RAPID DETECTION OF *VIBRIO PARAHAEMOLYTICUS* IN FROZEN SHRIMP BY USING NESTED PCR

SUPORNWIT PUNGCHITTON

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Nested PCR-based assay with two sets of primers was developed for the detection of lecithin dependent hemolysin gene (*ldh*) of *Vibrio parahaemolyticus* directly in 103 frozen shrimp samples. All food samples were obtained from a frozen food factory in Chachoengsao province from April to May, 1999. The nested PCR assay produced a specific product size of 196 bp and detected the lowest genomic DNA of *ldh* reference strain, *V. parahaemolyticus* BG26 ($tdh^{-}trh^{-}$) at 1 fg corresponding to 1.7 cells per PCR reaction. All 21 strains of other *Vibrio* spp. and enteric bacteria did not give a specific DNA band of 196 bp but all tested *V. parahaemolyticus* strains did give positive band, indicating 100% specificity by nested PCR assay. It was demonstrated that 79.6% (82/103) of frozen shrimp enrichment samples were contaminated with *V. parahaemolyticus* using the conventional method and 88.3% (91/103) by the detection of *ldh* of *V. parahaemolyticus* in the enrichment samples using the primary PCR. The detection rates were significantly different between these 2 methods ($p=0.012$). Furthermore, the detection of *ldh* of *V. parahaemolyticus* directly from the samples by the nested PCR was 33.9% (35/103) and was significantly higher than that of the primary PCR (7.7%; 8/103)($p<0.001$). In contrast, using the conventional method, only one sample was found positive for *V. parahaemolyticus* with the count of 10^2 CFU/g of food sample, which was acceptable according to acceptable limit of *V. parahaemolyticus* by the criteria of International Commission on Microbiological Specification for Food (ICMSF). However, if other criteria of microbiological quality standards (such as total viable count, coliform counts and *E. coli* counts) were considered, 37.9%(39/103) of the samples were unacceptable. This study successfully developed the nested PCR assay for rapid detection (less than 8 hr) of *V. parahaemolyticus* in frozen shrimp samples without a prior enrichment step. However, enrichment of the frozen food samples before using PCR method was necessary to improve the detection of viable but nonculturable *V. parahaemolyticus.* Application of nested PCR assay could give a significant screening method for detection of *ldh* of *V. parahaemolyticus* contamination in frozen food as specificity and positive predictive value of the test, as compared with primary PCR, were as high as 85.7% and 91.4%, respectively.

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ศุภรวิทย์ พึ่งจิตต์ตน : การตรวจหา *Vibrio parahaemolyticus* อย่างรวดเร็วในกุ้งแช่แข็งโดยใช้วิธี nested PCR (RAPID DETECTION OF *VIBRIO PARAHAEMOLYTICUS* IN FROZEN SHRIMP BY USING NESTED PCR) คณะกรรมการควบคุมวิทยานิพนธ์ : อรษา สุตเธียรกุล Ph.D., คล้ายอัปสร พงศ์รพีพร Ph.D., กนกรัตน์ ศิริพานิชกร M.D., M.P.H., ปรารถนา สถิตย์วิภาวี M.P.H. 167 หน้า ISBN 974-664-233-2

วิธีการเพิ่มจำนวนสารพันธุกรรมด้วยปฏิกิริยาลูกโซ่โดยใช้ไพรเมอร์ 2 ชุด (nested PCR) ได้พัฒนาขึ้น เพื่อตรวจหายีนซึ่งกำหนดการสร้าง lecithin dependent hemolysin (*ldh*) ของ *Vibrio parahaemolyticus* ในตัวอย่างกุ้งแช่แข็ง จำนวน 103 ตัวอย่างที่เก็บจากจากโรงงานอาหารแช่แข็งแห่งหนึ่งในจังหวัดฉะเชิงเทรา ระหว่างเดือนมีนาคม ถึง เดือนพฤษภาคม 2542 จากการศึกษาพบว่า วิธี nested PCR สามารถขยายปริมาณดีเอ็นเอที่มีขนาด 196 คู่เบส และสามารถตรวจปริมาณดีเอ็นเอของเชื้อสายพันธุ์มาตรฐานน้อยที่สุดประมาณ 1 เฟมโตกรัม หรือประมาณ 1.7 เซลล์ต่อปฏิกิริยา PCR ความจำเพาะของวิธี nested PCR พบร้อยละ 100 โดยให้ผลบวกกับ *ldh* ของ *V. parahaemolyticus* ที่ตรวจทั้งหมด และให้ผลลบกับดีเอ็นเอของวิบริโอเชื้อสายอื่นๆและเอนเทอโรแบคทีเรียซึ่งใช้ทดสอบรวมทั้งหมด 21 สายพันธุ์ ผลจากการเพาะเชื้อตัวอย่างกุ้งแช่แข็งในอาหารเสริม ซึ่งเป็นวิธีมาตรฐาน พบว่ามีการปนเปื้อน *V. parahaemolyticus* ร้อยละ 79.6 (82/103) แต่การตรวจหายีน *ldh* ของ *V. parahaemolyticus* ด้วยวิธี primary PCR ในตัวอย่างกุ้งแช่แข็งจากอาหารเสริม พบร้อยละ 88.3 (91/103) ซึ่งสูงกว่าที่ตรวจพบโดยวิธีเพาะเชื้อจากอาหารเสริมอย่างมีนัย สำคัญทางสถิติ (p=0.012) นอกจากนั้นการตรวจหายีน *ldh* ของ *V. parahaemolyticus* ในตัวอย่างกุ้งแช่แข็งโดยตรงที่ไม่ได้เพาะในอาหารเสริมด้วยวิธี nested PCR พบตัวอย่างที่ให้ผลบวกถึงร้อยละ 33.9 (35/103) และสูงกว่าวิธี primary PCR ที่ตรวจพบร้อยละ 7.7 (8/103) อย่างมีนัยสำคัญทางสถิติ (p<0.001) จากการศึกษาครั้งนี้การตรวจด้วยวิธีมาตรฐาน มีเพียง 1 ตัวอย่างที่ตรวจพบ *V. parahaemolyticus* และนับได้ 10^2 CFU/กรัมอาหาร ซึ่งการปนเปื้อนของ *V. parahaemolyticus* ในระดับนี้อยู่ในระดับที่ยอมรับได้ตามเกณฑ์ของ International Commission on Microbiological Specification for Food (ICMSF) แต่ถ้าพิจารณาดัชนีมาตรฐานตัวอื่นๆ เช่น Total viable count, Coliforms, และ *E. coli* counts พบว่าร้อยละ 37.9 (39/103) ของตัวอย่างไม่ผ่านเกณฑ์ความปลอดภัยของอาหารแช่แข็ง การศึกษาครั้งนี้ได้พัฒนาวิธี nested PCR ได้สำเร็จและเป็นครั้งแรกที่สามารถตรวจ *V. parahaemolyticus* อย่างรวดเร็วในตัวอย่างกุ้งแช่แข็งได้โดยตรงภายใน 8 ชั่วโมงโดยไม่ต้องผ่านการเพาะในอาหารเสริม อย่างไรก็ตาม ขั้นตอนการเพาะเชื้อในอาหารเสริมสำหรับการตรวจอาหารแช่แข็งก่อนใช้วิธี PCR ยังมีความจำเป็นสำหรับการตรวจเชื้อที่ยังไม่ตายแต่เพาะเลี้ยงไม่ขึ้น ซึ่งจะช่วยเพิ่มความอัตราการตรวจพบ *V. parahaemolyticus* ในอาหารแช่แข็งได้มากขึ้น วิธี nested PCR อาจนำไปใช้เป็นวิธีคัดกรองในการตรวจการปนเปื้อนของ *V. parahaemolyticus* ในอาหารแช่แข็ง เนื่องจากเป็นวิธีที่ให้ความจำเพาะ และ positive predictive value สูงถึงร้อยละ 85.7 และร้อยละ 91.4 ตามลำดับเมื่อเปรียบเทียบกับวิธี primary PCR

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LIST OF ABBREVIATIONS

bp = Base pair

CFU = Colony forming unit

cm = Centrimetre

CTAB = Cetyl trimethylammonium bromide

DDW = Deionized distilled water

DNA = Deoxyribonucleic acid

dATP = Deoxyadenosine triphosphate

dCTP = Deoxycytosine triphosphate

dGTP = Deoxyguanine triphosphate

dNTP = Deoxyribonucleotide triphosphate

dTTP = Deoxythymine triphosphate

EDTA = Ethylenediaminetetra-acetic acid

ELISA = Enzyme link immunosorbent assay

et al. = Et alli (Latin), and other

fg = Femtogram

e. g. = For example

g = Gram

GM = Geometric mean

hr = Hour

Kda = Kilodalton

KP = Kanagawa phenomenon

L = Litre

LIST OF ABBREVIATIONS (CONT.)

LDH = Lecithin dependent hemolysin

ldh = Lecithin dependent hemolysin gene

M = Molar

mg = Milligram

min = Minute

ml = Millilitre

mM = Millimolar

mm = Millimetre

M.W. = Molecular weight

ng = Nanogram

nm = Nanometre

OD = Optical density

P = P-value

PCR = Polymerase chain reaction

pg = Picogram

pmole = Picomolar

RNA = Ribonucleic acid

RNAase = Ribonuclease

rpm = Revolution per minute

TBE buffer = Tris-Borate buffer

TE buffer = Tris-EDTA buffer

Tris-HCl = Tris hydrochloride buffer

LIST OF ABBREVIATIONS (CONT.)

TVC	=	Total viable count
U	=	Unit
µg	=	Microgram
µl	=	Microlitre
VNC	=	Viable but nonculturable cell
v / v	=	Volume per volume
w / v	=	Weight per volume

CHAPTER I

INTRODUCTION

A. Statement of Problems

Vibrio parahaemolyticus is an important cause of acute gastroenteritis and traveller's diarrhea after consumption of raw or partially cooked seafood (1,2). *V. parahaemolyticus* causing food borne outbreak has been reported from several countries including United States, Canada, Japan, Taiwan and Thailand which located in coastal and estuarine region (3-6). The incidence rates of *V. paarahaemolyticus* infected patients were reported in high percent and found in Japan (50%), Taiwan (29%) and Thailand (60%) (6-8). In Thailand, *V. parahaemolyticus* were isolated in frozen seafood products (26.5%). The highest percentage was found in frozen raw shrimp (36.9%), followed by frozen squid (21.3%), frozen fresh shrimp (10%) and frozen raw fish (6.1%) (9). Raw seafood in Bangkok contaminated with *V. parahaemolyticus* ranged from 49-78%. Mussel was the most common (56.7-100%), followed by squid (56%), prawn (56-68%), fish (53%), cockle (47-96%), clam (46%) and crab (28-51%) (10,11). *V. parahaemolyticus* may present in the seafood at the time of harvest according to it is a halophilic organism that distributed in marine water and estuarine environment.

At present, fresh and frozen shrimp products is one of the important export of Thailand which export to Asia, Europe, Australia, the United States and

Canada. In 1998, it made incomes more than 58,000 million Baht (12). However, the frozen shrimp was faced the over acceptability limit problem of the contamination of foodborne pathogens which led to rejection of the export products and devastating economic loss (13). The International commission on Microbiological Specifications for Foods (ICMSF) has recommended an acceptability limit for *V. parahaemolyticus* of 10^2 CFU/g in frozen shrimp (14). Except the industry problem, some export food caused the foodborne outbreak that faced the imported public health and medical problems in that countries (15). Generally, the common method for the detection of *V. parahaemolyticus* is a culture based procedure which employs enrichment in liquid media and the subsequent isolation of colonies on selective plating media. Unfortunately, a number of other *Vibrio* spp. are taxonomically similar to *V. parahaemolyticus* necessitating the utilization of additional biochemical tests or reliable identification (16-18). This method had limitation due to low sensitivity and required several days to interpret the results. In addition, the culture method could not detect the viable but nonculturable (VNC) state of *V. parahaemolyticus* affected by several factors such as chilling, freezing, heat, pH and starvation (19-21). These VNC of *V. parahaemolyticus* were capable of growth and multiplication with limited nutrients at an extraordinary rate when the temperature was upshifted. Therefore, the quality control of laboratory in a seafood-processing industry needs rapid and sensitive method to detect their presence as soon as the raw material is received. Furthermore, with the adoption of quality management systems such as hazard analysis critical control point (HACCP), rapid technique are necessary to identify and monitor step points in the seafood processing chain that allow the contamination of *V. parahaemolyticus* and other foodborne pathogens.

Several methods for detection of thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) which are encoded by the *tdh* and *trh* genes, respectively, have been developed. These methods were ELISA, DNA colony hybridization and polymerase chain reaction (PCR) (22-28). All methods mentioned above were examined *V. parahaemolyticus* by using hemolysin gene. Generally, the *tdh* gene was absent in most environmental strains of *V. parahaemolyticus* but *tdh* and/or *trh* genes were present in clinical strains of *V. parahaemolyticus* (29-30). Thus, these specific hemolysin gene probes could be detected in most of clinical isolates of *V. parahaemolyticus* but be unfavourable to detect in food or environmental isolates (23,25,31).

Several species specific gene fragments including lecithin depedent hemolysin gene (*ldh*) were developed for detection of environmental strains of *V. parahaemolyticus*.. Lecithin dependent hemolysin (*ldh* gene) is one of species specific gene fragments of *V. parahaemolyticus.* It was thermolabile hemolysin, with the nucleotide sequence of 1.5 kb. The LDH was presented in all strains of *V. parahaemolyticus* but it was no responsibility for the pathogenesis in *V. parahaemolyticus* infection (32,33). Suthienkul *et al.* (unpubished data) used the designed primers for *ldh* to detect the identified *V. parahaemolyticus* isolates from seafood samples by PCR. The results showed that 98% of these identified *V. parahaemolyticus* isolates were positive for *ldh* gene. Kachornchaiyakul (34) investigated the *ldh* gene of *V. parahaemolyticus* in frozen shrimp by PCR and DNA hybridization with digoxigenin labeled probes. The results of PCR gave higher efficiency than DNA hybridization assay. Using PCR to detect *V. parahaemolyticus* isolates from frozen shrimp samples, sensitivity and specificity were increased to 98.6% and 100%, respectively. Moreover, the detection of *ldh* of *V. parahaemolyticus* directly from all of the 111 frozen shrimp samples gave negative results. When the enriched samples which were incubated for 18-24 hr were used, only 68.5% were found to be positive by PCR.

The PCR protocol mentioned above and other studies have still required an enrichment step for a certain level of detection of the foodborne pathogen bacteria in food samples which was time consuming (34-36). Moreover, PCR product could not be detect by gel electrophoresis, if the amount of target DNA was low. The development of nested PCR had been reported to detect foodborne pathogens in food and environmental samples (37-39). Winters *et al.* (38) performed the nested PCR protocol to detect *C. jejuni* directly from chicken washes, without prior enrichment steps for PCR. The result showed that the sensitivity of nested PCR could detect *C. jejuni* cells of 10^2 CFU/ml within a day. In 1998, Lee *et al.* (39) established the nested PCR method and developed a single step DNA extraction method for direct detection of *V. vulnificus* in clinical samples. The result showed high sensitivity and could detect as little as 1 fg of chromosomal DNA or even one cell. In order to shorten detection time with sensitive and specific result within several hours for direct detection of low level of *ldh* gene of *V. parahaemolyticus* in frozen shrimp, nested PCR was developed for alternatively rapid method to detect *V. parahaemolyticus* directly in frozen shrimp samples.

B. Objectives

1. General objective

To study the prevalence of *ldh* gene of *V. parahaemolyticus* directly in frozen shrimp samples by using nested PCR.

2. Specific objectives

2.1 To develop the nested PCR for determining the minimum amount of *ldh* gene of *V. parahaemolyticus.*

2.2 To detect the contamination level of *V. parahaemolyticus* in frozen shrimp samples by culture method.

2.3 To examine *ldh* gene of *V. parahaemolyticus* directly in frozen shrimp samples by using nested PCR.

2.4 To study the prevalence *ldh* gene of *V. parahaemolyticus* in direct and enriched frozen shrimp samples by using primary PCR.

2.5 To determine the sensitivity, specificity, efficiency and correlation of nested PCR and primary PCR compared with the culture method as the gold standard.

2.6 To compare the agreement rate for detection of *ldh* of *V. parahaemolyticus* from the samples directly between nested PCR and primary PCR.

3. Hypothesis

3.1 The results of nested PCR for the detection of *V. parahaemolyticus* directly in frozen shrimp samples method were not different from conventional method.

3.2 The detection of *V. parahaemolyticus* directly from the frozen shrimp samples between nested PCR and primary PCR was not different.

CHAPTER II

LITERATURE REVIEW

A. History

Vibrio parahaemolyticus is a halophilic bacteria which was first identified as an agent of food poisoning outbreak in Japan associated with the consumption of "shirasu" (semi-dried young sardines) (40,41). In 1953, Fujino *et al.* (42) isolated an organisms which they named *Pasturella parahaemolyticus* from stools of patients and intestinal contents of autopsied cases. The poisoning was thought to result from a mixed infection with *Proteus morganii*. Later, Takikawa (43) isolated a bacteria from an outbreak of food poisoning at the National Yokohama hospital and they named *Pseudomonas enteritis*. He compared his isolates with the strain isolated by Fujino *et al.* and thought that the organisms were the same species. In 1962, Miyamoto *et al.* (44) studied pathogenic halophilic bacteria together with the strains isolated from human and marine sources on the basis of halophilism and fermentative utillization of glucose. They proposed a new generic name *Oceanomonas parahaemolyticus*. Subsequently, the definitive studies on the morphological, cultural and chemical properties of this organism, as well as its taxonomic position, were reported by Sakazaki *et al.* (45). The genus was established as *Vibrio* and named it *Virbio parahaemolyticus*.

B. Characteristics

1. Morphology

V. parahaemolyticus is a Gram-negative, generally straight, although occasionally slightly curved rod, 0.5 to 0.8 µm in diameter and 1.4 to 2.6 µm in length. It is motile by mean of a single polar flagellum. Additionally, it produces peritrichous flagella on solid media (46). It is a mesophilic organism, requires a minimum of 0.5% (w/v) NaCl in media for growth and is distinguished from other mesophilic, halophilic vibrios by its inability to ferment sucrose and its range of salt tolerance (0.5-10%) (47).

2. Physiological characteristics

V. parahaemolyticus is unable to grow in the absence of NaCl. Because of it is a halophilic bacterium. The upper and lower tolerance limits of NaCl for growth are 10% and 0.5%, respectively, with the best growth occurring in the 2-4% although other cations such as Li^+ and K^+ have been shown to have a sparing effect or the specific Na^+ requirment. On synthetic medium this organisms have a minimal essential Na^+ is approximately 0.003-0.07 M for the growth requirement (48). The optimal concentration of salt for growth may be influenced by the nature of the medium used. Enriched media such as blood agar or brain heart infusion agar may usually support good growth without the need for additional salt (41). The organisms grow well at 24°C to 44°C, the optimum being 37°C (49-51). The organism cannot proliferate below 15°C and the minimum growth temperature ranged from 9.5° C to 10.5° C (51,52). The organism can survive at an even lower temperature although it could not proliferate (53). The maximum temperature for growth of *V. parahaemolyticus* was 44°C to 45°C (50,51). *V. parahaemolyticus* dies when exposed to temperature <5-7° C; the rate of mortality is highest between 0°C and 5°C (47). Initial direct viable count of organisms declined rapidly upon cold storage, while remaining cells can survive for long periods. The surviving cells were capable of growth when increasing temperature (21,47,54). *V. parahaemolyticus* is very sensitive to heat, beginning to die slowly at 47°C, at 60°C killed the organisms in 15 min while heating at 100°C destroyed the organisms immediately (41,47). Similar to other member of the genus *Vibrio*, *V. parahaemolyticus* grow well under alkaline condition, a characteristic used to advantage in many selective isolation media. The pH range for growth was 4.8 to 11, with an optimum being 7.6 to 8.6 (55). Acidic condition were lethal to *V. parahaemolyticus*, viable cells being destroyed within 1 hr in vinegar and a few minutes in 0.5% acetic acid (41). The optimum of water activity (a_w) for growth corresponding to shortest generation time was found to be 0.992 (2.9% NaCl in tryptic soy broth). Employing the latter medium at 29°C and various solutes to control a_w, minimum values were 0.937 (glycerol), 0.945 (KCl), 0.948 (NaCl), 0.957 (sucrose), 0.983 (glucose), and 0.986 with propylene glycol (55). The vibrio is very sensitive to ionizing radiation. Dose of 1-3 KGY of gamma irradiation have been reported to be required for the elimination of vibrios from frozen shrimp (47,56). Under optimal conditions, the generation time of *V. parahaemolyticus* in the log phase of growth has been estimated at about 10 min (41).

2. Serological characteristics

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 vibrio, thus they have not been used for serotyping (48). The somatic (O) antigen is thermostable and is not destroyed by treatment with 50% ethanol and n-HCl at 37°C for 24 hr. The K antigen is a capsule or envelope antigen that is thermolabile and susceptible to heating at 100°C for 1 to 2 hr (4,57,48). The Committee on the Serological Typing of *Vibrio parahaemolyticus* extended an antigenic scheme of O and K antigen (Table 1).

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. Although numerous environmental and some clinical isolates are untypable by the K antigen, the majority of the clinical strains can usually be categorized according to the O type (48).

Serotyping is usually limited to strains from outbreaks or to special studies and should be performed in reference laboratory (59). The epidemiological study of *V. parahaemolyticus* in Japan showed that distribution of the strains was found among the O groups 1 to 8 and O4 antigen occurred most frequently. These extensive bacteriological studies have resulted in classification of strains of this organism into O antigen groups ranging from O1 to O13. It is not certain, however, that all strains of these antigen groups exhibit same degree of pathology (60).

The first isolation of *V. parahaemolyticus* in Kenya was from seafood and

Table 1 Antigenic scheme of *Vibrio parahaemolyticus* (58)

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

estuarine environment even there has no clinical disease has been reported (61). The main serovars were O3:K37, O3:K40, 08:K39, O10:K23, O19:K52 and O11:K40.

Kaysner *et al.* (62) tested a total of 173 urease positive strains of *V. parahaemolyticus* from water, oysters and sediment samples from Willapa Bay, Washington DC USA. The most common antigenic groups was O5. Serotype O5:K17 was the highest frequency.

In Thailand, the dominant serovar of *V. parahaemolyticus* isolates from patients with gastroenteritis was O4:K10 in 1992 and O3:K29 in 1994 (63,64). The dominant serovar of *V. parahaemolyticus* contaminated in raw seafood was O5:K17 and O3 (10,11).

C. Epidemiology

1. Outbreak

Food poisoning caused by *V. parahaemolyticus* commonly occurs in estuarine waters throughout the world. It is easily isolated from estuarine, coastal water, sediment, marine fish and shellfish (65,66). The illness is almost exclusively associated with seafood which is consumed raw, semi-cooked, or cooked but recontaminated. Several researchers reported an outbreak of *V. parahaemolyticus* in the world-wide. Food poisoning due to *V. parahaemolyticus* in Japan was reported by Ministry of Health and Welfare. It was found that the incidences were about 20% to

30% of all case of food poisoning (66). An epidemiological study of food poisoning in Korea and Japan during the period from 1971 to 1990. The result showed that outbreaks in Korea most frequently involved home-made foods in 48%, while in Japan, restaurants accorded for 32.7%. Foods most commonly incriminated both in Korea and Japan were seafood. The majority of bacterial causes in Korea and Japan were *Vibrio* spp. in 37.6% and 47.3%, respectively (67). In 1999, WHO Surveillance Programme reported the food poisoning outbreak in Japan during 1996-1998. The monthly outbreaks of *V. parahaemolyticus* food poisoning trend to be more prevalent in summer with a peak in August and few outbreaks in winter. There were 496 outbreaks during these three years: 102 in 1996; 160 in 1997; and 234 in 1998. Only 6% of the outbreaks involving more than 500 cases occurred only twice. Recently, *V. parahaemolyticus* food poisoning outbreaks in Japan tended to be small in scale but rather frequent. The most serovars caused of food poisoning outbreaks since 1996, was O3:K6. In contrast, the predominant serovar in previous outbreaks was O4:K8 (68). Okuda *et al.* (69) reported that active surveillance of *V. parahaemolyticus* infection among hospitalized patients in Calcutta, India was initiated in January 1994 and *V. parahaemolyticus* isolated strains of those were examined for serovar. The result showed that emergence of a unique O3:K6 serovar was found highly in 50 to 80% from February to August 1996. Furthermore, these strains were compared with O3:K6 serovar of *V. parahaemolyticus* isolated from travelers arriving in Japan from Southeast Asia. The result showed that both strains were indistinguishable.

Matsumoto *et al.* (70) reported that the *V. parahaemolyticus* isolated strains from hospital in Bangladesh between 1996 and 1998, and from clinical sources in

Taiwan, Laos, Japan, Thailand, Korea and the United States between 1997 and 1998 were shown to belong to the new O3:K6 clone. These new O3:K6 clones differed from the former O3: K6 strains isolated before 1995 by using AP-PCR method.

In 1996, Pan *et al.* (71) investigated 102 outbreaks of foodborne disease involving 4,726 cases reported to the Taiwan Department of Health in 1994. Of these outbreaks 72.5% (74/102) were caused by enteric pathogens and *V. parahaemolyticus* was found the highest in 56.7% (42/74). Additionally, Pan *et al.* (72) reported that between 1986 and 1995, 852 outbreaks of foodborne disease involving 26,173 cases and 20 deaths occurred in Taiwan. About 80% of the outbreaks occurred in the warmer months. Of these reported outbreaks, 65% (555/852) were caused by bacterial pathogens and *V. parahaemolyticus* was dominantly found in 35% (197/555).

