna (37) reported the isolation of *V. parahaemolyticus* during 1970 to 1973 from diarrheal patients who admitted in the Infectious Disease Hospital near Bangkok and found that 10.7% in cool season and 22.5% in summer. Sriratanaban and Reinprayoon (48) studied a major cause of traveller's diarrhea in Bangkok and suggested that was *V. parahaemolyticus*. This organism has been a common cause of gastroenteritis from diarrheal patients in Bamrasnaradura Infectious Disease Hospital in 1983 to 1996. It was ranged from 4.5 to 7.6% of all diarrheal cases (7). Foodborne disease due to *V. parahaemolyticus* is the most important problem of public health in Thailand. Recently, in June 2000, a small food poisoning outbreak occurred in Nachonrachasima province which 104 individuals were affected and *V. parahaemolyticus* were isolated from their stools (49). Three major causes of foodborne and food poisoning outbreaks during 1992 to 2001 that have been reported, *V. parahaemolyticus* (45-60%) was ranking in the first followed by *Salmonella* (29-39%), and *Staphylococcus aureus* (4.3-26%) are shown in Table 1, Fig. 1 (50).

Table 1 Occurrence of food poisoning-related cases reported to Department of Epidemiology, Office of The Permanent of Secretary For Public Heath, Ministry of Public Heath, 1992-2001 (50).

Year	<i>V. parahaemolyticus</i>	<i>Salmonella</i>	<i>Staph.aureus</i>	<i>C. perfringens</i>	Total cases
1992	52.9	33.01	13.69	0.36	833
1993	45.5	39.61	14.9	0.59	3732
1994	53.48	34.60	11.52	0.16	3646
1995	54.84	29.32	14.42	1.42	4929
1996	60.10	3.72	4.30	1.88	3888
1997	55.28	30.90	11.54	2.28	4958
1998	56.00	33.77	8.21	2.02	5141
1999	57.89	14.86	26.93	0.31	323
2000	57.38	18.83	23.34	0.45	644
2001	60.18	30.74	7.0	2.08	914

Percents



—◆— *V. parahaemolyticus* —■— *Salmonella* —▲— *Staph. Aureus* —●— *C. perfringens*

Fig. 1 Occurrence of food poisoning-related cases reported to Department of Epidemiology, Office of The Permanent of Secretary For Public Heath, Ministry of Public Heath, 1992-2001

Isolation and identification

Vibrio species are currently recognized, 12 of which are known to be human pathogens (Table 2) (51). The most widely recognized for their role in human intestinal infection, *V. cholerae* and *V. parahaemolyticus* diarrhea are important and occurred world wide disease (30). *V. parahaemolyticus* grow well on ordinary laboratory media if 1 to 7% sodium chloride is added, but they grow very poorly or do not grow at all on or in media containing no salt (2). Although several selective media have been devised for the isolation of *V. parahaemolyticus*, thiosulfate citrate bile salt sucrose (TCBS) agar (52) or bromthymol blue (BTB)-teepool agar (53), which is available from commercial sources, is recommended for purpose. After overnight (18-24 h) incubation at 37°C on TCBS agar, *V. parahaemolyticus* forms round bluishgreen colonies 3 to 5 mm in diameter. On direct plating of food or fecal specimens, some other bacteria such as *Proteus*, *Aeromonas*, *Pseudomonas*, and *Escherichia* might grow on both media, but their colonies are smaller than those of *V. parahaemolyticus*, although some of them from greenish colonies (2, 14, 30) as shown in Table 3.

When direct isolation from fecal specimens was unsuccessful and also when food samples were examined, enrichment of samples should be performed. One percent peptone water containing 3% NaCl is usually used for an enrichment medium (54). Since the halophilic *V. parahaemolyticus* is the most important enteropathogenic causes of food poisoning throughout the world, this pathogen must be considered in the differential diagnosis for all acute diarrheal persons after ingestion of seafood (10). These are several tests that can use in the initial identification a *Vibrio* isolate (Table 4). Vibrios can be easily confused with other genera, including *Aeromonas*, *Plesiomonas*, *Pseudomonas* and some Enterobacteriaceae. *V. parahaemolyticus* is different from other species by several physiological and biochemical characteristics.

Table 2 *Vibrio* species that may be found in human clinical specimens (51)

species	Occurance in human clinical specimens	
	Intestinal	Extraintestinal
<i>V. alginolyticus</i>	+	++
<i>V. carchariae</i>	-	+
<i>V. cholerae</i>		
O1	++++	+
Non-O1	++	++
<i>V. cincinnatiensis</i>	-	+
<i>V. damsela</i>	-	++
<i>V. fluvialis</i>	++	-
<i>V. furnissii</i>	++	-
<i>V. hollisae</i>	++	-
<i>V. metschnikovii</i>	-	+
<i>V. mimicus</i>	++	+
<i>V. parahaemolyticus</i>	++++	+
<i>V. vulnificus</i>	+	+++

The symbols +, ++, +++ and ++++ give the relative frequency of each organism in the specimens, - = not found

Table 3 Colony differentiation of vibrio group from other organisms on TCBS agar isolated from human stools (6)

Organisms	Colony	
	Size	Appearance
<i>V. parahaemolyticus</i>	Large	Deep blue or green center
<i>V. alginolyticus</i>	Large	Yellow
<i>V. cholerae</i>	Rather small	Pale yellow
<i>Proteus</i>	Small or NG ^a	Yellow, black or greenish
Coliforms	NG or very poor	-
<i>Aeromonas</i>	Small or NG	Yellow
<i>Pseudomonas</i>	Small or NG	Colorless or pale green
Enterococci	Small or NG	Compact, yellowish white

NG^a = No growth

Table 4 Biochemical tests and other characteristics of 12 *Vibrio* species found in human clinical specimens (55)

Test ^a	% Positive ^b											
	1	2	3	4	5	6	7	8	9	10	11	12
Indole production ^a (HIB ^c , 1%NaCl)	99	98	20	8	97	0	13	11	85	98	97	100
Methyl red (1%NaCl)	99	99	96	93	0	100	96	100	75	80	80	100
Voges-Proskauer ^a (1%NaCl, Barritt)	75	99	96	0	0	95	0	0	95	0	0	50
Citrate, Simmons	97	99	75	21	0	0	93	100	1	3	75	0
H ₂ S on TSI	0	0	0	0	0	0	0	0	0	0	0	0
Urea hydrolysis	0	1	0	0	0	0	0	0	0	15	1	0
Phenylalanine deaminase	0	0	0	0	0	0	0	0	1	1	35	NG
Arginine, Moeller ^a (1% NaCl)	0	0	60	0	0	95	93	100	0	0	0	0
Lysine, Moellera ^a (1%NaCl)	99	100	35	57	0	50	0	0	99	100	99	100
Ornithine, Moeller ^a (1% NaCl)	99	99	0	0	0	0	0	0	50	95	55	0
Motility (36°C)	99	98	74	86	0	25	70	89	99	99	99	0
Gelatin hydrolysis (1% NaCl, 22°C)	90	65	65	0	0	6	85	86	90	95	75	0
KCN test (% that grow)	10	2	0	0	0	5	65	89	15	20	1	0
Malonate utilization	1	0	0	0	0	0	0	11	0	0	0	0
D-Glucose, acid production ^a	100	100	100	100	100	100	100	100	100	100	100	50
D-Glucose, gas production ^a	0	0	0	0	0	0	0	100	0	0	0	0
Acid production from :												
D-Adonitol	0	0	0	0	0	0	0	0	1	0	0	0
D-Arabinose ^a	0	1	0	100	97	0	93	100	1	80	0	0
D-Arabitol ^a	0	0	0	0	0	0	65	89	0	0	0	0
Cellobiose ^a	8	0	9	100	0	0	30	11	3	5	99	50
Dulcitol	0	0	0	0	0	0	0	0	0	3	0	0
Erythritol	0	0	0	0	0	0	0	0	0	0	0	0
D-Galactose	90	82	45	100	100	90	96	100	20	92	96	0
Glycerol	30	13	100	100	0	0	7	55	80	50	1	0
<i>myo</i> -Inositol	0	0	40	100	0	0	0	0	0	0	0	0
Lactose ^a	7	21	50	0	0	0	3	0	0	1	85	0
Maltose ^a	99	99	100	100	0	100	100	100	100	99	100	100
D-Mannitol ^a	99	99	96	100	0	0	97	100	100	100	45	50
D-Mannose	78	99	100	100	100	100	100	100	99	100	98	50
Melibiose	1	0	0	7	0	0	3	11	1	1	40	0
α -Methyl-D-glucoside	0	0	25	57	0	5	0	0	1	0	0	0
Raffinose	0	0	0	0	0	0	0	11	0	0	0	0
D-Rhamnose	0	0	0	0	0	0	0	45	0	1	0	0
Salicin	1	0	9	100	0	0	0	0	4	1	95	0
D-Sorbitol	1	0	45	0	0	0	3	0	1	1	0	0
Sucrose	100	0	100	100	0	5	100	100	99	1	15	50
Trehalose	99	94	100	100	0	86	100	100	100	99	100	50
D-Xylose	0	0	0	43	0	0	0	0	0	0	0	0

Table 4 Biochemical tests and other characteristics of 12 *Vibrio* species found in human clinical specimens (continued)

Test ^a	% Positive ^b											
	1	2	3	4	5	6	7	8	9	10	11	12
Mucate, acid production	1	0	0	0	0	0	0	0	0	0	0	0
Tartrate, Jordan	75	12	35	0	65	0	35	22	95	93	84	50
Esculin hydrolysis	0	0	60	0	0	0	8	0	3	1	40	0
Acetate utilization	92	78	25	14	0	0	70	65	0	1	7	0
Oxidase ^c	100	100	00	100	100	95	100	100	100	100	100	100
Nitrate→nitrite	99	10	50	100	100	00	100	100	100	10	100	100
Dnase, 25°C	93	55	100	79	0	75	100	100	95	92	50	100
Lipase (corn oil) ^a	92	17	50	36	0	0	90	89	85	90	92	0
ONPG test ^a	94	90	0	86	0	0	40	35	0	5	75	0
Yellow pigment 25°C	0	0	5	0	0	0	0	0	0	0	0	0
Tyrosine clearing	13	30	0	3	0	65	45	70	77	75	0	0
Growth in nutrient broth												
With												
0% NaCl	100	100	0	0	0	0	0	0	0	0	0	0
1% NaCl	100	100	100	100	99	100	99	99	99	100	99	100
6% NaCl	53	49	78	100	83	95	96	100	100	99	65	100
8% NaCl	1	0	44	62	0	0	71	78	94	80	0	0
10% NaCl	0	0	4	0	0	0	4	0	69	2	0	0
12% NaCl	0	0	0	0	0	0	0	0	17	1	0	0
Swarming (marine agar, 25°C)	-	-	-	+	-	-	-	-	-	+	-	100
String test	100	100	100	80	100	80	100	100	91	64	100	100
O/129, zone of inhibition ^d	99	95	90	25	40	90	31	0	19	20	98	100
Polymyxin B, zone of inhibition	22	88	100	92	100	85	100	89	63	54	3	100

^aTest is recommended as part of the routine set for *Vibrio* identification

^bAfter 48 h of incubation at 36°C (unless other conditions are indicated). Most positive reaction occur during the first 24 h NG, no growth (probably because NaCl concentration is too low); +, most strains (generally about 90 to 100%) positive; -, most strains negative (generally about 0 to 10% positive)

^cHIB, heart infusion broth; 1%NaCl added to the standard medium to enhance growth; TSI, triple sugar iron agar.

^dDisk potency, 150µg

- | | |
|------------------------------|--------------------------------|
| 1. <i>V. cholerae</i> | 7. <i>V. fluvialis</i> |
| 2. <i>V. mimicus</i> | 8. <i>V. furnissii</i> |
| 3. <i>V. metschnikovii</i> | 9. <i>V. alginolyticus</i> |
| 4. <i>V. cincinnatiensis</i> | 10. <i>V. parahaemolyticus</i> |
| 5. <i>V. hollisae</i> | 11. <i>V. vulnificus</i> |
| 6. <i>V. damsela</i> | 12. <i>V. carcharia</i> |

The minimal characteristics for identification of *V. parahaemolyticus* proposed by Hugh and Sakazaki (56) are shown in Table 5. Most of strains show an alkaline slant and an acid butt without gas formation and fail to produce hydrogen sulfide in the butt of Kligler's iron or triple sugar iron (TSI) agar. They show positive reaction for oxidase, gelatinase, lysine and ornithine decarboxylase; they demonstrate indole production, motility, nitrate reduction. They produce acid from D-mannose and grow in 3 to 8% NaCl; they show a negative reaction for the Voges-Proskauer and arginine dihydrolase. They do not grow in 0 and 10% NaCl. They ferment glucose, maltose, mannose, and mannitol without gas formation but fail to acidify lactose, raffinose, rhamnose, sucrose, xylose, adonitol, dulcitol, inositol, sorbitol, and salisin. Fermentation of arabinose varies with different strains (2, 14, 39). The urease production strongly correlates with the possession of the *trh* gene (16).

The Heiberg fermentation scheme (57) gave a limited number of groups of different kinds of vibrios, under fermentation on sucrose, arabinose, or mannose (Table 6). From the Heiberg classification of vibrios, they could be subdivided into six groups. Smith and Goodner (58) studied and added for two groups of Heiberg classification. *V. parahaemolyticus* was classified into Heiberg's group V and VII which was different from arabinose fermentation (56).

Table 5 Minimal number of characteristics for identification of *Vibrio parahaemolyticus* strains (56)

Characteristics	Reaction
Gram-negative, asporogenous rod	+
Indophenol oxidase	+
Glucose, acid under mineral oil seal	+
Glucose, gas	-
D-mannitol, acid	+
Sucrose, acid	-
Acetylmethylcarbinol	-
Hydrogen sulfide (Kligler)	-
Lysine decarboxylase	+
Arginine dihydrolase	-
Ornithine decarboxylase	+
Growth in 1% trypticase broth	-
Growth in 1% trypticase broth with 8% NaCl	+
Growth in 1% trypticase broth with 10% NaCl	-
Growth at 42°C	+

Table 6 The Heiberg's classification (57)

Heiberg's group	Fermentation		
	Sucrose	Arabinose	Mannose
I	+	-	+
II	+	-	-
III	+	+	+
IV	+	+	-
V	-	-	+
VI	-	-	-
VII ^a	-	+	+
VIII ^a	-	+	-

^a Added by Smith and Goodner (58)

Serotypes

Serotyping is usually limited to strains from outbreaks or to special studies and should be performed in reference laboratory (59). Serological typing of *V. parahaemolyticus* is based on two major antigenic structure, O and K antigens. The H or flagella antigens are common to all strains and to other marine vibrios, thus they have not been used for serotyping (38). The somatic (O) antigen is thermostable. The K antigen is a capsule or envelope antigen that is thermolabile and susceptible to heating at 100°C for 1 to 2 h (14, 38). There is no correlations between *V. parahaemolyticus* and other vibrios in those cases when heterologous K antigens are apparent, definitive typing can be accomplished with the O antigen. However the majority of the clinical strains can usually be classified by the O type, numerous environmental and some clinical isolates are untypable by the K antigen (38).

The epidemiological studies of *V. parahaemolyticus* in Japan have resulted in classification of strains of this organism into O antigen groups ranging from O1 to O13 (32, 38, 60). It is not certain, however, that all these antigen groups exhibit same degree of pathology. At present it can be determined into 13 groups by O antigen and 75 types by K antigens are shown in Table 7 (60).

In 1970, Fishbein *et al.* (61) isolated *V. parahaemolyticus* from the processed meat of Chesapeake Bay Blue crabs (*Callinectes sapidus*). A total 56 strains of *V. parahaemolyticus* found that 34 strains (61%) were untypable. Five of these strains failed to react with the monovalent K antisera, 7 strains reacted with more than one antisera, and 22 *V. parahaemolyticus* strains (39.4%) responded monospecifically with fairly strong agglutination (2⁺ to 4⁺). The dominant serovar of the typable (22 strains) were O2:K3 (7 strains). The first isolation of *V. parahaemolyticus* in Kenya was from seafood and estuarine environment even there has no clinical disease has been reported in 1982 (62). The main serovars were O3:K37, O3:K40, O8:K39, O10:K23, O19:K52, and O11:K40.

