RISK ASSESSMENT OF *VIBRIO PARAHAEMOLYTICUS* IN WHITE SHRIMP (*LITOPENAEUS VANNAMEI*) FROM RETAIL MARKET

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การประเมินความเสี่ยงเชื้อวิบริโอ พาราฮิโมไลติคัส ในกุ้งขาวจากตลาดค้าปลีก

น.ส.ประภัสสร หยกยิ่งยง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	RISK ASSESSMENT OF *VIBRIO PARAHAEMOLYTICUS* IN WHITE SHRIMP (*LITOPENAEUS VANNAMEI*) FROM RETAIL MARKET
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Field of Study	Veterinary Public Health
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ประภัสสร หยกยิ่งยง : การประเมินความเสี่ยงเชื้อวิบริโอ พาราฮิโมไลติคัส ในกุ้งขาวจากตลาดค้าปลีก. (RISK ASSESSMENT OF *VIBRIO PARAHAEMOLYTICUS* IN WHITE SHRIMP (*LITOPENAEUS VANNAMEI*) FROM RETAIL MARKET) อ.ที่ปรึกษาหลัก : รศ. น.สพ. ดร. ศุภชัย เนื้อนวลสุวรรณ

วิบริโอ พาราฮิโมไลติคัส เป็นสาเหตุของการเกิดโรคทางเดินอาหารอักเสบในหลายประเทศทั่วโลก รวมถึงประเทศไทย เชื้อนี้เป็นเชื้อแบคทีเรียแกรมลบ ต้องการเกลือในการเติบโต โดยสามารถพบได้ในน้ำทะเล และในอาหารทะเล การติดเชื้อวิบริโอ พาราฮิโมไลติคัส ส่วนใหญ่มักพบได้จากการบริโภคอาหารทะเลดิบ กึ่งสุกกึ่งดิบ หรือกระทั่งจากการปนเปื้อนระหว่างอาหารที่ปรุงสุกแล้วกับอาหารที่ยังไม่ผ่านการปรุง การประเมินความเสี่ยงคือเครื่องมือทางวิทยาศาสตร์ที่สามารถนำมาใช้ในการประเมินความเจ็บป่วยจากการติดเชื้อวิบริโอ พาราฮิโมไลติคัสได้ โดยในการศึกษานี้ได้ทำการประเมินความเสี่ยงจากการบริโภคกุ้งขาวแปซิฟิกดิบ (*Litopenaeus vannamei*) โดยมีวัตถุประสงค์คือการหาความชุกและระดับการปนเปื้อนของเชื้อวิบริโอ พาราฮิโมไลติคัส ในแต่ละชนิดของร้านค้าและ 6 จังหวัดในประเทศไทยโดยใช้วิธี MPN และประเมินความเสี่ยงจากการบริโภคกุ้งดิบ โดยใช้ข้อมูลความชุกและระดับการปนเปื้อนของเชื้อวิบริโอ พาราฮิโมไลติคัสที่ก่อโรค ซึ่งได้กำหนดโดยการตรวจหายีนสร้างสารพิษ คือ *tdh* และ *trh* ความชุกของเชื้อวิบริโอ พาราฮิโมไลติคัส และวิบริโอ พาราฮิโมไลติคัสก่อโรค คือร้อยละ 66 และ ร้อยละ 1.4 ตามลำดับ เชื้อวิบริโอ พาราฮิโมไลติคัสที่มียีน *tdh* พบร้อยละ 0.93 และเชื้อวิบริโอ พาราฮิ-โมไลติคัสที่มียีน *trh* พบร้อยละ 0.46 โดยตรวจไม่พบเชื้อวิบริโอ พาราฮิโมไลติคัสที่มีทั้งสองยีน การประมาณความเสี่ยงจากการบริโภคกุ้งดิบที่ปนเปื้อนเชื้อวิบริโอ พาราฮิโมไลติคัสคือ 1.02×10^{-4} คิดเป็นผู้ป่วย 3,711 รายต่อประชากรหนึ่งแสนคนต่อปี ผลการวิเคราะห์ความอ่อนไหวในการศึกษานี้ พบว่าความน่าจะเป็นของการเจ็บป่วยมีผลต่อการประเมินความเสี่ยงอย่างมาก (ค่าสัมประสิทธิ์ = 0.94) ตามด้วยระยะเวลาจากร้านค้าจนบริโภค (ค่าสัมประสิทธิ์ = 0.22) ระดับการปนเปื้อนเชื้อ ณ เวลาที่บริโภค (ค่าสัมประสิทธิ์ = 0.16) และปริมาณการได้รับเชื้อวิบริโอ พาราฮิโมไลติคัสก่อโรค (ค่าสัมประสิทธิ์ = 0.15) ยิ่งไปกว่านั้นจากการศึกษาพบว่าการให้ความร้อนที่เหมาะสมแก่กุ้งเช่นที่อุณหภูมิ 60 องศาเซลเซียส เป็นเวลา 2 นาทีสามารถลดความเสี่ยงจากเชื้อวิบริโอ พาราฮิโมไลติคัสได้

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Praphatsorn Yokyingyong : RISK ASSESSMENT OF *VIBRIO PARAHAEMOLYTICUS* IN WHITE SHRIMP (*LITOPENAEUS VANNAMEI*) FROM RETAIL MARKET. Advisor: Assoc. Prof. Suphachai Nuanualsuwan, Ph.D.