In 1999, Wong *et al.* (73) 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.

The first outbreak of food poisoning caused by *V. parahaemolyticus* occurred in Maryland, USA. in 1971. About 320 patients suffered from acute gastroenteritis, a result of improperly steamed crabs (74). In 1978, the largest outbreak in the United States occurred during the summer month and 1,133 of 1,700 persons ill when attending a dinner in Port Allen, La. The food implicated was boiled shrimp (56).

During July-August 1997, an outbreak of *V. parahaemolyticus* infections occurred in North America. Illness in 209 persons was associated with eating raw oysters harvested from California, Oregon, and Washington in the United State and from British Columbia (BC) in Canada; one person died (3). Subsequently, an outbreak of *V. parahaemolyticus* was reported by New York State Department of Health in 1998. A total 23 ill persons suffered from *V. parahaemolyticus* infection associated with consumption of oysters and clams harvested from Long Island Sound. The serovar of *V. parahaemolyticus* clinical isolated was O3:K6 (75).

In Peru, Begue *et al.* (76) reported that it was an outbreak of *V. parahaemolyticus* gastroenteritis among military personal in Lima, 74% of food specimens (208/249) were analyzed, and *V. parahaemolyticus* was identified in 40% (84/208) as well as from a sample of sea water.

In Southeast Asia, Bonang *et al.* (77) examined rectal swab from 514 ill persons with acute gastroenteritis. *V. parahaemolyticus* was identified in 3.7% (19/514). In Vietnam, the isolation rate of *V. parahaemolyticus* from patients with gastroenteritis was 8.5 to 15% (78).

In Thailand, the first outbreak of *V. parahaemolyticus* gastroenteritis was reported by the Southeast Asian Treaty Organization Medical Research Laboratory in

1970 (79). Gastroenteritis caused by this organism was up to 2.5% in diarrheal patients. In 1974, Atthasampanna (80) reported the isolation rate of *V. parahaemolyticus* from 7,930 diarrheal patients who admitted in the Infectious Diseases Hospital near Bangkok during November 1970 to June 1973, was 10.7% (850/7,930). Subsequently, Sriratanaban *et al.* (1) studied traveller's diarrhea in Bangkok and suggested that *V. parahaemolyticus* was a major cause of traveller's diarrhea. Additionally, this organism has been a common cause of gastroenteritis among diarrheal patients in Bamrasnaradura Infectious Diseases Hospital (BIDH). Since 1983 to 1996, the isolation rate of *V. parahaemolyticus* ranged from 4.5% to 7.6% of all diarrheal cases (81).

2. Source of infections

V. parahaemolyticus is a halophilic microorganism, it requires sodium chloride for growth. Because of its halophilic nature, the organism is widely distributed in marine environments but is seldom associated with freshwater or non-marine environments. The organism frequently present in fish and shellfish caught in estuarine and inshore coastal water (82-84). *V. parahaemolyticus* is usually present not only on the surface of the fish but also in the gills, stomach and intestines. Additionally, it is not found, as a rule, on the open ocean or in deep ocean fish (85).

V. parahaemolyticus infection is particularly associated with consumption of raw or undercooked seafood often prepared in the traditional Japanese culinary style. Several types of seafood implicated in foodborne vibrio infections (Table 2) (86).

Table 2 **Sources and types of food implicated in foodborne vibrio infections (86)**

Sources	Types of food	*V. cholerae*	*V. parahaemolyticus*	*V. vulnificus*
Bivalve Molluscan Shellfish	Mussels	+	+	
	Clams	+	+	
	Oysters	+	+	+
	Cockles	+	+	
Crustacean Shellfish	Prawns	+	+	
	Crab	+	+	+
	Lobster	+	+	
Fish	Sardines	+		
	Lagoon fish	+		
	Raw fish	+	+	
	Dried fish		+	
Water	Vegetables	+		
	Lettuce	+		
	Bottled water	+		
	Cooked rice	+		
	Potato salad	+		
Infected food Handlers	Cooked meats	+		
	Prawns	+		
	Chicken/rice	+		
	Cooked Squid	+		

In countries where seafoods are not often consumed raw, contamination of foods most likely occurs via insufficient cooking to destroy natural contamination, and cross contamination of cooked food by raw seafood or seawater. Subsequently, foods are mishandled being held at inappropriate temperature for excessive periods of time, allowing the organisms to proliferate and reach the level of an infectious dose (17). Baker *et al* (87) 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. These three mechanisms serve to show that the majority of outbreaks and sporadic cases of gastroenteritis can be prevented by attention to corrective measures.

Several reports proposed the isolation of *V. parahaemolyticus* from coastal water, estuarine sediment, plankton, sea mud and sea fish (44,52,88). Subsequently, the study on 3-years survey of 716 seafood samples taken in North Carolina, found 46% to be positive for *V. parahaemolyticus.* Notable were unshucked oysters (79% positive), unshucked clams (83% positive), unpeeled shrimp (60% positive), and live crabs (100% positive) (56,89). Similarly, Fang *et al.* (90) found 45.7% of seafood samples collected from the retail markets in coastal of Republic of China (Taiwan) contaminated with *V. parahaemolyticus.* In Hong Kong, Chan *et al.* (91) investigated the prevalence of *V. parahaemolyticus* and other halophilic vibrios in seafood from Hong Kong markets. The result showed that oyster was the highest contamination with

V. parahaemolyticus in 22%, followed by mussel 12%, clam 10%, crab 8.6%, prawn 8%, rabbit fish and grouper 10%.

In 1989, the study on gastroenteritis in Louisiana, it was found that 100% of the raw oysters were contaminated with *V. parahaemolyticus* and 66% contained *V. vulnificus.* Interestingly, 50% of the cooked oysters tested also contained *V. parahaemolyticus* (56,92).

In 1997-1998, multistage outbreaks of *V. parahaemolyticus* infection occurred in the United States. Illness in 232 persons ill associated with consumption of raw or undercooked oysters and clams harvested from Pacific Northwest and Long Island Sound. One person died from these outbreak (3,75).

In Thailand, from 1971 to 1972, SEATO Medical Research Laboratory (79) surveyed natural sources for *V. parahaemolyticus,* they detected *V. parahaemolyticus* 52.9% (138/261) in environment and 77.48% (46/595) in seafood. Subsequently, Phayakvichien *et al.* (9) reported the isolation rate of *V. parahaemolyticus* from several types of frozen seafood. They found that the raw shrimp was the highest contaminated with *V. parahaemolyticus* in 36.9% (38/103), followed by boiling clam 33.3% (1/3), all seafood 25% (1/4), raw squid 21.3% (10/47), fresh shrimp 10% (1/10) and fish 6.1% (2/33). Pumiprapat (10) found that 49% (245/500) of raw seafood samples were contaminated with *V. parahaemolyticus.* Mussel was the highest contaminated food 56.7%, followed by prawn and squid 56%, fish 53%, cockle 47%, clam 46% and crab 28%. In 1995, Limuthaitip (11) isolated 78% (155/200) of raw seafood samples in Bangkok contaminated with *V. parahaemolyticus.* The highest detection of *V. parahaemolyticus* was found in mussel 100%, followed by cockle 96%, shrimp 68% and mangrove crab 51%.

3 Seasonal variation

V. parahaemolyticus is common in oceanic and coastal water. Its detection is related to water temperature. It was highest isolated during the summer to early autumn months (48,55,82-84). In the other hand, isolation of the organism from shellfish, sediment, and water is infrequent or rare when water temperature fall below 13-15°C, thus outbreak from the organism did not occur during the winter (4,82).

The organism's affinity for chitin, which is a constituent of the exoskeleton of many plankton species, is an important factor that influences the survival of *V. parahaemolyticus* during winter (82,83). The organism adsorbs to chitinous material of plankton which sinks to the estuarine bottom during the winter. As the temperature increases the following year, the organism reappears in the water column as part of the annual cycle of sedimentation and resuspension (82).

The distribution of *V. parahaemolyticus* has been closely associated with the salinity of the water. The optimum NaCl concentration for growth are 2 - 4%. The occurrence of *V. parahaemolyticus* in water of low salinity has been distributed to tidal transport or to coincidental contamination from human infection. The incidence and count of *V. parahaemolyticus* were consistently highly in estuarine area (salinity 15.5

a_w) with the highest incidence of various serotypes (93). However, the natural habitat of *V. parahaemolyticus* is not exclusively associated with marine environments, as the organisms have been detected in island salt lakes in Florida (94). In contrast, the organisms are not detected in the open sea or at deep water levels where high salinity, together with low temperature and nutrient availability and hydrostatic pressure also exert effects (48).

D. Pathogenesis

V. parahaemolyticus is a halophilic Gram-negative bacterium which causes not only gastroenteritis but also secondary septicemia, wound and ear infection in human (Table 3). It was categorized as moderate hazard group, limited spread according to the severity of the potential hazard (Table 4) (56,95). Gastroenteritis due to *V.parahaelymoticus* is always associated with ingestion of seafood. The incubation period of *V. parahaemolyticus* infection is 3-24 hr, usually being about 10-15 hr. The main symptoms are diarrhea and abdominal, along with nausea, vomiting, fever and general fatigue. 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 watery, mucoid, bloody, or mucoid and bloody. *V. parahaemolyticus* infection is usually a self-limiting disease and clinical symptoms may last 1-2 weeks (96,97).

The experimental dosages required for initiation of gastroenteritis, volunteer studies have shown that ingestion of *V. parahaemolyticus* virulent strains ranges from 2×10^5 to 3×10^7 CFU can lead to the rapid development of gastroenteritis illness

Table 3 Association of *Vibrio* spp. with different clinical syndromes[a] (58)

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

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

Table 4 **Hazardous microorganisms and parasites grouped on the basis of risk severity (56)**

Severe hazards

- *Clostridium botulinum* types A, B, E, and F
- *Shigella dysenteriae*
- *Salmonella typhi, Salmonella paratyphi* A, *Salmonella paratyphi* B
- Enterohemorrhagic *Escherichia coli*
- *Brucella abortus, Brucella suis*
- *Vibrio cholerae* O1
- *Vibrio vulnificus*
- Hepatitis A and E virus
- *Taenia solium*

Moderate hazards : potentially extensive spread[a]

- *Listeria monocytogenes*
- *Salmonella* spp.
- *Shigella* spp.
- Other enterovirulent *Escherichia coli*
- *Streptococcus pyogenes*
- Rota virus
- Norwalk virus group
- *Entamoeba histolytica*

Table 4 Hazardous microorganisms and parasites grouped on the basis of risk severity (continued)

Moderate hazards : potentially extensive spread[a]

Diphyllobothrium latum

Ascaris lumbricoides

Cryptosporidium parvum

Moderate hazards : limited spread

Bacillus cereus

Campyrobacter jejuni

Clostridium perfingens

Staphylococcus aureus

Vibrio cholerae non-O1

Vibrio parahaemolyticus

Yersinia enterolitica

Giardia lamblia

Taenia saginata

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[a] Although these organisms are classified as moderate hazards, complications and sequelae may be severe in certain susceptible populations.

(56,82). Conversely, volunteers receiving as many as 1.6×10^{10} CFU of non-virulent strains exhibited no signs of diarrheal illness (56). Usually the number of *V. parahaemolyticus* on freshly caught seafoods are low, up to 10^2/g. However, in market shellfish, especially during summer, count of 10^3/g are not uncommon. Proper refrigeration of seafoods, both before and after cooking, is essential to control the growth of the organisms. Due to *V. parahaemolyticus* has a remarkably short generation time in seafood that has been temperature abused such as in raw squid and horse makerel, 13 min at 37°C and 15-18 min at 30°C, respectively, and in boiled octopus 12 min at 30°C (82). Thus, short period of temperature abuse allow a rapid increase in number of *V. parahaemolyticus* in seafood.

The mechanism of pathogenicity of *V. parahaemolyticus* have not been defined or fully understood but it was closely correlated with the Kanagawa phenomenon (KP^+) : the β hemolytic ability of strains cultured on Wagatsuma blood agar(29,97). Production of Kanagawa hemolysin or the thermostable direct hemolysin (TDH) is responsible for the Kanagawa phenomenon. Thus, TDH has been considered a major virulence factor (98,99). However, Honda *et al.* (7) reported that the clinical isolates of KP^- *V. parahaemolyticus* from traveller in Maldives produced newly hemolysin, named TDH-related hemolysin (TRH). This suggests that TRH may be an importance virulence factor and possibly cause diarrhea.

V. parahaemolyticus is considered to cause diarrhea by adhering to the intestinal epithelium and then releasing toxin. Carruthers (100) reported that a KP^+ *V. parahaemolyticus* adhered to a human fetal intestinal (HFI) cell line (HS 0074) *in vitro* much more rapidly than did a KP$^-$ strain. Subsequently, Yamamoto *et al.* (101) observed adherence abilities of *V. parahaemolyticus* strains to human small intestinal mucosa correlated roughly with the hemagglutination (HA) level of the strain under the test conditions, ideal lymphoid follicle epithelium (espicially M cells) provided the best adherence target for *V. parahaemolyticus*.

In 1990, Nagasone *et al.* (102) demonstrated that the purified pili of KP$^+$ *V. parahaemolyticus* (Ha 7) had the ability to adhere to the intestinal but the pretreatment of the intestine with purified pilli did not allow adherence of the organism to the intestines. These results suggested that pili of this *V. parahaemolyticus* strain played an importance role in colonization. Nagayama *et al.* (103) found a positive correlation between cell associated mannose-sensitive hemagglutination and adherence of *V. parahaemoluticus* to rabbits enterocytes. The cHA is located on the bacterial cell surfaces and is not associated with pili and involved in the adherence mechanisms of *V. parahaemolyticus* to the enterocytes and that the receptors for the cHA on the enterocytes appear to be a D-mannose containing compound. The purified cHA was found to be a heat-labile tetrameric protein consisting of four subunits (of 29 KDa each). Hemagglutinating activity was inhibited by D-mannose. A 17 KDa glycoprotein on the brush border of the rabbit enterocytes was identified as cHA-binding receptor. This cHA-binding glycoprotein was distributed locally on the upper and middle portion of the rabbit small intestine.

Although the hemolytic activity of TDH is a convenient assay of activity, it does not explain how this toxin might cause diarrheal illness. When administration orally to sucking mice at low levels, TDH causes diarrhea, and at higher level (e.g., 50 μg) it produces diarrhea and death. When TDH at dose of 100 μg was administered to ligated rabbit ileal loops, fluid accumulation was not observed, in contrast to cholera toxin (CT), with which this response was observed following inoculation of only 0.2 μg. At dose of 200 μg, TDH gives a positive response in rabbit ileal loop but with erosive lesion and desquamation of necrotic intestinal mucosa. Such histological changes were not observed when whole bacteria expressing more physiologically relevant TDH levels were tested in ileal loops. Using a KP^+ strain and isogenic TDH mutant, it was demonstrated that only the KP^+ parent strain was capable of inducing fluid accumulation in the rabbit ileal loop assay (56,104). When the complete *tdh* gene was returned to the isogenic mutant, restoration of activity was observed. Similar results were seen when culture supernatants of these strains were tested on rabbit ileal tissue mounted in Ussing chambers, a more sensitive measure of secretory activity. In this assay, the ability of TDH to alter ion transport in the intestinal tract was demonstrated at nanogram levels, with no histological changes (56). Subsequently, the study of effect of purified TDH in Ussing chamber was reported in 1995. The result demonstrated that TDH induces intestinal chloride ion secretion and that the trisialoganglioside GT_{1b} appears to be the cellular receptor. Furthermore, rather than cAMP or cGMP, TDH uses Ca^{2+} as an intracellular second messenger, thereby being the first bacterial enterotoxin for which the linkage between changes in intracellular calcium and secretory activity has been established (56,105).

In 1996, Wong *et al.* (106) elucidated the roles of iron acquisition in the pathogenesis of *V. parahaemolyticus* by examining the changes in the virulence of these iron-utillizing mutants by *in vitro* and *in vivo* assays. They proposed that iron-regulated outer membrane proteins and other unknown factors associated with iron utilization may have profound influences, besides iron acquisition, on the pathogenesis of *V. parahaemolyticus*.. In addition, Pace *et al.* (107) suggested that a bile acid-containing environment such as that found in the human host favors growth of virulent strains of *V. parahaemolyticus* and that bile acids enhance the expression of virulence factors. These effects seem to be mediated by a decrease in intracellular calcium.

E. Hemolysin

1. Thermostable direct hemolysin (TDH)

Gastroenteritis from *V. parahaemolyticus* is closly associated with Kanagawa Phenomenon positive (KP$^+$) (4,29). The KP was examined on the Wagatsuma agar and showed beta-hemolytic reaction (108). Kanagawa hemolysin or thermostable direct hemolysin (TDH) is a thermostable even at 100°C for 10 min. The hemolytic activity of 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 humans, rabbits, quinea pigs and chickens; slightly hemolytic for erythrocytes of sheep; and non hemolytic for erythrocytes of horse (29,109,110).

Sakurai *et al.* (110) reported the extensive purification and some characters of TDH. They found that it was a protein free from phospholipid and carbohydrate, and 43% of total amino acids were acidic amino acid, whereas 11% were basic amino acids. The molecular weight of this hemolysin was 42 KDa by using gel filtration. It has a dimer structure composed of two identicals subunit having molecular weight about 21 KDa (98). In 1992, Honda *et al.* (2) demonstrated that TDH acts as a "pore-forming toxin" in temperature-dependent and independent steps. The first temperature-dependent step required only about 1-2 min incubation at 37°C and made a "pore" with a functional diameter of approximately 2 mm. The pore size was deduced from the molecular diameter of the colloidal inhibitory polysaccharides. The second step, which was a temperature-independent lytic step, caused the erythrocytes to swell owing to a colloidal osmotic influx of water via the "pore" into cell, resulting in erythrocytes lysis (or rupture) owing to increase intracellular pressure.

The first report of the cloning of the gene encoding TDH (designated *tdh*) from a KP$^+$ strain of *V. parahaemolyticus* was reported by Kaper *et al.* (2112). Later, Nishibuchi *et al.* (112) reported that the functional *tdh* gene was localized to a 1.3 kilobase *Hind* III fragment. This fragment was sequenced, and the structural gene was found to encode a major protein of 165 amino acid residues. The mature protein sequence was preceded by a putative signal peptide sequence of 24 amino acid.

A recent review by Nishibuchi *et al.* (113), *V. parahaemolyticus* strains were shown to carry variant of the *tdh* gene encoding TDH. A KP-positive clinical strain contained two chromosomal *tdh* genes (designated *tdh*1 and *tdh*2) located on a 1.3 Kb *Hind* III fragment and 2.5 Kb *Hind* III fragment, respectively. The data obtained from the amino acid sequences as well as construction of a *tdh*1 – deficient yet hemolytic strain of *V. parahaemolyticus* suggested that extracellular TDH purified and characterized previously (114) was derived from the *tdh*2 gene. A KP-negative clinical strain contained one chromosomal *tdh* gene copy (designated *tdh*3) located on a 2.8 Kb *Hind* III fragment and another the plasmid *tdh* gene copy (designated *tdh*4) located on a 5.0 Kb *Hind* III fragment. All the gene copies encoded polypeptides composed of 189 amino acid resudues and the nucleotide sequences of the various *tdh* genes are well conserved (>97% identify). TDH was produced from the *tdh*2 gene rather than the *tdh*1 gene. The nucleotide homologies of *tdh*2 with *tdh*1, *tdh*3 and *tdh*4 were 97%, 98.6% and 98.6%, respectively.(112,115).

In 1991, Baba *et al.* (116) reported that *V. parahaemolyticus* AQ 3860 contained a various of the chromosomal *tdh* gene (designated *tdh*5). Nucleotide sequence analysis of the coding region of the *tdh*5 was highly homologous in 96.7% to 99.1% with the *tdh*1-*tdh*4 genes. It was indicated that the *tdh*5 gene is evolutionary any more closely related to the *trh* gene than to the *tdh*1- *tdh*4 gene group. These results suggested that the *tdh*5 and *trh* gene were not originally produced by gene duplication in AQ 3860 but rather that one of the two genes moved into AQ 3860 from an external source. Later, Tang *et al.* (117) employed *in vitro* mutagenesis to the gene for TDH (*tdh*) analyzed the critical amino acid residues for hemolytic activity. In the experiment obtained several mutant toxins of TDH that showed various deficiencies in hemolytic activity. Interestingly, R7 with a single amino acid substitution of serine for glycine 62, inhibited the hemolytic halo caused by wild-type TDH on a rabbit blood agar plate while its hemolytic activity decreased dramatically. They suspected that R7 might retained the ability to bind to erythrocytes and complete for binding sites with wild-type TDH. Another mutant TDH, H17, which has an amino acid substitution at glycine 86, showed moderately decreased hemolytic activity. Probably because of its deficiency in binding to erythrocytes 417 could not all inhibit the hemolytic halo caused by wild-type TDH on rabbit blood agar. Both mutant TDH, suggested that the confirmation of these mutant TDHs are substantially similar to that of wild-type TDH. They also analyzed the properties of R7 throughout the hemolytic process such as binding, permeabilization of the erythrocytes membrane, and hemolysis. The hemolytic activity of R7 decreased to less than 1/100 of wild-type TDH, and its mouse lethality was undetectable. However, this mutant showed a masked inhibitory effect on hemolysin by wild-type TDH. Enzyme immunoassay and flow cytometric analysis demontrated that R7 retained approximately 50% of the ability to bind to erythrocytes compared with that of wild-type TDH might be due to blocking the binding on the erythrocytes membrane. Wild-type TDH affected the erythrocytes membrane by causing an influx of calcium and propidium idodide, while R7 showed no detectable effects of these kinds. These results suggested that hemolysis by TDH consists of at least two steps, binding and postbinding, and that R7 was likely to be a postbinding activity deficient mutant toxin of TDH.

2. Thermostable direct hemolysin-related hemolysin (TRH)

Thermostable direct hemolysin-related hemolysin (TRH) has been considered as a virulence factor for *V. parahaemolyticus*. In 1987, the first report of KP-negative strains caused of gastroenteritis was proposed by Honda *et al.* (118,119). Later, Honda *et al.* (7) examined the hemolysin produced by a clinical isolate of KP-negative *V.*

parahaemolyticus. They found that it was a new hemolysin (named (Vp -TRH, for thermostable direct hemolysin [Vp-TDH]-related hemolysin) that was related to the Vp-TDH produced by ordinary Kanagawa phenomenon-positive *V. parahaemolyticus.* Vp-TRH was purified by ammonium sulfate fractionation and successive column chromatographies on DEAE-cellulose, hydroxylapatite and Mono Q. The molecular weight of Vp-TRH was estimated as 48,000 by Sephadex G-100 gel filtration, and the molecular weight of the subunit was estimated to be 23,000 by sodium dodecyl sulfate-slab gel electrophoresis. Thus, like Vp-TDH, Vp-TRH seems to be compound of two subunits. The isoelectric point of Vp-TRH was determined to be 4.6. Vp-TRH showed lytic activities different from those of Vp-TDH on erythrocytes from various animals, especially those from calves, chickens, and sheep. The hemolytic activity of Vp-TRH was labile on heat treatment at 60°C for 10 min, unlike that of Vp-TDH. The immunological similarities, but not the identities of Vp-TRH and Vp-TDH, were demonstrated by Ouchterlony, neutralization, and latex agglutination tests. Thus, we conclude that this clinical isolate of Kanagawa phenomenon-negative *V. parahaemolyticus* produces a new type of hemolysin that is similar, but not identical, to Vp-TDH.

The study on the properties and the characterization of Vp-TRH was also reported by Honda *et al.* (120). The purified Vp-TRH showed various biological activities, such as fluid accumulation in rabbit ileal loops, increase of rabbit skin vascular permeability, and cardiotoxicity on cultured myocardial cell. These biological properties not only were similar to Vp-TDH but also a hemolytic toxins produced by *V. hollisae* and *V. cholerae* non-O1. These results suggest that Vp-TRH played rules similar to Vp-TDH in the pathogenesis of *V. parahaemolyticus*. Various characteristics reported about Vp-TRH and Vp-TDH are compared in Table 5 (2).

The study on the gene encoding TRH (named the *trh* gene) was reported by Nishibuchi *et al.* (99). They proposed that the nucleotide sequence of *trh* gene like the *tdh* gene, encoded the hemolysin subunit composed of 189 amino acid residues. The *trh* gene had significant nucleotide sequent homology with the *tdh* gene (68.4% with the *tdh*1 gene copy and 68% with the *tdh2* gene copy). The amino acid sequences of the hemolysin subunits deduced from the nucleotide sequences of the *trh* gene and *tdh* gene werehomologous (61.9% homology with the *tdh*1 – encoded subunit and 63% homology with the *tdh*2 – encoded subunit) and contained the two cystein residues to form an intrachain band at the same positions, and their possible conformations appeared to be similar as determined by hydrophobicity-hydrophilicity analysis and a secondary structure prediction. The *trh* and *tdh* genes may have had a common ancestor and may have evolved by single-base changes so that they maintained the fundamental architecture of the molecular. Subsequently, Kishishita *et al*. (31) cloned the nucleotide sequence responsible for the very weak hybridization signals from a representative strain and analyzed the nucleotide sequence. The very weak hybridization signal was due to a variant of the *trh* gene (named *trh*2) which was 84% homologous to the *trh* gene (newly named *trh*1) and 54.8 to 68.8% homologous to the genes *(tdh)* encoding thermostable direct hemolysins showed that both the *trh*1 and *trh*2- carrying strains should be considered potentially virulent.