V. parahaemolyticus isolated from diarrheal patients have serotypes uncertainly pattern and serotypes may vary considerable from one area to another, and

Table 7 The serotyping scheme of *Vibrio parahaemolyticus* (60)

O-antiserum	K-antiserum
1	1, 5 ^a , 20 ^a , 25, 26, 32, 33 ^a , 38, 41 ^a , 56 ^a , 58 ^a , 60 ^a , 64, 69
2	3, 28
3	4 ^a , 5 ^a , 6, 7, 29 ^a , 30 ^a , 31, 33 ^a , 37, 43, 45, 48, 51 ^a , 54 ^a , 56 ^a , 57, 58 ^a , 59 ^a , 65 ^a , 72, 75
4	4, 8, 9, 10, 11, 12, 13, 29 ^a , 34, 42, 49, 53 ^a , 55, 63, 67, 73
5	15, 17, 19 ^a , 30 ^a , 47, 54 ^a , 60 ^a , 61 ^a , 68
6	18, 46 ^a
7	19 ^a , 52 ^a
8	20 ^a , 21, 22, 39, 41 ^a , 70, 74
9	23, 44
10	19 ^a , 24, 52 ^a , 66 ^a , 71
11	5 ^a , 19 ^a , 36, 40, 46 ^a , 50, 51 ^a , 61 ^a
12	19 ^a , 52, 53 ^a , 61 ^a , 66 ^a
13	59 ^a , 65 ^a
Total 13	75 serotypes

^a Occurs in more than one O groups

from time to time. The serotypes of *V. parahaemolyticus* found in Thailand were varied. The most dominant serotypes found in 1979 from April to June and July to December was O4:K8 and O3:K6 respectively (63). In 1983 and 1984 (64, 65), the Department of Medical Sciences Report, Ministry of Public Health reported that the most dominant serotypes isolated from diarrheal patients were O4:K10 and O5:KUT, found in 22.4% (338/1,735) and 10.2% (223/2,200), respectively.

In 1994, Ussawachairachan (66) studied the difference in serovars among 10 isolates of *V. parahaemolyticus* from individual diarrheal patients at BIDH, Thailand. In the study, 71% (71/100) of cases had same serovars in 10 isolates per case and 29% had 2 to 5 serovars in 10 isolates per case. The dominant of the same serovar were O3:K29 (8%) followed by O1:K56 (6%), O4:K55 (5%), O8:K39 (5%), and O8:K32 (5%).

Urease enzyme

The family of Enterobacteriaceae is routinely determined urease activity in the clinical bacteriological laboratory for differentiation and identification purposes (67). The genus in this family could produce urease enzyme are *Proteus*, *Klebsiella*, *Citrobacter*, *Yersinia* and some species of *Serratia* and *Enterobacter* (68, 69).

V. parahaemolyticus was commonly considered negative for urea hydrolysis (70). The first isolate of urease-positive strains was reported by Huq *et al.*(21) they found 11 urease-positive *V. parahaemolyticus* isolated in Bangladesh from case of gastroenteritis in 1975. Since this report, urease-positive *V. parahaemolyticus* have been reported from various countries such as Singapore, the United States, Brazil, Canada, Malaysia and Thailand (16, 66, 71-75).

In Singapore (71), there was the first reported case in which the vibrios demonstrated a positive urease reaction. There was isolated from the site of a perforated gangrenous appendix. The infected swab from the patient was taken from the peritoneal cavity and sent for the bacteriological investigation. The results of the

biochemical reaction indicated that this strain is similar but not identical to the classical *V. parahaemolyticus* strain, it could be included in minority group of *V. parahaemolyticus*. However, urease-positive *V. parahaemolyticus* has not been reported before, this strain was strongly urease positive on both normal and salted Christensen urea agar after overnight incubation.

In 1989, Kelly and Stroh (76) studied *V. parahaemolyticus* in patients and environment in the Pacific Northwest. They found 50% and 8% urease-positive strain from 13 patients and 221 environmental specimens. The biochemical characteristics of their clinical isolates were similar to these of standard *V. parahaemolyticus* (70); except for the urease and ornithine reaction. Their finding suggested that urease positive strains of *V. parahaemolyticus* associated with gastroenteritis in the Pacific Northwest.

In Thailand, Suthienkul *et al.*(16) found that 8% of the isolates (489) from clinical patients were positive for urease production and possessed the *trh* gene. The result indicated that the possession of the *trh* gene was strongly correlated with the urease production by *V. parahaemolyticus* strains.

Hemolysin

1. Thermostable direct hemolysin (TDH)

The hemolytic characteristic of *V. parahaemolyticus* was observed by Fujino (77) when he first isolated this bacterium. Kato *et al.*(78) and Miyamoto *et al.*(79) reported a close correlation of human pathogenicity with Kanagawa-phenomenon positive (KP⁺) strains of *V. parahaemolyticus*. The KP was examined on the special blood agar that called Wagatsuma agar and showed beta-hemolytic reaction (17). Kanagawa hemolysin or thermostable direct hemolysin (Vp-TDH) is a heat stable even at 100°C for 10 min. The molecular weight of this hemolysin was approximate 42 KD (80). The hemolytic activity of Vp-TDH could hemolyse on various kind of erythrocytes such as highly hemolytic for erythrocytes of rats, dogs, mice, and

monkeys; moderately hemolytic for erythrocytes of human, rabbits, guinea pigs and chickens; slightly hemolytic for erythrocytes of sheep; and non hemolytic for erythrocytes of horses (81).

The Vp-TDH is not only hemolytic activity (81) but also cytotoxic to human FL cells in cell culture (82), lethal toxin due to cardiotoxic to cultured mouse heart cells, and may reported the heart arrhythmia observed in some patients infected by *V. parahaemolyticus*. This explained the rapid death of animals after intravenous injection of the toxin (83). Several hemolytic Vp-TDH detection methods have been developed such as Wagatsuma agar (biological method), modified Elek test (immunological method) (18), enzyme-linked immunosorbent assay (ELISA) (19), DNA colony hybridization test (DCH) (20) and polymerase chain reaction (PCR) (16, 24).

2. Thermostable direct hemolysin-related hemolysin (TRH)

Kanagawa phenomenon negative clinical isolate of *V. parahaemolyticus* capable of causing disease have been found by Honda *et al.*(84). These clinical strains produce a new hemolysin named thermostable direct hemolysin-related hemolysin (Vp-TRH). It was purified by ammonium sulfate fractionation and successive column chromatographies on DEAE-cellulose, hydroxylapatite and Mono Q (15). The molecular weight of Vp-TRH was estimated as 48,000 by Sephadex G-100 gel filtration. The TRH is composed of two subunits, a molecular weight of each subunit was estimated to be 23,000 by SDS-PAGE. The hemolytic activities of VP-TRH on erythrocytes from various animals different from those TDH, especially from calves, chickens, mice and sheep. The hemolytic activity of Vp-TRH, unlike that of Vp-TDH was labile on heat treatment at 60°C or higher temperature for 10 min. Honda *et al.*(85) demonstrated that Vp-TRH exhibited similar biological activities to Vp-TDH, such as cardiotoxic (83) and enterotoxic (86) activities. These results suggested that TRH played rules similar to Vp-TDH in the pathogenesis of *V. parahaemolyticus*. Various characteristics reported about Vp-TDH and Vp-TRH were compared in Table 7 (87).

Table 8 Comparison of Vp-TDH and Vp-TRH toxin (87)

Property	Vp-TDH	Vp-TRH
Molecular weight		
- Holo toxin	46,000	47,000
- Subunit	23,000	23,000
PI	4.9	4.6
Heat stability	Stable at 100°C	Labile at 60°C
Antigenicity	Related but not identical	Related but not identical
Amino acid sequence homology	67%	67%
Biological activity:		
- Hemolytic activity	Rabbit, human > calf, sheep > horse	Calf, sheep > rabbit, human > horse
- Lethal activity (mouse)	similar	similar
- Fluid accumulation in rabbit ileal loop	250µg/loop	100µg/loop

3. Lecithin dependent hemolysin (LDH)

Lecithin dependent hemolysin (LDH) is one of species specific gene fragment of *V. parahaemolyticus*. The LDH gene is present in all *V. parahaemolyticus* strains and did not have any homologous sequences with genes of the α -hemolysin of *Staphylococcus aureus*, the enterotoxin of *V. cholerae*, the enterotoxin ST and hemolysin of *E. coli*. It was thermolabile hemolysin with the nucleotide sequence was 1.5 Kb. The preprotein and the mature protein consisted of 418 and 398 amino acids, and that the molecular weights were 47.5 KDa and 45.3 KDa, respectively. The GC content of LDH gene was 47.6% which is almost the same as that of *V. parahaemolyticus* genome (88).

Pathogenicity

V. parahaemolyticus is the most important enteropathogen that causes not only acute gastroenteritis but also secondary septicemia, wound, eye and ear infections (Table 8) (1-2, 89-90). The size of infecting dose necessary to produce clinical symptoms may vary with the strain, but it is probably about 10^6 - 10^9 viable cells (91-93). The incubation period in *V. parahaemolyticus* infections is usually 9 to 24 h, although symptoms may appear as soon as 2 h after ingesting contaminated seafood or be delayed for up to 96 h (89).

Diarrhea and abdominal cramps are the most frequent and earliest symptoms, accompanied by fever, nausea, vomiting, and headache. The frequency of diarrhea is usually less than 10 times a day, but in some cases it is more than 20 times. The diarrhea is explosive and watery with little mucus but sometimes bloody, although a dysentery-like, or gastroenteritis of sudden onset and during the first day. *V. parahaemolyticus* infection is usually a self-limiting disease and clinical symptoms may last 1-2 weeks (11, 12, 59), with little evidence of spread of the infection from one person to another (30, 70). Treatment is preferable supportive with fluid replacement.

Table 9 Association of *Vibrio* spp. with different clinical syndrome* (90)

Species	Clinical syndrome				
	Gastroenteritis	Wound infection	Ear infection	Primary septicemia	Secondary septicemia
<i>V. cholerae O1</i>	+++	+			
<i>V. cholerae non O1</i>	+++	++	+	+	+
<i>V. mimicus</i>	++		+		
<i>V. fluvialis</i>	++				
<i>V. parahaemolyticus</i>	+++	+	+		+
<i>V. alginolyticus</i>	(+)	++	++	+	
<i>V. cincinnatiensis</i>				+	
<i>V. hollisae</i>	++			+	
<i>V. vulnificus</i>	+	++		++	++
<i>V. furnissii</i>	(+)				
<i>V. damsela</i>		++			
<i>V. metschnikovii</i>	(+)			(+)	
<i>V. carchariae</i>		+			

*+++ = frequently reported, ++ = less common (6-100 reports), + = rare (1-5 reports), and (+) = association is unclear.

The mechanism of pathogenicity of *V. parahaemolyticus* was closely correlated with a clear hemolytic zone on Wagatsuma blood agar is called Kanagawa phenomenon positive (KP⁺) (13,14). The most strains isolated from human patients were KP⁺, while most strains isolated from sea fish and sea water were KP⁻ (6). However, Honda *et al.*(15) reported that the clinical isolates of KP⁻ *V. parahaemolyticus* from travelers in Maldives produced newly hemolysin, named TDH-related hemolysin (TRH). This suggests that TRH may be an importance virulence factor and possibly cause of diarrhea. Sanyal and Sen (31) demonstrated the enteropathogenicity of *V. parahaemolyticus* isolated from a case of food poisoning by feeding broth cultures of isolates to human volunteers and found that administration of relatively large numbers of KP⁻ cells ($1-2 \times 10^{10}$) did not cause any symptoms of gastroenteritis, on the other hand abdominal discomfort and diarrhea were observed after administration of $2 \times 10^5 - 3 \times 10^7$ cells of KP⁺ strains. In 1977, Carruthers (94) reported that a KP⁺ strain of *V. parahaemolyticus* adherenced to a human fetal intestine (HFI) cell line (HS 0074) in vitro much more rapidly than did a KP⁻ strain.

Although the mechanism of pathogenicity, colonization and multiplication of vibrios in the human intestine would seem a necessary precedent to disease. Because the intestinal tract effectively cleans itself by sheeding its surface coat and by undergoing peristalsis, a factor that promotes bacterial adherence to intestinal epithelium could be an important virulence factor. In 1990, Nakasone *et al.*(95) demonstrated that the purified pili of KP⁺ *V. parahaemolyticus* (Ha 7) had the ability to adhere to the intestinal. It is adhered to the intestinal epithelium and then releasing toxin. These results suggest that pili of this *V. parahaemolyticus* strain play an important role in colonization.

Human carriage

V. parahaemolyticus is an important pathogen of humans and aquacultured animals. Disease caused by this organism is widely distributed throughout the world. It is self-limiting, generally lasting only a few days (70). The principal reservoir for *V. parahaemolyticus* is seafoods and seawater, usually in costal areas. Although

persons with asymptomatic infections have been found, there are no reports of long-term carriage of this organism, and there is no evidence that infected foodhandlers have been a source of the organism in disease outbreaks (4).

In Japan, Zen-Yoji *et.al* (32) reported the detection of *V. parahaemolyticus* from the stools of asymptomatic persons, as well as from individuals with, or recovering from, actual illness, during the warm months of June to October. Carriage rates of 0.3% among healthy individuals and 2.5% among sushi chefs have been reported. Duration of carriage is short: 3 to 7 days in stools of healthy individuals and 10 to 16 days in those recuperating from diarrhea.

In Culcutta (33), the study of clinical and epidemiological features of *V. parahaemolyticus* infection was showed that 33.3% of 60 patients with *V. parahaemolyticus* associated gastroenteritis denied having eaten seafood during the previous seven days. About 15% of the healthy contact of these patient were detected to be carrier.

In Thailand, cross-sectional study on the epidemiology of *V. parahaemolyticus* in two villages of Samut Sakhon province was demonstrated that 87 and 97% of the positive cases did not have diarrhea in the previous month and apparently healthy during the time of the investigation. (34).

Recently, Bangtrakulnonth *et al.*(35) survey on foodborne pathogens from employee's feces in 18 frozen food manufacturers. The study reported about 2.0% of frozen seafood plants employees harboured this vibrio without showing any symptom of diarrhea. Thus they could be considered as healthy carriers of this bacteria.

Antimicrobial susceptibility

Antimicrobial resistance in enteric pathogens is great importance problem of public health in the developing world. In this time, most hospitals and clinics treat diarrheal infected patients with antibiotics prior to receiving definitive laboratory

results. The seriousness of the problem has prompted calls for improved surveillance for antimicrobial resistance in developing country, which could provide early warnings of the emergence of resistant bacterial strains (96). The genus *Vibrio* caused many diseases from seafood in Thailand especially *V. parahaemolyticus* and *V. cholerae*, that the important problem of frozen seafood for export.

Chatterjee *et al.*(97) studied sensitivity test of *V. parahaemolyticus* by disc method. The result indicated that *V. parahaemolyticus* were sensitive to chloramphenicol, tetracycline, streptomycin, kanamycin, polymyxin B, colistin and furazolidone but resistant to ampicillin.

In 1985, Farmer *et al.*(70) studied antimicrobial susceptibility from 144 strains of *V. parahaemolyticus*. They found that most of strains were susceptible to tetracycline, gentamicin, and nalidixic acid but resistance to penicillin, carbenicillin, ampicillin, sulfadiazine, colistin, cephalotin, streptomycin and kanamycin

Nettip (74) isolated *V. parahaemolyticus* from 526 diarrheal patients at Bamrasnaradura Infectious Disease Hospital, Nonthaburi, Thailand during April 1990 to March 1991 by disc diffusion method. All *V. parahaemolyticus* were assessed for their susceptibility to 8 antimicrobial agent. All strains 99.8% (525/526) were sensitive to cotrimoxazole, gentamicin, nalidixic acid, nitrofurantoin, tetracycline and chloramphenicol. Intermediate susceptibility to colistin and ampicillin were seen in 22.6% (119/526) and 1.0% (5/526), respectively.

In 1995, Ussawachairachan (66) studied antimicrobial susceptibility of *V. parahaemolyticus* from individual diarrheal patients at the same hospital (BIDH) during August 1992 to February 1993. One hundred rectal swab specimens were tested for their susceptibility to 8 antimicrobial agents by disc diffusion method. All strains were sensitive to cotrimoxazole, gentamicin, nalidixic acid, nitrofurantoin, tetracycline and norfloxacin. Intermediate susceptibility to colistin and ampicillin were seen in 65.0% (65/100) and 13.0% (13/100), respectively. The resistance of ampicillin and colistin were 86.0% and 6.0%, respectively.

Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is rapidly become established as one of the most widely used techniques of molecular biology. The basic reaction of this method is amplified a specific DNA fragment during a cyclic three step process in a closed tube using a simple thermo-cycler; (1) the target DNA is denatured at high temperature, (2) two synthetic oligonucleotides (primers) are annealed at opposite strand at a temperature that only allows hybridization to the correct target, and (3) polymerization is performed with the oligonucleotides as primers for the enzyme and the target DNA as template. When this process is repeated over and over with newly synthesized DNA as template in addition to the original target DNA, an exponential amplification of the DNA fragment between the two oligonucleotides is obtained.