Vibrio parahaemolyticus is the leading cause of seafood borne gastroenteritis in many countries including Thailand. It is a gram-negative, halophilic bacterium which was normally found in estuarine environment and seafood. The infection usually occurred from consuming raw, inadequate cooked, or cross-contaminated seafood. Risk assessment is a scientific tool to evaluate health hazard from *V. parahaemolyticus* infection. In this study, a risk of *V. parahaemolyticus* infection from raw shrimp consumption (*Litopenaeus vannamei*) was conducted. This study estimated the prevalence and level of *V. parahaemolyticus* in 2 retail types across 6 provinces nationwide by 3-tubes MPN. Risk assessment from raw shrimp consumption was estimated from prevalence and level of pathogenic *V. parahaemolyticus* carrying *tdh* and *trh* genes by multiplex PCR. The prevalence of total and pathogenic *V. parahaemolyticus* was 66% and 1.4%, respectively. Pathogenic *V. parahaemolyticus* carrying *tdh* and *trh* genes were 0.93% and 0.46%, respectively. *V. parahaemolyticus* isolates carrying both *tdh* and *trh* genes was not detected by multiplex PCR. Estimated daily risk for raw shrimp consumption was predicted at 1.02×10^{-4} equivalent to incidence rates per 100,000 people at 3,711 cases per year. Sensitivity analysis from simulation showed that risk estimates was highly correlated with probability of illness (correlation coefficient or r = 0.94), followed by time between retail to consumption (r = 0.22), concentration at consumption (r = 0.16), and dose (r = 0.15). Moreover, the study has found the proper cooking of shrimp such as 60°C for 2 minutes can reduce risk from *V. parahaemolyticus* to safe negligible level.

Field of Study:	Veterinary Public Health	Student's Signature .....
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CHAPTER 1 INTRODUCTION

1.1 Importance and Rationale

Vibrio parahaemolyticus (V. parahaemolyticus) is a gram negative, rod-shape, halophilic bacterium that habitats in marine or estuarine environment worldwide. *V. parahaemolyticus* is normally found in many marine animals. After being first discovered in Japan in 1950 (Xu et al., 2014), *V. parahaemolyticus* has been the major pathogen causing seafood-borne gastroenteritis around the world. The illness, that is caused by this organism, is mostly associated with consumption of raw or inadequate cook seafood and post contamination. In healthy people, the clinical symptoms of gastroenteritis are watery diarrhea, nausea, and fever. After the proper medical treatment, the clinical symptoms may be resolved within a few days (Su and Liu, 2007). However, children, elderly, or immunocompromised people can be shock or dead upon this foodborne infection (Su and Liu, 2007; Yamamoto et al., 2008). Only a few strains of *V. parahaemolyticus* are pathogenic to human health. The pathogenic *V. parahaemolyticus* is defined by carrying either *tdh* or *trh* genes, which produce thermostable direct hemolysin (TDH) toxin and thermostable direct hemolysin related hemolysin (TRH) toxin, respectively (Yamamoto et al., 2008). These hemolysin toxins cause the hemolytic and cytotoxic effects to the host cells resulting in host cell death (Matsuda et al., 2010).

Since the first outbreak in Japan caused more than 270 illnesses and up to 20 deaths from food poisoning (Fujino et al., 1953; Daniels et al., 2000). Afterwards, *V. parahaemolyticus* has been accounted for 20-30% of food poisoning cases in Japan (Alam et al., 2003). In 1997, a major outbreak in France, related to the shrimps imported from an Asian country, has been reported as foodborne disease with 44 infected

patients (Robert-Pillot et al., 2004). In the United States, the annual estimated illnesses were approximately 45,000 cases (CDC, 2019). In Thailand, *V. parahaemolyticus* has been ranked the highest reported foodborne pathogen since 2010 (BOE, 2018). The reports of *V. parahaemolyticus* gastroenteritis cases worldwide have emphasized the need to establish the effective tools to assess and reduce this health risk.

Risk assessment is a powerful tool to evaluate health risk resulting from the chemical, biological, or physical hazards. It provides necessary data, that are useful to support decision making in terms of risk management options, to develop effective control measures, to establish quality food standard, and to improve public health worldwide. Unlike the chemical risk assessment, the challenge of microbial risk assessment is the dynamic of levels of microbial contamination along food chains. Quantitative microbial risk assessment has been employed on several of food commodities to evaluate adverse health effect and to establish the effective control measures to immigrate the health risk.

V. parahaemolyticus infection related to seafood consumption occurred in many countries worldwide and became one of the most important foodborne bacteria in the United State and Asia. The molecular epidemiological studies and the risk assessment have been performed to evaluate and track the pathogenic strains in the outbreaks. However, to date quantitative risk assessment of *V. parahaemolyticus* has mainly focused on only oysters as a result of both previous foodborne disease outbreaks and larger amount of oyster consumption. Even in 2008, the joint research of Japan and Thailand formulated the models for risk assessment of *V. parahaemolyticus* in bloody clams, but the stochastic models are only applicable to the southern part of Thailand (Yamamoto et al., 2008). In Thailand, quantitative risk assessment of *V. parahaemolyticus* data in shrimp is limited. Therefore, statistics of prevalence and

levels of pathogenic *V. parahaemolyticus* contaminated in shrimp in Thailand is prerequisite to implement quantitative risk assessment.

In this study, the prevalence and levels of pathogenic *V. parahaemolyticus* in shrimp were investigated. The quantitative microbial risk assessment of *V. parahaemolyticus* was focused on the consumption of white shrimp (*Litopenaeus vannamei*), which is a popular seafood and sometimes lightly cooked or even consumed raw across different types of seafood retail dishes. The background risk estimate of *V. parahaemolyticus* nationwide can be beneficial for the risk managers (e.g. Department of Fishery, Ministry of Public Health) to determine an appropriate food safety policy, and eventually, to enhance seafood safety in Thailand.