Table 5 **Comparison of Vp-TDH and Vp-TRH (2)**

Properties	Vp-TDH	Vp-TRH
Molecular weight		
Holo toxin	45,000	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	250 µg/loop	100 µg/loop

Several studies have reported urease-positive *V. parahaemolyticus* isolates from clinical sources, and implicated these strains in human gastroenteritis (121-123). In 1995, Suthienkul *et al.* (26) examined 489 clinical strains of *V. parahaemolyticus* isolated in Thailand. was detected in the cloned sequence. The *trh*2 gene product showed a profile of hemolytic activities again various animal erythrocytes different from that of the *trh*1 gene product. The *trh*2 gene products was antigenically related (partially identical) to the *trh*1 and *tdh* gene products. The gene belonging to the *trh*2 group was detected not only in rare environment strains but also in a significant number of clinical strains. These results Of these strains, 8% were urease positive and possessed the *trh* gene. The result indicated that the urease-positive phenotype can be a convenient and useful marker in clinical diagnosis for predicting virulent (*trh*-gene possessing) *V. parahaemolyticus* strains.

3. Lecithin dependent hemolysin (LDH)

Lecithin dependent hemolysin (LDH) is one of species specific gene fragment of *V. parahaemolyticus*. 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 was 47.6% which is almost the same as that of *V. parahaemolyticus* strains and did not have any homologous sequences with genes of the α-hemolysin of *Staphylococcus aureus*, the enterotoxin of *Vibrio cholerae*, and the enterotoxin ST and the hemolysin of *E. coli* (32).

In 1991, Shinoda *et al.* (124) studied the purification and characterization of lecithin dependent hemolysin (LDH) of *V. parahaemolyticus*. It comprised of two LDH proteins with different molecular masses which were immunologically cross reactive and had the enzymic activity. The LDH was a phospholipase hydrolysing fatty acid esters of phospholipid, i.e. it hydrolyzed phosphotidyl choline (PC) to lysophosphotidyl-choline (LPC) and then LPC to glyserophosphosylcholine (GPC). From this point of view, LDH should be classified as phospholipase B. However, phospholipase B does not usually show hemolytic activity, because the intermediate (LPC, which is the natural hemolytic agent, is immediately hydrolyzed to the final product (GPC). On the other hand, LPC formed by LDH action was comparatively stable, because the rates of the two reactions catalyzed by LDH, PC to LPC and LPC to GPC, are almost the same. This is the reason that LDH shows hemolytic activity. Therefore, LDH of *V. parahaemolyticus* is an typical phospholipase to be designated as phospholipase A_2 / lysophospholipase.

4. δ-VPH hemolysin

In 1990, Taniguchi *et a.l* (33) cloned a new thermostable hemolysin (δ-VPH) gene from a Kanagawa-negative (KP^-) *Vibrio parahaemolyticus* strain into vector pBR 322 in *Escherichia coli* K12. The nucleotide and amino acid sequences had no homology with those of the thermostable direct hemolysin (TDH) which causes the Kanagawa phenomenon, and of the thermolabile hemolysin (TLH) of *V. parahaemolyticus..* The gene was present in all *V. parahaemolyticus* strains tested and also in one strain of *V. damsela.*

F. Isolation and Identification

The family Vibrionaceae includes the genera, *Vibrio*, *Aeromonas*, *Plesiomonas* and *Photobacterium*. Members of the family are usually found in aquatic environments but can also be isolated from man and other animals in which they may be pathogenic (46). *Vibrio* spp. occur often in association with plankton and their number increase in parallel with seasonal planktonic bloom (83,84,86).

1. Isolation

The recognition of *V. parahaemolyticus* infections as food infections and food poisoning particularly associated with seafoods stimulated the development of selective enrichment media for use in food laboratories. There exploit the ability of *V. parahaemolyticus* to grow at alkaline pH (8.6-9.4), its halophilism (1.7% NaCl) and its resistance to a wide variety of selective agents. These include detergents, such as teepol (0.2-0.4%), sodium dodecylsulphate (SDS; 0.13%), antibiotics, such as colistin (500 iu/ml) and polymyxin B (100-1000 iu/ml), chemotherapeutic agents, such as alkylbenzene sulphonate (0.2%), bile salts such as sodium lauryl sulphate (0.05%) and metachrome yellow II RD (0.125%). Some broths are made differential by exploitation of activities common to *Vibrio* spp. such as gelatin hydrolysis, starch hydrolysis and sucrose fermentation. The need for strongly selective enrichment broths is not generally accepted. There is a consensus that the following enrichment media are effective for the isolation of *V. parahaemolyticus*: APW; glucose salt teepol broth (GSTB); salt polymyxin broth (SPB) or salt colistin broth (SCB). GSTB can be modified by the substitution of SDS for Teepol (86).

After the recognition of *V. parahaemolyticus* as a halophile, it was at first common practice to isolate the organisms on conventional culture media supplemented with 3% NaCl (83,86). Competing microflora, however, obscured *V. parahaemolyticus* colonies on such media. As a consequence, a range of more selective plating media was developed, some of which are summarized in Table 6. Selective agents, such as bile salts, tellurite and teepol, used in enrichment media have also been exploited in plating media. The diagnostic system used for most plating is sucrose/bromthymol blue (86).

The isolation of *V. parahaemolyticus* generally involves a pre-enrichment step, and many such enrichment broths as mentioned previously. Alkaline peptone water has generally been shown to provide superior recovery of *V. parahaemolyticus* from a variety of fish and shellfish, even when the samples have been chilled or frozen (56,125). Furthermore, plating media was importance for recovery *V. parahaemolyticus.*

Thiosulphate Citrate Bile Salts Sucrose (TCBS) is a highly selective differential medium that is widely used not only for *V. cholerae* but all other pathogenic vibrios except *V. hollisae.* Its use was also recommended for the isolation of *V. parahaemolyticus* (86,126). Its selective system consists of ox bile (0.8%), NaCl (1%) and alkaline pH (8.6) which suppress the growth of most interfering organisms

Table 6 Main selective agars (86)

Selective agar	NaCl	pH	Carbohydrate	Selective agents	Indicators
Thiosulphate Citrate Bile Salt Agar (TCBS)	1%	8.6	Sucrose	Thiosulphate[a] Sodium+Ferric Citrate	BTB[b] TB[c]
Monsur's Gelatin Taurocholate Telluric Agar (GTT)	1%	8.5	-	Taurocholate	Tellurite[d]
Polymyxin Mannose Tellurite (PMT)	1%	8.4	Mannose	Polymyxin Tellurite SDS[e]	BTB
SDS Polymyxin Sucrose (SPS)	2%	7.6	Sucrose	SDS Polymyxin CR[f]	BTB
Cellobiose Polymyxin B Colistin (CPC)	2%	7.6	Cellobiose	Colistin Polymyxin	BTB CR
Bromothymol Blue Teepol Agar (BTBT)	4%	7.8	Sucrose	Teepol	BTB TB
Sucrose Tellurite Teepol Agar (STT)	0%	8.0	Sucrose	Teepol Tellutite	BTB
Trypticase Soya Agar Triphenyl tetrazolium (TSAT)	3%	7.1	Sucrose	Bile Salt	TTC[g]
Vibrio vulnificus Agar (VV)	1%	8.6	Salicin	Tellurite Crystal Violet Ox Gall	
Vibrio vulnificus enumeration (VVE)	2%	8.5	Cellobiose Lactose X-Gal	OX Gall Taurocholate[i] Tellurite	X Gal[h]

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[a] Thiosulphate = Sodium thiosulphate, [b] BTB=Bromothymol Blue, [c] TB=Thymol Blue, [d] Tellurite = Potassium tellurite, [e] SDS = Sodium dodecylsulphate, [f] CR=Cresol Red, [g] TTC = Triphenyl tetrazolium chloride, [h] X-Gal = 5 bromo-4-chloroindoxyl-β-D-galactopyranoside, [i] Taurocholate =Sodium taurocholate.

such as Enterobacteriaceae, pseudomonads, aeromonads, and Gram-positive bacteria (86,127). The advantage of TCBS is its sucrose/bromthymol blue diagnostic system which readily distinguishes sucrose-positive vibrios such as *V. cholerae* from other colonies (Table 7). On TCBS agar, *V. parahaemolyticus* forms round bluishgreen colonies 3 to 5 mm in diameter after 18-24 hr incubation at 37°C. Moreover, the 8th edition of the Bacteriological Analytical Manual of the U.S. FDA prescribes a 16 hr enrichment at 35°C to 37°C in alkaline peptone water, from which a loopful is streaked onto TCBS to obtain isolated colonies of *V. parahaemolyticus* (56).

2. Identification

Classic biochemical methods can be used for the identification of *Vibrio* species (Table 8). *V. parahaemolyticus* is characterized primarily by salt requirement. The addition of 1% NaCl to medium without salt is recommended to enhance the positivity of biochemical reactions (18,128). All strains produce catalase, indophenol oxidase but fail to produce hydrogen sulfide in the butt of Kligler's iron agar or triple sugar iron (TSI) agar. They liquefy gelatin, produce indole, lysine and ornithine decarboxylase but not arginine dihydrolase. They give a negative reaction in the Voges-Proskauer and phenylalanine deaminase tests. They generally utilize citrate as a sole carbon source but fail to decompose malonate. They ferment glucose, maltose, mannose, trehalose, and mannitol without gas production but fail to attack lactose, raffinose, rhamnose, sucrose, xylose, adonitol, dulcitol, inositol, sorbitol, and salicin. Fermentation of arabinose varies with different strains. Cellobiose is usually

Table 7 Appearance of *Vibrio* species on TCBS (18)

Organisms	Colony color	Growth and plating efficiency
V. alginolyticus	Yellow	Good
V. carchariae	Yellow	Good
V. cholerae	Yellow	Good
V. cincinnatiensis	Yellow	Very good
V. fluvialis	Yellow	Good
V. furnissii	Yellow	Good
V. metschnikovii	Yellow	May be reduced
V. damsela	Green[a]	Reduced at 36°C
V. hollisae	Green	Very poor
V. mimicus	Green	Good
V. parahaemolyticus	Green[b]	Good
V. vulnificus	Green[c]	Good

[a] 5% Yellow

[b] 1% Yellow

[c] 10% Yellow

Table 8 **Biochemical and other characteristics of 12 *Vibrio* species found in human clinical specimens (18)**

Test^a	% Positive^b											
	1	2	3	4	5	6	7	8	9	10	11	12
Indole production^c (HIB, 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^c (1% NaCl, Barritt)	75	9	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_2S 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^c (1% NaCl)	0	0	60	0	0	95	93	100	0	0	0	0
Lysine, Moeller^c (1% NaCl)	99	100	35	57	0	50	0	0	99	100	99	100
Ornithine, Moeller^c (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^c	100	100	100	100	100	100	100	100	100	100	100	50
D-Glucose, gas production^c	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^c	0	1	0	100	97	0	93	100	1	80	0	0
D-Arabitol^c	0	0	0	0	0	0	65	89	0	0	0	0
Cellobiose^c	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
myo-Inositol	0	0	40	100	0	0	0	0	0	0	0	0
Lactose^c	7	21	50	0	0	0	3	0	0	1	85	0
Maltose^c	99	99	100	100	0	100	100	100	100	99	100	100
D-Mannitol^c	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
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

Table 8 Biochemical 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
Oxidase[c]	100	100	0	100	100	95	100	100	100	100	100	100
Nitrate → nitrite	99	10	0	100	100	100	100	100	100	10	100	100
DNase, 25°C	93	55	50	79	0	75	100	100	95	92	50	100
Lipase (corn oil)[c]	92	17	100	36	0	0	90	89	85	90	92	0
ONPG test[c]	94	90	50	86	0	0	40	35	0	5	75	0
Yellow pigment 25°C	0	0	0	0	0	0	0	0	0	0	0	0
Tyrosine clearing	13	30	5	0	3	0	65	45	70	77	75	0
Growth in nutirent 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

[a] HIB, heart infusion broth; 1% NaCl added to the standard medium to enhance growth; TSI, triple sugar iron agar.

[b] After 48 hr of incubation at 36°C (unless other conditions are indicated). Most positive reaction occur during the first 24hr 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)

[c] Test is recommended as part of the routine set for *Vibrio* identification

[d] Disk potency, 150 µg

1. *V. cholerae*
2. *V. mimicus*
3. *V. metschnikovii*
4. *V. cicinatiensis*
5. *V. hollisae*
6. *V. damsela*
7. *V. fluvialis*
8. *V. furnissii*
9. *V. alginolyticus*
10. *V. parahaemolyticus*
11. *V. vulnificus*
12. *V. carchariae*

not acidified within 24 hr (41). The urease production is based on *V. parahaemolyticus* strain habouring *trh* gene (26). Furthermore, the using eight key differential tests provide a convenient starting point for further species identification (Table 9).

3. Immunodiagnosis

Several method have been reported for the rapid detection of *V. parahaemolyticus* such as a modified Elek test (22), reversed passive hemagglutination assay (129), fluorogenic assay base on trypsin-like activity (130) and latex agglutination assay (131). Enzyme-linked immunosorbent assay (ELISA) was developed to detect TDH of *V. parahaemolyticus* (132). The result showed high sensitivity but some disadvantage of this method due to the anti-TDH-alkaline phosphatase conjugate must be prepared in the laboratory. Later, in 1995, Chen *et al.* (133) developed an ELISA for detection of two outer membrane protein of *V. parahaemolyticus*. The detection limit of this assay was 10 ng/ml and need for 18 hr enrichment step.

4. DNA hydridization

An assay based on the DNA probe and DNA hybridization were developed to detect *tdh*, *trh* and other species specific gene of *V. parahaemolyticus*. Nishibuchi *et al.* (23) used the DNA colony hybridization method to examine the presence or absence of the *tdh* or related DNA sequences in *V. parahaemolyticus* and other *Vibrio*. The result showed that all KP^+ strains were *tdh* gene positive and 16% of KP^- strains also reacted with the gene probe labeled with ^{32}P. Subsequently, Nishibuchi *et al.* (24)

Table 9 **Eight key differential tests for dividing the 12 clinically significant *Vibrio* species into six groups (18)**

Biochemical tests	Reaction of species in[a] :											
	Group 1		Group 2	Group 3	Group4	Group 5			Group 6			
	VC[b]	VM[c]	VME[d]	VCI[e]	VH[f]	VD[g]	VFL[h]	VFU[i]	VA[j]	VP[k]	VV[l]	VCA[m]
Growth in nutrient Broth :												
With no NaCl added	+	+	-	-	-	-	-	-	-	-	-	-
With 1% NaCl added	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase			-	+	+	+	+	+	+	+	+	+
Nitrate → nitrite		.	-	+	+	+	+	+	+	+	+	+
myo-Inositol Fermentation	-	-	V	+	-	-	-	-	-	-	-	-
Arginine dihydrolase					-	+	+	+	-	-	-	-
Lysine decarboxylase					-				+	+	+	+
Ornithine decarboxylase					-							

[a] Key test results are boxed . All data are for reactions within 2 days at 37°C unless otherwise specified. Symbols, +, most strains (generally about 90 to 100%) positive -, most strains negative (generally about 0 to 10% positive); V. strain-to-strain variation.
[b]=*V.cholerae*, [c]= *V. mimicus*, [d] =*V.metschnikovii*, [e] =*V. cincinnatiensis*, [f]=*V. hollisae*, [g]=*V. damsela*, [h]=*V. fluvialis*, [i]=*V. furnissii*, [j]=*V. alginolyticus*, [k]=*V. parahaemolyticus*, [l]=*V. vulnificus*, [m]=*V. carchariae*

used synthetic oligodeoxyribonucleotide probe (19 to 28 base long) labeled with ^{32}P as hybridization probe in the DNA colony hybridization technique to examine the association between KP and *tdh* gene detection. This method could distinguish potentially significant mutations in the *tdh* gene and was specific for KP^+ strains. Furthermore, it was more suitable for definitive determination than hybridization with the longer gene probe. However, the DNA hybridization probe had some limitation due to radioisotope labeling which have several disadvantage in routine use such as the short halflife and difficult to handle (25). Later, the alkaline phosphatase conjugated oligonucleotide probe was developed to detect the *tdh* and *trh* gene of *V. parahaemolyticus*. The results showed that *tdh* probe was high specificity and sensitivity in 100 and 93%, respectively, and those of *trh* probe was 93 and 86%, respectively. In Thailand, Oon-ob (134) used *tdh* and *trh* probe labeled with digoxigenin for rapid detection of *tdh* and *trh* gene directly from crude DNA extracted from stool specimens. The sensitivity and specificity of this study were both 100%. Other studies by using hemolysin gene probe or recombinant plasmid pR72H probe were developed for detection of *V. parahaemolyticus* (8,135). However, these DNA hybridization had limitation due to it required about 10^6 colony forming units to yield a positive signal (135,136).

5. Polymerase chain reaction (PCR)

PCR is used increasingly in research in food microbiology. By this method, a specific DNA-fragment is amplified during a cyclic three step process : (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 is repeated over and over with newly synthesized DNA as template in additional to the original target DNA, an exponential amplification of the DNA-fragment between the two oligonucleotides is obtained. Since the inclusion of a thermostable DNA-polymerase from *Thermus aquaticus*, PCR can be performed in a closed tube using a simple thermo-cycler.

An important factor in evaluating any DNA–based test is the specificity of the DNA sequences chosen for the gene and strains of interest. Size of the amplicon is often used as evidence that the PCR is positive. However, the identify of the amplified fragment should be confirmed by hybridization to a specific DNA probe, digestion with a restriction enzyme or DNA sequencing of the DNA fragment, as some oligonucleotides prime the formation of DNA-fragment of several sizes and base composition (136).

The widespread use of the PCR technique has been applied to detect hemolysin gene both *tdh* and *trh* of *V. parahaemolyticus* in clinical and environmental samples. In 1992, Toda *et al.* (137) established PCR protocols for specific detection of *tdh* and *trh* genes of *V. parahaemolyticus.* These protocols could detect 400 fg (100 cells) of cellular DNA carrying the respective gene. Furthermore, they were also spike experiments in faecal samples. 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 extraction or enrichment of the faecal sample in alkaline peptone water for 4 hr before using PCR for directly detecting of faecal samples. Later, Lee *et al.* (138) designed olignucleotide primers to detect *tdh* target gene in stool samples by PCR technique. The detection limit for the *tdh* gene by PCR amplification was 40 pg of total DNA, or broth culture containing 1000 viable cells. In 1995, Suthienkul *et al.* (26) used PCR for detection of *tdh* and *trh* genes of *V. parahaemolyticus* isolated from the diarrheal patients. Eighty-one percent of the isolates were positive for *tdh*, only 6% had both *tdh* and *trh* , and 2% had the *trh* gene.

Subsequently, PCR protocol was performed for detection of *tdh* gene of *V. parahaemolyticus* in artificially contaminated seafood. The sensitivity of the assay could be improved to detect less than 10 cells of *V. parahaemolyticus* by performing PCR after 8 hr enrichment in alkaline peptone water (27).

In Thailand, Pumiprapat (11) used PCR technique for detection of *V. parahaemolyticus* strains isolated from 200 raw seafood samples. The results showed that 2 samples (1%) were positive for *tdh* and *trh* gene. The using of digoxigenin labeled PCR product probes were performed to detect *tdh* and *trh* directly from frozen shrimp samples by Kowcachaporn (28). It was found that 13.5% of *tdh* positive from direct samples were positive increasingly to 31.5% when using the enriched samples. Another species specific gene fragments were developed for detection of *V. parahaemolyticus* from food and environmental samples. For examples, pR72H fragment was developed for detection of *V. parahaemolyticus* in shellfish by PCR (139). The sensitivity of PCR detection for a pure culture of *V. parahaemolyticus* and purified chromosomal DNA were 10 cells and 2.6 fg, respectively. Later, Kachornchaiyakul (34) investigated the *ldh* gene of *V. parahaemolyticus* in frozen shrimp by PCR and DNA hybridization with digoxigenin label probes. By PCR technique, all samples were negative results but positive results were increased to 68.5% of enrichment samples. The detection limit of genomic DNA of *V. parahaemolyticus* was 122 fg corresponding to 175 cells. In 1998, Venkateswaran *et al.* (140) employed the gyrase B gene (*gyr B*) as a molecular diagnostic probe to examine *V. parahaemolyticus* in shrimp by PCR technique. The lower detection limit was 5 CFU for live cells, and 4 pg for purified DNA and initial inoculum of *V. parahaemolyticus* was 1.5 CFU/g of shrimp homogenate after 18 hr enriched step.

6. Nested PCR

Nested PCR primers are ones that are internal to the first primer pair. The larger fragment produced by the first round of PCR is used as the template for the second PCR. Nested PCR can also be performed with one of the first primer pair and a single nested primer. The sensitivity and specificity of both DNA and RNA amplification can be dramatically increased by using the nested PCR method. The specificity is particularly enhanced because this technique almost always eliminates any spurious nonspecific amplification products. This is because after the first round of PCR any nonspecific products are unlikely to be sufficiently complementary to the nested primers to be able to serve as a template for further amplification, thus the desired target sequence is preferentially amplified. However, the increased risk of contamination is a drawback of this extreme sensitivity, and great care must be taken when performing such PCRs, particularly in a diagnostic laboratory (141). In order to enhance the sensitivity and specificity of PCR, nested PCR was develop for this purpose. Several investigators reported nested PCR technique for rapid detection of bacterial pathogens both clinical and environmental samples such as *Listeria monocytogenes, Vibrio vulnificus, Escherichia coli, Neisseria gonorrhoeae* and *Campylobacter jejuni* (37,38,142-144). In the other hand, the detection of *V. parahaemolyticus* by using nested PCR technique have not been reported.

In clinical samples, nested PCR assay was developed for rapid detection of *N. gonorrhoeae* from air-dried gentital samples. The optimized assay had a detection limit of less than 0.3 cell. The nested PCR assay provided high degrees of both sensitivity and specificity and could apply to air-dried genital samples, permitting the detection of DNA after long-term storage at ambient temperature (144). In 1998, Rappelli *et al.* (145) reported the development of a nested PCR-assay for the detection of *Cryptococcus neoformans* in cerebrospinal fluid. These results demonstrate that the nested PCR is a sensitive, specific, and reproducible technique. It gave positive and negative results which correlated with conventional technique. Subsequently, Lee *et al.* (39) established a nested PCR protocol for direct identification of *V. vulnificus* in clinical specimens. The nested PCR method could detect as little as 1 fg of chromosomal DNA and single CFU of *V. vulnificus*. The nested PCR was applied to examine a total of 39 serum specimens and bulla aspirates from septicemia patients. Seventeen (94.4%) of the 18 *V. vulnificus* culture-positive specimens were positive by the nested PCR. Eight (42.1%) of the 19 culture-negative samples gave positive nested PCR results.

The development of nested PCR assay was proposed for direct detection of foodborne pathogens in food and environmental samples. In 1995, Herman *et al.* (142) reported that a method for direct detection of *L. monocytogenes* in 25 ml of raw milk is presented. The detection limit can be situated between 10 and 5 CFU. The detection method is based on chemical extraction of the milk components and PCR amplification with two nested pairs of primers specific for *L. monocytogenes.* The nested PCR was also reported to detect *V. vulnificus* in fish farms by using universal primers flanking the *V. vulnificus* specific sequences directed against 23 S rRNA genes. This specific assay detected 10 fg of DNA or 12 to 120 cells in artificially inoculated samples without enrichment and within 24 hr (37). Furthermore, nested PCR was applied to detect *E. coli* in potable water by filter concentration. This protocol can detect 1-10 bacterial cells/50 ml water sample within 6-8 hr (143). Later, nested PCR was developed for direct detection of *C. jejuni* in chicken washes. This sensitive nested PCR assay, could detect 10^2 CFU/ml of *C. jejuni* cells and also performed within 1 day (38).

In 1999, Lee *et al.* (146) established two stage nested PCR for direct detection of *V. vulnificus* in natural samples. The nested PCR amplification, coupled with direct extraction of template DNA, revealed improved sensitivity sufficient for detection of 1 to 10 CFU in 1ml of seafood homogenates, eliminated the need for enrichment culturing, and could report the result within 10 hr.

Nested PCR assay is not only rapid, taking less than 1 day to identify bacterial pathogens, but also specific and sensitive. Therefore, the nested PCR assay would be alternative approach to the detection of foodborne pathogens especially *V. parahaemolyticus* in contaminated food or frozen food products.

CHAPTER III

MATERIALS AND METHODS

A. Samples

The cross-sectional study was to carry out in frozen shrimp samples which were obtained from the frozen food factory located in Chacheongsao province, from April to May, 1999. The sample size was calculated by the formula: $n = Z^2\alpha/_2\, PQ\, /d^2$ (147) by using 64 % prevalence of contamination rate of *V. parahaemolyticus* that reported in the study of Kowcachaporn (28). A total of 103 frozen shrimp samples were used in this study and categorized into two groups as follow :

1. The finished products were frozen shrimp samples passed the process and were also divided into two types : the first type was peeled frozen shrimp, divided with or without tail; the second type was unpeeled frozen shrimp, head-on and/or headless.
2. The raw materials were the frozen shrimp which were not passed the process and were also divided into two types similar to those of the finished products.

All samples were transported to the laboratory immediately in an ice box and kept at -20°C until tested.