The widespread use of the PCR technique has been applied to detect hemolysin genes both *tdh* and *trh* of *V. parahaemolyticus* in clinical and environmental samples. Toda *et al.*(24) established PCR protocols for specific detection of *tdh* and *trh* genes of *V. parahaemolyticus*. They found that the sensitivity of the established PCRs were reduced by a factor of 10^4 - 10^5 by an inhibitor(s) present in a normal faecal sample, indicating the need for either DNA or enrichment of the faecal sample in alkaline peptone water for 4 h before using PCR for directly detecting the DNA in faecal samples. Later, Lee *et al.*(25) designed oligonucleotide primers to detect *tdh* gene in stool samples. The detection limit for the *tdh* gene was 40 pg of total DNA, or broth culture containing 1000 viable cells.

In Thailand, Suthienkul *et al.*(16) used PCR for detection of *tdh* and *trh* genes of *V. parahaemolyticus* isolated from the diarrhea patients. They found that eighty-one percent of the isolates were positive for *tdh*, 2% had the *trh* and only 6% had both *tdh* and *trh* genes. Recently, Bej *et al.*(98) developed multiplex-PCR for rapid detection of the genus-specific (*tl* or *ldh*), and hemolysin-producing *V. parahaemolyticus*; thermostable direct hemolysin (*tdh*), and thermostable direct hemolysin-related hemolysin (*trh*) in shellfish. This method has been spent a lower cost, less time, rapid to detect and identify *V. parahaemolyticus* by conventional culture method and primary or nested PCR.

CHAPTER 3

MATERIALS AND METHODS

Study location

The study were performed at the Department of Microbiology, Faculty of Public Health, Mahidol University, Bangkok.

Study samples

Rectal swab specimens were collected from individual healthy person who is working in the frozen seafood and frozen processed seafood plants at Samut Prakan province during July 2002 to August 2003. Duration of present job, history of illness, signs, symptoms were obtained using a pretest questionnaire (see Appendix A). Ethical Clearance of this study was approved by the Committee on Human Rights Related to Human Experimentation, Mahidol University.

Specimen collection

A rectal swab was collected with a cotton swab and kept into Cary-Blair medium. Frequency of sample collection was followed by protocol of each plant. In frozen seafood plant, sample were collected every 3 months but sample in frozen processed seafood plant was collected every 2 weeks.

Detection of *Vibrio parahaemolyticus*

1. Culture media

APW with 3% NaCl was used as enrichment medium for *V. parahaemolyticus*. TCBS (Eiken Co. LTD., Tokyo) was the selective medium for isolation *Vibrio* spp.

Motility test medium was used for the stock medium of *V. parahaemolyticus*. Triple sugar iron agar (TSI; BBL, Cockeysville, MD, U.S.A) and lysine indole motility (LIM) were used for screening *V. parahaemolyticus*. Other biochemical tests for confirmation were arginine dihydrolase, ornithine decarboxylase, lysine decarboxylase, urease test, citrate test, methyl red - Voges-Proskauer (MR-VP), fermentation of sugar (glucose, lactose, sucrose, mannose, arabinose, mannitol) and salt tolerance test (APW with 0%, 3%, 6%, 8% and 10% NaCl). All media mentioned above except APW contained 1% sodium chloride for the growth requirement of *V. parahaemolyticus*.

2. Isolation

After enrichment the rectal swab were directly enriched in alkaline peptone water (APW) with 3% NaCl and incubated at 37°C for 4-6 h, the cultures were streaked on TCBS agar plates (Fig. 2) and incubated at 37°C for 18-24 h. On TCBS agar plate, *V. parahaemolyticus* formed the round bluishgreen colonies, 3 to 5 mm in diameter. The suspected colonies were counted as presumptive *V. parahaemolyticus* and were further identified. On the other hand, *V. mimicus*, *V. vulnificus* a related organism formed green colonies. Other vibrio colonial appearance are shown in Table 10

3. Identification

Approximately, ten suspected bluishgreen colonies of *V. parahaemolyticus* per sample were picked and subcultured into TSI and LIM for screening test. After overnight incubation, the typical reaction of *V. parahaemolyticus* on TSI were acid (yellow) in butt and alkaline (red) in slant and no production of hydrogen sulfide (H₂S). For confirmation, *V. parahaemolyticus* were identified with biochemical tests as described by Kelly *et al.*(59). The biochemical test results of *V. parahaemolyticus* and other vibrios are shown in Table 5.

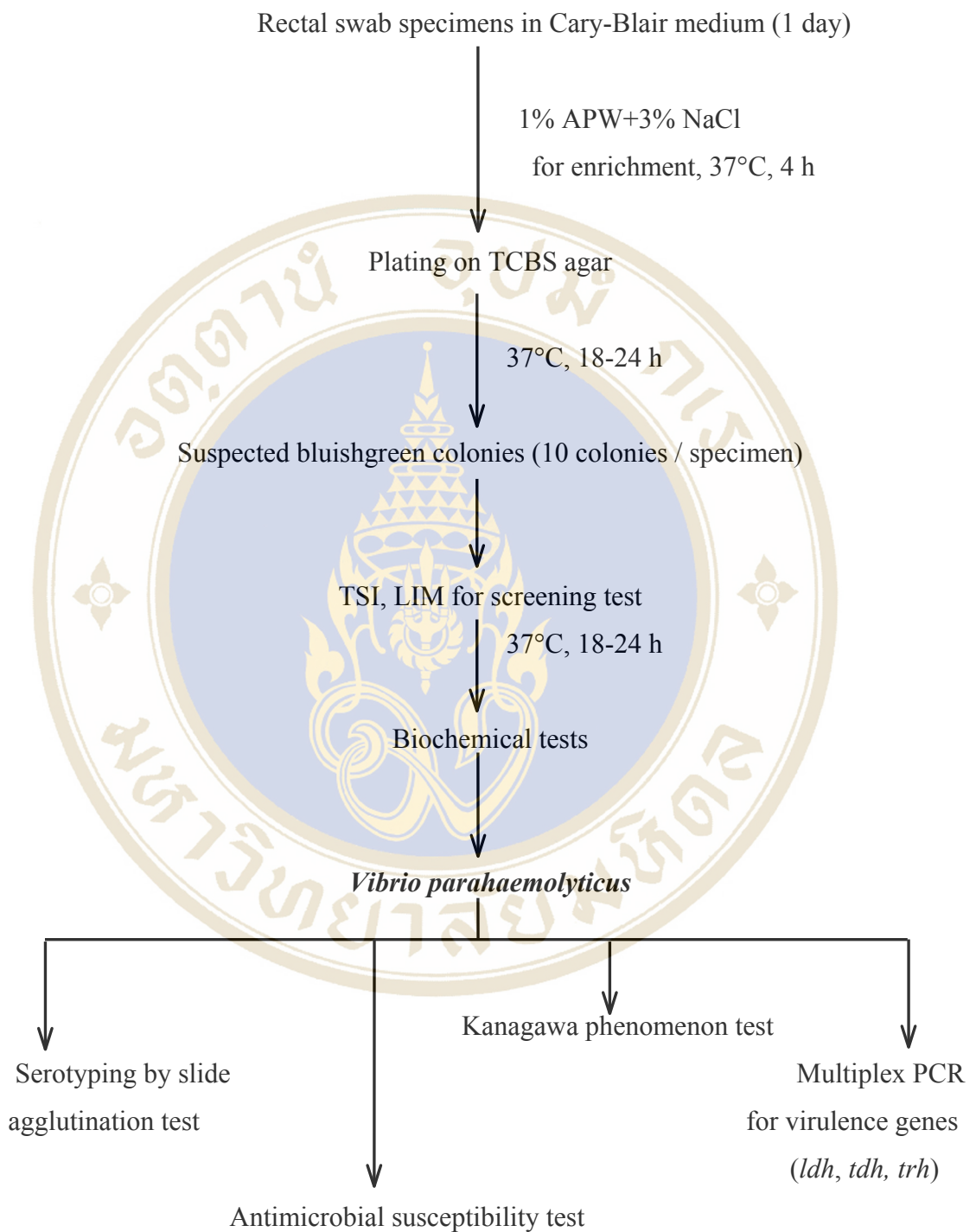


Fig. 2 Isolation and identification of *Vibrio parahaemolyticus* from rectal swab

Table 10 Colony differentiation of vibrio group on TCBS agar (82)

Organisms	Colony appearance on TCBS agar		Growth-plating efficiency
	(%)		
	Green	Yellow	
<i>V. parahaemolyticus</i>	99	1	good
<i>V. mimicus</i>	100	0	good
<i>V. vulnificus</i>	90	10	good
<i>V. hollisae</i>	100	0	very poor
<i>V. damsela</i>	95	5	reduced at 36° C
<i>V. alginolyticus</i>	0	100	good
<i>V. cholerae</i>	<1	>99	good
<i>V. fluvialis</i>	0	100	good
<i>V. furnissii</i>	0	100	good
<i>V. metchnikovii</i>	0	100	may be reduced
Marine vibrios	variable	variable	variable

Hemolysin assay

Hemolytic activity was determined by Kanagawa phenomenon test. The clear hemolysis zone was observed on a special blood agar medium called Wagatsuma's medium (17).

Kanagawa phenomenon test

1. Medium

Wagatsuma's medium (Eiken, Co. LTD., Tokyo) which contained 5% washed human O-erythrocytes was used as medium for detecting the production of hemolysin.

2. Assay

V. parahaemolyticus tested isolates from the stock medium were inoculated on Wagatsuma's medium containing 5% washed human O-erythrocytes. The plate was incubated at 37°C for 24 h. The isolates producing clear hemolytic zone around the colonies were identified as Kanagawa phenomenon-positive or thermostable direct hemolysin (TDH) positive, those with no hemolytic zone were identified as Kanagawa phenomenon-negative. *V. parahaemolyticus* strains AQ 4613 (TDH⁺) and AQ 4023 (TRH⁺, TDH⁻), kindly provided by Dr. Masanori Ishibashi, Department of Food Microbiology, Osaka Prefectural Institute of Public Health, Osaka, Japan were used as positive and negative controls, respectively.

Slide agglutination test for serotyping

1. Medium

Tryptic soy agar (TSA; Difco, Detroit, Michigan, USA) with 3% NaCl was used for preparing overnight culture of *V. parahaemolyticus* for serotyping.

2. Antisera

V. parahaemolyticus O and K antisera (Denka Seiken Co., LTD., Tokyo) were used for serotyping of *V. parahaemolyticus*. The scheme of *V. parahaemolyticus* serotyping is shown in Table 1 and Table 11.

3. Preparation of antigen

V. parahaemolyticus tested strains were overnight cultured on TSA with 3% NaCl agar slants. A slant was washed with 2 ml of normal saline solution. Cell suspension was separated into two tubes. One tube of cell suspension was autoclaved for 15 min at 121°C and used as O antigen. Unautoclaved suspension in another tube represented as K antigen. All tubes were centrifuged at 2,500 rpm for 20 min. After centrifugation, the supernate was discarded, the precipitate was served as the heavy suspension for further slide agglutination test.

4. Slide agglutination test

To perform O agglutination test, slide was divided into 14 equal compartments using wax pencil. One drop of each 13 O-group antisera was placed onto each compartment. One drop of normal saline instead of O-antisera was placed onto the 14th compartment to serve as an autoagglutination control. Then a small drop of heavy suspension (O-antigen) was added into each compartment. Slide was gently tilted back and forth until thoroughly mixed. A positive agglutination was recognized by the prompt formation of fine granules or large aggregates. The control and any negative tests were shown a homogenous turbidity.

For K agglutination test, 9 polyvalent K-antisera were tested with live packed cells by slide agglutination test as described above. Monovalent K-antisera corresponding to the positive polyvalent K-antisera were subsequently tested (Table 11). The serotype of tested strain of *V. parahaemolyticus* was recorded by the combination of both O-group and K-monovalent antiserum as shown in Table 1. The isolates that did not agglutinate with a specific O or K group antisera were considered nontypable.

Antimicrobial susceptibility assay

1. Antimicrobial agents

The following antimicrobial discs were used for antimicrobial susceptibility assay.

Table 11 Polyvalent and monovalent K-antiserum of *Vibrio parahaemolyticus* *

Polyvalent K-antiserum	Monovalent K-antiserum						
I	1	3	4	5	6	7	8
II	9	10	11	12	13	15	17
III	18	19	20	21	22	23	24
IV	25	26	28	29	30	31	32
V	33	34	36	37	38	39	40
VI	41	42	43	44	45	46	47
VII	48	49	50	51	52	53	54
VIII	55	56	57	58	59	60	61
IX	63	64	65	66	67	68	69
	70	71	72	73	74	75	

* Denka Seiken Co., LTD., Tokyo

- 1.1 Ampicillin disc (10 µg, AMP-10; Oxoid, Basingstoke, England)
- 1.2 Colistin disc (10 µg, CT-10; Oxoid, Basingstoke, England)
- 1.3 Gentamicin disc (10 µg, CN-10; Oxoid, Basingstoke, England)
- 1.4 Nalidixic acid disc (30 µg, NA-30; Oxoid, Basingstoke, England)
- 1.5 Nitrofurantoin disc (300 µg, F-300; Oxoid, Basingstoke, England)
- 1.6 Tetracycline disc (30 µg, TE-30; Oxoid, Basingstoke, England)
- 1.7 Cotrimoxazole disc (combination of Trimetoprim-Sulfamethoxazole; 1.25 µg / 23.75 µg, SXT-25; Oxoid, Basingstoke, England)
- 1.8 Norfloxacin disc (10 µg, NOR-10; Oxoid, Basingstoke, England)

2. Medium

Mueller Hinton agar (MH; BBL, Cockeysville, MD, U.S.A) was used as the culture medium for antimicrobial susceptibility assay. Tryptic soy broth (TSB; BBL, Cockeysville, MD, U.S.A) with 3% NaCl was used as an enrichment medium.

3. Method

Disc diffusion technique (99) was used for testing susceptibility of *V. parahaemolyticus* to antimicrobial agents. *V. parahaemolyticus* strains from the stock medium were inoculated into test tubes containing 4 ml of TSB with 3% NaCl and incubated for 4 h at 37°C. The suspension was diluted with 0.9% normal saline to a density visually comparable to a MacFarland standard number 0.5. The sterilized cotton swab was soaked with the bacterial suspension and dried by pressing and rotating the swab against the side of tube above the fluid level. The swab was streaked in 3 planes on the surface of Muller Hinton agar plates. Disc of AMP-10, CT-10, CN-10, NA-30, F-300, TE-30, SXT-25, NOR-10 were aseptically placed on the surface of Muller Hinton plates. Later, the plates were incubated at 37°C overnight. The inhibition zone diameter were measured, then recorded and interpreted according to the standard listed in Table 12.

Table 12 Interpretation of susceptibility tests (89)

Antimicrobial agents	Disc potency (µg)	Zone diameter (mm)		
		Resistant	Intermediate	Sensitive
Ampicillin	10	≤ 11	12-13	≥ 14
Colistin	10	≤ 8	9-10	≥ 11
Gentamicin	10	≤ 12	13-14	≥ 15
Nalidixic acid	30	≤ 13	14-18	≥ 19
Nitrofurantoin	300	≤ 14	15-16	≥ 17
Tetracycline	30	≤ 14	15-18	≥ 19
Cotrimoxazole	1.25/23.75	≤ 10	11-15	≥ 16
Norfloxacin	10	≤ 12	13-16	≥ 17

Multiplex PCR

Multiplex PCR was used to confirm the species identification (*tl* or *ldh*) and detect the virulence gene of *V. parahaemolyticus*, *tdh* and *trh* genes. In this study multiplex PCR amplification was modified the method mentioned previously by Bej *et al.* (98).

1. Oligonucleotide primers for multiplex PCR

Two PCR oligonucleotide primers were designed according to the DNA sequences of the genes encoding the TDH and TRH of *V. parahaemolyticus* (23, 101). The DNA sequences of the species-specific, *ldh* genes were 5' – AGC TAC TCG AAA GAT GAT CCA GCG A – 3' and 5' – ACC TAA GAA CCA GCT GTT CGG GT -3' (98). The sequences of oligonucleotide primers for the *tdh* gene were 5' - GTA CCG ATA TTT TGC AAA - 3' and 5' - ATG TTG AAC TGT ACT TGA - 3', the *trh* gene were 5' - CTC TAC TTT GCT TTC AGT - 3' and 5' - TAC CGT TAG ATA GTC GCT TA - 3'. The predicated size of the amplified DNA from the *ldh* gene was 450 bp, *tdh* gene was 382 bp and the *trh* gene was 276 bp (Table 13). The primers were synthesized with an automatic oligonucleotide synthesizer at the Bioservice Unit, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency (NSTDA), Bangkok.