1.2 Objectives of this Study

1. To determine the prevalence and levels of *V. parahaemolyticus* contaminated in white shrimp (*Litopenaeus vannamei*) in retail types and in six provinces in Thailand.
2. To determine probabilities of illness of *V. parahaemolyticus* from white shrimp (*Litopenaeus vannamei*) consumption in cooking preferences, retail types and provinces using stochastic models.

CHAPTER 2 LITERATURE REVIEW

2.1 Microbiology of *V. parahaemolyticus*

V. parahaemolyticus is a member in family of *Vibrionaceae*. It is a Gram negative, facultative anaerobic, asporogenous rod or comma-shaped bacterium. This organism has two flagella systems. Polar flagella are used for bacterial motility while lateral flagella are responsible for survival in different environment (Broberg et al., 2011). *V. parahaemolyticus* can be classified by the somatic (O) and capsular (K) antigens, containing more than 12 O types by 70 K types of antigen (Xu et al., 2014). *V. parahaemolyticus* normally grows in bacteriological media containing 1-8% sodium chloride and thus referring as halophilic bacterium. The colony size is approximately 2-3 millimeters, round, opaque, green or blue-green on thiosulphate citrate bile salts sucrose (TCBS) agar. The optimal growth condition for *V. parahaemolyticus* is approximately 42^{o}C. Besides, this bacterium is positive for indole, oxidase, and glucose fermentation test (Jones et al., 2012).

Three pathogenic species of Genus *Vibrio* are *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, are widely known as foodborne disease causative agents in human (Gopal et al., 2005). Microbiological differentiation of these 3 bacterial spp. can be characterized by Table 1.

Table 1 Differential characteristics of *V. cholerae, V. parahaemolyticus*, and *V. vulnificus*

Characteristics	*V. cholerae*	*V. parahaemolyticus*	*V. vulnificus*
Growth at 0% NaCl (w/v)	+	-	-
3% NaCl (w/v)	+	+	+
6% NaCl (w/v)	-	+	+
8% NaCl (w/v)	-	+	-
10% NaCl (w/v)	-	-	-
TCBS agar	Yellow	Green	Green
Chromogenic agar	Blue	Mauve	Blue
TSI slant	K/a	K/A	K/A
Oxidase test	+	+	+
Indole test	+	+	+
Glucose Fermentation	+	+	+
Lactose Fermentation	Variable	-	+
Sucrose Fermentation	+	-	-

TSI : Triple sugar iron slant;

CHROM agar : chromogenic agar

TCBS : thiosulphate citrate bile salts sucrose agar

K/a : Slant alkaline - Bottom slightly **a**cidic; K/A : Slant alkaline - Bottom **A**cidic

V. parahaemolyticus inhabits in coastal and estuarine water and has been isolated from many species of marine animals such as fish, crab, shrimp, and shellfish

(Su and Liu, 2007). The distribution of this microorganism in the environment is related or determined by the sea water temperatures. Study shows that, for the sea water temperature less than 15°C, *V. parahaemolyticus* can only be survived in the sea sediment and rarely detected in sea water (Colwell et al., 1973). However, when the water temperature increased to 25°C, the organism densities could rise up to 10 cells/ml in the environment (Colwell et al., 1973; DePaola et al., 1990). Additionally, a positive correlation between *V. parahaemolyticus* in environment and sea water temperatures was also found in Oregon between November 2002 and October 2003 (Duan and Su, 2005). The rise of water temperatures did not only amplify the level of *V. parahaemolyticus* in sea water but also contaminate marine animals (Su and Liu, 2007). A survey of *V. parahaemolyticus* in oysters in US found a seasonal distribution with high level of this microorganism in summer (Cook et al., 2002). Some studies have shown the rapidly increase of this organism in oysters when exposed to the optimal temperature. According to Gooch (2002), if the sea water temperature after harvest reach up to 26°C, the level of *V. parahaemolyticus* contamination could rise up to 50-790 folds within 24 hours compared to the original level.

The growth and survival of *V. parahaemolyticus* was affected by temperature, pH, water activity and salinity. The temperature limit for growth of this pathogen in seafood was reported from 3 to 13 °C depending on the growth substrate and conditions in the study (Beuchat, 1975). Some studies reported that number of *V. parahaemolyticus* in whole peeled deveined shrimp decreased sharply during the first 2 days of storage at 3, 7, 10, and -18°C. Unlike whole shrimp, homogenized shrimp has a slight increase of pathogen in the first 12 hours at the same condition except -18°C. However, there was no differences of population changes between whole shrimp and homogenized shrimp (Vanderzant and Nickelson, 1972). For laboratory media, pathogen growth was affected by pH and the temperature limit reported from 5 to 9 °C under pH 7.1 to 7.7 (Beuchat,

1975). In 2009, study showed that *V. parahaemolyticus* strains could survive in tryptic soy broth supplement with 2% NaCl held at 5 °C without multiplication. But when stored at 8 and 10 °C, the pathogen can multiply (Burnham et al., 2009).