B. Study Location

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

C. Bacterial Strains

In order to determine the sensitivity and specificity of primary PCR and nested PCR; the reference, clinical and environmental strains of *V. parahaemolyticus, Vibrio* spp. and enteric bacteria were included in this study. *V. parahaemolyticus* strains; containing BG 26 (*tdh*$^{-}$*trh*$^{-}$), AQ 4613 (*tdh*$^{+}$) and AQ 4023 (*trh*$^{+}$) provided from Dr. Masanori Ishibashi, Department of Food Microbiology, Osaka Prefectural Institute for Public Health, Osaka, Japan were used as controls for sensitivity and specificity of primary PCR and nested PCR. *V. cholerae* 01 El Tor Ogawa AQ 1034 (*ct*$^{+}$), *V. cholerae* 01 EL Tor AQ 1002 (*ct*$^{-}$), *V. vulnificus* 2219009 and *Salmonella typhimurium* RIMD 1980051 were kindly provided from Professor Dr. Takeshi Honda, Research Institute for Microbial Diseases, Osaka University, Japan. *V. parahaemolyticus* strains each harbouring *tdh*$^{+}$, *trh*$^{+}$, *tdh*$^{+}$*trh*$^{+}$ from clinical isolates, and *V. hollisae, V. mimicus* and *V. alginolyticus* from environmental isolates were provided from Associate Professor Dr. Orasa Suthienkul, Department of Microbiology, Faculty of Public Health, Mahidol University.

Other *Vibrio* spp., *Aeromonas* spp. and enteric bacteria such as *V. cholerae* O139, *V. cholerae* non O1, *Aeromonas hydrophila, E. coli, Shigella* spp. and

Salmonella were obtained from the Bamrasnaradura Infectious Diseases Hospital, Nonthaburi and the WHO National Salmonella and Shigella Typing Center, Division of Clinical Pathology, National Institute of Health, Nonthaburi, Thailand (Table 10).

D. Development of Primary PCR and Nested PCR

1. Reagents

Buffers and reagents were mentioned in Appendix A.

2. Enumeration and genomic DNA extraction of *Vibrio parahaemolyticus* reference strain

V. parahaemolyticus reference strain BG 26 (*tdh*$^-$ *trh*$^-$) was grown in 1.5 ml of LB broth with 3% NaCl. After incubation with shaking at 37°C for 3 hr, the bacterial suspensions of *V. parahaemolyticus* were measured to give on optical density of 0.8 at the wavelength of 600 nm in a spectrophotometer (Spectronic 3000 Array, Milton Roy, Rochester, NY, USA). These bacterial suspensions were enumerated with plate count agar containing 1% NaCl by using spread plate method. Viable counts of the reference strain was calculated by CFU/ml.

The 1.5 ml of bacterial culture (OD=0.8) in a microcentrifuge tube were used for genomic DNA extraction by the following procedure of Maniatis *et al.*(148). The cell pellets were harvested by centrifugation (Himag Centrifuge, Hitachi Kiko, Tokyo, Japan) at 12,000 rpm for 1 min and resuspended in 582 ul of TE buffer. The cell pellet was digested with 15 ul of 20% SDS (Amersco, Solon, Ohio, USA) and 3

Table 10 Bacterial strains used for sensitivity and specificity test of primary PCR and nested PCR

Type of bacteria	Strains/source	No. of isolates
Vibrio parahaemolyticus BG 26 (*tdh*$^-$*trh*$^-$)	Reference/OPIPH[a]	1
Vibrio parahaemolyticus AQ 4613 (*tdh*$^+$)	Reference/OPIPH	1
Vibrio parahaemolyticus AQ 4023 (*trh*$^+$)	Reference/OPIPH	1
Vibrio cholerae AQ 1034 (*ct*$^+$)	Reference/RIMD[b]	1
Vibrio cholerae AQ 1002 (*ct*$^-$)	Reference/RIMD	1
Salmonella typhimurium 1980051	Reference/RIMD	1
Vibrio vulnificus 2219009	Reference/RIMD	1
Vibrio parahaemolyticus (*tdh*$^+$)	Clinical isolates/ PHM[c]	2
Vibrio parahaemolyticus (*tdh*$^+$*trh*$^+$)	Clinical isolates/ PHM	2
Vibrio parahaemolyticus (*trh*$^+$)	Clinical isolates/PHM	1
Vibrio cholerae O 139	Clinical isolates/BIDH[d]	1
Vibrio cholerae O1 Eltor Ogawa	Clinical isolates/BIDH	1
Vibrio cholerae non O1	Clinical isolates/BIDH	1
Vibrio fluvialis	Clinical isolates/BIDH	1
Aeromonas hydrophila	Clinical isolates/BIDH	1
Aeromona sobria	Clinical isolates/BIDH	1
Plesiomonas shigelloides	Clinical isolates/BIDH	1
Escherichia coli	Clinical isolates/BIDH	3
Shigella spp.	Clinical isolates/NIH[e]	2
Salmonella	Clinical isolates/NIH	2
Vibrio hollisae	Environmental isolates/OPIPH	1
Vibrio mimicus	Environmental isolates/PHM	1
Vibrio alginolyticus	Environmental isolates/PHM	1

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[a] Osaka Prefectural Institute for Public Health, Osaka, Japan.

[b] Research Institute for Microbial Diseases, Osaka University, Japan.

[c] Faculty of Public Health, Mahidol University, Bangkok, Thailand.

[d] Bamrasnaradura Infectious Diseases Hospital, Nonthaburi, Thailand.

[e] The WHO National *Salmonella* and *Shigella* Typing Center, Division of Clinical Pathology, National Institute of Health, Nonthaburi, Thailand.

ul of 20 mg/ml proteinase K (Amersco, Solon, Ohio, USA), mixed thoroughly and incubated at 37°C for 1 hr or more. Ten microliters of 10 mg/ml RNase A (Pharmacia Biotech, USA) was added and incubated at 37°C for 1 hr. Then, 100 ul of 5 M NaCl (Merck, Darmstadt, Germany) and 80 ul of 10% hexadecyltrimethyl ammonium bromide (CTAB, Merck, Darmstadt, Germany) in 0.7 M NaCl solution was added, mixed thoroughly and incubated at 65°C for 10 min. An equal volume of chloroform : isoamyl alcohol (Merck, Darmstadt, Germany) (24:1) was added, mixed thoroughly for 5 min by inverting the tube, spin for 5 min and the upper phase was drained into a new microcentrifuge tube. Each 300 ul of phenol (Ameresco, Solon, Ohio, USA) and chloroform (Merck, Darmstadt, Germany) was added in a microcentrifuge tube, mixed thoroughly and centrifuged for 5 min. The upper phase was transferred to a new microcentrifuge tube. The phenol and chloroform extraction step was repeated twice. Then, 600 ul of chloroform was added, mixed thoroughly and then centrifugated again. The upper phase was transferred into a new microcentrifuge tube. The genomic DNA was precipitated with 0.6 volume of isopropanol (Merck, Darmstadt, Germany), mixed by rotating and inverting the tube until a stringy white DNA precipitate became clearly visible. After centrifugation at 15,000 rpm for 5 min, the supernate was discarded. DNA pellet was then washed twice with 1 ml of 70% ethanol. The DNA pellet was dried in a vacuum desiccator (Bel-Art Product, New Jersey, USA) and then resuspensed the pellet in 100 μl of TE buffer. DNA concentration was determined by measuring the optical density (OD) at the wavelength of 260 nm by a spectrophotometer (Spectronic, 3000 Array, Millton Roy, Rochester, NY, USA). One OD value of extracted DNA was equaled 50 μg/ml of double-strand DNA. Additionally, the purity was determined by calculating the ratio of OD at the wavelength of 260/280 nm. The ratio that more than 1.8, the extracted DNA was pure DNA; In contrast, the ratio that less than 1.6, the extracted DNA was contaminated with protein, phenol or other organic compounds (149). The DNA was kept at –20°C as DNA stock, and the quality of the extracted DNA was analyzed by using 0.8% gel electrophoresis.

3. Nested *ldh* primers design

The design *ldh* primers were derived from the published sequence of Taniguchi *et al.* (32) which was 1581 bp in length (Fig 1). The sequence of primary PCR primers and nested PCR primers were designated as LD_1-LD_2 and LD_3-LD_4, respectively. Primary PCR primers (LD_1-LD_2) were used in the study of Kachornchaiyakul (34) and produced amplified products of 372 bp derived from the nucleotide sequence starting at the position 283 to 654. The nested or internal primers (LD_3-LD_4,) were designed by using free software program of Williamstone Enterprises; http:// www. Williamstone. com/ primers/ nojavascript/ primers. cgi (150).

The nested PCR primers were 22 bp each in length; melting temperature was 72°C, 68°C;GC content was 63.6%, 54.5%, respectively. The nested primers produced specific amplified products of 196 bp from the nucleotide sequence starting at the position 357 to 552. Both primary PCR primers were synthesized by Bioservice Unit (BSU), National Center of Genetic Engineering and Biotechnology, National Science and Technology Development Agency (NSTDA), Ministry of Science, Technology

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   1   tcgactgtct   ggagtattta   ctcagaatta   gagggtttag   tgagaaaaat   tctcatctaa
  61   acaatgttat   agccaagtat   tttttcaatg   tgcttgggtc   aataaccatt   acaagaagag
 121   tgaatgatga   aaaaaacaat   cacactatta   actgcattac   tcccgcttgc   ttctgcagtt
 181   gccgaagagc   caaccttatc   accagaaatg   gtttcagcgt   ctgaagtgat   cagcacgcaa
 241   gaaaaccaaa   cctataccta   tgttcgctgt   tggtatcgca   ccagctactc   gaaagatgat
 301   ccagcgaccg   attgggaatg   ggcaaaaaac   gaagatggta   gctacttcac   cattgacggc
 361   tactggtgga   gctccgtttc   atttaaaaac   atgttctaca   ccaacacgtc   gcaaaacgtt
 421   atccgtcagc   gttgtgaagc   aacattagat   ttggcgaacg   agaacgcaga   cattacgttc
 481   ttcgccgctg   acaatcgctt   ctcatacaac   cacacgatct   ggagcaacga   cgcagcaatg
 541   cagccagatc   aaatcaacaa   agtggttgca   ctcggtgaca   gcttgtctga   tacaggcaac
 601   atctttaacg   catcacaatg   gcgcttccct   aacccgaaca   gctggttctt   aggtcacttc
 661   tccaacggtt   ttgtgtggac   agaatacatt   gccaaagcga   agaaccttcc   gctctacaac
 721   tgggcagttg   gcggcgcggc   tggtgagaac   caatacatcg   cgctaacagg   ggttggtgat
 781   caagtttctt   cgtacttaac   ctacgcaaaa   ctggcgaaga   actacaaacc   agcaaacacc
 841   ttgtttacgc   ttgagtttgg   tttgaatgac   ttcatgaact   acaaccgtgg   cgttccagaa
 901   gtgaaagcgg   attatgcaga   agcactgatt   cgtttgacgg   acgcaggtgc   gaagaacttc
 961   atgttgatga   cactgccaga   tgcgacgaaa   gcgcctcagt   ttaagtactc   aacacaagaa
1021   gagatcgaca   aaattcgtgc   gaaagtgctt   gagatgaacg   agttcatcaa   ggcacaagcg
1081   atgtactaca   aagcgcaagg   ttacaacatc   acgttgtttg   atactcacgc   cttgttcgag
1141   acgctaactt   ctgcgcccga   agagcacggt   ttcgtgaacg   cgagcgatcc   ttgtttggac
1201   atcaaccgct   catcgtctgt   cgattacatg   tacacccacg   cattgcgctc   tgagtgtgca
1261   gcgtctggtg   ctgagaagtt   tgtgttctgg   aatgtcacgc   atccaacaac   agcaactcac
1321   cgctatgttg   cagagaaaat   gctagaaagt   agcaacaact   tagccgagta   ccgtttctaa
1381   ccggacacgg   cttctgagtt   gaaaccttat   cttcgtacac   acgttgataa   cgaacacatc
1441   gtggccattt   ttatcgaagg   aacgttgtgg   tcacagcagt   cacaacgcta   aacaagttac
1501   agtggcgcga   cgtcggttcc   ccctaaagac   tggtaaagcg   taacctgagt   gagaaactgg
1561   ttgtagcggt   tctccaacaa   agaagc
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Fig. 1 The nucleotide sequence of lecithin dependent hemolysin *(ldh)* of *V. paarahaemolyticus* derived from Taniguchi *et al.* (32).

and Environment, Bangkok. These primers used in this study are shown in Table 11.

4. Optimization of PCR

4.1 Primary PCR

The optimal PCR condition was set up from varying several parameters such as dNTP, magnesium chloride, primers, and *Taq* DNA polymerase (151,152). Annealing temperature was calculated from melting temperature (Tm) of *ldh* oligonucleotide primers which was 72°C. The genomic DNA of *V. parahaemolyticus* BG 26 (*tdh*$^-$*trh*$^-$) was varied from 10 ng/µl to 0.01 fg/µl and used as the DNA template for the optimization of PCR condition in the final volume of 25 µl. The constituents of PCR mixture were adjusted by varying the concentration of each ingredient as follow :

Annealing temperature was varied from 54°, 60°, 65° and 70°C to select the best annealing temperature which gave high yield, bright clearly visible band of correct size and non-specific reaction did not occur. Concentration of magnesium chloride (Merck, Darmstadt, Germany) was varied from 1.0, 1.5, 2.0, 2.5 and 3.0 mM. Concentration of deoxynucleotide triphosphate (dNTPs) (Promega, Madison, WI, USA) was also varied from 100, 150, 200, 250 and 300 µM. Additionally, *Taq* DNA polymerase (Pharmacia Biotech, USA) was diluted to amount of 0.5, 1.0, 1.5 and 2.0 units. Concentration of primary PCR primers was also varied from 3, 5, 7.5, 10 and 20 pmole in the final volume of 25 µl PCR mixture.

Table 11 Oligonucleotide sequences of primary PCR and nested PCR primers for *ldh* gene

Primers set/name	Primers sequence 5' to 3'	Position	Products (bp)
Primary PCR primers[a]			
LD_1	AGC TAC TCG AAA GAT GAT CCA GCG A	283-307	372
LD_2	ACC TAA GAA CCA GCA GTT CGG GT	635-654	
Nested PCR primers			
LD_3	CGG CTA CTG GTG GAG CTC CGT T	357-378	196
LD_4	TTG ATC TGG CTG CAT TGC TGC G	531-552	

[a] From Kachornchaiyakul (34).

All the concentration of substances as mentioned above was added in the final volume of 25 µl PCR mixture containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl and 0.1% Triton-X. After that, a drop of mineral oil (Sigma, ST. Louis, MO, USA) was overlaid to avoid evaporation during thermal cycling. The primary PCR amplifications were carried out in a DNA thermal cycler (Perkin Elmer 480, Perkin Elmer Corporation, Norwalk, CT, USA). The pre-PCR cycle for denaturation was done at 94°C for 5 min; annealing temperature was selected from the results of the optimization determination for 1 min; and extension temperature was done at 72°C for 1 min. Then, 30 cycles of amplification target sequences were conducted in the same temperatures of pre-PCR each for 1 min, including a final extension at 72°C for 10 min.

4.2 Nested PCR

One microliter of primary PCR products obtained from the amplification using primary PCR primers at 3, 5, 10 pmole and each concentration of genomic DNA of *V. parahaemolyticus* BG 26 varied from 10 ng/µl to 0.01 fg/µl were added to new tubes of reaction mixture. These reaction mixtures were contained 10 pmole/reaction of nested primers concentration. The optimal condition used for nested PCR was followed by the same condition as mentioned in the primary PCR section D-4.1.

The result of nested PCR products gave high sensitivity, bright clearly specific band of 196 bp and non-specific did not occur, was selected as an optimal condition for the detection of *ldh* of *V. parahaemolyticus* in direct frozen shrimp samples.

5. Sensitivity of PCR

5.1 Primary PCR

The serial ten-fold dilution of genomic DNA of *V. parahaemolyticus* BG 26 (*tdh*$^-$*trh*$^-$) was performed in nuclease free distilled water starting from the concentration of 10 ng/µl to 0.01 fg/µl. One microliter of each DNA concentration was used as the DNA template. The amplification condition for primary primers was performed as mentioned previously. After amplification, PCR products were analyzed by using gel electrophoresis.

5.2 Nested PCR

In order to perform the sensitivity of nested PCR, one microliter of each DNA concentration from primary PCR was used as template in nested PCR. Amplification condition was carried out as described by optimization of nested PCR. Electrophoresis and visualization were performed in the same manner as previously described in section D-7.

6. Determination of specificity of nested PCR

The specificity of nested PCR for amplification of *ldh* was determined by using the crude lysate cells of 29 totally bacterial strains including *V. cholerae* O1, *V. cholerae* non O1, *V. cholerae* O139, *V. vulnificus*, *V. fluvialis*, *V. hollisae*, *V. mimicus*, *V. alginolyticus*, *Aeromonas* spp., Enteropathogenic *E. coli*, *Shigella* spp. and *Salmonella*. These bacterial strains are shown in Table 10. One microliter of crude lysate cell DNA was used as the DNA template, and amplification condition was performed as mentioned previously in section D-5.2.

7. Electrophoresis

The quality of the extracted DNA or PCR products were analyzed by gel elctrophoresis (Mupid , Tokyo, Japan). Then, 10 µl of extracted DNA or PCR products with 2 µl of loading dye were electrophoresed on a 0.8 % and a 1.5% agarose gel (Seakem LE, FMC Bioproducts, Rockland, ME, USA.), respectively. Electrophoresis was performed at the constant 100 voltage for 30 min. Ten microliter of 100 ng of DNA molecular weight (Boehringer, Mannheim, Germany) and 50 ng of 100 bp DNA Ladder (Pharmacia Biotech, USA) were used as a standard size markers for extracted DNA and PCR products, respectively. After electrophoresis, the gel was removed, then stained with 0.5 µg/ml ethidium bromide solution (Merck, Darmstadt, Germany) for 10-15 min and destained in distilled water for 30 min before visualization under an UV transilluminator (Fotodyne, Hartland, WI, USA).

8. Control procedure

To minimize the false positivity and carry over contamination, sample preparation, pre- PCR preparation, thermal cycling, and post PCR processing were done in separated rooms and separated tools as recommended by standard protocols. At every step of the processing, aeroseal advantage filter pipette tips (Scientific^{TM}/USA) were used. False positivity was strictly excluded by employing negative control at every step (151,153). Genomic DNA of *V. parahaemolyticus* BG26 (*ldh*$^+$,*tdh*$^-$*trh*$^-$) and *V. cholerae* AQ 1034 (*ldh*$^-$,*ct*$^+$) (10 pg/reaction) were used as positive and negative DNA controls, respectively. In addition, sterile distilled water was used as a negative reagent control (no DNA template) in each PCR experiment.

E. Application of the Nested PCR to Detect *ldh* of *Vibrio parahaemolyticus* in Frozen Shrimp Samples

1. Bacterial isolation from frozen shrimp samples

1.1 Media

Media and biochemical tests are mentioned in Appendix A

1.2 Preparation of samples

One hundred and three frozen shrimp samples were obtained from frozen food factory as indicated in section A. To ensure a more even distribution, 100 g of frozen shrimp samples were collected from different parts of the samples, then cut into small pieces under sterile condition. Four hundred milliliters of 1% NaCl were added to frozen shrimp samples, mixed by shaking in a large mouth bottle with a screw cap (1:5 dilution) (154). One hundred milliliters of frozen shrimp samples were mixed again with an equal volume of 1% NaCl solution to finally make 1:10 dilution and followed by performing serial ten-fold dilution for enumeration of presumptive *V. parahaemolyticus* by direct method and enrichment method as the following (Fig 2).

1.3 Direct method

One hundred microliters of each appropriate dilution were spread on PCA, m-FC, MC (Difco, Detroit, USA) and TCBS agar (Merck, Darmstdt, Germany) for enumerating total viable count, coliforms count, *E. coli* count and *V. parahaemolyticus* count, respectively. All agar plates were incubated at 37°C for 18-24 hr except MC incubated at 44°C overnight. The suspected colonies of *V. parahaemolyticus* were bluishgreen colonies, round, 2-3 mm in diameter with a dark green center on TCBS agar. These colonies were enumerated as presumptive *V. parahaemolyticus* count. For presumptive *E. coli* count was enumerated from suspected pink with precipitated zone around colonies on MC agar. Both presumptive *V. parahaemolyticus* and *E. coli* were further identified as described by McLaughlin (18).

1.4 Enrichment method

One hundred milliliters of the same homogenized samples were added in an equal volume of 2X alkaline peptone water (APW) and incubated at 37°C for 18-

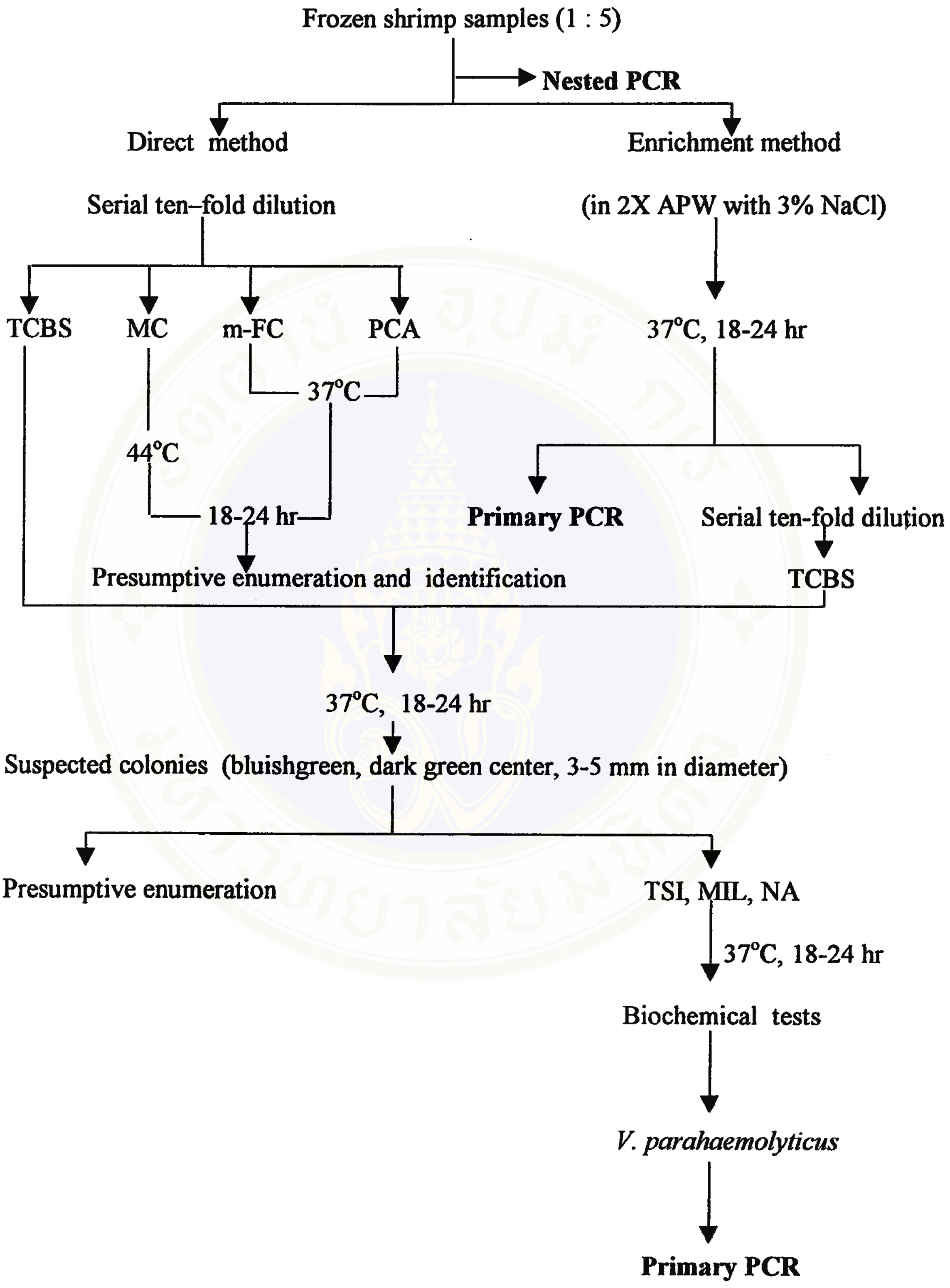

Fig 2. Isolation and identification of *Vibrio parahaemolyticus* in frozen shrimp samples.

The enriched sample was cultured on TCBS agar by spread plate technique and incubated at 37°C, overnight. The suspected colonies of *V. parahaemolyticus* were enumerated and further identified as described by McLaughlin (18).

1.5 Identification

At least 5 suspected colonies of *V. parahaemolyticus* were picked and inoculated on triple sugar iron (TSI) agar (Difco, Detroit, USA), motility indole lysine (MIL) medium (Difco, Detroit, USA) and nutrient agar (NA) (Difco, Detroit, USA). After incubation at 37°C for overnight, the typical reaction on TSI of *V. parahaemolyticus* was acid (yellow) in butt and alkaline (red) in slant, no production of hydrogen sulfide (H_2S) and gas. In MIL medium, *V. parahaemolyticus* showed positive result of motility, indole and lysine reaction. Furthermore, they also showed positive reaction for oxidase test by using colonies from NA. The other biochemical tests of *V. parahaemolyticus* were further examined as described by McLaughlin (18).