2. DNA extraction

V. parahaemolyticus strains from semisolid stock culture (1 loop) were grown in 5 ml of Luria-Bertani broth (LB broth) supplemented with 3% NaCl, shaking overnight at 37°C. Twenty microliter of the overnight culture was inoculated into new 5 ml of LB broth tube, shaking again at 37°C for 2 h approximate optimal density (OD) of 0.4 at 600 nm. Bacterial culture of 1 ml was transferred into a 1.5 ml microcentrifuge tube, then centrifuged at 8,000 rpm for 3 min, and supernatant was discarded. The cells were washed twice with 200 µl of sterile TE buffer, resuspended with 200 µl of TE buffer and boiled at 95°C for 10 min, then immediately cooled in ice for 5 min. The cell pellet was separated by centrifugation at 12,000 rpm for 3 min. The supernatant containing bacterial DNA was used as template for PCR amplification (Fig. 3).

Table 13 PCR oligonucleotide primers and product size of *Vibrio parahaemolyticus*

Taget gene	PCR primers sequences (5'-3')	Product size (bp)
<i>ldh</i>	AGC TAC TCG AAA GAT GAT CCA GCG A ACC TAA GAA CCA GCT GTT CGG GT	450
<i>tdh</i>	GTA CCG ATA TTT TGC AAA ATG TTG AAC TGT ACT TGA	382
<i>trh</i>	CTC TAC TTT GCT TTC AGT ACC TAA GAA CCA GCT GTT CGG GT	276

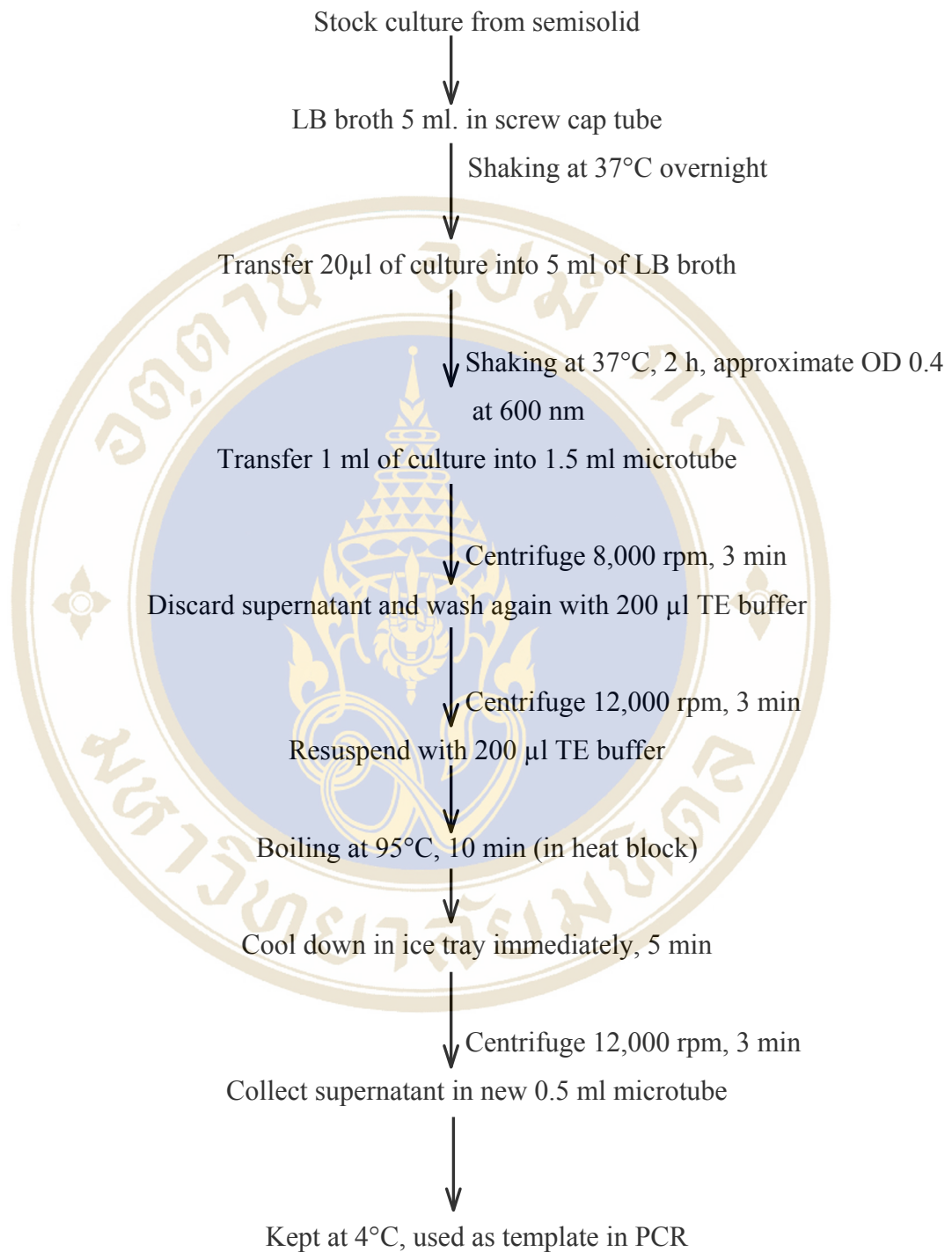


Fig. 3 DNA extraction from *Vibrio parahaemolyticus* stock culture

3. PCR condition

Multiplex PCR amplification was performed on 1 μ l of *V. parahaemolyticus* DNA template in a 24 μ l reaction mixture consisting of 2, 10, 18 μ mol/ μ l of *ldh*, *tdh* and *trh* genes oligonucleotide primers, respectively, 2.5 μ l of 10 X PCR buffer (1X PCR buffer consisted of 50 mM KCl, 10 mM Tris HCl, pH 8.8, 2.5 mM MgCl₂), 200 μ M of the dNTPs (Pharmacia), 1 unit of *AmpliTaq* DNA polymerase (Perkin Elmer, Norwalk, U.S.A) and appropriate volume of sterile MilliQ water (Millipore).

All multiplex PCR amplifications were performed in a DNA thermal cycler (Perkin Elmer, Norwalk, U.S.A) using the following temperature-cycling parameters: initial denaturation at 94°C for 3 min followed 30 cycles of amplification; each cycle consisted of denaturation at 94°C for 1 min, primer annealing at 51°C for 1 min, and primer extension at 72°C for 1 min. The final extension was performed at 72°C for 10 min. All PCR products were analyzed by electrophoresis (Mupid II, Tokyo, Japan).

After amplification, 5 μ l of the PCR product was mixed with 1 μ l of loading dye. The mixtures were loaded into horizontal 1.5% agarose gel and run electrophoresis in Tris-borate buffer at the constant 100 voltage. The electrophoresis was continued until the bromphenol blue tracking dye approached the end of the running gel. The amplified DNA bands were visualized after ethidium bromide staining and photographed under UV transilluminator (Fotodyne, Hartland, U.S.A.). The 100-bp ladder (Pharmacia Biotech, U.S.A) was used as a marker for determining the size of the amplification products.

Data analysis

The isolation rates, biochemical reaction, serotypes, urease activity, hemolysin genes, hemolysin production, antimicrobial susceptibility of *V. parahaemolyticus* in healthy carriers were presented by number and percentage. In order to compare the isolation rates of *V. parahaemolyticus* in healthy carriers between two plants were analysed by Chi-square test.

CHAPTER 4

RESULTS

Detection rates of *Vibrio parahaemolyticus*

The detection rates of *V. parahaemolyticus* were determined from rectal swab specimens of the healthy workers in the frozen seafood and frozen processed seafood plants at Samut Prakan Province, during July 2002 to August 2003. In the frozen seafood plant, rectal swab samples were collected at 2-3 months interval. Participated workers were ranged from 790 to 1,097 during 6 times of sample collection. Carrier rates of *V. parahaemolyticus* were 0.6-2.6% in the frozen seafood plant, with the average of 1.3%. In the frozen processed seafood plant, rectal swab examination was determined twice a month. There were 150-232 healthy workers participated during each collection. Table 14 shows the monthly results of *V. parahaemolyticus* positive samples. *V. parahaemolyticus* detection rates were ranged from 0.3% to 3.1% with the average of 1.6% at the frozen processed seafood plant. Overall *V. parahaemolyticus* detection rate among the healthy seafood workers was 1.4%. The detection rates of *V. parahaemolyticus* of healthy workers were not significantly difference between these 2 plants ($P>0.05$). All of the carriers did not have any signs and symptoms of gastroenteritis or diarrhea.

It was observed that the positive samples were high (1.95-3.1%) during July to August of both years (Fig. 4). All healthy carriers found in this study were transient carriers, i.e. *V. parahaemolyticus* was found in the stool only once and not upon repeated investigation. However, upon followed up of these carriers, 5 of them were *V. parahaemolyticus* positive two to three times but with different serotypes (Table 15).

Table 14 Detection rates of *Vibrio parahaemolyticus* in healthy workers at the frozen seafood and frozen processed seafood plants, July 2002-August 2003

Number/Date of Sample collection	No. of rectal swab specimen	No. (%) of positive samples	No. of isolates
Frozen seafood plant (every 2-3 months interval of investigation)			
1 Jul/02	809	16 (1.9)	71
2 Sep/02	848	7 (0.8)	64
3 Nov/02	793	6 (0.8)	58
4 Feb/03	790	6 (0.8)	42
5 May/03	905	5 (0.6)	5
6 Aug/03	1097	28 (2.6)	121
	5,242	68 (1.3)	361
Frozen processed seafood plant (every 2 weeks interval of investigation)			
1 Aug/02*	321	10 (3.1) ^a	100
2 Sep/02	316	2 (0.6)	20
3 Oct/02	305	1 (0.3)	10
4 Nov/02	313	3 (0.9) ^b	30
5 Dec/02	328	4 (1.2) ^{a,c}	40
6 Jan/03	334	3 (0.9) ^{d,e}	30
7 Feb/03	384	7 (1.8) ^{c,d}	52
8 Mar/03	413	5 (1.2) ^{b,e}	50
9 Apr/03	435	4 (0.9) ^a	4
10 May/03	439	8 (1.8)	80
11 Jun/03	460	10 (2.2) ^e	75
12 Jul/03	462	13 (2.8)	90
13 Aug/03	462	9 (1.9) ^c	57
	4,972	79 (1.6)	638
Total	10,214	147 (1.4)	999

* Data were monthly results of the two sample collections.

^{a,b,c,d,e} *V. parahaemolyticus* were repeatedly detected from 5 transient healthy carriers for 2-3 times during the study period.

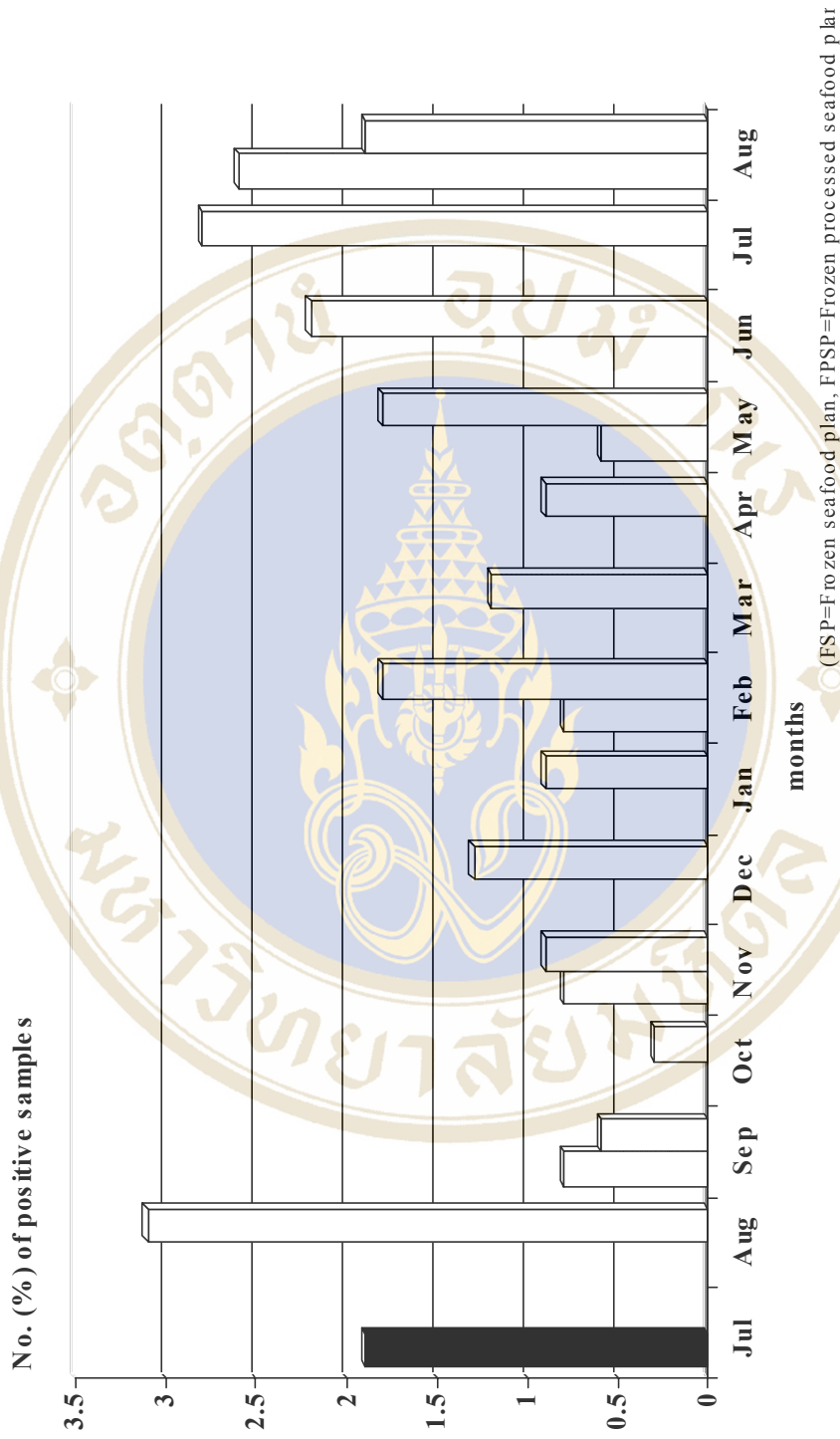


Fig. 4 Seasonal variation in the isolation of *Vibrio parahaemolyticus* from healthy workers at the frozen seafood and frozen processed seafood plants during July 2002 – August 2003

Table 15 Detection of 5 transient healthy carriers of *Vibrio parahaemolyticus* with different serotypes at the frozen processed seafood plant, July 2002-August 2003

Carriers	2002					2003							
	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug
A	O3:K46 ^a O8:KUT				O1:K55				O11:KUT				
B				O8:K47				O4:K63					
C					O3:K30 O10:K5 O11:K22		O3:K4						O1:KUT
D						O4:KUT	O5:KUT						
E						O3:K3 O5:KUT O11:KUT		O1:K8 O4:K34 O11:K34			O1:KUT		

^a serotypes found from the same healthy carrier in one specimen

General characteristics of healthy carriers

The age of *V. parahaemolyticus* healthy carriers ranged from 18 to 42 years old. The mean age was 28.0 years old. The highest proportion of healthy carriers (37.4%) was in the age group of 26-30 years old, followed by 28.0% of the age group 21-25 years old, and 16.5% of the age group 31-35 years old (Table 16).

Seventy-seven percent of healthy carriers were female. The ratio of male to female was 1:3.3. This carrier rates of *V. parahaemolyticus* in male were significantly lower than those of female ($P < 0.05$). In the frozen seafood plant, all carriers were female (Table 16).

Serotyping

The serotyping of *V. parahaemolyticus* was based on O and K antigens of the cells. In this study, some isolates were untypable (O and/or K) and the typable isolates were found in 11 antigenic O groups (Table 17 and Fig. 5), 36 antigenic K types (Fig. 6), and 64 O:K serotypes (Table 18, 19).

The dominant O group was O1 which was found in the highest frequency (21.7%), followed by O3 (17.6%), O4 (14.1%), O5 (13.1%), O8 (10.0%), O10 (8.7%), and others. The lowest frequency of the O group was O7 (0.4%), while O12 and O13 were not found in this study (Table 17).

The dominant K type was K9 with the highest frequency (4.8%), followed by K8 (3.9%), K6 (3.5%), K5 (3.4%), K20 (2.5%), K4 (2.4%), K63 (2.1%), K55, K21 (2.0%) and 55.2% of the isolates were KUT. The lowest frequency (0.1%) of the K type was found in K7, K11, K24, K25, K48, K50, K51, K52, K61 and 34 antigenic K types were not found in this study (Table 18 and Fig. 6).