V. parahaemolyticus appear to be susceptible to the effect of chilling and freezing. However, these conditions did not warrant the effective inactivation of *V. parahaemolyticus* in seafood. Storage condition between 10 to -18 °C for 8 days can reduce *V. parahaemolyticus* for 1-2 log (Vanderzant and Nickelson, 1972). Thomson and Thacker (1973) also showed that multiplication of *V. parahaemolyticus* in oyster can reach unsafe levels when kept higher than 8 °C. Another study reported that saurel and mackerel storage at -10 and -16 °C for 4 day can eliminate all the pathogen (Temmyo, 1966). In contrast, a study turned out that survival of this pathogen in raw tuna at -10 °C and -20 °C was higher than at 0 °C (Asakawa, 1967). Johnson (1973) also demonstrated that *V. parahaemolyticus* could be isolated after held at 4 °C for 3 weeks with no noticeable decrease in population.

A wide range of thermal inactivations of *V. parahaemolyticus* has been reported. Temmyo (1966) showed that *V. parahaemolyticus* was inactivated at 60°C 5 minutes or 55 °C 10 minutes in peptone water. Vanderzant and Nickelson (1972) found that low populations (2.7 log CFU/ml) of pathogen were killed by heating shrimp homogenate at 60 °C, 80 °C, 100 °C for 1 minutes. For higher level population (6.3 log CFU/ml), some could still be isolated after heating at 60 °C and 80 °C for 15 minutes but cannot isolated at 100 °C for 1 minutes. There was a study noted that heat inactivation of this pathogen was pH specific. According to Beuchat (1973) and Goldmintz (1974), *V. parahaemolyticus* was more susceptible to heat treatment when the pH become too acidic or alkaline. The highest heat resistance has been reported at pH 7.0. Moreover, the presence of sodium chloride was seemed to be a protective factor. A study demonstrated that heating a crab meat-salt broth containing 6.2 log CFU/ml of

V. parahaemolyticus at 48 °C and 55 °C reduced 2.5 log and 4 log of pathogen, respectively while heat inactivation at 65 °C for 10 min can completely killed the pathogen (Goldmintz, 1974).

Covert and Woodburn (1972), reported that 12% sodium chloride in trypticase soy broth restore the pathogen at 48 °C. Therefore, sodium chloride in recovery media can fix thermally stressed cells (Beuchat, 1975).

2.2 Disease attributable to *V. parahaemolyticus*

The infection of *V. parahaemolyticus* is called Vibriosis. It can be divided into three medical conditions which are gastroenteritis, septicemia, and wound infections. Acute gastroenteritis is a common illness caused by *V. parahaemolyticus* demonstrating watery diarrhoea, abdominal pain, nausea, vomiting, fever, and headache. Consuming raw or inadequate cook or cross-contaminated seafood (after cooking) are among major means of getting this illness. The infection onset could be within 4-96 hours depending on the doses of *V. parahaemolyticus* and usually be promptly resolved with oral rehydration or other proper medical treatments. In contrast, the highly susceptible population, defined as children or immunocompromised people, can become more severe or even dead. This bacterium also manifest itself by watery diarrhoea, lowering blood pressure and shock (Broberg et al., 2011). Some other clinical symptoms are convulsions, pale or cyanotic, septicemia and death in patients with some underlying medical conditions (Nair et al., 2007).

Septicemia happened whenever the bacterium invaded the blood stream of host. The immune system is activated and followed by the inflammation and higher vascular permeability. Subsequently, patients with liver disease, diabetes or cancer were among the greatest health risk.

Wound infection is more common in occupational activities and normally infected through the skin cut. Cellulitis is a common form of *V. parahaemolyticus* wound infection, but it can be found with other lesions including inflammation and necrotic tissues.

2.3 Virulence factors of *V. parahaemolyticus*

V. parahaemolyticus has several virulence factors such as adhesion molecule, toxins, and secretion system.

2.3.1 Adhesion factors

Adhesion factors are important in host cell binding, they were present at the bacterial cell surface to create the pathway for effectors and toxins delivery. The multivalent adhesion molecule 7 (MAM7) is found in many gram-negative bacteria including *V. parahaemolyticus* and necessary for the initial attachment to the host cells (Letchumanan et al., 2014).

2.3.2 Toxins

It is clear that not all strains of *V. parahaemolyticus* are pathogenic to human depending on virulence genes. The *tdh* gene encodes toxin so-called the thermostable direct hemolysin (TDH) while *trh* gene produces the thermostable direct hemolysin-related hemolysin (TRH) toxin. TDH toxin can lyse red blood cells and demonstrate Beta haemolysis on Wagatsuma blood agar plate, called Kanagawa phenomenon (Tada et al., 1992). TDH toxin has been commonly reported in gastroenteritis patients, consequently, recognized as the important virulence factor of *V. parahaemolyticus* (Nishibuchi and Kaper, 1985). Besides, TRH toxin has also been considered as another significant virulence factor, since an outbreak in Maldives was reported by Kanagawa

negative strain (Honda et al., 1991). Some Kanagawa-negative *V. parahaemolyticus* strains may produce a hemolysin. This protein has some similar activities to TDH activity, and lyses human erythrocytes (Broberg et al., 2011). Both TDH and TRH toxins cause hemolytic and cytotoxic effects to the host cells by forming a pore on the membrane leading to a loss of permeability control and finally cell death (Raghunath, 2015). From previous studies, the isolates carrying the *tdh* or *trh* gene were of at risk for human. According to Nishibuchi and Kaper (1995), the presence of both *tdh* or *trh* genes was strongly associated with clinical patients.

2.3.3 The type III secretion systems (T3SS)

T3SS is a transmembrane protein that is responsible for transport effector proteins directly into the host cell. There are two types of T3SS system which are T3SS1 and T3SS2 (Makino et al., 2003). Each secretion system has many effectors thus having difference effect on host cells. The studies have shown that T3SS1 cause cytotoxic effects while T3SS2 is associated with enterotoxicity and cytotoxicity in the intestinal cells (Park et al., 2004; Ritchie et al., 2012).