2. Preparation of DNA template from frozen shrimp samples

One milliliter of all direct and/or enrichment frozen shrimp samples was centrifuged at 15,000 rpm for 5 min and discarded the supernatant. The cell pellet was resuspended in 0.5 ml of 6% Chelex-100 solution and incubated at 56°C for 30 min. Then, the suspension was boiled at 100°C for 10 min and centrifuged at 12,000 rpm for 3 min (Fig 3). The supernatant was collected, stored at –20°C and used as DNA template for PCR and nested PCR.

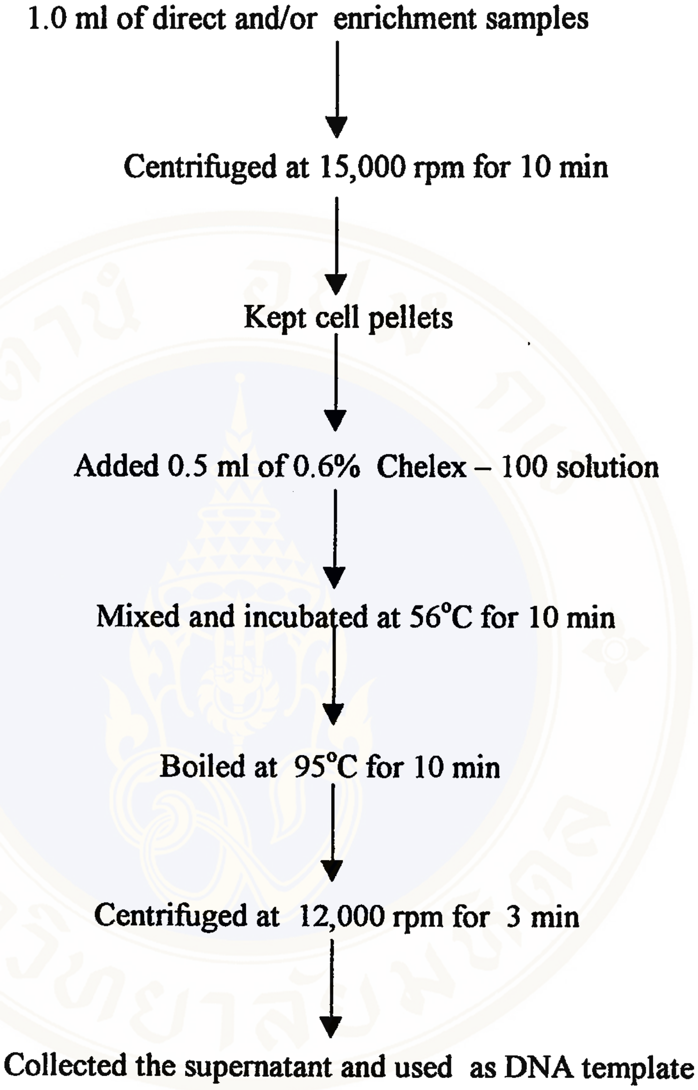

Fig 3. Preparation of frozen shrimp samples for primary PCR and nested PCR assay.

3. Direct detection of *ldh* gene from frozen shrimp samples by nested PCR

One microliter of extracted direct frozen shrimp samples prepared as mentioned in section E-2 was used as DNA template and the amplification condition was used the same protocol as mentioned in section D-4. The nested PCR products were further analyzed by agarose gel electrophoresis as mentioned previously in section D-7.

4. Detection of *ldh* gene from *Vibrio parahaemolyticus* isolated by primary PCR

All *V. parahaemolyticus* strains isolated from frozen shrimp samples were subcultured on Mueller Hinton agar with 3% NaCl. After overnight incubation at 37°C, 5 colonies were picked from individual samples and resuspened in 200 µl of TE buffer. Then, the bacterial cell suspensions were lyzed by heating at 95°C for 10 min and kept immediately on an ice box. The supernatant was collected by centrifugation at 12,000 rpm for 3 min and used as the DNA template for primary PCR. The amplification conditions for PCR were performed as mentioned in section D-4. The analysis of primary PCR products was conducted by agarose gel electrophoresis as mentioned previously in section D-7.

F. Data Analysis

All bacterial count value was transformed to base 10 logarithms and average value of bacteria was presented by antilog of mean (geometric mean). The contamination rate of *V. parahaemolyticus* in frozen shrimp samples was analyzed by percentage. The McNemar Chi-square test was used for analysis of association of *V. parahaemolyticus* detection between nested PCR and enrichment culture as gold standard (155). The agreement rate to detect *V. parahaemolyticus* from frozen shrimp samples between nested PCR and primary PCR was analyzed by kappa measure of agreement (156). The McNemar Chi-square test and kappa measure of agreement were determined by using SPSS version 7.5 program. P-value <0.05 was used for determining the significant difference.

CHAPTER IV

RESULTS

A. Polymerase Chain Reaction

1. Enumeration and genomic DNA extraction

Enumeration and genomic DNA extraction were determined by testing with reference bacterial strains including *V. parahaemolyticus* BG 26 (*tdh*$^-$*trh*$^-$), *V. cholerae* AQ 1034 (*ct*$^+$). The enumeration of viable count and concentration of genomic DNA of these reference strains were 4.2 x 10^8 CFU/ml, 365 µg/ml and 1.0 x 10^9CFU/ml, 492 µ g/ml, respectively (Table 12). The quality of all genomic DNA was also analyzed by 0.8% agarose gel electrophoresis and then stored at –20°C for further experiments.

2. Optimization of the polymerase chain reaction (PCR) condition

2.1 Primary PCR

Genomic DNA of *V. parahaemolyticus* BG 26 (*tdh*$^-$*trh*$^-$) and *V. cholerae* AQ 1034 (*ct*$^+$) was used for optimization of PCR condition. The optimal PCR condition was set up from several parameters including dNTPs, magnesium chloride ($MgCl_2$), primers, *Taq* DNA polymerase and annealing temperature

Table 12 Enumeration of viable count and concentration of reference genomic DNA

Reference strains	Viable count CFU/ml	Concentration of DNA µg/ml
V. parahaemolyticus BG 26 (*tdh*$^-$*trh*$^-$)	4.2×10^8	365
V. cholerae AQ 1034 (*ct*$^+$)	1.0×10^9	492

which were adjusted from the calculation of melting temperature (Tm) of *ldh* oligonucleotide primers. In this study, Tm, denaturation and extension temperatures were 72° C, 95°C and 72°C, respectively.

Annealing temperature was varied from 54°, 60°, 65° and 70°C. It was indicated that the optimal annealing temperature was 60°C for primary *ldh* primers (Fig. 4).

$MgCl_2$ concentration was varied from 1.0, 1.5, 2.0, 2.5 and 3.0 mM. The optimal $MgCl_2$ concentration was 2.0 mM (Fig 5).

Deoxynucleotide triphosphate (dNTPs) concentration was varied from 100, 150, 200, 250 and 300 µM. It was revealed that all concentration of dNTPs gave the same results. Therefore, the lowest concentration of dNTPs that gave the optimal condition was 200 µM (Fig. 6).

The primers concentration was varied from 3, 5, 7.5, 10 and 20 pmole. the results indicated that the primers concentration at 3, 5 and 10 pmole were selected for optimal concentration of primary PCR (Fig. 7). In addition, the PCR products amplified from these primer concentration were further tested for optimization of nested PCR.

Taq DNA polymerase was also varied from 0.5, 1, 1.5 and 2 units (U)in. The results showed no differences in all amount of *Taq* DNA polymerase which gave

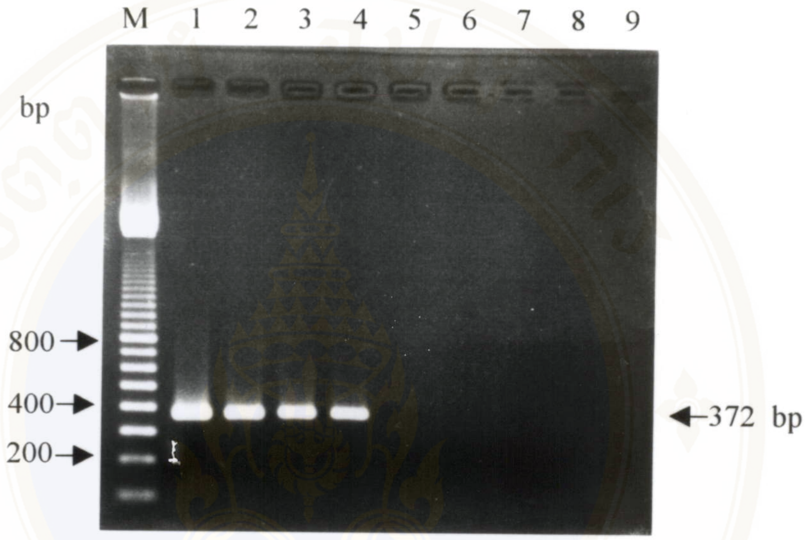

Fig. 4 1.5 % agarose gel electrophoresis showing 372 bp amplification products of *ldh* for the optimization of annealing temperatures. Lanes 1 to 4, 54, 60, 65 and 70° C, respectively; Lanes 5 to 8, negative DNA controls (*V. cholerae* AQ 1034)(*ldh*$^-$,*ct*$^+$)54, 60, 65, and 70° C, respectively; Lane 9, reagent control (no DNA template); Lane M, 100 bp DNA ladder (Pharmacia Biotech, USA).

Fig. 5 1.5% agarose gel electropheresis showing 372 bp amplification products of *ldh* for the optimization of $MgCl_2$ concentration. Lanes 1 to 5, 1.0, 1.5, 2.0, 2.5 and 3.0 mM, respectively; Lanes 6 to 10, negative DNA controls (*V. cholerae* AQ 1034) (ldh^{-}, ct^{+}) 1.0, 1.5, 2.0, 2.5, and 3.0 mM, respectively; Lanes 11, a negative control (no DNA template); Lane M, 100 bp DNA ladder (Pharmacia Biotech, USA).

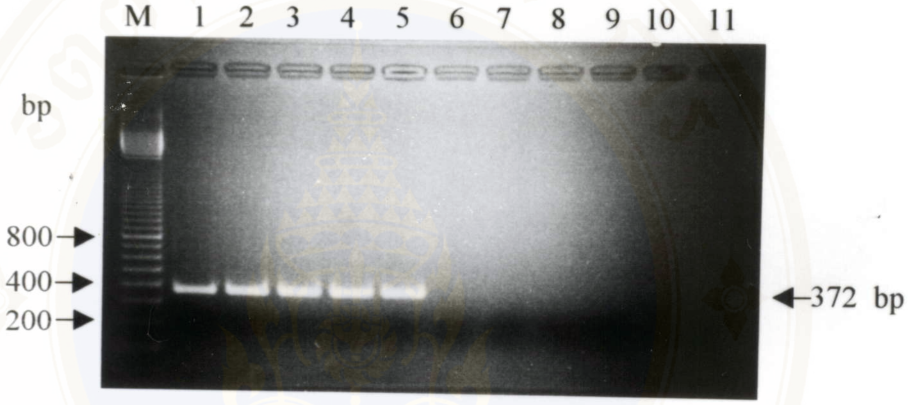

Fig. 6 1.5% agarose gel electrophoresis showing 372 bp amplification products of *ldh* for the optimization of dNTPs concentration. Lanes 1 to 5, 100, 150, 200, 250 and 300 µM, respectively; Lanes 6 to 10, negative DNA controls (*V. cholerae* AQ 1034) (*ldh*$^-$,*ct*$^+$)100, 150, 200, and 300 uM, respectively; Lane 11, a negative control (no DNA template); Lane M, 100 bp DNA ladder (Pharmacia Biotech, USA).

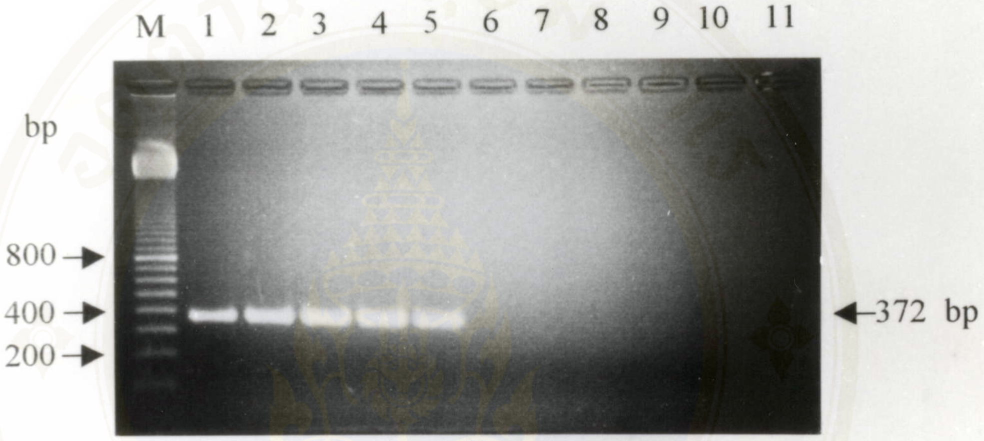

Fig. 7 1.5 % agarose gel electrophoresis showing 372 bp amplification products of *ldh* for the optimization of primer concentration. Lanes 1 to 5, 3, 5, 7.5, 10 and 20 pmole; Lane 6 to 10, negative DNA controls (*V. cholerae* AQ1034)(ldh^{-},ct^{+}) 3, 5, 7.5, 10, and 20 pmole, respectively; Lane 11, a negative control (no DNA template); Lane M, 100 bp DNA ladder (Pharmacia Biotech, USA).

bright and clearly positive band. Thus, 1 U of *Taq* DNA polymerase was used in both primary PCR and nested PCR (Fig. 8).

2.2 Nested PCR

The concentration of nested primers in nested PCR protocol was determined by using the same concentration of primary primers at 10 pmole / 25 ul PCR mixture. After that, the primary PCR products obtained from an amplification of primary PCR primers varying concentration at 3, 5, and 10 pmole were used as DNA template in nested PCR protocol. The nested PCR products which were obtained from each concentration of primary primers in primary PCR were analyzed by using 1.5% agarose gel electrophoresis.

The results of nested PCR products gave two product sizes; the first product size was 372 bp amplicon obtained from the primary primers concentration and DNA template excess, the second product size was 196 bp amplicon amplified by nested primers. When using concentration of primary primers at 10 and 5 pmole, the results showed strong, sharp product of 372 bp and non-specific occurred (Fig 9,10). On the other hand, when using 3 pmole of primary primers, it was revealed that non-specific bands was absent or faint bands, and amplicon of 372 bp was found when using 10 ng to 1 pg of DNA template. Furthermore, the amplification products of 196 bp showed sharp, clear and only specific one band when using100 fg to 1 fg of DNA template (Fig.11).

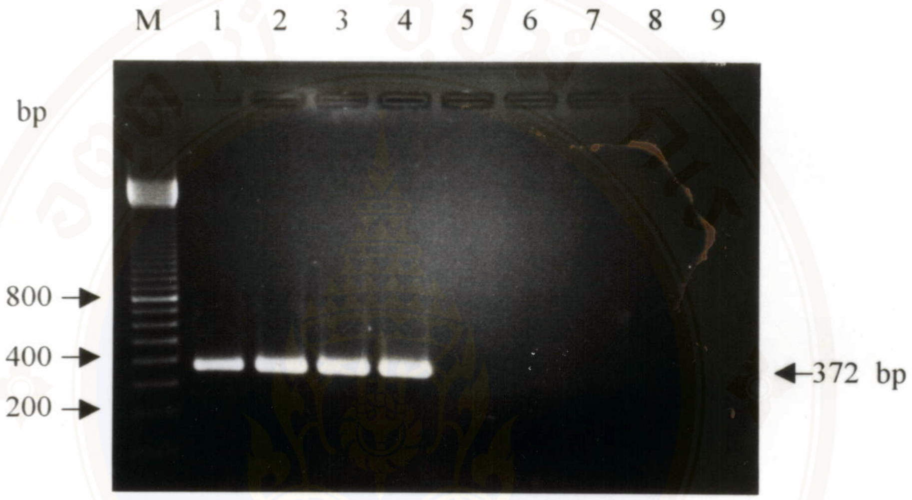

Fig. 8 1.5 % agarose gel electrophoresis showing 372 bp amplification products of *ldh* for the optimization of *Taq* DNA polymerase. Lanes 1 to 4, 0.5, 1.0, 1.5 and 2.0 U, respectively; Lanes 5 to 8, negative DNA controls (*V. cholerae* AQ 1034) (ldh^{-},ct^{+}) 0.5, 1.0, 1.5 and 2.0 U, respectively; Lane 9, a negative control (no DNA template); Lane M, 100 bp DNA ladder (Pharmacia Biotech, USA).

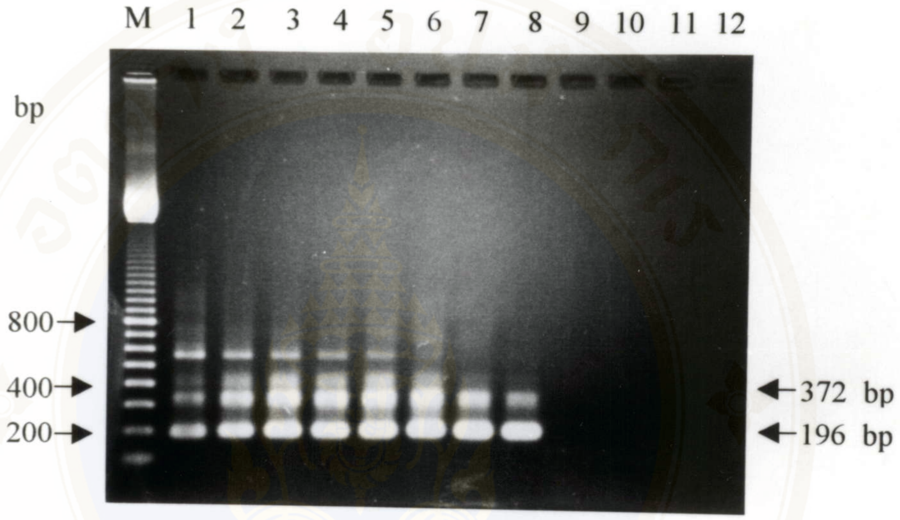

Fig. 9 Sensitivity test of nested PCR detection by 1.5 % agarose gel electrophoresis showing 196 bp amplification products of *ldh* when using DNA template obtained from primary PCR products amplified with concentration of primary primers at 10 pmole. Lanes 1 to 10, serial ten-fold dilution of genomic DNA of *V. parahaemolyticus* BG 26 (*ldh*$^+$,*tdh*$^-$*trh*$^-$), 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, 0.1 fg and 0.01 fg, respectively; Lane 11, a negative control from primary PCR cycles repeated for nested PCR; Lane 12, a negative control (no DNA template); Lane M, 100 bp DNA ladder (Pharmacia Biotech, USA).

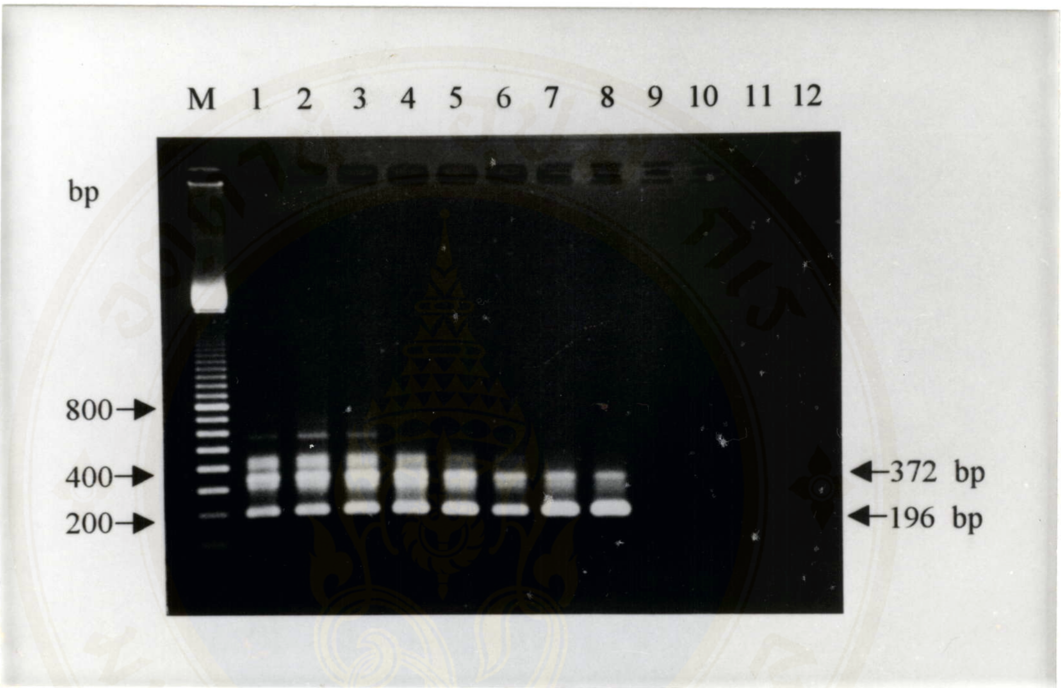

Fig. 10 Sensitivity of nested PCR detection by 1.5 % agarose gel electrophoresis showing 196 bp amplification products of *ldh* when using DNA template obtained from primary PCR products amplified with concentration of primary primers at 5 pmole. Lanes 1 to 10, serial ten-fold dilution of genomic DNA of *V. parahaemolyticus* BG 26 (*ldh*$^+$,*tdh*$^-$*trh*$^-$), 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, 0.1 fg and 0.01 fg, respectively; Lane 11, a negative control from primary PCR cycles repeated for nested PCR; Lane 12, a negative control (no DNA template); Lane M, 100 bp DNA ladder (Pharmacia Biotech, USA).

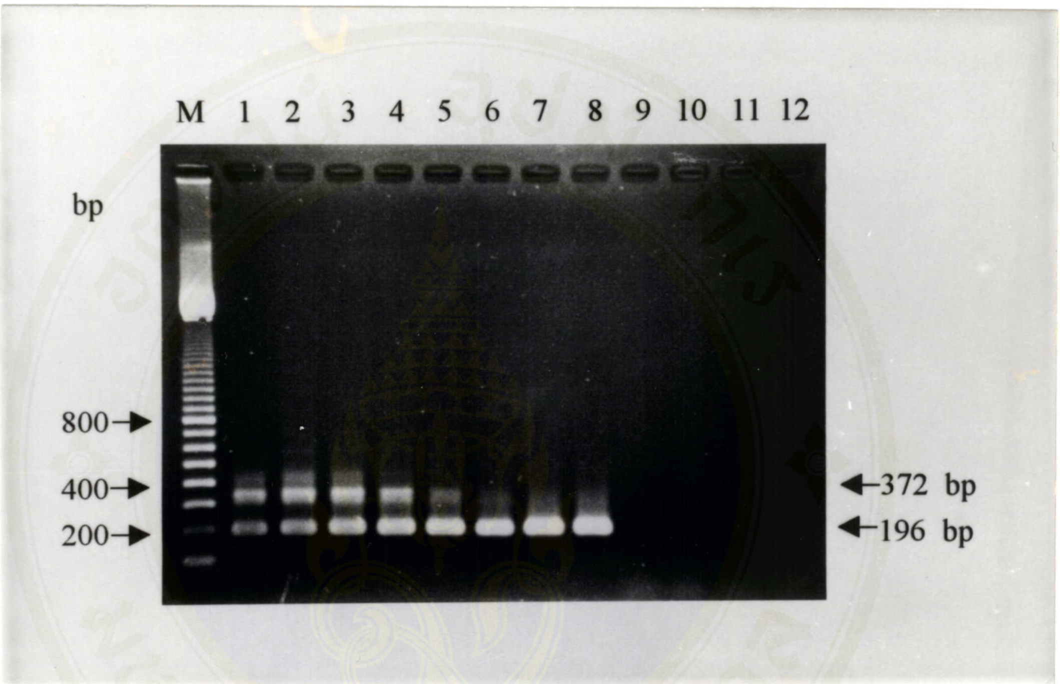

Fig. 11 Sensitivity of nested PCR detection by 1.5 % agarose gel electrophoresis showing 196 bp amplification products of *ldh* when using DNA template obtained from primary PCR products amplified with concentration of primary primers at 3 pmole. Lane 1 to 10, serial ten-fold dilution of genomic DNA of *V. parahaemolyticus* BG 26 (*ldh*$^{+}$,*tdh*$^{-}$*trh*$^{-}$), 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, 0.1 fg and 0.01 fg, respectively; Lane 11, a negative control from primary PCR cycles repeated for nested PCR; Lane 12, a negative control (no DNA template); Lane M, 100 bp DNA ladder (Pharmacia Biotech, USA).

Therefore, the optimum condition of nested PCR protocol was as followed: The primary PCR step was performed in a final volume of 25µl of PCR mixture which was composed of 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.1% Triton X- 100, 2.0 mM $MgCl_2$, 200 µM dNTP each, 1.0 U *Taq* DNA polymerase, 3 pmole each for primary primers and 10 pg of *V. parahaemolyticus* genomic DNA as DNA template. Amplification was performed in a DNA thermal cycler; a pre-PCR cycle was consisted of denaturation at 94°C for 5 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. PCR cycle consisted of 94°C, 60°C and 72°C each for 1 min for 30 cycles, with a final extension at 72°C for 10 min. For nested PCR, 1 µl of the primary PCR products was added to the second reaction tube contained 10 pmole each for nested primers and appropriate concentration of the other constituents similar as mentioned in the primary PCR. The nested PCR protocol was used for detection of *ldh* of *V. parahaemolyticus* in direct frozen shrimp samples.