Table 16 Age and sex distribution of healthy carriers of *Vibrio parahaemolyticus* at the frozen seafood and frozen processed seafood plants

Characteristics	No.(%) of healthy carriers		Proportion of all carriers (N=139)
	Frozen seafood plant (N=68)	Frozen processed seafood plant (N=71)	
Age (years)			
≥ 20	3 (4.4)	6 (8.5)	9 (6.5)
21-25	21 (30.9)	18 (25.3)	39 (28.0)
26-30	29 (42.6)	23 (32.4)	52 (37.4)
31-35	8 (11.8)	15 (21.1)	23 (16.5)
36-40	6 (8.8)	7 (9.9)	13 (9.4)
41-45	1 (1.5)	2 (2.8)	3 (2.2)
Total	68 (100.0)	71 (100.0)	139 (100.0)
Mean age	27.7	28.4	28.0
Sex			
Male	0	32 (45.1)	32 (23.0)
Female	68 (100.0)	39 (54.9)	107 (77.0)
Total	68 (100.0)	71 (100.0)	139 (100.0)

Table 17 Frequencies of *Vibrio parahaemolyticus* isolated from the healthy carriers at the frozen seafood plants by O group classification

O group	No. (%) of isolates		No. (%) of total isolates (n=999)
	Frozen seafood plant (N=361)	Frozen processed seafood plant (N=638)	
1	68 (18.8)	149 (23.3)	217 (21.7)
2	11 (3.0)	12 (1.9)	23 (2.3)
3	77 (21.3)	98 (15.4)	175 (17.6)
4	49 (13.6)	92 (14.4)	141 (14.1)
5	43 (11.9)	88 (13.8)	131 (13.1)
6	4 (1.1)	11 (1.7)	15 (1.5)
7	4 (1.1)	0	4 (0.4)
8	19 (5.3)	81 (12.7)	100 (10.0)
9	0	10 (1.6)	10 (1.0)
10	34 (9.4)	53 (8.3)	87 (8.7)
11	50 (13.9)	34 (5.3)	84 (8.4)
UT ^a	2 (0.5)	10 (1.6)	12 (1.2)
total	361 (100.0)	638 (100.0)	999 (100.0)

UT^a = untypable

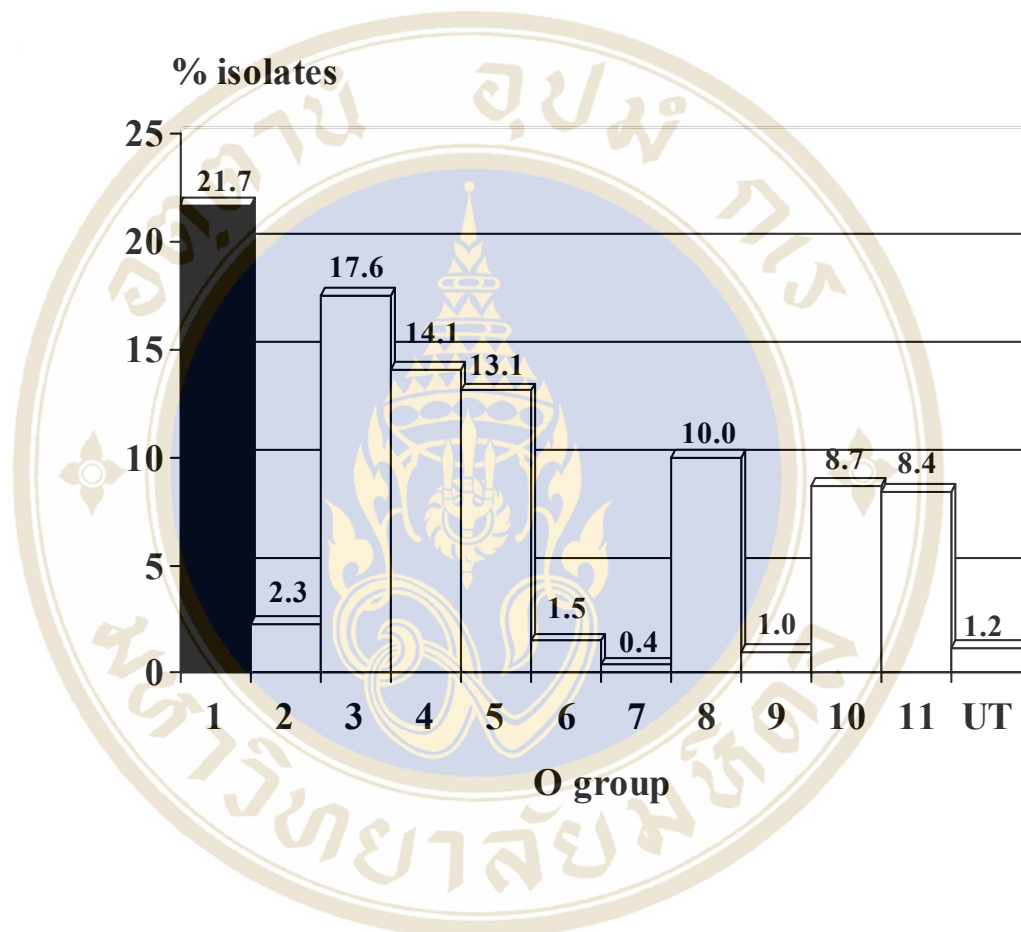


Fig. 5 Proportion (%) of *Vibrio parahaemolyticus* serogroups O1-O11 and untypable found among 999 isolates from the healthy carriers during July 2002 to August 2003

Table 18 Frequencies of *Vibrio parahaemolyticus* isolated from healthy carriers at the frozen seafood and frozen processed seafood plants by K type classification

K type	No. (%) of isolates		No. (%) of total isolates (N=999)
	Frozen seafood plant (N=361)	Frozen processed seafood plant (N=638)	
1	0	10 (1.6)	10 (1.0)
3	10 (2.8)	3 (0.5)	13 (1.3)
4	0	24 (3.8)	24 (2.4)
5	8 (2.2)	26 (4.1)	34 (3.4)
6	15 (4.2)	20 (3.1)	35 (3.5)
7	0	1 (0.2)	1 (0.1)
8	15 (4.2)	24 (3.8)	39 (3.9)
9	2 (0.5)	46 (7.2)	48 (4.8)
11	1 (0.3)	0	1 (0.1)
12	4 (1.1)	14 (2.2)	18 (1.8)
13	6 (1.7)	0	6 (0.6)
17	3 (0.8)	1 (0.1)	4 (0.4)
18	0	2 (0.3)	2 (0.2)
20	0	25 (3.9)	25 (2.5)
21	0	20 (3.1)	20 (2.0)
22	0	3 (0.5)	3 (0.3)
24	0	1 (0.1)	1 (0.1)
25	0	1 (0.1)	1 (0.1)
28	3 (0.8)	2 (0.3)	5 (0.5)
29	2 (0.5)	0	2 (0.2)
30	0	15 (2.4)	15 (1.5)
33	0	8 (1.3)	8 (0.8)
34	4 (1.1)	6 (0.9)	10 (1.0)
41	2 (0.5)	14 (2.2)	16 (1.6)
46	14 (3.9)	4 (0.6)	18 (1.8)
48	0	1 (0.1)	1 (0.1)
50	0	1 (0.1)	1 (0.1)
51	1 (0.3)	0	1 (0.1)
52	1 (0.3)	0	1 (0.1)
55	0	20 (3.1)	20 (2.0)
56	0	13 (2.0)	13 (1.3)
58	10 (2.8)	0	10 (1.0)
61	1 (0.3)	0	1 (0.1)
63	11 (3.1)	10 (1.6)	21 (2.1)
67	0	10 (1.6)	10 (1.0)
68	0	10 (1.6)	10 (1.0)
UT ^a	248 (68.7)	304 (47.6)	552 (55.2)
Total	361 (100.0)	638 (100.0)	999 (100.0)

^a untypable

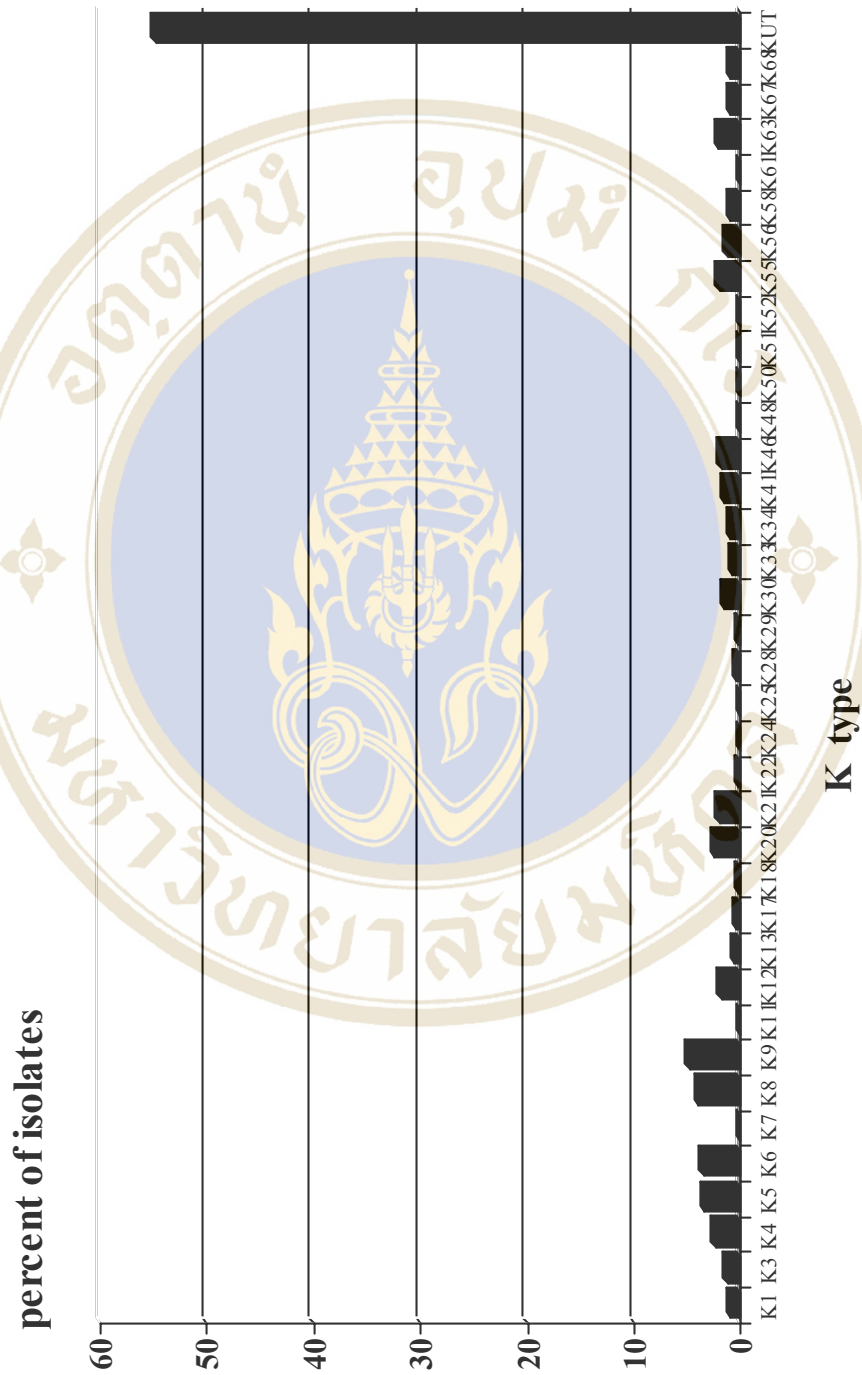


Fig. 6 Proportion (%) of serogroups K type among 999 isolates of *Vibrio parahaemolyticus*

Isolation rates of O:K serotypes is shown in Table 19. The serotype O1:KUT was found in the highest frequency (11.9%). Other O:K serotypes found in high frequency were O5:KUT (11.5%), O10:KUT (8.0%), O11:KUT (7.1%), O8:KUT (5.6%) and O3:KUT (5.0%). The lowest frequency (0.1%) was found in 13 serotypes O1:K25, O3:K48, O3:K51, O5:K12, O6:K18, O6:K61, O10:K7, O10:K11, O10:K24, O10:K52, O11:K5, O11:K18, O11:K50. The untypable serotype (OUT:KUT) was found in 1.0%.

Variation of O:K serotypes isolated from individual healthy carriers is shown in Table 20. Most of the healthy carriers (68.7%) had only one serotype from both plants (67.6% in the frozen seafood plant and 69.6% in the frozen processed seafood plant) while 31.3% of the carriers had from 2 to 5 serotypes with different ratios in one rectal swab sample. Three healthy carriers had five serotypes of *V. parahaemolyticus* in the stools (Table 20).

Heiberg's biotyping

All 999 isolates of *V. parahaemolyticus* from 139 healthy workers were also examined for its Heiberg's biotypes (V and VII). Heiberg biotypes VII was the most common isolated (64.0%) from 139 carriers. There were 25 (18.0%) of the carriers had both biotypes V and VII (Table 21). The isolation rates of the Heiberg's biotypes from both plants were not significantly different ($P>0.05$).

Table 19 Serotypes of *Vibrio parahaemolyticus* in healthy carriers at the frozen seafood and frozen processed seafood plants in Samut Prakan province

Serotypes	FSP*	FPSP#	No. (%) of isolates		Serotypes	FSP*	FPSP#	No. (%) of isolates	
			N=999					N=999	
O1:K1	0	10	10	(1.0)	O4:K68	0	10	10	(1.0)
O1:K8	0	4	4	(0.4)	O4:KUT	11	8	19	(1.9)
O1:K9	0	10	10	(1.0)	O5:K12	0	0	1	(0.1)
O1:K12	0	10	10	(1.0)	O5:K17	3	1	4	(0.4)
O1:K20	0	10	10	(1.0)	O5:K20	0	11	11	(1.1)
O1:K25	0	1	1	(0.1)	O5:KUT	40	76	115	(11.5)
O1:K33	0	10	10	(1.0)	O6:K18	0	1	1	(0.1)
O1:K55	0	20	20	(2.0)	O6:K61	1	0	1	(0.1)
O1:K56	0	13	13	(1.3)	O6:KUT	3	10	13	(1.3)
O1:K58	10	0	10	(1.0)	O7:KUT	4	0	4	(0.4)
O1:KUT	58	61	119	(11.9)	O8:K4	0	4	4	(0.4)
O2:K3	2	2	4	(0.4)	O8:K21	0	20	20	(2.0)
O2:K28	3	2	5	(0.5)	O8:K41	0	10	10	(1.0)
O2:KUT	6	8	14	(1.4)	O8:K67	0	10	10	(1.0)
O3:K3	8	1	9	(0.9)	O8:KUT	19	37	56	(5.6)
O3:K4	0	10	10	(1.0)	O9:K30	0	10	10	(1.0)
O3:K5	8	22	30	(3.0)	O10:K5	0	3	3	(0.3)
O3:K6	15	20	35	(3.5)	O10:K7	0	1	1	(0.1)
O3:K9	0	10	10	(1.0)	O10:K11	1	0	1	(0.1)
O3:K29	2	0	2	(0.2)	O10:K24	0	1	1	(0.1)
O3:K30	0	5	5	(0.5)	O10:K52	1	0	1	(0.1)
O3:K41	0	4	4	(0.4)	O10:KUT	32	48	80	(8.0)
O3:K46	14	4	18	(1.8)	O11:K5	0	1	1	(0.1)
O3:K48	0	1	1	(0.1)	O11:K18	0	1	1	(0.1)
O3:K51	1	0	1	(0.1)	O11:K20	0	2	2	(0.2)
O3:KUT	29	21	50	(5.0)	O11:K22	0	3	3	(0.3)
O4:K4	0	10	10	(1.0)	O11:K34	4	1	5	(0.5)
O4:K8	15	20	35	(3.5)	O11:K50	0	1	1	(0.1)
O4:K9	2	26	28	(2.8)	O11:KUT	46	25	71	(7.1)
O4:K12	4	3	7	(0.7)	OUT:K41	2	0	2	(0.2)
O4:K13	6	0	6	(0.6)	OUT:KUT	0	10	10	(1.0)
O4:K34	0	5	5	(0.5)					
O4:K63	11	10	21	(2.1)	Total	361	638	999	(100.0)

* Frozen seafood plant

Frozen processed seafood plant

Table 20 Variation of serotypes of *Vibrio parahaemolyticus* isolated from individual healthy carriers in the frozen seafood and frozen processed seafood plants

No. of serotypes found in one sample and ratio of the two or more	No. (%) of positive samples (carriers)			Serotypes
	Total N=147	FSP* N=68	FPSP# N=79†	
One	101 (68.7)	46	55	
Two	30 (20.5)	18	12	
1:1		3	1	O3:KUT ,O3:K6; O10:K11,O1:KUT; O2:K28,O8:KUT; O3:KUT ,O5:KUT
1:2		1	0	O2:K28,O10:KUT
1:3		1	0	O5:K17,O10:KUT
1:4		4	1	O1:KUT ,O3:K46; O1:KUT ,O7:KUT; O10:KUT,O11:KUT O8:KUT,O4:K12; O1:KUT ,O4:KUT
1:9		1	2	O4:K63,O1:KUT; O4:KUT, O10:KUT ; O10:K7, O10:KUT
2:2		2	1	O3:KUT ,O6:KUT; O3:K5 , O3:KUT , O2:K3, O3:K5
2:3		2	0	O3:K6,O10:KUT; O5:KUT,O11:KUT
2:5		1	0	O2:K3, O1:KUT
2:8		3	4	O3:K3,O3:KUT;O11:KUT,OUT:K41; O10:KUT , O5:KUT , O1:KUT, O10:KUT ;O4:KUT, O5:KUT (2); O1:K20,O1:K33
3:4		0	1	O4:K12,O10:KUT
4:6		0	2	O3:K46,O8:KUT; O3:KUT,O4:K9
Three	10 (6.8)	3	7	
1:1:2		2	0	O3:K51,O10:KUT,O5:KUT; O6:K61,O6:KUT,O8:KUT
1:1:3		1	1	O1:K56, O3:KUT ,O8:KUT; O3:KUT ,O11:KUT,O10:KUT
1:1:8		0	1	O3:K3,O5:KUT,O11:KUT
1:2:2		0	1	O2:KUT,O5:KUT,O11:KUT
1:2:7		0	1	O4:KUT O3:KUT,O8:KUT
1:4:5		0	1	O1:K8,O4:K34,O11:K34
2:3:5		0	2	O3:K30,O10:K5,O11:K22; O2:K28,O2:KUT,O10:KUT
Four	3 (2.0)	1	2	
1:1:4:4		0	1	O3:K41,O8:K4,O10:KUT,O11:K9
1:2:2:5		0	1	O6:K18,O8:KUT,O10:KUT,O11:K18
2:2:2:4		1	0	O1:KUT,O3:KUT,O8:KUT,O10:KUT
Five	3 (2.0)	0	3	
1:1:1:1:2		0	1	O1:K25,O1:KUT,O2:KUT,O10:K24,O10:KUT
1:1:1:2:5		0	2	O5:K12, O5:KUT ,O8:KUT,O10:KUT,O11:K5; O5:K17,O5:K20, O5:KUT ,O11:K20, O11:KUT

* Frozen seafood plant, # Frozen processed seafood plant, † A total of 71 carriers with additional 8 specimen positive for *Vibrio parahaemolyticus* from the same persons but different times of collections.