2.3.4 The type VI secretion systems (T6SS)

T6SS has been recently discovered and presented in many *Vibrio* species including *V. parahaemolyticus, V. cholerae, V. alginolyticus,* and *V. harveyi* (Pukatzki et al., 2007). The function of this system is to deliver the effector proteins into the host cell. *V. parahaemolyticus* contains two types of T6SS systems, T6SS1 and T6SS2 (Boyd et al., 2008) and both systems are important to the host cell adhesion step (Yu et al., 2012).

2.4 Incidence of *V. parahaemolyticus*

The first foodborne disease outbreak of *V. parahaemolyticus* was traced back in 1950 as the cause of acute gastroenteritis in Osaka, Japan. More than 270 persons were ill and up to 20 persons were dead from food poisoning. The implicated food of this foodborne disease outbreak was sardines (Takeda, 1982; Su and Liu, 2007). Afterwards, *V. parahaemolyticus* has been accounted for 20-30% of food poisoning cases reported in Japan, associated with a variety of seafoods(Jahangir Alam et al., 2002). Moreover, in many Asian countries including India, Korea, Malaysia, Taiwan and China, *V. parahaemolyticus* was the major cause of foodborne disease associated with seafood consumption (Letchumanan et al., 2014). Additionally, antimicrobial resistance *V. parahaemolyticus* have been isolated from cockles and shrimps in Malaysia (Al-Othrubi et al., 2011). Also, studies in India have found multidrug resistance and pathogenic *V. parahaemolyticus* strains (Reyhanath and Kutty, 2014; Sudha et al., 2014).

V. parahaemolyticus was first introduced into the United States during 3 major outbreaks in Maryland in 1971. The foodborne illness was related to crab consumption (Molenda et al., 1972). During 1970s and 1990s, approximately 40 sporadic outbreaks were reported to the Centers for Disease Control and Prevention (Daniels et al., 2000). Most of these outbreaks happened in the warm months of the year and were associated with seafood. Since then, approximately 40% of vibriosis have been described as *V. parahaemolyticus* infection annually in the US (Letchumanan et al., 2014).

Unlike Asian countries and the United States, *V. parahaemolyticus* has been rarely reported in the European countries. A sporadic outbreak has been reported in some countries such as UK, Denmark, Greece, Spain, and Turkey since 1989. These sporadic outbreaks were related to fish, shellfish, crab and raw oyster consumption with

almost 160 infected patients (Letchumanan et al., 2014). A major outbreak occurred in France in 1997, affecting 44 patients and was related to the shrimps imported from an Asian country (Robert-Pillot et al., 2004). Additionally, *V. parahaemolyticus* has also been described in wound infections, and frequently caused septicemia in severe cases (Wang et al., 2015). For instance, in Denmark, seven cases of skin infection linked to bathing have been reported (Baker-Austin et al., 2010).

2.5 Epidemiology of *V. parahaemolyticus* in Thailand

An importance infection from *V. parahaemolyticus* in Thailand was reported in 1999 (Yamamoto et al., 2008). The researchers collected stool samples from two hospitals in the southern provinces of Thailand. This study found that one third of the *V. parahaemolyticus* isolates belonged to the pandemic strains some of which were O3:K6, O1:K25, O1:K41, and O4:K12 (Laohaprertthisan et al., 2003). Among these pandemic strains, O3:K6 was found to be the most predominant of more than 70% of the isolates (Vuddhakul et al., 2006). Later on during 2000 and 2002, the distribution of pandemic strains of *V. parahaemolyticus* and their molecular relationship were conducted in seafood in Hat Yai, Songkhla, Thailand (Vuddhakul et al., 2006). After that, quantitative risk assessment of *V. parahaemolyticus* was established in southern Thailand, which assessed the prevalence and concentration of the bacterium in bloody clams by the same research group (Yamamoto et al., 2008). Moreover, in Thailand as major shrimp producer and exporter, not only virulent was *V. parahaemolyticus* isolated, but also antimicrobial resistance *V. parahaemolyticus* has been investigated in white leg shrimp and black leg shrimp (Yano et al., 2014). In Thailand, *V. parahaemolyticus* has been reported as the main pathogen responsible for food poisoning between 1990s and 2000s. Furthermore, in 2014, the Bureau of Epidemiology in Thailand has unprecedentedly and surprisingly reported *V. parahaemolyticus*-contaminated chicken

blood. To date, *V. parahaemolyticus* has been ranked number one food poisoning in Thailand (BOE, 2018).

2.6 Food Safety Risk analysis

Nowadays, one of the major issues of international public health concern is food safety. The challenge of food safety is the dynamic of agricultural practice, human behavior, ecology, climate, trading and technology. These considerations increased the demand of an advanced food safety system. In order to meet these requirements, any health risks are obliged to be controlled by risk assessment and management.

Risk in the context of food safety is defined as a probability of adverse health effects resulting from biological, physical or chemical hazards. The collaboration between Food and Agriculture Organization of United Nation (FAO) and World Health Organization (WHO) has developed a scientific tool to promote food safety control system and to provide standards or guidelines used for domestic and international trades. A potential tool, used to support this food safety system, is so-called food risk analysis. It is composed of 3 major components (Fig 1), which are risk assessment, risk management and risk communication (FAO/WHO, 2006).