3. Sensitivity of primary PCR

The sensitivity test of primary PCR was examined by performing serial ten- fold dilution of genomic DNA of *V. parahaemolyticus* BG 26 (*tdh*$^{-}$*trh*$^{-}$) starting from 10 ng/µl to 0.01 fg/µl and 1 µl of each DNA dilution used as DNA template. Each DNA template was amplified with the individual concentration of primary primers at 3, 5 and 10 pmole in PCR reaction. The condition of primary PCR was performed as mentioned previously (Table 13). The amplified products were analyzed by 1.5% agarose gel electrophoresis. The results showed that the minimum amount of the genomic DNA to give the positive PCR reaction was 100 fg corresponding to175 cells

Table 13 Optimal condition of primary PCR and nested PCR

Substances	Concentration / 25 µl PCR reaction	
	Primary PCR[a,b]	Nested PCR[b]
Tris-HCl (pH 8.8) (mM)	10	10
KCl (mM)	50	50
Triton X-100 (%)	0.1	0.1
$MgCl_2$ (mM)	2	2
d NTPs (each) (µM)	200	200
Primers (each) (pmole)	3	10
Taq DNA polymerase (U)	1	1

[a] Using for detection of *ldh* in enrichment frozen shrimp samples and isolated colonies from culture method.

[b] PCR amplification for 30 cycles and annealing temperature at 60°C.

of the amplified products detected when using primers concentrations at 3, 5 and 10 pmole. All of these concentration products showed quite the same results. However, the high yield, bright and clearly specific band of 196 bp without non- specific bands for the nested PCR was obtained from the reaction using 3 pmole primary primers (Fig.11). Therefore, the lowest concentration of genomic DNA at 100 fg corresponding to 175 cells when using 3 pmole of primary primers was chosen to be used throughout the experiment (Fig. 12). Two negative controls which no DNA template, one obtained from primary PCR cycles repeated for nested PCR and the other used for nested PCR cycles were included.

4. Sensitivity of nested PCR

In order to access the sensitivity of nested PCR, the primary PCR products obtained from genomic DNA of *V. parahaemolyticus* BG 26 and varied from 10 ng to 0.01 fg were used for DNA template in nested PCR. The optimal condition and amplification process were performed by the optimized condition (Table 13). Then, nested PCR products were electrophoresed on a 1.5% agarose gel and visualized under an UV transilluminator. The nested PCR product of *ldh* showed bright and clearly specific DNA band of 196 bp. The sensitivity of nested PCR showed that the minimum amount of genomic DNA used for amplification by nested PCR step was as little as 1 fg corresponding to approximately 1.7 cells (Fig. 11).

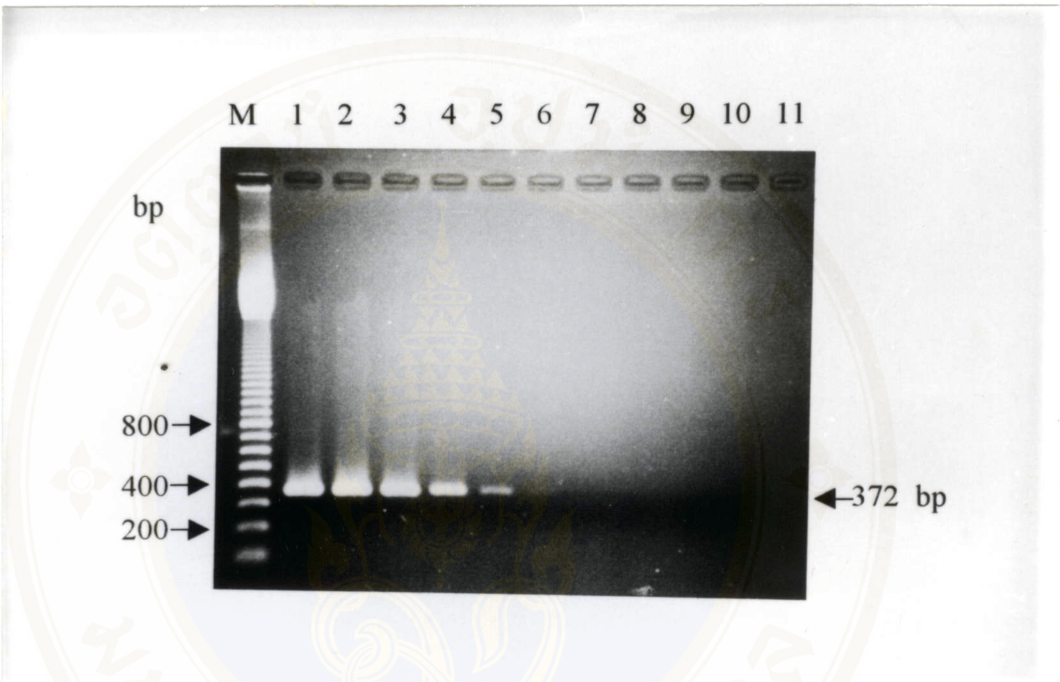

Fig. 12 Sensitivity test of primary PCR detection by 1.5 % agarose gel electrophoresis showing 372 bp amplification products of *ldh* when using primary primers concentration at 3 pmole. Lanes 1 to 10, serial ten-fold dilution of genomic DNA of *V. parahaemolyticus* BG 26 (*ldh*$^+$,*tdh*$^-$*trh*$^-$), 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, 0.1 fg and 0.01 fg, respectively; Lane 11, a negative control from primary PCR cycles; Lane 12, a negative control (no DNA template); Lane M, 100 bp DNA ladder (Pharmacia Biotech, USA).

5. Specificity of nested PCR

Specificity of nested PCR was determined by using DNA extracted from other bacteria. Fig. 13 and 14 showed that the result of the predicted 196 bp fragment was amplified only from *V. parahaemolyticus* target DNA but not from other bacterial target DNA including *V. cholerae* O1, *V. cholerae* non O1, *V. cholerae* O 139, *V. vulnificus*, *S. typhimurium*, *V. fluvialis*, *V. hollisae*, *V. mimicus*, *V. alginolyticus*, *A. hydrophila* , *A. sobria*, *P. shigelloides*, *E. coli*, *Shigella* spp. and *Salmonella* spp.. It was concluded that the specificity of nested PCR primer was 100 %.

B. Bacterial Isolation from Frozen Shrimp Samples

1. Enumeration of suspected bacteria detected from frozen shrimp samples

The level of bacterial contamination of 103 frozen shrimp samples were enumerated by using plating media. The total viable count (TVC) of all frozen shrimp (100%) was contaminated with bacteria and ranged from 1.0×10^3 to 3.0×10^6 CFU/g. The geometric mean (GM) was 3.8×10^4 CFU/g. The coliforms count ranged from 5.0×10 to 2.9×10^3 CFU/g and GM was 2.2×10^2 CFU/g. The suspected *E. coli* was not detected in all 103 frozen shrimp samples but the suspected *V. parahaemolyticus* in 1 (0.9%) of 103 samples was detected and enumerated colonies were 1.0×10^2 CFU/g (Table 14).

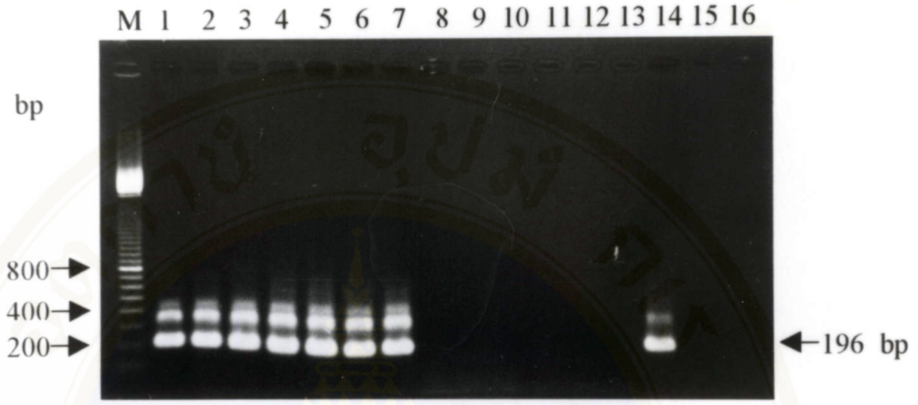

Fig. 13 Specificity of nested PCR detection by 1.5 % agarose gel electrophoresis showing 196 bp amplification products of *ldh*. Lanes 1 to 13, *V. parahaemolyticus* AQ 4613 (*tdh*$^+$), *V. parahaemolyticus* AQ 4023 (*trh*$^+$), two strains of *V. parahaemolyticus* (*tdh*$^+$), two strains of *V. parahaemolyticus* (*tdh*$^+$*trh*$^+$), *V. parahaemolyticus* (*trh*$^+$), *V. cholerae* AQ 1034 (*ct*$^+$), *V. cholerae* AQ 1002 (*ct*$^-$), *S. typhimurium* 1980051, *V. vulnificus* 2219009, *V. cholerae* O 139 and *V. cholerae* O1 Eltor Ogawa, respectively; Lane 14, *V. parahaemolyticus* BG 26 (*ldh*$^+$,*tdh*$^-$*trh*$^-$) as a positive control; Lane 15, a negative control from primary PCR cycles repeated for nested PCR; Lane 16, a negative control (no DNA template); Lane M, 100 bp DNA ladder (Pharmacia Biotech, USA).

Fig. 14 Specificity of nested PCR detection by 1.5 % agarose gel electrophoresis showing 196 bp amplification products of *ldh* (*Vibrio* spp. and other bacteria). Lanes 1 to 13, *V. cholerae* non 01, *V. fluvialis*, *V. hollisae*, *V. mimicus*, *V. alginolyticus*, *A. hydrophila*, *A. sobria*, *P. shigelloides*, *E. coli* (EHEC; *vt*$^+$), *E. coli* (ETEC; *inv*$^+$), *S. sonnei*, *S. flexneri and S. anatum*, respectively; Lane 14, *V. parahaemolyticus* BG 26 (*ldh*$^+$,*tdh*$^-$*trh*$^-$) as a positive control; Lane 15, a negative control from primary PCR cycles repeated for nested PCR; Lane 16, a negative control (no DNA template); Lane M, 100 bp DNA ladder (Pharmacia Biotech, USA).

From enrichment samples, the contamination of *V. parahaemolyticus* was found in 82 (79.6%) samples. The contamination ranged from 1.0×10^5 to 7.5×10^8 CFU/g, and GM was 5.4×10^6 CFU/g. In addition, 24 (23.3%) of frozen shrimp samples contaminated with *E. coli* ranged from 2.2×10^6 to 4.0×10^8 CFU/g, and GM was 3.6×10^7 CFU/g. Other *Vibrio* spp. were also detected in 60 (58.2%) frozen shrimp samples (Table 14).

2. Microbiological quality in frozen shrimp samples

According to the Japan External Trade Organization (JETRO, Specifications and standards for foods, food additives, under the food sanitation law, 1997). The microbiological specification value for TVC was not more than 10^5 CFU/g; the coliform group including *E. coli* was absent. In addition, the International Commission on Microbiological Specification for food (ICMSF) has recommended an acceptable limit for *V. parahaemolyticus* was 10^2 CFU/g; (Table 15). In this study, 74 (71.8%) and 29 (28.2%) of frozen shrimp samples were acceptable and unacceptable for TVC, respectively. Moreover, 77(74.8%) of frozen shrimp samples were acceptable and 26 (25.2%) were unacceptable according to specification value of coliforms group. Furthermore, all 103 frozen shrimp samples were also acceptable for *E. coli* and *V. parahaemolyticus.*

Considering all the microbiological criteria, 39(37.9%) of 103 frozen shrimp samples were unacceptable according to the microbiological specification of JETRO and ICMSF for frozen products (Table 15).

Table 14 **Enumeration of bacterial contamination from 103 frozen shrimp samples**

Organism	No (%) of contaminated samples	Range of contamination (CFU/g)[a]	Geometric mean (CFU/g)
Direct samples			
Total viable bacteria	103 (100)	$1.0 \times 10^3 - 3.0 \times 10^6$	3.8×10^4
Coliforms	26 (25.2)	$5.0 \times 10 - 2.9 \times 10^3$	2.2×10^2
Suspected *E. coli*	0	0	-
Suspected *V. parahaemolyticus*	1 (0.9)	1.0×10^2	1.0×10^2
Enrichment samples			
Suspected *E. coli*	24 (23.3)	$2.2 \times 10^6 - 4.0 \times 10^8$	3.6×10^7
Suspected *V. parahaemolyticus*	82 (79.6)	$1.0 \times 10^5 - 7.5 \times 10^8$	5.4×10^6
Other *Vibrio* spp.[b]	60 (58.2)	ND	ND

[a] Colony Forming Unit.

[b] See Table 17

ND = not determined

Table 15 Comparison of bacterial contamination from frozen shrimp samples with microbiological quality standard of frozen shrimp products

Microbiological criteria	Standard value CFU/g	No.(%)of frozen shrimp samples	
		Acceptable	Unacceptable
Total viable count [a]	$<10^5$	74 (71.8)	29(28.2)
Coliforms [a]	Absent	77 (74.8)	26 (25.2)
E. coli[a]	Absent	103 (100)	0
V. parahaemolyticus [b]	10^2	103 (100)	0 (0)
Total [c]		64(62.1)	39(37.9)

[a] Japan External Trade Organization (JETRO).

[b] International Commission on Microbiological Specification for food (ICMSF).

[c] All of microbiological criteria together.

3. Distribution of enteric bacteria contamination according to type of frozen shrimp samples

Table 16 shows the distribution of enteric bacteria contamination according to types of frozen shrimp. All 103 frozen shrimp samples comprised of 54 finished products and 49 raw materials. Fifty-four finished products were divided into two groups : unpeeled finished products (UPFP) and peeled finished products (PFP). These finished products were not contaminated with any microorganisms by direct culture method. However, enteric bacteria were discovered from 74.1% (40/54) of finished products after enrichment method. It was found that 87.5% (35/40) of positive samples were contaminated with *V. parahaemolyticus;*12.5%(5/40) were contaminated with *V. parahaemolyticus* and *E. coli.* It was obvious that 95%(19/20) of PFP types were positive for enteric bacteria and higher than UPFP type (61.8%,21/34). Among the group of 21 UPFP positive for enteric bacteria, 80.9% (17/21) were contaminated with *V. parahaemolyticus* only, and 19.0 % (4/21) were contaminated with both *V. parahaemolyticus* and *E. coli.* The same manner was found in the group of 19 PFP positive for enteric bacteria, 94.7%(18/19) were contaminated with *V. parahaemolyticus* only, 5.3% (1/19) were contaminated with *V. parahaemolyticus* and *E. coli.*

The other 49 frozen shrimp samples type categorized as raw materials were also divided into two groups : unpeeled raw materials (UPRM) and peeled raw materials (PRM). These raw materials were contaminated with *V. parahaemolyticus* only 2.0% (1/49) by direct culture method. After enrichment, 91.8% (45/49) of raw materials were contaminated with enteric bacteria. It was found that 57.8% (26/45) were contaminated with *V. parahaemolyticus*; 35.5% (16/45) were contaminated with *V. parahaemolyticus* and *E. coli* and only 6.7% (3/45) were contaminated with *E. coli*. It was 94.1%(16/17) of PRM samples were positive for enteric bacteria and higher than UPRM samples (90.6%, 29/32). Among 29 UPRM samples positive for enteric bacteria, 55.2% (16/29) were contaminated with *V. parahaemolyticus only*, 34.5% (10/29) were contaminated with *V. parahaemolyticus* and *E. coli*, and 10.3% (3/29) were contaminated with *E. coli*. As for the group of 16 PRM samples positive for enteric bacteria, 62.5% (10/16) were contaminated with *V. parahaemolyticus* only, 37.5% (6/16) were contaminated with *V. parahaemolyticus* and *E. coli*. The contamination of enteric bacteria in raw material frozen shrimp samples (91.8%,45/49) by enrichment method were significantly higher than that of finished product frozen shrimp samples (74.1%,40/54) ($P=0.017$;χ^2 test). However, the prevalence of contamination of *V. parahaemolyticus* from total raw material frozen shrimp samples (85.7%,42/49) by enrichment method were not significantly different from that of total finished product frozen shrimp samples (74.1%,40/54) ($p=0.143$; χ^2 test).

4. Prevalence of enteric bacteria in frozen shrimp samples

Bacterial contamination of frozen shrimp samples other than *V. parhaemolyticus* was Gram negative bacteria. The list of the Gram negative bacteria is shown in Table 17. *V. parahaemolyticus* was found in the highest percent (79.6%, 82/103) of frozen shrimp samples, followed by *V. alginolyticus* 28.1% (29/103), *E. coli* 23.3% (24/103), *V. vulnificus* 12.6% (13/103), *V. cholerae* non 01 5.8% (6/103), *V. mimicus* 1.9% (2/103), *P. shigelloids* 5.8% (6/103) and *Aeromonas* spp. 4.8% (5/103).

Table 16 Distribution of enteric bacteria contamination according to type of frozen shrimp samples

Type of frozen shrimp samples	Total no. of tested samples	No. (%) of culture positive samples from							
		Direct method				Enrichment method			
		Total positive	EC[a]	VP[b]	EC+VP	Total positive	EC	VP	EC+VP
Finished products	**54**	**0**	**0**	**0**	**0**	**40(74.1)[c]**	**0**	**35(64.8)**	**5(9.3)**
Unpeeled (UPFP)	34	0	0	0	0	21(61.8)	0	17(50.0)	4(11.8)
Peeled (PFP)	20	0	0	0	0	19(95.0)	0	18(90.0)	1(5.0)
Raw materials	**49**	**1(2.0)**	**0**	**1(2.0)**	**0**	**45(91.8)[c]**	**3(6.1)**	**26(53.1)**	**16(32.6)**
Unpeeled (UPRM)	32	1(3.1)	0	1(3.1)	0	29(90.6)	3(9.4)	16(5.0)	10(31.2)
Peeled (PRM)	17	0	0	0	0	16(94.1)	0	10(58.8)	6(35.3)
Total	**103**	**1(0.9)**	**0**	**1(0.9)**	**0**	**85(82.5)**	**3(2.9)**	**61(59.2)**	**21(20.4)**

[a] *Escherichia coli.*
[b] *Vibrio parahaemolyticus.*
[c] $p=0.017$; χ^2test

Table 17 The prevalence of Gram negative bacteria in 103 frozen shrimp samples by culture method and subsequent biochemical tests for species characteristics

Type of bacteria	No. of isolated samples		No. (%) of	No. of
	Direct	Enrichment	contaminated samples	Isolates
V. parahaemolyticus	1	82	82 (79.6)	258[a]
V. alginolyticus	0	29	29 (28.1)	46[a]
E. coli	0	24	24 (23.3)	119
V. vulnificus	1	13	13 (12.6)	28
V. cholerae non 01	0	6	6 (5.8)	9[a]
V. mimicus	0	2	2 (1.9)	2
P. shigelloides	0	6	6 (5.8)	10
Aeromonas spp.	0	5	5 (4.8)	9[a]

[a] Taken for further tested by primary PCR.

All 258 isolates of *V. parahaemolyticus* were collected for further determination of *ldh* by primary PCR. Furthermore, isolates of *V. alginolyticus, V. cholerae* non O1 *and Aeromonas* spp., also collected for determining *ldh* by primary PCR for specificity test.

C. Application of Primary PCR and Nested PCR for Detecting *Vibrio parahaemolyticus* in Frozen Shrimp Samples

1. Detection of *ldh* gene of *V. parahaemolyticus* directly from frozen shrimp samples by primary PCR

A total of 103 direct samples and their correspondent enrichment samples were determined the prevalence of *ldh* by primary PCR. The results of *ldh* gene detection are shown in Table 18. It was found that only 7.7 % (8/103) from direct frozen shrimp samples were positive for *ldh* of *V. parahaemolyticus*. Among these direct samples, 16.0% (4/25) were positive for *ldh* detected from direct samples that showed negative upon culture for *V. parahaemolyticus* but positive for other microorganisms and 5.2% (4/77) were positive for *ldh* detected from direct samples which no any microorganisms grew on TCBS (Table 18). The representation of primary PCR results from direct frozen shrimp samples is shown in Fig. 15. In contrast, 88.3 % (91/103) of enrichment samples were positive for *ldh* of *V. parahaemolyticus*; 98.8% (81/82) from enrichment culture positive for *V. parahaemolyticus* were positive for *ldh* by primary PCR and 50% (10/20) of the enrichment samples cultured negative for *V. parahaemolyticus* but positive for other microorganisms were positive for *ldh* by primary PCR. The representation of primary PCR results from enrichment frozen shrimp samples is shown in Fig. 16. The *ldh* detection rate was significantly improved after the enrichment process from 7.7% of direct samples to 88.3% of the enrichment samples ($p<0.001$).

2. Direct detection of *ldh* gene of *V. parahaemolyticus* from frozen shrimp samples by nested PCR

The nested PCR was determined only from 103 direct samples. The result showed that 33.9% (35/103) of all samples were positive for *ldh* of *V. parahaemolyticus* (Table 18). It was found that 40.0% (10/25) of samples negative for *V. parahaemolyticus* but positive for other microorganisms by culture method were *ldh* positive. Furthermore, 31.1% (24/77) from the direct culture which no any microorganisms grew on TCBS, gave positive results for *ldh* and 1 (100%, 1/1) from samples positive for culturing *V. parahaemolyticus* was positive for *ldh* by nested PCR but negative by primary PCR. The representation of nested PCR results is shown in Fig. 17. However, only enrichment cultures (12 from 103) that were negative for *ldh* by primary PCR were further determined for *ldh* by nested PCR. The results showed that all samples were also negative for *ldh*. Moreover, direct frozen shrimp samples positive for *ldh* of *V. parahaemolyticus* by nested PCR were detected in higher percent than that of primary PCR ($P< 0.001$).

Table 18 Detection of *ldh* of *Vibrio parahaemolyticus* directly in frozen shrimp samples

Microorganism growth on TCBS	No. of samples	No (%) of samples positive for *ldh* detected by	
		Primary PCR	Nested PCR
Direct samples	**103**	**8 (7.7)**[c]	**35 (33.9)**[e]
no growth[a]	77	4 (5.2)	24 (31.1)
Negative for *V. parahaemolyticus*	25	4 (16.0)	10 (40.0)
Positive for *V. parahaemolyticus*	1	0	1 (100.0)
Enrichment samples	**103**	**91 (88.3)**[d]	-[b]
no growth	1	0	-
Negative for *V. parahaemolyticus*	20	10 (50.0)	-
Positive for *V. parahaemolyticus*	82	81 (98.8)	-

[a] No any microorganisms isolated on TCBS.

[b] Only the negative samples (12 samples) for *ldh* by primary PCR were further determined for *ldh* by nested PCR; all samples were also negative for *ldh*..

[cd ; ce] $p<0.001$; χ^2test

Fig. 15 1.5% agarose gel electrophoresis showing 372 bp amplification products of *ldh* of primary PCR from direct frozen shrimp samples. Lanes 1 to 13, samples number FS 084-99/D to FS 096-99/D; Lane 14, *V. parahaemolyticus* BG 26 (*ldh*$^+$,*tdh*$^-$*trh*$^-$) as a positive control; Lane 15, a negative control (no DNA template); Lane M, 100 bp DNA ladder (Pharmacia Biotech, USA).

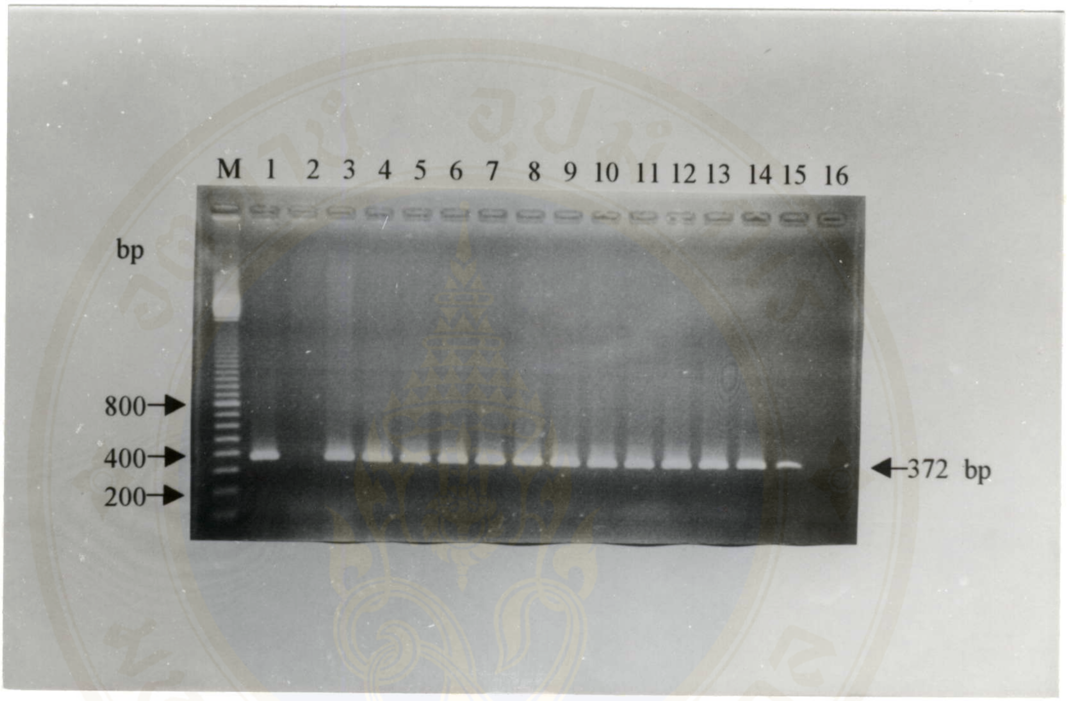

Fig. 16 1.5% agarose gel electrophoresis showing 372 bp amplification products of *ldh* of primary PCR from enrichment frozen shrimp samples. Lanes 1 to 13, samples number FS 084-99/E to FS 096-99/E; Lane 14, *V. parahaemolyticus* BG 26 (*ldh*$^+$, *tdh*$^-$*trh*$^-$) as a positive control; Lane 15, a negative control (no DNA template); Lane M, 100 bp DNA ladder (Pharmacia Biotech, USA).