Table 21 Heiberg's biotypes of *Vibrio parahaemolyticus* isolated from healthy carriers at the frozen seafood and frozen processed seafood plants in Samut Prakan province

Heiberg's biotype	No. (%) of healthy carriers		No. (%) of total carriers
	Frozen seafood plant	Frozen processed seafood plant	
V	17 (25.0)	8 (11.3)	25 (18.0)
VII	40 (58.8)	49 (69.0)	89 (64.0)
V + VII	11 (16.2)	14 (19.4)	25 (18.0)
Total	68 (100.0)	71 (100.0)	139 (100.0)

Kanagawa phenomenon assay

All 999 isolates of *V. parahaemolyticus* from healthy carriers were tested for hemolysin activity (TDH) or Kanagawa phenomenon on Wagatsuma's medium (Fig. 7). Clear hemolysis zone around the colony on this agar was observed as positive (KP⁺). Only 32.8% (328) of 999 isolates from healthy carriers gave positive results of hemolytic activity (KP⁺) while 67.2% gave negative results (Table 22).

In the frozen seafood plant, 33.2% (120/361) of isolates showed positive hemolytic activity (TDH⁺) on Wagatsuma's medium and 66.8% (241/361) showed negative hemolytic activity (TDH⁻). In the other plant, 32.6% (208/638) of isolates were Kanagawa phenomenon positive (TDH⁺), and 67.4% (430/638) of isolates gave negative results (Table 22). The proportion of positive and negative of hemolysin activity (TDH⁺ and TDH⁻) were not significantly difference between the 2 plants (P>0.05).

Urease enzyme hydrolysis

All isolates were tested for urea hydrolyzing activity (TRH). In this study, 92% of all *V. parahaemolyticus* isolates from healthy carriers of both plants showed negative results for urea hydrolysis (TRH⁻). Only 10% of 361 isolates from the frozen seafood plant and 6.9% of 638 isolates in the frozen processed seafood plant produced urease enzyme with the average of 8% urease positive (Table 22). The urease production (TRH⁺) of *V. parahaemolyticus* from healthy carriers were not significantly different between the 2 plants (P>0.05).

TDH and urease enzyme hydrolysis

In this study, 60.9% of 999 isolates of *V. parahaemolyticus* showed KP⁻ and Urease⁻. Only 1.7% (17/999) gave positive to both KP and urease hydrolysis. There was 31.1% (311/999) of isolates produced only TDH and 6.3% produced only urease enzyme. Hemolysin and urease production of *V. parahaemolyticus* isolated from

healthy carriers were not significantly different between the two plants ($P>0.05$) (Table 22).

Hemolysin genes

A total of 269 representative isolates of *V. parahaemolyticus* were selected from all positive samples (999 isolates) to determine hemolysin genes (*tdh* and *trh*) by multiplex PCR (Fig 8, 9). These isolates were selected because of their differences in biochemical reactions and serotypes that found among 10 isolates per sample. The isolates with *tdh*⁺*trh*⁻ of *V. parahaemolyticus* were found 25.3%, followed by *tdh*⁺*trh*⁺ 4.8%, and *tdh*⁻*trh*⁺ 4.8%. About sixty-five percent (175/269) of *V. parahaemolyticus* isolates did not harbour any hemolysin genes (Table 23).

Among 81 isolates positive for *tdh*, 92.6% (75/81) were KP⁺ and 7.4% (6/81) were KP⁻. As of 188 isolates negative for *tdh* gene, all of them were KP⁻. Relationships between urease production and the presence of *trh* gene were determined among 269 isolates of *V. parahaemolyticus*. All 26 isolates positive for *trh* gene were also positive for urease production (Table 24).



Fig. 7 Themostable direct hemolysin (TDH⁺) or Kanagawa-phenomenon positive showed clear hemolysis zone on Wagatsuma's medium; AQ 4613 and AQ 4020 were positive and negative reference strains, respectively

Table 22 Hemolysin production on Wagatsuma's medium and urea hydrolyzing reaction of *Vibrio parahaemolyticus* isolated from healthy carriers at the frozen seafood and frozen processed seafood plants in Samut Prakan province, July 2002 to August 2003

Characteristics	No. (%) of isolates		Total No. (%) of isolates
	Frozen seafood plant	Frozen processed seafood plant	
Kanagawa phenomenon			
KP ⁺	120 (33.2)	208 (32.6)	328 (32.8)
KP ⁻	241 (66.8)	430 (67.4)	671 (67.2)
Total	361 (100.0)	638 (100.00)	999 (100.0)
Urea hydrolyzing			
U ⁺	36 (10.0)	44 (6.9)	80 (8.0)
U ⁻	325 (90.0)	594 (93.1)	919 (92.0)
Total	361 (100.0)	638 (100.0)	999 (100.0)
Hemolysin production^a			
KP ⁺ U ⁺	7 (1.9)	10 (1.6)	17 (1.7)
KP ⁺ U ⁻	123 (34.1)	188 (29.5)	311 (31.1)
KP ⁻ U ⁺	29 (8.0)	34 (5.3)	63 (6.3)
KP ⁻ U ⁻	202 (56.0)	406 (63.6)	608 (60.9)
Total	361 (100.0)	638 (100.0)	999 (100.0)

^a Thermostable direct hemolysin (TDH) or Kanagawa phenomenon (KP) detected by Wagatsuma's medium and thermostable direct hemolysin-related hemolysin (TRH) determined by urease enzyme hydrolysis.

Table 23 Hemolysin genes of 269 *Vibrio parahaemolyticus* isolates from the healthy carriers in the frozen seafood and frozen processed seafood plants

Hemolysin genes ^a	No. (%) of isolates from healthy carriers		Total no. (%) of isolates from healthy carriers
	Frozen seafood plant	Frozen processed seafood plant	
<i>tdh</i> ⁺ <i>trh</i> ⁺	8 (6.8)	5 (3.3)	13 (4.8)
<i>tdh</i> ⁺ <i>trh</i> ⁻	34 (29.1)	34 (22.4)	68 (25.3)
<i>tdh</i> ⁻ <i>trh</i> ⁺	4 (3.4)	9 (5.9)	13 (4.8)
<i>tdh</i> ⁻ <i>trh</i> ⁻	71 (60.7)	104 (68.4)	175 (65.1)
Total	117 (100.0)	152 (100.0)	269 (100.0)^b

^a Thermostable direct hemolysin (TDH) and thermostable direct hemolysin-related hemolysin genes (TRH) detected by multiplex polymerase chain reaction.

^b A total of 269 isolates of *V. parahaemolyticus* were selected from all 999 isolates, which showed the difference in biochemical reactions and serotypes among 10 isolates/sample from individual healthy carrier.



Fig. 8 1.5% Agarose gel electrophoresis showing the hemolysin genes (*tdh*, *trh*) of *Vibrio parahaemolyticus* isolated from reference strains. Lane M, 100 Base-Pair Ladder; Lane 1,8, positive control for *ldh*, *tdh* and *trh* from clinical strain; Lane 2, AQ 4613 (*tdh*⁺*trh*⁻); Lane 3, 5-6, BG 26 (*tdh*⁻*trh*⁻); Lane 4, AQ 4020 (*tdh*⁻*trh*⁺); Lane 7, negative control

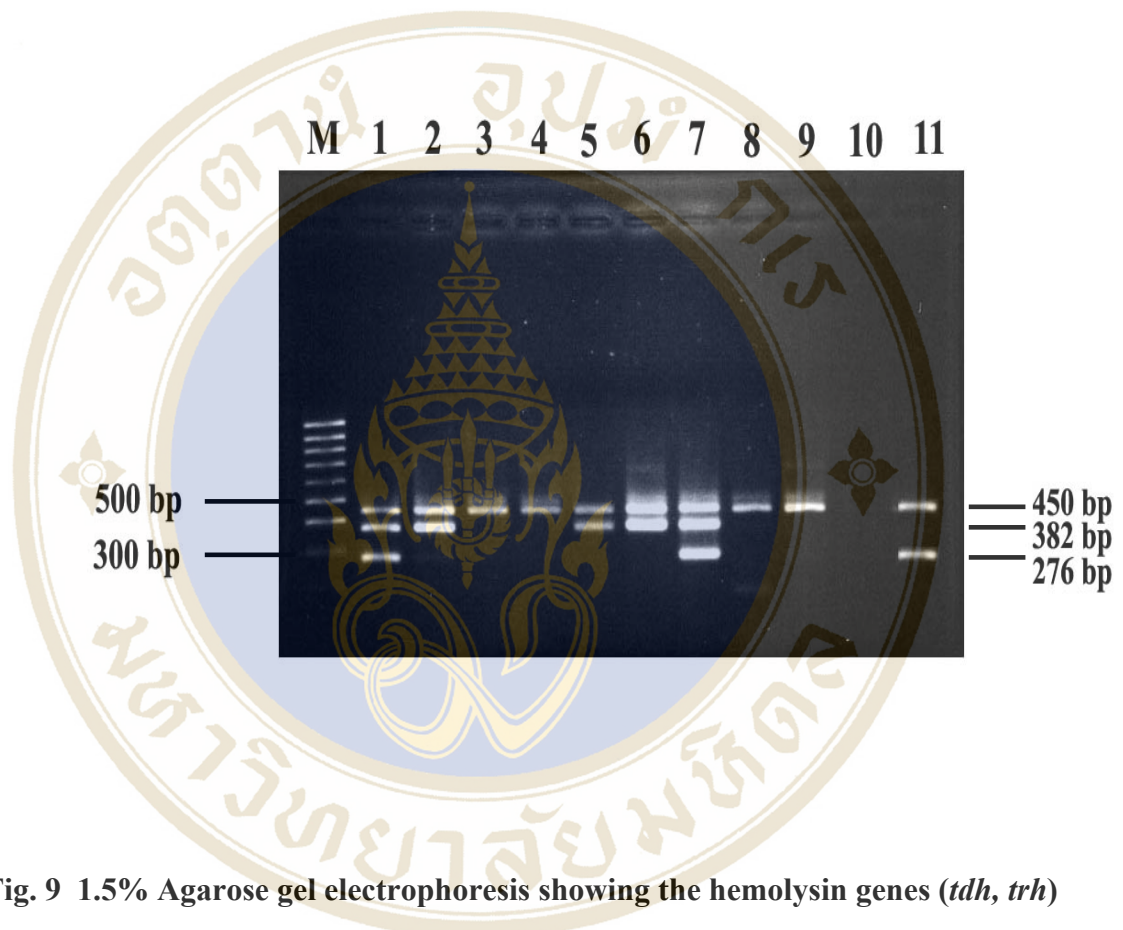


Fig. 9 1.5% Agarose gel electrophoresis showing the hemolysin genes (*tdh*, *trh*) of *Vibrio parahaemolyticus* isolated from healthy carriers in frozen seafood and frozen processed seafood plants, July 2002-August 2003. Lane M, 100 Base-Pair Ladder; Lane 1, positive control for *ldh*, *tdh* and *trh*; Lane 2-9, 11, *Vibrio parahaemolyticus* isolated from healthy carriers; Lane 10, negative control

Table 24 Relationship between urease production, Kanagawa phenomenon and the presence of *tdh* and *trh* genes of 269 *Vibrio parahaemolyticus* isolated from healthy carriers

Hemolysin genes			No. (%) of isolates with given urease activity	
<i>tdh</i>	<i>trh</i>	KP	positive	negative
+	+	+	7 (2.6)	0
+	+	-	6 (2.2)	0
-	+	-	13 (4.8)	0
+	-	+	0	68 (25.3)
+	-	-	0	0
-	-	-	0	175 (65.1)
Total			26 (9.6)	243 (90.4)

Antimicrobial susceptibility

Two hundred and sixty nine isolates of *V. parahaemolyticus* were selected from all 999 isolates to assess antibiotic susceptibility by disc diffusion method (Table 25).

In this study, all tested isolates of *V. parahaemolyticus* from 2 frozen seafood plants employees showed the similar susceptibility patterns. More than 96% of isolates were sensitive to 6 of 8 antimicrobial agents. All isolates were sensitive to nitrofurantoin (100%) and norfloxacin (100%). Ninety-nine point six percent were sensitive to nalidixic acid, 99.3% to tetracycline, 98.1% to gentamicin and 97.4% to cotrimoxazole. Intermediate susceptibility to colistin, ampicillin, gentamicin, cotrimoxazole and nalidixic acid were found in 37.6%, 26.4%, 1.5%, 0.7% and 0.4%, respectively. Ampicillin and colistin resistance were 27.9% and 17.8%, respectively. In frozen processed seafood plant, it was found that 15 isolates of 152 (9.9%) *V. parahaemolyticus* showed multiple drug resistance, 13 of 15 (86.7%) isolates were resistant to ampicillin and colistin, another 2 isolates were resistant to ampicillin and cotrimoxazole. Multidrug resistant patterns of isolates from the frozen seafood plant were ampicillin and colistin (3/117); ampicillin and tetracycline (2/117); and ampicillin and gentamicin (1/117) (Table 26). All together 7.8% (21/269) of all isolates showed multidrug resistant patterns.

Table 25 Antimicrobial susceptibility of *Vibrio parahaemolyticus* isolated from healthy carriers at the frozen seafood and frozen processed seafood plants, July 2002 to August 2003

Antimicrobial agents	No. (%) of isolates											
	Frozen seafood plant (N=117)			Frozen processed seafood plant (N=152)			Total no. (%) of isolates (N=269)					
	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant
Ampicillin	47 (40.2)	27 (23.1)	43 (36.7)	76 (50.0)	44 (29.0)	32 (21.0)	123 (45.7)	71 (26.4)	75 (27.9)			
Colistin	59 (50.4)	51 (43.6)	7 (6.0)	61 (40.1)	50 (32.9)	41 (27.0)	120 (44.6)	101 (37.6)	48 (17.8)			
Gentamicin	113 (96.6)	3 (2.5)	1 (0.9)	151 (99.3)	1 (0.7)	-	264 (98.1)	4 (1.5)	1 (0.4)			
Nalidixic acid	117 (100.0)	-	-	151 (99.3)	1 (0.7)	-	268 (99.6)	1 (0.4)	-			
Nitrofurantoin	117 (100.0)	-	-	152 (100.0)	-	-	269 (100.0)	-	-			
Tetracycline	115 (98.3)	-	2 (1.7)	152 (100.0)	-	-	267 (99.3)	-	2 (0.7)			
Cotrimoxazole	114 (97.4)	1 (0.9)	2 (1.7)	148 (97.4)	1 (0.7)	3 (1.9)	262 (97.4)	2 (0.7)	5 (1.9)			
Norfloxacine	117 (100.0)	-	-	152 (100.0)	-	-	269 (100.0)	-	-			

Table 26 Multi-drug resistance of *Vibrio parahaemolyticus* isolated from healthy carriers at frozen seafood and frozen processed seafood plants

Antimicrobial resistance					FSP*	FPSP#	Total
AMP	CT	SXT	TE	CN	N=117	N=152	N=269
+	+	-	-	-	3 (2.5)	13 (8.6)	16 (5.9)
+	-	+	-	-	0	2 (1.3)	2 (0.7)
+	-	-	+	-	2 (1.7)	0	2 (0.7)
+	-	-	-	+	1 (0.8)	0	1 (0.4)
Total					6 (5.1)	15 (9.9)	21 (7.8)

AMP = Ampicillin

CT = Colistin

SXT = Cotrimoxazole

TE = Tetracycline

CN = Gentamicin

* Frozen seafood plant

Frozen processed seafood plant

Transient healthy carriers

V. parahaemolyticus was isolated from 139 workers throughout the year. Only five persons in the frozen processed seafood plant were transient carriers who carried *V. parahaemolyticus* 2-3 times during the investigations (Table 27). The age of the transient healthy carriers of *V. parahaemolyticus* in this study was ranged from 21 to 30 years old. Transient healthy carriers of *V. parahaemolyticus* were female 20% (1/5), and males 80% (4/5).