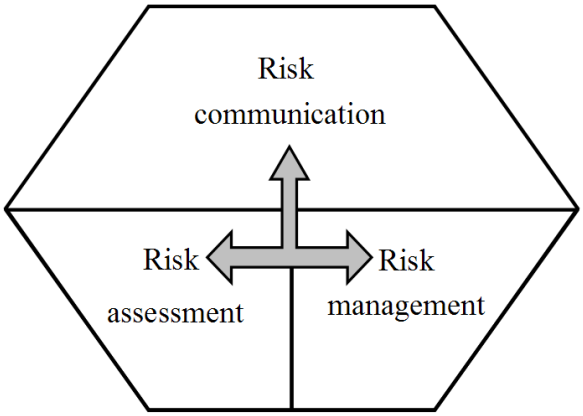

Figure 1 Component of food risk analysis

2.6.1 Risk assessment

Risk assessment is a science-based process that consists of 4 steps, which are hazard identification, exposure assessment, hazard characterization and risk characterization (Fig 2). This system provides a framework for evaluation of foodborne hazard.

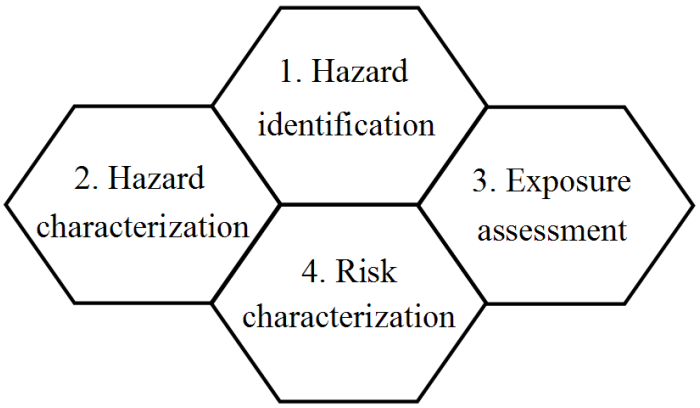

Figure 2 The steps in risk assessment

2.6.2 Risk management

Risk management is a process that concern of both scientific data and other factors including social, economic, and cultural factor to decide the appropriate risk management options. It is comprised of 4 phases. The first phase is preliminary risk management study. In this phase, risk managers can prioritize risk controls based on available scientific information to determine the need of risk assessment. The risk ranking maybe carried out either with or without risk assessment. The second phase is to identify and prioritize several possible measures for managing risk. Information and opinions from affected stakeholders are worth for decision making process. When the risk control has been selected, it must be implemented and validated by relevant

stakeholder. And for the last phase, monitoring outcomes and reviewing control measure should be performed. If the monitoring phase indicated the needed to review risk management options, these processes should be carried out all over again.

2.6.3 Risk communication

Risk communication is an importance process, to identify the food safety issue or describe the risk assessment finding and risk management options, the effective communicate among risk assessor, risk manager, stakeholders are needed. In risk assessment process, risk communication helps to obtain relevant and accurate information from members of risk analysis team and stakeholders to improve knowledge of risk issues. For risk management, it provides opinions and feedback of affected stakeholders for making an effective choice to manage the risk. Besides, informing the public with clear and timely data about food safety risk and the management, this process should be concern. To communicate successfully among different audiences, substantial knowledge, cautious planning and specialized skills are required.

2.7 Microbial risk assessment

Microbial risk assessment (MRA) scientifically evaluates adverse consequences on human health of contaminated microbial pathogens in foods or drinks. Unlike chemical contaminants, levels of microbial contamination are dynamic throughout the food supply chains. This poses a great challenge to both risk assessors and managers. According to Codex Alimentarius Commission (CAC), MRA could be performed either qualitatively or quantitatively.

Qualitative microbial risk assessment describes the degree of risk by scoring system. This approach requires less complicated calculation methods and the risk can be easily communicated with the regulators and the public. Levels of exposure and

potential of infection are rated using texts such as “low”, “medium” or “high” and then the risks are characterized by combining these two elements using a matrix table.

In quantitative microbial risk assessment (QMRA), on the other hand, the risk estimates are numerically calculated and expressed by utilizing complex mathematical equations and/or statistical models. The numerical outputs from QMRA are applicable for establishing the control measures in terms of risk-based microbiological metric e.g. food safety objectives (FSO) or appropriate level of protection (ALOP).

Risk assessors may conduct QMRA by using either deterministic or stochastic (probabilistic) approaches (Wooldridge, 2008). Deterministic approach to QMRA requires only a single value (point estimate) of each variable and parameter and establish only one possible risk estimate. Worst-case and best-case scenarios are frequently examined by using this approach. In stochastic QMRA, the probability distributions and mathematical models are utilized to characterize variability and uncertainty of variables and model parameters. The stochastic approach is more realistic since it considers wider ranges of possible outcomes of model parameters and different possible scenarios are assessed. In addition, MRA should be reassessed whenever more novel relevant information is available (FAO/WHO, 2006).

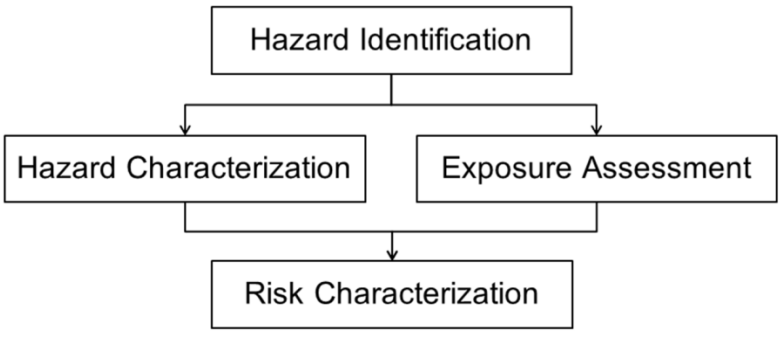

Figure 3 A structural framework of MRA established by CAC

2.7.1 Hazard identification

Hazard identification is an initial step of MRA where the biological hazards in foods or commodity is addressed. Microbiological, epidemiological and clinical information of the pathogen of interest and its characteristics along the food supply chain should be acquired from scientific evidences (FAO/WHO, 2009).