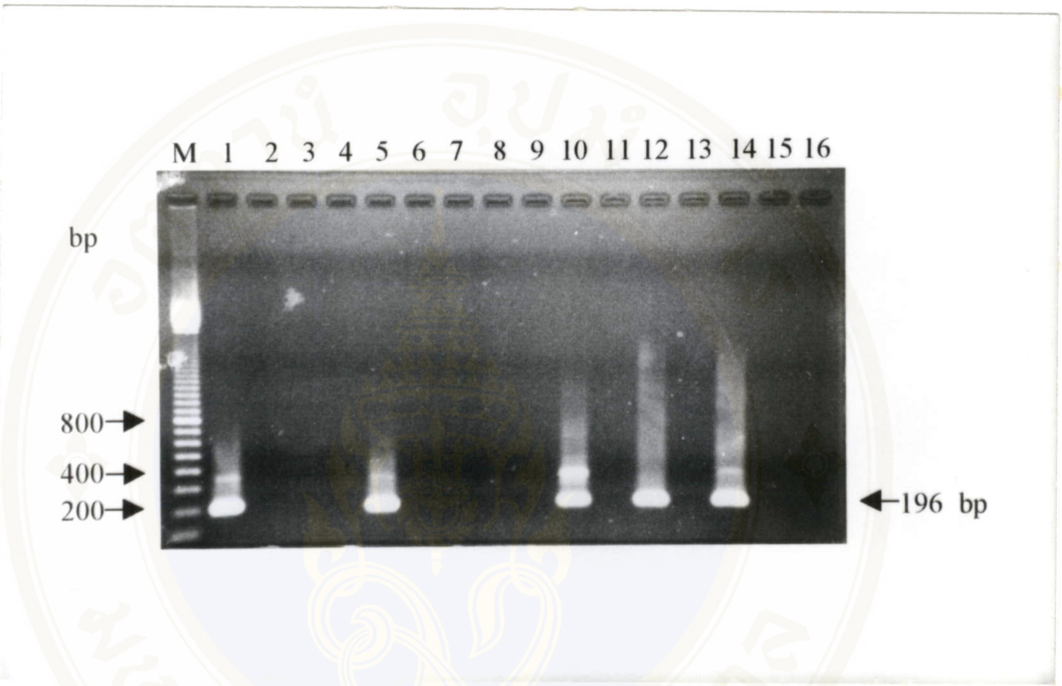

Fig. 17 1.5% agarose gel electrophoresis showing 196 bp amplification products of *ldh* of nested PCR from direct frozen shrimp samples. Lane 1 to 13, samples number FS 084-99/D to FS 096-99/D; Lane 14, *V. parahaemolyticus* BG 26 (*ldh*$^{+}$,*tdh*$^{-}$ *trh*$^{-}$) as a positive control; Lane 15, a negative control from primary PCR cycle repeated for nested PCR; Lane 16, a negative control (no DNA template); Lane M, 100 bp DNA ladder (Pharmacia Biotech, USA).

3. Distribution of *ldh* gene of *V. parahaemolyticus* according to type of frozen shrimp samples

Of those 103 frozen shrimp samples; 88.3% of frozen shrimp enrichment samples were positive for *ldh* by primary PCR while 33.9% of frozen shrimp samples from direct detection were positive for *ldh* by nested PCR. Forty-four of 54 (81.4%) samples from finished products were positive for *ldh* by primary PCR (Table 19). The detection of *ldh* from PFP was 95% (19/20) which was significantly higher than that of UPFP, (73.5 %, 25/34), by primary PCR ($p = 0.049$). Forty-seven of 49 (95.9%) raw material samples gave positive for *ldh* detection by primary PCR. The UPRM was detected *ldh* in a little higher percent (96.9 %, 31/32) than that of PRM (94.1 %, 16/17). However, the detection of *ldh* from raw materials frozen shrimp type (95.9%) was significantly higher than that of finished products frozen shrimp type (81.4%) by primary PCR ($p = 0.023$; χ^2 test).

By nested PCR, it was found that 35.2% (19/54) of finished products frozen shrimp type were positive for *ldh* of *V. parahaemolyticus*. It was obvious that *ldh* detection from PFP (75%;15/20) were about six times higher than that from UPFP (11.8%; 4/34), ($p < 0.001$). In raw material frozen shrimp type, 32.6% (16/49) were positive for *ldh* by nested PCR. The presence of *ldh* from PRM and UPRM were 35.3% (6/17) and 31.2% (10/32), respectively (Table 19). The association of *ldh* detected from finished product frozen shrimp type (35.2%) was not significantly higher than that of raw material frozen shrimp type (32.6%) by nested PCR ($p = 0.786$; χ^2 test).

Table 19 **Distribution of *ldh* of *Vibrio parahaemolyticus* according to type of frozen shrimp samples**

Type of frozen shrimp samples	No. of samples	No (%) of samples positive for *ldh* detected by	
		Primary PCR (enriched samples)	Nested PCR (direct samples)
Finished products	**54**	**44 (81.4)[a]**	**19 (35.2)[c]**
Unpeeled (UPFP)	34	25 (73.5)[b]	4 (11.8)[d]
Peeled (PFP)	20	19 (95.0)[b]	15 (75.0)[d]
Raw materials	**49**	**47 (95.9)[a]**	**16 (32.6)[c]**
Unpeeled (UPRM)	32	31 (96.9)	10 (31.2)
Peeled (PRM)	17	16 (94.1)	6 (35.3)
Total	**103**	**91 (88.3)**	**35 (33.9)**

[a] $p<0.023$; χ^2 test

[b] $p<0.049$; χ^2 test

[c] $p<0.786$; χ^2 test

[d] $p<0.001$; χ^2 test

4. Detection of *ldh* gene of *V. parahaemolyticus* isolates by primary PCR

Two hundred and fifty-eight isolates of *V. parahaemolyticus* collected from a total of 82 positive culture samples; 81 samples were primary PCR positive but only 1 sample was primary PCR negative. Eleven strains of *V. alginolyticus*, 3 strains of *V. cholerae* non O1 and 2 strains of *Aeromonas* spp. collected from enrichment samples were determined for *ldh* gene by primary PCR. The results are shown in Table 20. Of those 258 strains of *V. parahaemolyticus*, 97.3% (251/258) were positive for *ldh* while 2.7% (7/258) were negative for *ldh*. The representation of primary PCR results of *V. parahaemolyticus* isolates is shown in Fig 18. Among these *ldh* negative strains, 4 strains were from enrichment samples which gave primary PCR positive and the other 3 strains were frozen enrichment samples but gave primary PCR negative. When these strains of *ldh* negative were repeated for detection, they were still *ldh* negative. It was found that 10 strains (90.9%) of *V. alginolyticus*, all strains of *V. cholerae* non 01 and *Aeromonas* spp. were negative for *ldh* by primary PCR.

5. Relationship between culture method and presence of *ldh* of *V. parahaemolyticus* by primary PCR and nested PCR

Table 21 shows the relationship between culture method and present of *ldh* of *V. parahaemolyticus* by primary PCR and nested PCR from 103 frozen shrimp samples. Samples showing positive for *V. parahaemolyticus* and/or *ldh* by PCR methods were found 89.3%, and samples showing negative for *V. paraahaemolyticus*

Table 20 **Prevalence of possessing *ldh* gene in bacterial strains isolated from frozen shrimp samples**

Microorganisms	Total no. of tested strains	No. (%) of tested strains	
		ldh$^+$	*ldh*$^-$
V. parahaemolyticus	258[a]	251 (97.3)	7 (2.7)
V. alginolyticus	11[b]	1 (9.1)	10 (90.9)
V. cholerae non O1	3[b]	0	3 (100)
Aeromonas spp.	2[b]	0	2 (100)

[a] Isolated colonies obtained from all 82 culture positive samples for *V. parahaemolyticus.*

[b] Obtained from 10 enrichment samples negative for *V. parahaemolyticus*, but positive for other microorganisms which gave positive for *ldh* by primary PCR.

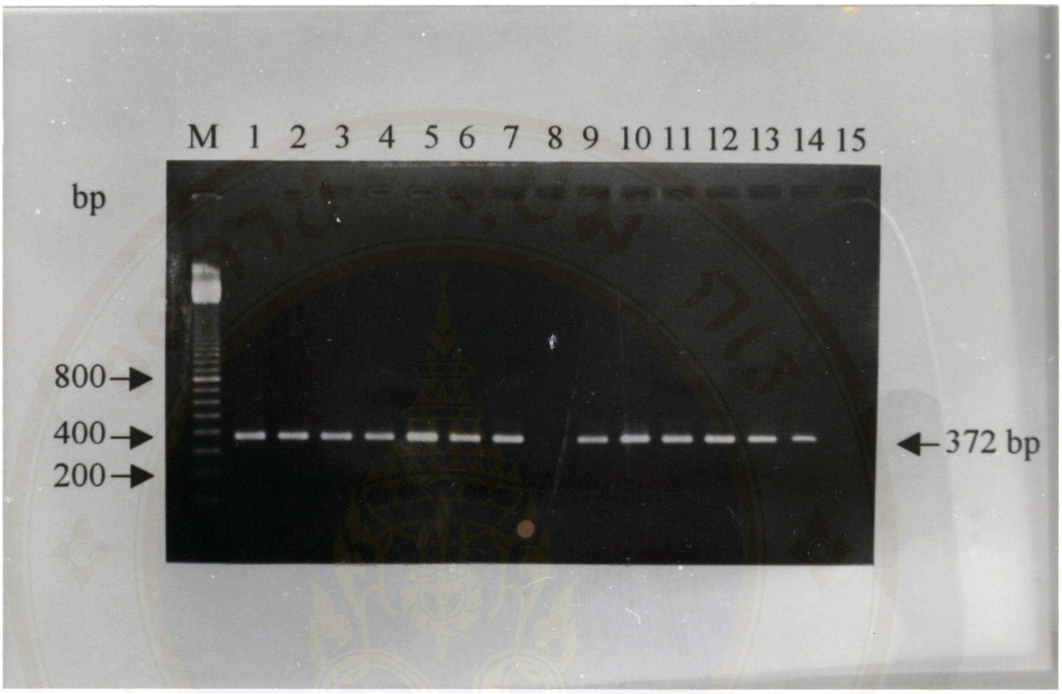

Fig. 18 1.5% agarose gel electrophoresis showing 372 bp amplification products of primary PCR of *V. parahaemolyticus* isolates from positive culture of frozen shrimp samples. Lane 1, isolated colonies from sample number FS 001-99/E; Lane 2, isolated colonies from sample number FS 002-99/D; lanes 3 to 6, isolated colonies from sample number FS 002-99/E; Lanes 7 to 11, isolated colonies from sample number FS 004-99/E; Lanes 12 to 14, isolated colonies from sample number FS-005-99/E; Lane 15, *V. parahaemolyticus* BG 26 (*ldh*$^+$,*tdh*$^-$*trh*$^-$)as a positive control; Lane 16, a negative control (no DNA template); Lane M, 100 bp DNA ladder (Pharmacia Biotech, USA).

by all studies method were 10.7%. *V. parahaemolyticus* was found in only 1 sample by direct culture method. This sample was also found positive for *ldh of V. parahaemolyticus* by nested PCR but not by primary PCR. Using nested PCR was also able to detect *ldh* directly in 3(2.9%) samples that found negative by culture method directly and after enrichment of the samples. Furthermore, primary PCR was able to detect *ldh* of *V. parahaemolyticus* from 10(9.7%) enriched samples which found negative by culture method. However, one enriched sample was found positive for *V. parahaemolyticus* by culture method but found negative by both PCR methods.

D. Comparison of Nested PCR and Primary PCR

Table 22 shows the detection of *ldh* of *V. parahaemolyticus* by nested PCR compared with the culture method from enrichment samples. Of those 82 frozen shrimp samples from enrichment culture positive for *V. parahaemolyticus*, only 32 samples were positive for *ldh* by nested PCR. Among 21 samples from enrichment culture negative, 3 samples were positive by nested PCR. The sensitivity, specificity and efficiency of nested PCR were 39.0%, 85.7% and 48.5%, respectively. Positive predictive value of using nested PCR on direct samples as a screening test was 91.4%, but negative predictive value was only 26.5%, as the false positive were smaller than false negative samples. Therefore, the nested PCR and enrichment culture methods for detection of *V. parahaemolyticus* were significantly different ($p < 0.001$; McNemar χ^2 test).

Table 21 **Relationship between culture method and presence of *ldh* of *Vibrio parahaemolyticus* by primary PCR and nested PCR from 103 frozen shrimp samples**

Growth on TCBS		Direct samples		Enrichment samples	No (%) of samples
Direct	Enrichment	Primary PCR	Nested PCR	Primary PCR	
+[a]	+	-	+	+	1 (0.9)
-	+	+	+	+	8 (7.8)
-	+	-	+	+	23 (22.4)
-	+	-	-	+	49 (47.6)
-	+[a]	-	-	-[b]	1 (0.9)
-	-	-	+	+	3 (2.9)
-	-	-	-	+	7 (6.8)
-	-	-	-	-[b]	11 (10.7)
Total 1 (0.9)	82 (79.6)	8 (7.7)	35 (33.9)	91 (88.3)	103 (100)

[a] An arabinose negative isolate identified as *V. alginolyticus* ; an negative for *ldh* when tested by using nested PCR.

[b] Also negative for nested PCR.

Table 22 Comparison of the detection of *Vibrio parahaemolyticus* by using nested PCR and by using enrichment method in 103 frozen shrimp samples

Nested PCR	Enrichment culture		Total
	Positive	Negative	
Positive	32	3	35
Negative	50	18	68
Total	82	21	103

Sensitivity $\frac{32}{82} \times 100 = 39.0\%$

Specificity $\frac{18}{21} \times 100 = 85.7\%$

Positive predictive value $\frac{32}{35} \times 100 = 91.4\%$

Negative predictive value $\frac{18}{68} \times 100 = 26.5\%$

Efficiency $\frac{(32 + 18)}{103} \times 100 = 48.5\%$

McNemar χ^2 test ; $p < 0.001$

The result of the detection of *ldh* from direct samples by primary PCR compared with the detection of *V. parahaemolyticus* from enrichment culture as shown in Table 23. Among 82 from enrichment culture positive for *V. parahaemolyticus,* only 8 samples were positive for *ldh* by primary PCR, but 74 samples were negative for *ldh.* Similar to nested PCR method, primary PCR gave sensitivity, specificity and efficiency 9.7%, 100% and 28.1%, respectively. The positive predictive value of primary PCR on direct samples as a screening test was 100% and negative predictive value was 22.1%. The detection of *ldh* by using the primary PCR from direct samples and the detection of *V. parahaemolyticus* from enrichment culture was significantly different ($p< 0.001$; McNemar χ^2 test)

Table 24 shows the comparison for detection of *ldh* from enrichment samples by primary PCR and detection of *V. parahaemoyticus* from enrichment samples by culture method. Of those 82 samples from enrichment culture positive for *V. parahaemolyticus*, 81 samples were positive for *ldh* by primary PCR that gave sensitivity of the test 98.8%. Moreover, 21 samples obtained from enrichment culture negative samples, 10 samples were also *ldh* positive by primary PCR suggesting the gene detection method was able to improved *V. parahaemolyticus* detection rate and more rapid than culture method. The sensitivity, specificity and efficiency of primary PCR when compared with culture method were 98.8%, 52.4% and 89.3%, respectively. The result was indicated that the primary PCR from enrichment samples was able to enhance the detection of *V. parahaemolyticus* in higher percent than enrichment culture. Using primary PCR for detection of *V. parahaemolyticus* from enrichment

Table 23 Comparison of the detection of *Vibrio parahaemolyticus* by using primary PCR in direct samples and by using enrichment method in 103 frozen shrimp samples

Primary PCR from Direct samples	Enrichment culture		Total
	Positive	Negative	
Positive	8	0	8
Negative	74	21	95
Total	82	21	103

Sensitivity $\frac{8}{82} \times 100 = 9.7\%$

Specificity $\frac{21}{21} \times 100 = 100\%$

Positive predictive value $\frac{8}{8} \times 100 = 100\%$

Negative predictive value $\frac{21}{95} \times 100 = 22.1\%$

Efficiency $\frac{(8+21)}{103} \times 100 = 28.1\%$

McNemar χ^2 test ; $p < 0.001$

Table 24 Comparison of the detection of *Vibrio parahaemolyticus* by using primary PCR in enrichment samples and by using enrichment method in 103 frozen shrimp samples

Primary PCR from enrichment samples	Enrichment culture		Total
	Positive	Negative	
Positive	81	10	91
Negative	1	11	12
Total	82	21	103

Sensitivity $\frac{81}{82} \times 100 = 98.8\%$

Specificity $\frac{11}{21} \times 100 = 52.4\%$

Positive predictive value $\frac{81}{91} \times 100 = 98.0\%$

Negative predictive value $\frac{11}{12} \times 100 = 91.7\%$

Efficiency $\frac{(81 + 11)}{103} \times 100 = 89.3\%$

McNemar χ^2 test ; p = 0.012

samples were significantly different enrichment culture method (P = 0.012; McNemar χ^2 test).

E. Association and Agreement Rate between Nested PCR and Primary PCR

The association and agreement rate between nested PCR and primary PCR from direct frozen shrimp samples are shown in Table 25. Among 103 frozen shrimp samples, 8 samples were *ldh* positive both by nested PCR and primary PCR. Twenty-seven samples were positive by using nested PCR but negative by using primary PCR from direct samples. The result was revealed that nested PCR was able to detect *ldh* of *V. parahaemolyticus* from direct samples approximately four times higher than primary PCR. Consequently, the association between nested PCR and primary PCR from direct samples was significantly different by McNemar χ^2 test ($p < 0.001$). The kappa measure of agreement of both methods was poor (k=0.28).

Table 25 Association and agreement rate between nested PCR and primary PCR from direct samples for detection of *ldh* of *Vibrio haemolyticus* from 103 frozen shrimp samples

Nested PCR	Primary PCR from direct samples		Total
	Positive	Negative	
Positive	8	27	35
Negative	0	68	68
Total	8	95	103

Kappa = 0.28

McNemar χ^2 test $P < 0.001$

CHAPTER V

DISCUSSION

Several investigators have been reported the detection of *V. parahaemolyticus* by using molecular biology technique. These techniques such as DNA hybridization and PCR were employed to examine *tdh* and *trh* hemolysin gene of *V. parahaemolyticus* (25,27,135,137,138). Generally, the distribution of *tdh* gene of *V. parahaemolyticus* isolated from environmental samples was very few but *tdh* and *trh* genes were commonly presented in clinical strains of *V. parahaemolyticus*(13,23-25, 29-30). Therefore, hemolysin genes, as the virulence factors detected in food or environmental samples were unfavorable markers for detection of *V. parahaemolyticus* (23-25,31)

This study was the first in the field to develop nested PCR successfully to detect *ldh* gene of *V. parahaemolyticus* in frozen shrimp. Kachornchaiyakul (34) reported the detection of *ldh* of *V. parahaemolyticus* in frozen shrimp by primary PCR with the sensitivity of her technique was 122 fg/µl of genomic DNA, or broth containing 175 cells. In this study, the nested PCR which combined two sets of primers, was able to detect the lowest concentration of *V. parahaemolyticus* genomic DNA as little as 1 fg/reaction corresponding to approximately 1.7 cells by agarose gel electrophoresis. The results are similar to that of Lee SE, *et al.* (39) which reported that the sensitivity of nested PCR to detect *V. vulnificus* in clinical specimens was 1 fg of chromosomal DNA or single CFU. In addition, the results were similar to the study described by Lee JY, *et al.* (146), nested PCR could detect 1 to 10 CFU/ml of *V. vulnificus* in 1 ml of seafood homogenates. However, the sensitivity of nested PCR in this study (1fg) was also more sensitive than the study of Arias *et al.* (10 fg) (37) who developed the nested PCR for detection of *V. vulnificus* and of Herman *et al.* (142) who developed nested PCR for detection of *Listeria monocytogenes* (5 to 10 CFU) from raw milk.

The nested PCR products in this study gave additional band of 372 bp and non-specific band beside the predicted products of 196 bp. It was possible that the primary PCR products of 372 bp were amplified with the excess amount from the primary PCR primers (LD_1-LD_2) and DNA template. This correspond with the report of Winters *et al.*(38) who found an additional band of nested PCR products in their experiments. They proposed that an additional band was probably due to a larger amount of product in the initial cycling set being further amplified, since the first primers set were transferred to the second reaction when the sample was added.

Nested *ldh* primers were expected to specifically amplified a 196 bp amplicon. It showed 100 specificity for the tested strains of *Vibrio* spp. and other Gram negative bacilli. This showed the similar results as the study of Kachornchaiyakul (34) who examined the specificity of *ldh* primers by PCR assay. These results also corresponded to those of McCarthy *et al.* (157) who evaluated an alkaline phosphatase and digoxigenin labeled probes for detection of the thermolabile hemolysin (*tlh*) gene of *V. parahaemolyticus*. The *tlh* gene probe can identify the presence of both pathogenic and non-pathogenic strains of *V. parahaemolyticus*. The *ldh* gene were found in 97.3% of *V. parahaemolyticus* isolated from 103 frozen shrimp samples. In addition one *V. parahaemolyticus* isolated from the same set of samples was found to be ldh^+.

In this study, total viable count (TVC) were enumerated in all 103 frozen shrimp samples. It ranged from 1.0×10^3 to 3.0×10^6 CFU/g. The coliforms count ranged from 5.0×10 to 2.9×10^3 CFU/g. Similar results were reported as described by Kowcachaporn (28). The majority of TVC (71.8%) and coliforms (74.8%) of these frozen shrimp samples was acceptable according to specification and standards for frozen food products by JETRO criteria. Only 1 sample was detected *V. parahaemolyticus* of 1.0×10^2 CFU/g by direct plate count. The number of *V. parahaemolyticus* in this samples was acceptable according to IMCSF criteria (14). Comparison of prevalence of *V. parahaemolyticus* contaminated in raw fresh shrimp samples, Limuthaitip(10) reported 68% of raw shrimp contaminated with *V. parahaemolyticus* ranged from 5.0×10 to 4.0×10^4 CFU/g. While, *V. parahaemolyticus* was detected in 79.6% (82/103) of frozen shrimp samples after one-night enrichment in APW medium of this study. These results were supported that *V. parahaemolyticus* in cold, chilled or frozen samples which was inactivated at cold or freezing temperature were able to recover after culturing in the enriched medium especially APW (48,56,83,125). Generally, *V. parahaemolyticus* was commonly isolated from fish and shellfish caught in estuarine and inshore coastal water (43). Therefore, it was possible that raw material frozen shrimp types (91.8%) which were not passed the processing plant, the contamination of *V. parahaemolyticus* or other

Gram negative bacteria was higher than the finished product frozen shrimp types. (74.1%). The finished product frozen shrimp types passed the food processing steps such as rinsing and mechanical process in killing and washing away some microorganisms, thus reducing the number of *V. parahaemolyticus* present in the samples of finished products.

Halophilic vibrios are one of the major groups of bacteria found in the marine environment. Several researchers reported the prevalence of *Vibrio* spp. isolated from seafood and other environmental samples (11,91,158,159). Matte´ *et al.* (158) reported the distribution of vibrios in oysters originating from the coast of Brazil. The prevalence of *V. alginolyticus, V. parahaemolyticus, V. cholerae* non-O1, *V. fluvialis, V. furnissii, V. mimicus* and *V. vulnificus* was 81%, 77%, 31%, 27%, 19%, 12% and 12%, respectively. In this study, 58.2% (60/103) of frozen shrimp samples were contaminated with *Vibrio* spp. and *V. alginolyticus* was the most common isolates 28.1% from frozen shrimp samples, followed by *V. vulnificus* 12.6%, *V. cholerae* non O1 and *P. shigelloides* 5.8%, *Aeromonas* spp. 4.8% and *V. mimicus* 1.9%. Additionally, *E. coli* was also contaminated in 23.3% of these samples. These bacteria currently recognized as human pathogens and also indicators of health risk. A number of these bacteria also produced extracellular heat-labile or heat-stable hemolysin and cytotoxins (160), e.g. *V. cholerae* non O1 and *V. mimicus* produced hemolysin which were immunologically related to the thermostable direct hemolysin (TDH) of *V. parahaemolyticus* (115,161). In addition, *V. vulnificus* was phenotypically similar to *V. parahaemolyticus* and has been recognized as a highly virulent pathogen. It can result in life-treatening human infections when involved in wound infections, septicemia and foodborne gastroenteritis (125,162-164). Therefore, the most important mean of safeguarding against the public health hazard caused by these organisms is to adopt measures to control its multiplication in frozen shrimp and recommend proper food cooking and food holding before consuming to prevent the foodborne disease caused by these microorganisms.