The detection of 5 transient healthy carriers of *V. parahaemolyticus* is shown in Table 15. Two carriers were identified from December to March. Only one carrier were identified in August and November 2002, April, June and August 2003. All of 5 transient carriers had different isolates of *V. parahaemolyticus* as characterized by Heiberg's biotypes, hemolysin production and genes, susceptibility to antibiotics, and serotypes as shown in Table 27. These 5 carriers had Heiberg biotype VII but only 2 persons (Carrier A and C) also had biotype V in one out of three times of the investigations.

It was found that only one (Carrier A) person had TDH⁺ and *tdh*⁺ isolates among 2 out of 3 times of investigations, while Carrier C had TRH⁺ and *trh*⁺ in 1 out of 3 times of investigations. All the isolates from the remainders had neither production nor genes of both hemolysins.

Most of isolates from each individual carrier were sensitive to 6 of 8 antibiotics agents as mentioned before. Multiple drug resistance (ampicillin and colistin) was found only in one carrier (Carrier A). However, the resistant strain did not produce hemolysins nor had any hemolysin gene.

All isolates of transient carriers were typable and found in 7 antigenic O groups, 11 antigenic K type, and 17 O:K serotypes. Serotypes of *V. parahaemolyticus* from 5 transient carriers were found in various groups. Only 3 serotypes (O1:KUT, O5:KUT, and O11:KUT) were repeatedly isolated from transient carriers. Serotype O3:K46 and O8:KUT were found in Carrier A in the first positive sample collection,

while in 2nd and 3rd positive detection belonged to O1:K55 and O11:KUT, respectively. Carrier B was found positive twice (November 2002, March 2003) with different serotypes, O8:K47 and O4:K63, respectively. Carrier C was positive for *V. parahaemolyticus* three times (December 2002, February and August 2003) with 5 different serotypes (O3:K30; O10:K5; O11:K22; O3:K4; and O1:KUT). *V. parahaemolyticus* were detected in Carrier D twice in January and February 2003 with also different serotypes (O4:KUT and O5:KUT). Carrier E was found positive for *V. parahaemolyticus* 3 times in January, March and June 2003 with 7 different serotypes (O3:K3; O5:KUT; O11:KUT; O1:K8; O4:K34; O11:K34; and O1:KUT).

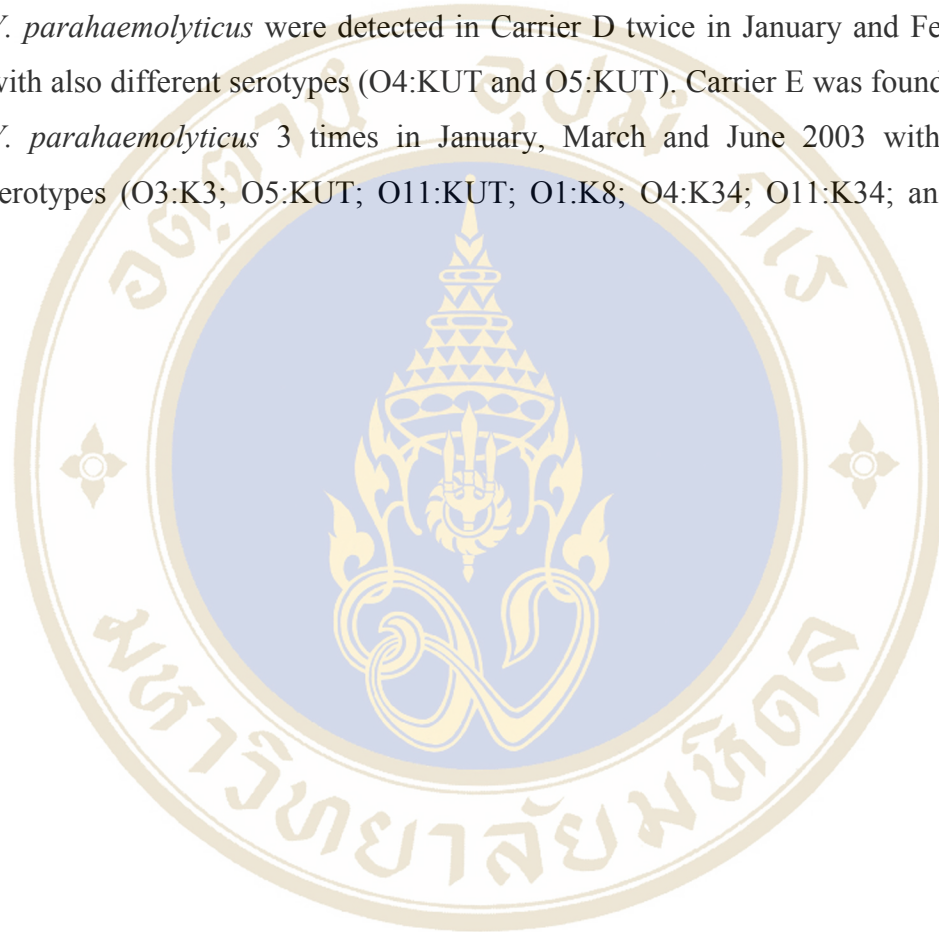


Table 27 Comparison on characteristics of 5 transient healthy carriers of *Vibrio parahaemolyticus* in frozen processed seafood plant at Samut Prakan province, August 2002-2003

Characteristic	Carrier A		Carrier B		Carrier C		Carrier D		Carrier E			
	13347/02	21113/02	17750/2	4404/03	21349/02	1075/03	12900/03	188/03	692/03	596/03	3805/03	9948/03
Sex		Male 27	Male 30	Male 30	Male 23	Male 23	Male 23	Male 26	Male 26	Female 21	Female 21	Female 21
Age	VII	V	VII	VII	VII	VII	V	VII	VII	VII	VII	VII
Heiberg's type	-	-	-	-	-	-	+	-	-	-	-	-
Urease	+	+	-	-	-	-	-	-	-	-	-	-
Hemolysin	-	-	-	-	-	-	-	-	-	-	-	-
TDH	-	-	-	-	-	-	-	-	-	-	-	-
TRH	+	-	-	-	-	-	+	-	-	-	-	-
Hemolysin genes												
<i>Tdh</i>	+	+	-	-	-	-	-	-	-	-	-	-
<i>Trh</i>	-	-	-	-	-	-	+	-	-	-	-	-
Antibiotics												
Ampicillin	S	S	I	I	S	S	I	S	S	S	I	R
Colistin	S	I	R	R	R	R	I	R	R	R	I	I
Gentamicin	S	S	S	S	S	S	S	S	S	S	S	S
Nalidixic acid	S	S	S	S	S	S	S	S	S	S	S	S
Nitrofurantoin	S	S	S	S	S	S	S	S	S	S	S	S
Tetracycline	S	S	S	S	S	S	S	S	S	S	S	S
Cotimoxazole	S	S	S	S	S	S	S	S	S	S	S	S
Norfloxacin	S	S	S	S	S	S	S	S	S	S	S	S
Serotypes	O3:K46	O1:K55	O8:K47	O4:K63	O3:K30	O3:K4	O1:KUT	O4:KUT	O5:KUT	O3:K3	O1:K8	O1:KUT
	O8:KUT				O10:K5					O5:KUT	O4:K34	
					O11:K22					O11:KUT	O11:K34	

CHAPTER 5

DISCUSSION

Vibrio parahaemolyticus is an important human pathogen responsible for foodborne gastroenteritis throughout the world. In Thailand, the first outbreak of food poisoning due to *V. parahaemolyticus* was reported by the Southeast Asian Treaty Organization (SEATO) in 1970 (36). This organism was recovered in 25% of diarrheal patients (36). The isolation rate of *V. parahaemolyticus* from diarrheal patients at Bamrasnaradura Infectious Disease Hospital during 1983 to 1996 were ranged from 4.5% to 7.6% (7). Three most common causes of foodborne and food poisoning outbreaks during 1992 to 2001 in Thailand that have been reported, were *V. parahaemolyticus* (45-60%), followed by *Salmonella* (29-39%), and *Staphylococcus aureus* (4.3-26%) (58). Although persons with asymptomatic *V. parahaemolyticus* infections have been found, there are no reports of long-term carriage of the organism. Among the Japanese, *V. parahaemolyticus* was isolated from 0.3% of healthy carriers among 2,000 Japanese population and 2.5% of healthy sushi chef (4, 32). Recently, Bangtrakulnonth *et al.*(35) reported about 2.0% of frozen seafood and 3.7% of frozen processed seafood plants employees harboured this vibrio without showing any symptom of diarrhea. The present study the isolation rate of *V. parahaemolyticus* in healthy workers from frozen seafood and frozen processed seafood plants were found in 1.3% and 1.4%, respectively. Only five persons of asymptomatic carriers harboured this organism 2 to 3 times during thirteen-month study period. The carrier rates from both plants in this study were lower than those reported by Bangtrakulnonth *et al.*(35). This variation could be due to the frozen seafood plants of the present study exported all their products and manufacturing processes were adopted to GMP and HACCP (9, 35,106, 107).

V. parahaemolyticus are classified into 2 Heiberger's biotypes. Heiberger type VII (arabinose positive) was more common than type V (arabinose negative) found in clinical strains as well as in environmental strains (66, 70, 74). In this study, Heiberger's biotypes isolated from healthy carriers have similar patterns in 2 plants. *V. parahaemolyticus* Heiberger type VII (arabinose positive) was found higher (64.0%) than those of type V (arabinose negative) (18%). The Heiberger's biotypes found in this study were similar to the other studies (66, 70, 74).

In this study, the dominant O group was O1 which was found in the highest frequency (21.7%) followed by O3 (17.6%) and O4 (14.1%). This finding was different from other reports that found O4 in the highest frequency among Thai diarrheal patients (63-65). In Japan, the study on epidemiology of *V. parahaemolyticus* also found that O4 was the most frequent serotype (32), whereas O1 and O4 were common isolates from patients during US Pacific North-West outbreaks (102). Therefore, O1 and O4 serotypes might be responsible for *V. parahaemolyticus* outbreaks in the particular geographic areas. In this study, O3 was the dominant (21.3%) group in the carriers of frozen seafood plant but O1 (23.3%) was predominate in the carriers of the frozen processed seafood plant. Distribution of O1, O3 and O4 groups should be surveillanced in the seafood and environmental samples, as they were important outbreak serogroups.

The dominant K type of *V. parahaemolyticus* found in the previous studies were varied. The study of Atthasampunna P. (37) showed that more than 50% of antigenic *V. parahaemolyticus* isolated from diarrheal patients were K untypable and no common K type was found in Thailand. In 1979, K8 was found to be the most common K type (10%) followed by K55 (6%) and K untypable (56%) (63). However, the dominant K type of *V. parahaemolyticus* isolated from patients with gastroenteritis was K10 (9%) in 1992 and K29 (10.5%) in 1994 (66, 74) while in individual healthy carrier, this K type has never been reported. In the present study, majority of K type from 2 plants was difference. In the frozen seafood plant, K8 (4.2%) was found in the highest frequency, K9 (7.2%) was found the highest in the frozen processed seafood plant, and K untypable (55.2%) was the most common found from all carriers. The

shift of K types were observed from various studies but the mechanism of K type shifting was still unknown.

Serotyping can differentiate isolates of *V. parahaemolyticus* into 13 O types and 75 K types by using the available commercial antisera (60). In 1973, O4:K11 *V. parahaemolyticus* was the dominant serotype found in three food poisoning outbreaks in Maryland, U.S.A (46). O:K serotypes predominantly found in other reports from clinical isolates were O4:K8, O4:K6, O4:K10, O5:KUT and O3:K29 (63, 64, 66, 74). Outbreaks of food poisoning caused by *V. parahaemolyticus* have increased globally in the last 5 years (103-104). Normally, a wide variety of serovars are involved in outbreaks. The most frequent serovars clinically isolated during 1992-1995 in Taiwan were 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%) (105). However, a predominant serovar O3:K6 appeared after 1996. Recent studies have revealed the emergence and pandemic spread of a single serovar, O3:K6. This serovar accounts for more than half of the *V. parahaemolyticus* isolates from diarrheal patients in Japan (44). Since 1998, other two serovars, O4:K68 and O1:KUT have also been isolated with increasing frequency from diarrheal patients (44, 104). In Bangladesh, the studied of serotypes of *V. parahaemolyticus* strains isolated from in-patients from January 1998 to December 2000 were found that O3:K6 being the dominant serotype 42.4%, followed by O4:K68 (19.7%), O1:KUT (9.1%), and O5:KUT (6.1%).

In present study, O3:K6, O4:K68, O1:KUT, and O5:KUT were found in the many healthy carriers from both frozen seafood plants. All of them did not have any signs and symptoms of gastroenteritis or diarrhea. *V. parahaemolyticus* was isolated from 139 individual healthy workers from 2 frozen seafood plants. About 16.5% (165/999) of the isolates had the pandemic serovars. These isolates belonged to four pandemic serovars with the newly emerged O1:KUT being dominant (11.9%) followed by O3:K6 (3.5%), O4:K68 (1.0%), and O1:K25 were found in the lowest frequency (0.1%). O1:KUT and O5:KUT were also isolated from 3 of 5 transient carriers.

In this study, the dominant O1:KUT was found in the highest frequency (11.9%) followed by O5:KUT (11.5%), O10:KUT (8%), O11:KUT (7.1%), O8:KUT (5.6%), O3:KUT (5%). The dominant serotypes of *V. parahaemolyticus* isolated from the healthy carriers in this study were different from those isolated from the patients during the outbreaks in the past. However, O1:KUT and O5:KUT have been isolated increasingly from the patients since 1998. Both serotypes were also the most common isolates among our carriers. Therefore, risk assessment and virulence factors of these isolates could be important to understand the role of carrier in pathogenesis of disease.

The mechanism of pathogenicity of *V. parahaemolyticus* have not been defined or fully understood. The presence of thermostable direct hemolysin (TDH) which demonstrated by a clear hemolytic zone on Wagatsuma blood agar, Kanagawa phenomenon positive (KP⁺), has been considered a major virulence factor (23, 80). In clinical laboratories, Wagatsuma's medium is routinely used for testing hemolysin production of isolated organism. Previously, most isolates from patients with diarrhea are Kanagawa positive, while most environmental isolates are Kanagawa negative (6, 92). In this study, All strains (999) of *V. parahaemolyticus* from healthy carriers were tested for hemolysin activity (TDH) on Wagatsuma's medium. Only 32.8% (328) of all isolates from healthy carriers were KP positive (TDH⁺) while 67.2% (671) of these isolates were KP negative. The presence of TDH⁺ *V. parahaemolyticus* isolated from the healthy carriers indicated that TDH alone may not be enough to cause the clinical signs and symptoms and hence disease. On the other hand, Honda *et al.* (15) reported that the clinical isolates of KP⁻ *V. parahaemolyticus* from travelers in Maldives produced newly hemolysin, named TDH-related hemolysin (TRH). This suggests that TRH may be an importance virulence factor and possibly cause of diarrhea. Since the first isolate of urease-positive strains was reported by Huq *et al.* (21), many studies reported urease hydrolysis positive strains from clinical and environmental isolates (16, 66, 71-76). Urease-positive strains were found in the studied of Kelly and Stroh in 1989. They found 50% and 8% urease-positive strains in admitted patients and environmental samples (76). Several studies have reported urease-positive *V. parahaemolyticus* isolates from clinical sources and implicated these strains as causes of human gastroenteritis (71, 76). In 1995, Suthienkul *et al.* (16) examined 489

clinical strains of *V. parahaemolyticus* isolated in Thailand and found 8% of urease-positive isolates possessed *trh* gene. The result indicated that the urease positive phenotype can be a convenient and useful marker in clinical diagnosis for predicting virulent strains of *V. parahaemolyticus*. In this study, urease positive strains were found in 8% of 999 isolates, which was similar to a previous study. These 60.9% of all 999 isolates absent of both virulence factors (KP⁻ and U⁻) in the highest frequency followed by 31.1% produced only KP⁺ and 1.7% produced both virulence factors. Patterns of presence and absence of virulence factors in healthy carriers from both frozen seafood plants were similar.