2.7.2 Exposure assessment

Exposure assessment is an appraisal of total amount and frequency of population exposure to the contaminated microbial hazards during a certain point of time. The major influences of exposure estimates are food consumption patterns, prevalence and concentration of bacterial contamination in foods.

A modular process risk model (MPRM) is frequently developed for food chain exposure pathway. MRPM provides a structured approach to exposure assessment by dividing the food production supply chain into some modules. Each module describes contamination dynamics, total number and prevalence of the pathogens, in a food production step (Nauta, 2008). In addition, predictive microbiological models are commonly used in QMRA to describe the contamination dynamics from microbial growth and inactivation throughout the food production chains (FAO/WHO, 2009).

2.7.3 Hazard characterization

Hazard characterization, previously known as dose-response assessment, describes the relationship between the level of exposed pathogen and the probability of adverse health consequences. In QMRA, dose-response models are expressed as mathematical functions. The dose-response relationships are established using information of either human subject or laboratory animal experiments (FAO/WHO, 2009).

2.7.4 Risk characterization

Risk characterization expresses the risk estimate as a product of the outcomes from exposure assessment and hazard characterization. Risk estimates could be presented as risk per serving, individual-based risk or population-based risk. In stochastic MRA, Monte Carlo simulation technique allows risk assessors to evaluate the risk across all different possible scenarios (FAO/WHO, 2009).

CHAPTER 3 MATERIALS AND METHODS

3.1 Evaluation of pathogenic *V. parahaemolyticus* contamination in white shrimps

3.1.1 Sample size calculation

The sample size is calculated by EpiTools program (Sergeant, 2019). Based on 50% prevalence which is represent a worst-case scenario to giving the largest sample size, the calculated sample size was 480.

3.1.2 Sample collection

The shrimp samples were collected from 6 provinces (Bangkok, Kanchanaburi, Chon Buri, Nakhon Ratchasima, Chiang Mai, and Prachuap Khiri Khan) representing 6 regions of Thailand by convenience sampling. For each individual province, 4 fresh markets and 2 supermarkets were visited during April 2016 to May 2017. Two sets of 100 grams of white shrimps were obtained from 3 vendors in each market (Figure 4). A total of 648 samples was collected from three visits with four months apart in order to account for the seasonal effect. The sample collections were also shown in Table 2

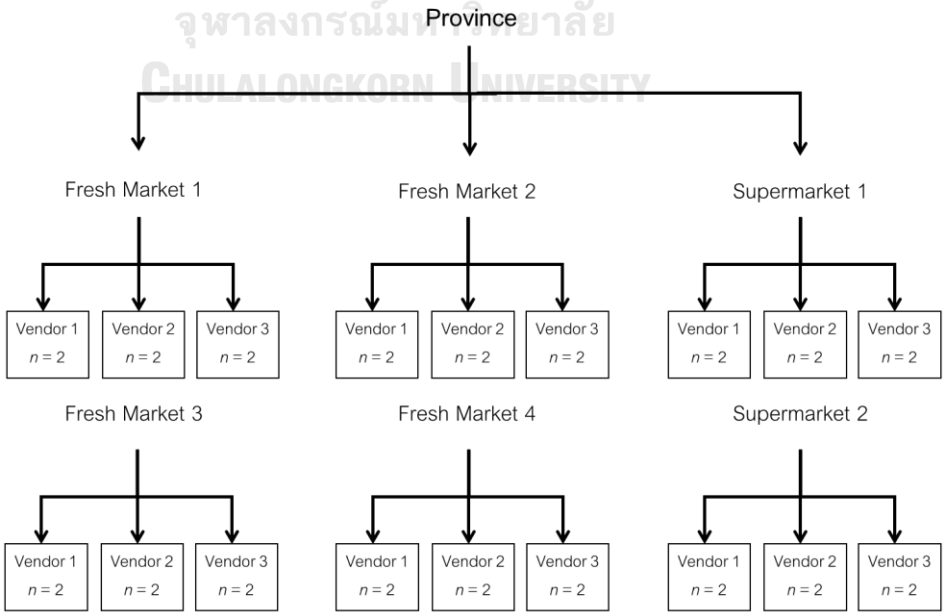

Figure 4 Diagram of sample collection of white shrimps by province

Table 2 Shrimp sample collection in 6 provinces

Province	Market		Vendor	Sample/season	Total
	Type	No.			3 seasons
1.Bangkok	Fresh	4	×3	24	108
	Supermarket	2	×3	12	
2.Chiang Mai	Fresh	4	×3	24	108
	Supermarket	2	×3	12	
3.Chon Buri	Fresh	4	×3	24	108
	Supermarket	2	×3	12	
4.Kanchanaburi	Fresh	4	×3	24	108
	Supermarket	2	×3	12	
5.Nakhon Ratchasima	Fresh	4	×3	24	108
	Supermarket	2	×3	12	
6.Prachuap Khiri Khan	Fresh	4	×3	24	108
	Supermarket	2	×3	12	
Total					**648**

3.1.3 Sample shipping and handling

The samples were collected in sterile double-sealed plastic bags and stored in the container with ice (2-8ºC). All samples were transported and analyzed for *V. parahaemolyticus* within 24 hours after collection.