PCR assays have been developed for detection of foodborne pathogens in both clinical and environmental samples. In this study, PCR and nested PCR were used for detection of *ldh* of *V. parahaemolyticus* in frozen shrimp samples. Using primary PCR to detect *ldh* of *V. parahaemolyticus* was able to detect *ldh* of *V. parahaemolyticus* 7.7% (8/103) of direct frozen shrimp samples. These results were contrast to the study of Kachornchaiyakul (34) that the PCR assay could not detect *ldh* gene in direct frozen shrimp samples. However, *V. parahaemolyticus* was isolated from 82 of 103 frozen shrimp samples by enrichment method. Of those 82 enrichment samples, 98.8% (81/82) were positive for *ldh* by primary PCR. It was obvious that 20 samples of *V. parahaemolyticus* culturing negative also gave positive results for *ldh* in 50% (10/20) by primary PCR. It corresponded with the report of Wang *et al.* (36) who established a PCR protocol for detection of foodborne pathogen required an enrichment step. These results indicated that the PCR assay was able to detect *ldh* gene even if *V. parahaemolyticus* cells were injured and low target DNA or viable but noncultural that could not be detectd by conventional method. When nested PCR was employed to detect *ldh* of *V. parahaemolyticus* in direct samples, 33.9%(35/103) of direct frozen shrimp samples were positive for *ldh*. These nested PCR results gave *ldh* positive significantly higher than those of primary PCR.($p< 0.001$). It was due to nested PCR composed of two rounds of PCR which could increase the sensitivity even the presence of low bacterial target DNA. Furthermore, The original results positive for *ldh* of nested PCR were only 24.3% (25/103) due to in the experiment of nested PCR protocol took 1 µl of sample diluted 1:5 , used as DNA template. When the samples were diluted as 1:10 dilution, the positive results were increased to 33.9%(35/103). These results could be explained that the complex composition of food matrices could inhibit the PCR reaction (36) or high level of template DNA usually inhibit PCR reaction. Thus, a proper dilution of sample was required (138).

In raw material frozen shrimp sample type, the *ldh* of *V. parahaemolyticus* was detected significantly higher than the finished product frozen shrimp sample type. It was possible that *V. parahaemolyticus* was a worldwide distribution in the estuarine and coastal environment and has been isolated from many species of fish, shellfish crustaceans and shrimp (165). They usually present on the surface of shellfish due to their affinity for chitin (82). Furthermore, the detection of *ldh* by both primary PCR and nested PCR in peeled finished products (PFP) were higher than that of unpeeled finish products (UPFP)($p<0.05$). These results might be due to (a) since shrimp had survived only a few minutes after removal from their natural habitat, microbial spoilage occurred immediately through marine bacteria on the surface, particularly their heads carrying approximately 75% of bacterial number (166); these reasons caused the increasing of DNA templates contained in food matrices of UPFP which might have higher than that of PFP; (b) these food matrices included the chitin coat could interfere and inhibit PCR reaction more than that of PFP, even chelex-100 used in the experiment could eliminate inhibitors; (c) the contamination of *V. parahaemolyticus* in PFP was higher than that of UPFP from the beginning, since the 2 groups of samples came from different batch.

The prevalence of possessing *ldh* gene of a total of 258 *V. parahaemolyticus* isolates were determined by primary PCR. The result showed that 97.3% (251/258) *V. parahaemolyticus* strains were positive for *ldh* while 2.7%(7/258) of *V. parahaemolyticus isolates* were negative for *ldh*. Among these 7 strains of *ldh* negative, all isolates showed negative for arabinose fermentation. It was corresponded with the report of Chan *et al.* (91). They suggested that fermentation of arabinose is an important feature for distinguishing *V. alginolyticus* and *V. parahaemolyticus* isolated from seafood. The results showed that 90.3% (28/31) of *V. parahaemolyticus* isolates gave positive reaction for arabinose fermentation. Furthermore, 16S rRNA sequences revealed 99.7% homology between both organisms (140). Thus, it was possible that 7 strains which were identified as *V. parahaemolyticus* by conventional method but gave *ldh* negative by primary PCR, were *V. alginolyticus*. Alternatively, *V. alginolyticus*, 90.9% (10/11) were negative for *ldh* but only 1 strain gave positive for *ldh*. The possible explanations for the absence of *ldh* in *V. parahaemolyticus* and presence of *ldh* in *V. alginolyticus* strains might be due to (a) *V. alginolyticus* isolates are closely related in biochemical reaction, and the *V. alginolyticus* isolates might be misidentified as *V. parahaemolyticus* (18) and vice versa; (b) this isolated strain might be an atypical *V. parahaemolyticus* showing yellow colony on the TCBS agar (91); (c) owing to, *ldh* is a species specific gene and present only in *V. parahaemolyticus* (32) this confirmed by the report of MaCarthy (157) who evaluated specificity of thermolabile haemolysin (*tlh)* gene probe against totally 816 strains of suspected *V. parahaemolyticus* and 98 strains of non-*Vibrio* spp. and *Vibrio* spp. including *V. alginolyticus.* The result showed high specificity and no-cross hybridization. In addition, all strains of *V. cholerae* non O1 and *Aeromonas* spp. gave negative for *ldh.*

V. parahaemolyticus isolates were detected in 82 (79.6%) enrichment samples while *ldh* of *V. parahaemolyticus* were detected in 91 (88.3%) enrichment samples. The detection of *V. parahaemolyticus* increased when using primary PCR. These reasons indicated that PCR assay was able to detect *ldh* of *V. parahaemolyticus* not only viable cells but also viable but noncultural cells (167). These viable but noncultural cells can exist in a state where they are viable, but can not be cultured by normal microbiological methods. This differentiation of vegetative cells into a dormant viable but nonculturable (VNC) state is a survival strategy for many nonsporulating species. The VNC state is morphologically different from the "normal" vegatative cell. During the transition to the VNC state, rod-shaped cells shrink and become small spherical bodies which are totally different from bacillus and clostridial spores (168,169). It takes from 2 days to several weeks for an entire population of vegetative cells to become VNC (169,170).

Because the VNC state is most often induced by nutrient limitation in aquatic environments, it might appear irrelevant of the nutrient-rich milieu of food. However, the VNC state can also be induced by changes in salt concentration, expose to hypochlorite, and shift in temperature (20,171,172).

Resuscitation of VNC cells is demonstrated by an increase in culturability that is not accompanied by an increase in the total number of cells. The return to culturability can be induced by temperature shifts or gradual return of nutrients. It can take several days for the population to fully recover its culturability. The same population of bacteria can go through multiple cycles of the VNC and culturable states in the absence of growth (169). Inhibitors of protein or peptidoglycan synthesis prevent VNC cells from resuscitating (172). Increased awareness of the VNC state should lead to a reexamination of concept of viability, dependence on enrichment culture to isolate pathogens, and reliance on established cultural methods to monitor microbes in the environment (171-173).

In this study, the use of enrichment method was able to detect *V. parahaemolyticus* in 79.6% (82/103) from frozen shrimp samples. In contrast to primary PCR could detect *V. parahaemolyticus* only 7.7% (8/103) from the samples directly. Previously, PCR assay could not detect foodborne pathogens from direct samples (34,36). In this study, primary PCR could detect *ldh* of *V. parahaemolyticus* in 8 (7.7%) direct samples this might be due to the samples contained cells or DNA enough to be able to detect the presence of *V. parahaemolyticus* (at least 175 cells or 100 fg genomic DNA.)

In addition, nested PCR was able to detect *ldh* of *V. parahaemolyticus* in direct frozen shrimp samples without prior enrichment step. Of 103 samples, 35 (33.9%) samples were positive for *ldh* of *V. parahaemolyticus* by nested PCR which was higher than that of primary PCR. It might be explained that the minimum of genomic DNA used for amplification by nested PCR was 100 time less than that of primary PCR. However, sensitivity and specificity of the tests were 39% and 85.7%, respectively. Sensitivity of nested PCR seemed to be low. The reasons might be explained that (a) the complex composition of food samples using in processing plant could inhibit the activity of *Tag* DNA polymerase in PCR reaction as mentioned previously (34,174,175); (b) chelex-100 was used to eliminate the PCR inhibiting components presenting in direct and enrichment frozen shrimp samples in this study might not fully eliminated; chelex-100 is known to stabilize and prevent the damage of the genomic DNA in boiling water by maintaining the ionic strength of the sample (152); (c) low densities of target DNA of *V. parahaemolyticus* in direct samples might be less than 1 fg or 1.7 cells which was the minimum amount for detecting *V. parahaemolyticus* by nested PCR. However, for enrichment culture negative for *V. parahaemolyticus,* 3 samples showed positive for *ldh* by nested PCR which was similar to the report of Lee *et al.* (39). They found that 40% of culture negative for *V. vulnificus* became positive by nested PCR.

On the other hand, the detection of *V. parahaemolyticus* by using primary PCR in enrichment samples gave positive results in 88.3% (91/103) which were higher than those of enrichment cultures (p=0.012). This method showed high sensitivity in 98.8% (Table 15). It was interestingly that 21 samples of enrichment culture negative for *V. parahaemolyticus,* 10 samples were positive for *ldh* by primary PCR from enrichment samples. The results could be explained that, primary PCR after enrichment step was able to detect not only viable cells but also viable but nonculturable cells

(167). In addition, enrichment culture procedure may be helpful distinguish the live cells from the dead cells, as only live cells can grow and give positive PCR (36,167).

As nested PCR becomes more widely used for the detection of infectious agent directly both in clinical and environment samples (38,144,146). In this study, we determined the association and agreement rate between nested PCR from direct samples and primary PCR from enrichment samples. The result showed poor agreement (k=0.127). Furthermore, association between nested PCR and primary PCR to detect *ldh* of *V. parahaemolyticus* was significantly different ($p<0.001$). The reasons for low detection of *ldh* of *V. parahaemolyticus* by nested PCR might be due to some inhibitors in food matrix or substances used to eliminate the inhibitor were insufficient as mentioned previously. It was interestingly that of 35 frozen shrimp samples which gave positive for *ldh* by nested PCR were also positive for *ldh* by primary PCR in enrichment samples and did not show any false positive by nested PCR. These results indicated that nested PCR was able to detect *V. parahaemolyticus* not only viable cells but also viable but nonculturable cells directly in food samples which was similar to primary PCR.

Although the nested PCR assay in this experiment showed low sensitivity to detect *ldh* of *V. parahaemolyticus* when compared with enrichment method. It was successfully developed for rapid to detecting target DNA directly from frozen shrimp samples, without prior enrichment step, and could report the results within 8 hr. Therefore, the nested PCR might be useful and further applied to detect of other foodborne pathogens in food and other environmental samples. However, the use of new substances such as Triton X-100 (36,39), guanidine isothiocyanate (146,174) to eliminate inhibitors should be investigated to improve the nested PCR protocol in comparison with chelex-100 for its activity. The extraction method to obtain highly sensitive and specific results within several hours should also be further studied.

CHAPTER VI

CONCLUSION

The rapid detection of foodborne pathogens including *V. parahaemolyticus* was carried out based on the principles of PCR assay. The nested PCR, in which internal primers complementary to the sequences amplified by the primary reaction were used in a secondary reaction, leading to increase the sensitivity as well as the specificity of the reaction. Therefore, nested PCR was successfully developed for rapid detection of *ldh* of *V. parahaemolyticus* in frozen shrimp samples with the lowest detectable genomic DNA of 1fg or 1.7 cells. In this study, 79.6% of enrichment samples were contaminated with *V. parahaemolyticus* by conventional method. This demonstrated that the enrichment step was important and necessary for recovery of *V. parahaemolyticus.* The enrichment step was also important and necessary when using primary PCR for detecting *ldh* of *V. parahaemolyticus* [*ldh* detection in enrichment samples (88.3%; 91/103) was significantly higher than that of direct samples (7.7%, 8/103) ($p<0.001$)]. Furthermore, the conventional method to identify *V. parahaemolyticus* from food samples had faced the problems of misidentifying *V. parahaemolyticus* as *V. alginolyticus;* or *V. alginolyticus* as *V. parahaemolyticus.* Therefore, the *ldh* primers were proved to be more specific for identifying *V. parahaemolyticus* in food samples than the conventional culture method. It was emphasized that the detection of *ldh* of isolated *V. parahaemolyticus* strains from food and environmental samples by PCR should be required for confirming the identification. Enrichment of the samples before using PCR method to detect *ldh of V. parahaemolyticus* was necessary to improve the detection of viable and nonculturable *V. parahaemolyticus* as compared with PCR method directly on (from 7.7% to 88.3%). The detection of *ldh* of *V. parahaemolyticus* from direct samples by using nested PCR (33.9%, 35/10) was significantly higher than that by primary PCR (7.7 %;8/103) ($p<0.001$). The nested PCR is a rapid method, taking less than 8 hr to identify *ldh* of *V. parahaemolyticus* in frozen shrimp samples. Conversely, conventional method and primary PCR require 4-5 days and 18-24 hr, to detect *V. parahaemolyticus*, respectively. However, the nested PCR should be further improved and developed in a) the food sample preparation step to eliminate PCR inhibitor; b) increasing the degree of sensitivity; c) reducing the risk of cross-contamination by using one-tube nested PCR (144) resulting in less time-consuming, and cost-saving to detect *V. parahaemolyticus* in food samples. The nested PCR assay then could be used as a screening method particularly on seafood samples with high prevalence of bacterial contamination as specificity and positive predictive value of the test were as high as 85.7% and 91.4%, respectively. Moreover, the nested PCR or the primary PCR, and the conventional method should be combined to increase the sensitivity and specificity in detecting *V. parahaemolyticus* from food samples for aiding food processors, regulators in establishing, controlling and monitoring critical control points in food processing plants. Nevertheless, the frozen shrimp samples may be a good model for VNC state of *V. parahaemolyticus*. The methods for detection *ldh* (marker for the whole *V. parahaemolyticus* species) by PCR or nested PCR can be further applied for detecting *V. parahaemolyticus* or other bacteria not only in food samples but also in other environmental and clinical samples as well.

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APPENDIX A

Preparation of Reagents and Media

1. Reagents for genomic DNA extraction

1.1 TE buffer pH 8.0 was composed of 10 mM Tris-Cl,1MEDTA.Tris-HCl (Sigma, LT. Louis, MO, USA) 1.57g and EDTA (USB, Cleveland, Ohio, USA) 0.37g were dissolved in 900 ml of deionized distilled water. After adjusted pH to 8.0 with concentration HCl, adjusted volume to 1,000 ml. The solution was sterilized by autoclaving at 121°C, 15 min and stored at 4°C.

1.2 20% SDS was consisted of 200 g of electropheresis grade of sodium dodecyl sulfate (Ameresco, Solon, Ohio, USA) in 900 ml of deionized distilled water. The solution was heated at 68°C for completely dissolving. After adjusted pH to 7.2 with concentrated HCl, deionized distilled water was added to 1,000 ml and sterilized by filtration. The solution was stored at room temperature.

1.3 Protinase K (20 mg/ml) (Ameresco, Solon, Ohio, USA). The solution was consisted of 20 mg of Protinase K and dissolved in 1 ml TE buffer. Then, it was dispensed into aliquots and stored at -20°C.

1.4 Ribonuclease I "A" bovine pancrease (10 mg/ml) was composed of 10 mg pancreatic RNase (Pharmacia Biotech, USA), dissolved in 1 ml of deionized distilled water. After boiled at 100°C, the solution was allowed to cool at room temperature, dispensed into aliquots and stored at –20°C. It was used to remove contaminating RNA from plasmid or genomic DNA preparation.

1.5 CTAB/NaCl solution (10% CTAB in 0.7 M NaCl) was composed of 4.1 g of NaCl (Merck, Darmstadt, Germany) in 80 ml of deionized distilled water. Ten grams of hexadecyltrimethyl amonium bromide (CTAB, Merck, Darmstadt, Germany) was slowly added to NaCl solution while stirring and heating to 65°C, and then, adjusted the volume to 100 ml with deionized distilled water. CTAB is a cation detergent that will bind protein and polysaccharide. These complex will be appeared as a white interphase after adding chloroform isoamyl alcohol and centrifugation.

1.6 Phenol (Ameresco, Solon, Ohio, USA). Hydroxyquinoline was added to phenol at a final concentration of 0.1%. This compound is an anti-oxidant, a partial inhibitor of RNase and a weak chelator of metal ion. In addition, its yellow color provides a convenient way to identify the organic phase. Phenol is necessary to equilibrate to pH > 7.8 because DNA will be partition into the organic phase at acid pH. The phenol solution was stored under 0.1 volume of 0.1 M Tris-HCl pH 8.0 containing 0.2% β-mercaptoethanol in a tight-light bottle at 4°C for a period up to 1 months.

1.7 Chloroform : isoamyl alcohol was composed of 24 parts of chloroform (Merck, Darmstadt, Germany) and 1 part of isoamyl alcohol (Merck, Darmstadt, Germany). It was used for removing any lingering traces of phenol.

1.8 Phenol : chloroform : isoamyl alcohol (25:24:1) was composed of equal part of equilibrated phenol and chloroform : isoamyl alcohol (24:1). It was used to remove proteins from preparation of nucleic acid. The chloroform denature protein and facilitates the separation of the aqueous and organic phase, and the isoamyl alcohol reduces foaming during extraction.

1.9 Isopropanol (Merck, Darmstadt, Germany) It was used for DNA preparation and stored at room temperature.

1.10 70% Ethanol was consisted of 70 ml of absolute ethanol (Merck, Darmstadt, Germany) and 30 ml of deionized distilled water. It was used to concentrate the nucleic acid and removed the remaining isopropanol which is difficult to evaporate.

1.11 Luria-Bertani (LB) broth was composed of 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl in 1000 ml deionized distilled water and sterilized by autoclaving at 121°C for 15 min. It was used to culture the reference and tested strains.

1.12 Chelex-100 solution was composed of 0.6 g Chelex-100 resin (Bio-Rad Laboratory, California, USA) in 100 ml deionized distilled water and sterilized by autoclaving at 121°C for 15 min.

2 Reagents for polymerase chain reaction

2.1 10 x PCR buffer was compound of 100 mM Tris-HCl (Sigma, ST.Louis, MO, USA) pH 8, 500 mM KCl (USB, Cleveland, Ohio, USA), 1% Triton X-100 (Ameresco, Solon , Ohio, USA). Ten ml of sterile 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.

2.2 25 mM $MgCl_2$ (Merck, Darmstadt, Germany) was consisted of 2.5 ml of sterile 1 M $MgCl_2$ 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.

2.3 Deoxyribonucleotide triphosphate (dNTPs, Promega, Madison, WI, USA). 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 this solution was added in 25 µl of PCR mixture to make a final concentration of 200 uM dNTP each.

2.4 *Taq* polymerase (Pharmacia Biotech, USA) *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.

2.5 Primers Two set of primers; LD_1-LD_2 and LD_3–LD_4 were synthesized by Bioservice Unit, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Bangkok.

LD_1 p mole/ OD = 4000

11.25 OD purified in 1 DDW

Thus, The LD_1 primer contained 400 x 11.25 = 45,000 pmole/ml After that, diluted the stock of LD_1 primer to make a final concentration at 3 pmole/µl in 100 µl sterile deionized distilled water by using the following formula :

$$N_1 V_1 = N_2 V_2$$

$$45{,}000 \text{ pmole/ml} \times 10^{-3} \times V_1 = 3 \text{ pmole}/100\ \mu\text{l}$$

$$V_1 = 6.7\ \mu\text{l}$$

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

LD_2 pmole / OD = 4348

11.1 OD purified in 1 ml DDW

Thus, the LD_2 primer contained 4348 x 11.1 = 48,262.80 pmole/ml then, diluted the stock of LD_2 primer, by taking 6.2 µl of LD_2 primers and sterile deionized distilled water were added to 100 µl to make a final concentration at 3 pmole/µl. One µl of this diluted primer was used in 25 µl of PCR mixture..

LD_3 pmole/OD = 4545

7.5 OD purified in 1 ml DDW

Thus, the LD_3 primer contained 4545 x 7.5 = 34,087.50 pmole/ml then, diluted the stock of LD_3 primer, by taking 29.3 µl of LD_3 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 PCR mixture.

LD_4 pmole /OD = 4545

9.25 OD purified in 1 ml DDW

Thus, the LD_4 primer contained 4545 x 9.5 = 43,177.5 pmole/ml then, diluted the stock of LD_4 primer, by taking 23.2 µl of LD_4 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.

3 Reagents for electrophoresis

3.1 1 x TBE powder (Ameresco, Solon, Ohio, USA) 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.

3.2 0.8% and 1.5% agarose gel (Seakem LE, FMC Bioproducts, Rockland, ME, USA) was composed of 0.8 g or 1.5 g agarose in 100 ml 1 x TBE buffer, respectively.

3.3 6x loading buffer was consisted of 2.5% bromophenol blue (USB, Cleveland, Ohio, USA) and 40% sucrose (Mallinckrod, ST, Louis, MO, USA) w/v in water. The buffer was dispensed into aliquots and stored at 4°C.

3.4 100 Base-Pair Ladder (Pharmacia Biotech, USA) 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 appeare 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.

3.5 Stock ethidium bromide solution (10 mg/ml Merck, Darmstadt, Germany) Five µl of stock ethidium bromide were diluted with 100 ml deionized distilled water to make working solution (5 µg/ml) for staining agarose gel after electrophoresis.

4. Media and biochemical tests

4.1 Thiosulfate citrate bile salt sucrose (TCBS) agar (Marck, Darmstadt, Germany) was the selective medium used for isolating *Vibrio* spp.

4.2 M-Fc agar (Difco, Detroit, Michigan, USA) was the medium used for enumerating coliforms group.

4.3 MacConkey (MC) agar (Difco, Detroit, Michigan, USA) was the differential medium used for enumerating *E. coli*.

4.4 Plate count (PC) agar (Difco, Detroit, Michigan, USA) was the medium used for enumerating the total bacteria in frozen shrimp samples.

4.5 Alkaline peptone water (APW) broth with 3% NaCl was the enrichment medium for *V. parahaemolyticus.*

4.6 Triple sugar iron (TSI) and **motility-indole-lysine (MIL)** medium (Difco, detroit, Michigan, USA) were used for screening of *V. parahaemolyticus.*

4.7 Biochemical confirmation tests were lysine decarboxylase, arginine dihydrolase, ornithine decarboxylase, Voges-Proskauer (VP), urea hydrolysis, simmons citrate, fermentation of lactose, sucrose, arabinose, salicin and the salt tolerance of 0%, 3%, 6%, 8% and 10% NaCl. In addition, semi-solid medium was used for stock medium. All biochemical test were purchased from Difco laboratory (Difco, Detroit, Michican, USA) and contained 1% NaCl which was the growth requirement of *V. parahaemolyticus.*

All kind of media as mentioned were prepared by the operation procedure of manufactory.

APPENDIX B

Statistical analysis

1. Sample size

Sample size was calculated by using the following formula (147)

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

n = Sample size

Z = Standard normal deviation at $\alpha = 0.05$

P = Proportion of event in population

Q = Proportion of failure in population

d = Allowable error = 15% of proportion of even in population

This study used the contamination rate of *Vibrio parahaemolyticus* in frozen shrimp samples obtained from the study of Kowcachaporn (28), the proportional of contamination rate in raw frozen shrimp samples was 64%

$$n = \frac{(1.96)^2 (0.64) (0.36)}{(0.15 \times 0.64)^2} = 96.04$$

Thus, 103 frozen shrimp samples were included in this study.

2. The sensitivity, specificity, positive predictive value, negative predictive value and efficiency test

Test measurement	Standard tests		Total
	Positive	Negative	
Positive	a	b	a + b
Negative	c	d	c + d
Total	a + c	b + d	N

% Sensitivity = (a/a+c) x 100

% Specificity = (d/b+d) x 100

% Positive predictive value = (a/a+b) x 100

% Negative predictive value = (d/c+d) x 100

% Efficiency = (a+d/N) x 100

a (True positive) = The number of true positive samples

b (False positive) = The number of false positive samples

c (False negative) = The number of false negative samples

d (True negative) = The number of true negative samples

N (Grand total) = The total number of the samples

3. Comparison of two correlated proportions between the two tests by McNemar test (155)

Assumption Ho : The results of both tests were not different

Ha : The results of both tests were different

Test B	Tests A		Total
	Positive	Negative	
Positive	a	b	a + b
Negative	c	d	c + d
Total	a + c	B + d	N

$$\chi^2 = \frac{\Sigma\,(O-E)^2}{E}$$

$$= \frac{(O_b-E_b)^2}{E_b} = \frac{(O_c-E_c)^2}{E_c}$$

E = The expected value of group

O = The observe value of group

E_b = $E_c = (b+c)/2$

4. Kappa measure of agreement between two tests (156)

Test B	Tests A		Total
	Positive	Negative	
Positive	a	b	a + b
Negative	c	d	c + d
Total	a + c	b + d	N

$$k = \frac{I_o - I_e}{1 - I_e}$$

I_o = The proportion of the same results from both tests

= (a+b)/N

I_e = The proportion of the expected value of the same result from both tests

= $(a_e + d_e)/N$

N = a + b + c + d (Grand total)

Interpretation of kappa (K) value

Value of K	Strength of agreement
< 0.20	Poor
0.21 - 0.40	Fair
0.41 - 0.60	Moderate
0.61 - 0.80	Good
0.81 - 1.00	Very good

BIOGRAPHY

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