The thermostable direct hemolysin (TDH) and TDH-related hemolysin of *V. parahaemolyticus* are encoded by the *tdh* and *trh* genes, respectively. PCR is commonly used to detect hemolysin genes from clinical and environmental samples. In 1995, Suthienkul *et al.*(16) used PCR for detection of *tdh* and *trh* genes of *V. parahaemolyticus* isolated from the diarrhea patients. They found that 81% of the isolates were positive for *tdh*, 2% had the *trh* and only 6% had both *tdh* and *trh* genes. In the present study, multiplex PCR was performed to detect *tdh* and *trh* genes in 269 isolates of *V. parahaemolyticus* from healthy carriers. It was found that 30.1% were positive for *tdh*, and 9.6% had *trh* and 4.8% had both *tdh* and *trh* genes. Thus, *tdh*⁺ positive isolates were mostly found in the clinical specimen than those of healthy carriers. While *tdh*⁻ *trh*⁻ isolates were most common among the healthy carriers of this study (65.1%). The relationship between *tdh/trh* genes and TDH/TRH in the present study were found that 7.4% (6/81) of isolates with *tdh*⁺ gene did not produce TDH and 46.2% (6/13) of isolates with both *tdh*⁺ and *trh*⁺ genes also did not produce hemolysin on Wagatsuma's medium. This might be due to the level of TDH produced by some strains that was too low to be detected (20) or some epigenetic factors that inhibit the expression *tdh* gene. All *trh*⁺ isolates produced urease enzyme in this study. Therefore, *trh* gene was closely related to urease enzyme production (16).

Antimicrobial susceptibility of *V. parahaemolyticus* was tested on Mueller Hinton agar. The results of antimicrobial susceptibility in this study found that this organism was sensitivity to all 8 antimicrobial agents. The resistance of ampicillin and

colistin were 27.9% and 17.8%, respectively. In the frozen processed seafood plant, it was found that some isolates of *V. parahaemolyticus* showed multiple drugs resistance, only to ampicillin and colistin in 9.9% (15/152). However, multi-drugs resistance in the frozen seafood plant was found in 3 forms, 2.6% to ampicillin and colistin, 1.7% to ampicillin and tetracycline, and 0.8% to ampicillin and gentamicin. The results were differed from previous studies that found higher frequency of resistant isolates from clinical specimen. The findings suggested that carriers isolates were less subjected to antimicrobial agents than clinical isolates. However, if the use of antimicrobial agents increased especially in aqua-agriculture (shrimp), the increasing trend of multidrugs strains of *V. parahaemolyticus* might be observed.

V. parahaemolyticus carrier stage was repeated among healthy persons with increasing prevalences among those associated with seafood or *V. parahaemolyticus* infected patients with clinical signs and symptoms (4, 32, 33). It was possible that seafood and patients were the most likely sources of *V. parahaemolyticus* among the healthy carriers. This study demonstrated that *V. parahaemolyticus* isolates from the healthy carriers had virulence factors (32.8% TDH⁺, 8.0% urease positive) and virulence genes (30.1% *tdh*⁺ and 9.6% *trh*⁺). Therefore, aside from virulence factor of *V. parahaemolyticus*, other factors must be important in the pathogenesis of the disease, such as infectious dose (91-93), the expression of virulence genes, and the host factors.

These healthy carriers in the frozen seafood plants could also be the source of *V. parahaemolyticus* contaminated seafood. The isolation or restraint them from working would not solve the problem effectively. Compliance of the industry with GMP and HACCP would improve quality of the seafood products and quality control of the manufacturing process (9, 106, 107). However, surveillance of foodborne pathogens among the employee would strengthen the GMP and provide the key information in hygiene training of the workers in food establishment and food industry. Moreover, the carrier stage of *V. parahaemolyticus* could be a model for the study on mechanism of disease, immune status of the carrier, vaccine development and epigenetic factor upon switching on and off of virulence gene expression.

Therefore, the carrier stage of *V. parahaemolyticus* should be further investigated why they are abortive infection in the carriers. Microbiological surveillance of seafood and employee should be properly designed to improve quality assurance in a cost-effective manner. Seasonal variation of the healthy carriers should be elucidated what were the sources of the microorganism.



CHAPTER 6

CONCLUSION

Vibrio parahaemolyticus carrier was studied among healthy employees of the frozen seafood and frozen processed seafood plants at Samut Prakan province. A total of 10,214 rectal swab samples were collected from July 2002 to August 2003. Detection rate of *V. parahaemolyticus* was 1.4% (147/10,214 samples) from 139 healthy workers throughout the year with highest detection rate in July and August (1.9%-3.1%). *V. parahaemolyticus* was repeatedly detected in rectal swabs of 5 healthy carriers two to three times. All carriers were apparently healthy without signs and symptoms of gastroenteritis. The detection rates were not significantly different between the 2 plants ($P>0.05$). Characterization of *V. parahaemolyticus* was determined among 999 isolates obtained from each positive sample (10 isolates per sample). Heiberg's biotypes VII and type V of *V. parahaemolyticus* were found in 64% and 18% of the samples, respectively. The isolates from another 18% of the samples were positive for both Heiberg types. A total of 64 O:K serotypes were identified among 999 isolates of *V. parahaemolyticus* from positive samples, with O1:KUT the most dominant serotype 11.9%, followed by O5:KUT 11.5%, O10:KUT 8.0% and O11:KUT 7.1%. Virulence factors namely thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) were determined by hemolysin activity on Wagatsuma's medium and urease enzyme hydrolysis activity, respectively. Only 1.7% of all 999 isolates were produced both thermostable direct hemolysin (TDH) and urease enzyme (TRH), 32.8% of isolates produced TDH and 8% produced urease enzyme. Furthermore, 269 of 999 isolates were selected on the basis of their different Heiberg types and serotypes from all positive samples to determine their virulence genes (*tdh* and *trh*) by using multiplex PCR. Both *tdh* and *trh* genes (*tdh*⁺*trh*⁺) were positive in 4.8% of the isolates, *tdh*⁺ only 25.3%, and *trh*⁺ only 4.8%. Majority of the isolates (65.1%) were *tdh*⁻*trh*⁻. All isolates with *trh*⁺ produced urease enzyme and

7.4% of *tdh*⁺ isolates did not produce TDH. Antimicrobial susceptibility of 269 isolates were tested with 8 antimicrobial agents, more than 96% of them were susceptible to norfloxacin, nalidixic acid, nitrofurantoin, tetracycline, cotrimoxazole and gentamicin. 27.9% and 17.8% of the isolates were resistant to ampicillin and colistin, respectively. Multidrug resistant patterns were also found in 7.8% of 269 isolates i.e. ampicillin and colistin 5.9%, ampicillin and tetracycline 0.7%, and ampicillin and cotrimoxazole 0.7%.

All healthy carriers in this study were transient carriers. Five of them, all from the frozen processed seafood plant had *V. parahaemolyticus* positive two to three times during the study period. However, all isolates from transient healthy carrier were of different serotypes and strains. A *tdh*⁺ isolate with TDH and another *trh*⁺ isolate with TRH were identified in two of these five carriers. This study had shown the significant findings and characteristics of *V. parahaemolyticus* among healthy carriers in the frozen seafood plants. Further research was suggested on the roles of carrier in transmission, mechanism of disease, virulence gene expression and immunology of the carriers.

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APPENDIX A
***Vibrio parahaemolyticus* record**

Code No.....

Date.....

Interviewed by.....

Name..... Sex male female Age.....years

Factory frozen seafood

frozen processed seafood

Position Duration of present jobyear(s)

1. Characteristic of stool at the time of stool collection ?

Normal stool frequency.....time (s)/day

Diarrhea frequency.....time (s)/day

feces bloody mucoid loose watery

color white yellow brown red

2. Signs and Symptom yes no If yes, answer 3

3. Clinical signs and symptoms observed **Yes** **No**

abdominal pain

abdominal distension

headache

nausea

vomiting

fever

if yes > 38°C ≤ 38°C



APPENDIX B

PREPARATION OF REAGENTS AND MEDIAS

1. Reagents for polymerase chain reaction

1.1 10 x PCR buffer was compound of 100 mM Tris-HCl (Sigma, ST. Louis, MO, U.S.A) pH 8, 500 mM KCl (USB, Cleveland, Ohio, U.S.A), 1% Triton X-100 (Ameresco, Solon, Ohio, U.S.A). Ten ml of sterile 1 M Tris-HCl pH 8, 50 ml of sterile 1 M KCl and 10 ml of sterile 10% Triton X-100 were mixed, and then sterile deionized distilled water was added to 100 ml. The solution was dispensed into aliquots and stored at -20°C. In 25 µl of PCR mixture contained 2.5 µl of 10 x PCR buffer.

1.2 25 mM MgCl₂ (Merck, Darmstadt, Germany) was consisted of 2.5 µl of sterile 1 M MgCl₂ and 97.5 ml of sterile deionized distilled water, and stored at 4°C. In 25 µl of PCR mixture contained 2 µl of this solution.

1.3 Deoxyribonucleotide triphosphate (dNTPs) (Promega, Medison, WI, U.S.A). The working stock of 1.25 mM dNTPs mixture contained 10 µl of 100 mM dNTPs each (ATP, TTP, GTP, CTP) and 760 µl of sterile deionized distilled water. The solution was dispensed into aliquots and stored at -20°C. Four µl of this solution was added in 25 µl of PCR mixture to make a final concentration of 200 µM dNTPs each.

1.4 Taq polymerase (Pharmacia Biotech, U.S.A). *Taq* polymerase (5 units/µl) was diluted to 1 unit/µl with sterile deionized distilled water and 1 µl of dilute *Taq* polymerase was used in 25 µl of PCR mixture.

1.5 Primers Three set of primers; **LD₁-LD₂**, **TD₁-TD₂** and **TR₁-TR₂** were synthesized by Bioservice Unit, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Bangkok.

LD₁ (No.0816880) pmole/OD = 4167

14.91 OD purified in 1 DDW

LD₁ primer contained $4167 \times 14.91 = 62,129.97$ pmol/ml. After that, diluted the stock of LD₁ primer to make a final concentration at 2 pmol/ml in 100 ml sterile deionized distilled water by using the following formula:

$$N_1V_1 = N_2V_2$$

$$62,129.97 \text{ pmole/ml} \times 10^{-3} \times V_1 = 10 \text{ pmole/100ml}$$

$$V_1 = 16.1 \mu\text{l}$$

0.2 μl of this diluted primer was used in 25 μl of PCR mixture.

LD₂ (No.0816881) pmol/OD = 4167

14.07 OD purified in 1 DDW

Thus, the LD₂ primer contained $4167 \times 14.07 = 58,629.69$ pmole/ml then, dilutes the stock of LD₂ primer, by tacking 17.06 μl of LD₂ primer and sterile deionized distilled water were added to 100 μl to make a final concentration at 2 pmole/ μl . 0.2 μl of this diluted primer was used in 25 μl of PCR mixture.

TD₁ (No. 1479) pmole/OD = 5556

18.35 OD purified in 1 DDW

Thus, the TD₁ primer contained $5556 \times 18.35 = 101,952.6$ pmole/ml then, dilutes the stock of TD₁ primer, by tacking 9.8 μl of TD₁ primer and sterile deionized distilled water were added to 100 μl to make a final concentration at 10 pmole/ μl . One μl of this diluted primer was used in 25 μl of PCR mixture.

TD₂ (No. 1480) pmole/OD = 5263

18.05 OD purified in 1 DDW

Thus, the TD₂ primer contained $5263 \times 18.05 = 94,997.15$ pmole/ml then, dilutes the stock of TD₂ primer, by tacking 10.53 μl of TD₂ primer and sterile deionized distilled water were added to 100 μl to make a final concentration at 10 pmole/ μl . One μl of this diluted primer was used in 25 μl of PCR mixture.

TR₁ (No. 1477) pmole/OD = 5556

19.35 OD purified in 1 DDW

Thus, the TR₁ primer contained $5556 \times 19.35 = 107,508.6$ pmole/ml then, dilutes the stock of TR₁ primer, by tacking 9.3 μl of TR₁ primer and sterile deionized distilled

water were added to 100 μl to make a final concentration at 18 pmole/ μl . One point eight μl of this diluted primer was used in 25 μl of PCR mixture.

TR₂ (No. 1478) pmole/OD = 5000

20.25 OD purified in 1 DDW

Thus, the TR₂ primer contained $5000 \times 20.25 = 101,250$ pmole/ml then, dilutes the stock of TR₂ primer, by tacking 9.9 μl of TR₂ primer and sterile deionized distilled water were added to 100 μl to make a final concentration at 18 pmole/ μl . One point eight μl of this diluted primer was used in 25 μl of PCR mixture.

2. Reagents for electrophoresis

2.1 1 x TBE powder (Ameresco, Solon, Ohio, U.S.A) 17 g was dissolved in 1,000 ml of sterile deionized distilled water. Single strength of 1 x TBE solution contained 0.089 M Tris-HCl, 0.089 M Borate and 0.002 M EDTA.

2.2 1.5% agarose gel (Seakem LE, FMC Bioproducts, Rockland, ME, U.S.A) was composed of 1.5 g agarose in 100 ml 1 x TBE buffer.

2.3 6 x loading buffer was consisted of 2.5% bromophenol blue (USB, Cleveland, Ohio, U.S.A) and 40% sucrose (Mallinckrod, ST. Louis, MO, U.S.A) w/v in water. The buffer was dispensed into aliquots and stored at 4°C.

2.4 100 Base-Pair Ladder (Pharmacia Biotech, U.S.A) is a mixture of two plasmids, each plasmids were harvested from *E. coli* K12 and were digested with restriction enzyme. The 800 base-pair band appear at twice the intensity of the other bands upon visualization after ethidium bromide staining. Ten μl of 1:10 100 Base-Pair ladder was electrophoresed in the same manner as sample.

2.5 Stock ethidium bromide solution (10 mg/ml, Merck, Darmstadt, Germany). Five μl of stock ethidium bromide were dilute with 100 ml deionized

distilled water to make working solution (5µg/ml) for staining agarose gel after electrophoresis.

3. Media for Kanagawa test (Wagatsuma's agar)

Ingredient	Amount (g/1000)
Yeast extract	3
Bacto-peptone	10
Sodium chloride (NaCl)	70
Dipotassium phosphate (K ₂ HPO ₄)	5
Mannitol	10
Crystal violet	0.001
Bacto-agar	15
Distilled water	1000 ml
Human or rabbit red blood cells, fresh (24 h) with anticoagulants	50 ml.

Mix fresh (within 24 h of drawing) human or rabbit blood with same or larger volume of physiological saline. Centrifuge cells at about 4000 X g at 4°C for 15 min. Pour off saline and wash 2 more times. After 3rd wash, pour off saline and resuspended cells to original volume with saline.

Suspend ingredients, except blood, in distilled water and boil to dissolve agar. Adjust to pH 8.0 ± 0.2. Steam 30 min. Do not autoclave. Cool to 50°-55°C. Add 50 ml of wash red blood cells to the cooled medium. Mix and pour into Petri dishes. Dry plates thoroughly and use promptly.



APPENDIX C

SAMMPLE SIZE

The following formula was used for calculating the sample size

$$n = \frac{Z^2 \alpha/2 PQ}{d^2}$$

- n = sample size
- Z = standard normal deviation at 0.05 = 1.96
- P = proportion of event in population (2.0%)
- Q = proportion of failure in population
- d = allowable error(1.0%)

In this study used the proportion of frozen seafood plants employees harboured this *V. parahaemolyticus* without showing any symptom of diarrhea as described previously (35).

$$n = \frac{(1.96)^2 (0.02) (0.98)}{(0.01)^2} = 752.95$$

The maximum calculated sample size was 753 samples. In frozen seafood plant was collected about 900 samples every 2 or 3 months but in frozen processed seafood plant was collected about 180 to 200 samples every 2 weeks.

BIOGRAPHY

NAME Capt. Thanuncha Athajariya

DATE OF BIRTH 8 April 1972

PLACE OF BIRTH Bangkok, Thailand

INSTITUTIONS ATTENDED Royal Thai Army Nurse College, 1993 :
Bachelor of Nursing
Mahidol University, 2004 :
Master of Science (Public Health)

RESEARCH-GRANT Support in part by the Thesis Grant,
Faculty of Graduate Studies,
Mahidol University

POSITION&OFFICE Pramongkutklao Hospital, Pediatrics
Department: Bangkok, Thailand
Position : Nurse

Tel. .0-2354-7661