3.1.4 *V. parahaemolyticus* enumeration

V. parahaemolyticus enumeration were adapted from ISO/TS 21872-1:2007 (Kaysner and DePaola, 2004). The microbiological procedures were described below.

3.1.4.1. Twenty-five grams of white shrimp samples were blended with 225 milliliters of alkaline peptone water (APW) (Oxoid™, UK) with 2% NaCl to achieve 0.1 dilution. One more 10-fold serial dilution was also done by APW (Figure 5).

3.1.4.2. Three-tubes most probable number (MPN) technique was performed to quantify the level of contamination by enrichment in APW with 2% NaCl having 1.0 ml, 0.1 ml, and 0.01 ml of shrimp sample, respectively (Figure 5) and then incubated at 35±2 ºC for 18-24 hour.

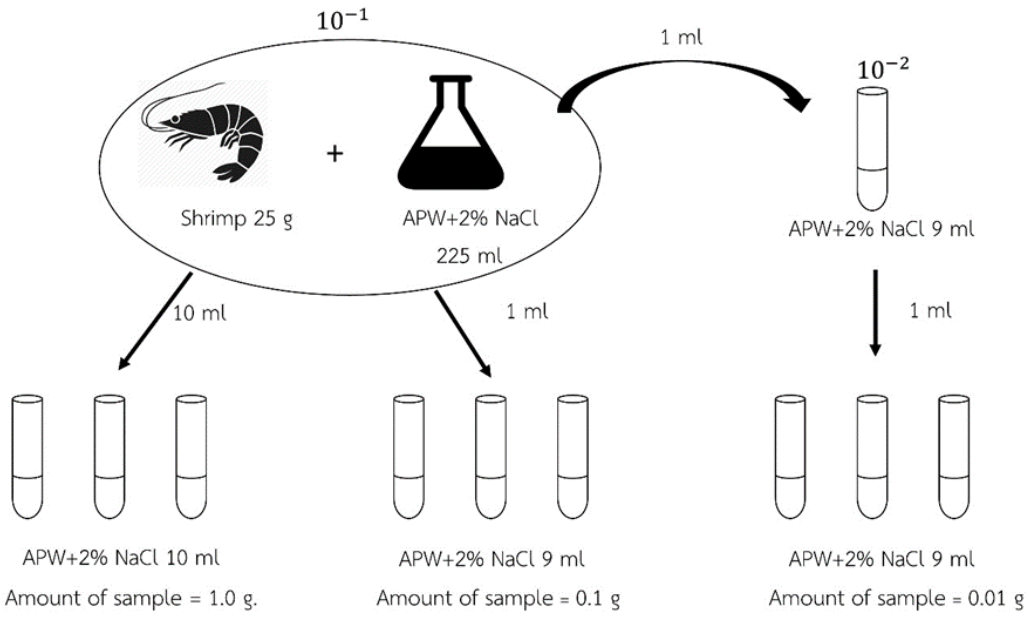

Figure 5 Diagram of 3-tubes MPN technique to estimate the density of *Vibrio parahaemolyticus* in shrimp samples.

3.1.4.3. A loopful of suspension from APW tubes, that turns turbidity, was streaked onto thiosulphate citrate bile salts sucrose (TCBS) agar (Scharlau™, Spain) and incubated at 35±2 ºC for 24 hours.

3.1.4.4. After incubation, the round, opaque, blue-green colonies from TCBS agar were chosen and streaked onto Vibrio chromogenic media (CHROMagar) (CHROMagar™Vibrio, France) and incubated overnight at 35±2 ºC.

3.1.4.5. Approximately 3-5 purple bacterial colonies on the surface of CHROMagar were transferred to triple sugar iron agar (TSI) (Difco™, France) with 2% NaCl and then incubated overnight at 35±2 ºC.

3.1.4.6. After incubated, the bacterial isolates on TSI that changed the slant color into red (alkaline) and the bottom color into yellow (acid) without hydrogen sulfide (H_2S) production (no black discoloration) were tentatively suspected as *V. parahaemolyticus*. All suspected colonies were then collected for further confirmation with molecular technique.

3.1.5 Confirmation of *V. parahaemolyticus* and virulence genes

All *V. parahaemolyticus* suspected isolates were tested for the presence of *tlh*, *tdh*, and *trh* genes by multiplex polymerase chain reaction (PCR) technique. The *tlh* gene is a species-specific marker of *V. parahaemolyticus* while *tdh* and *trh* genes are the indicator of pathogenic (virulent) *V. parahaemolyticus*. The primer pairs and the protocol in this study followed a previous study (Jones et al., 2012).

3.1.5.1 DNA extraction: To prepare the DNA template, the bacterial pellets were transferred to 150 µl of nuclease-free water and then, boiled at 100ºC for 10 min to break down the bacterial cells. After centrifuged at 12,000 × g for 5 minutes, the supernatant was transferred to a new microfuge tube and stored at -20 ºC.

3.1.5.2 Detection of of *tlh*, *tdh*, and *trh* genes: The sequence of primers and PCR condition used to detect these genes were shown in Tables 3 - 4. All

multiplex PCR amplifications were performed in a thermal cycle machine (Mastercycler personal, Eppendorf, USA) using 30 PCR cycles. The total volume reaction mixture was 20 µl comprised of 10 µl of 2x PCR Master mix solution (i-Taq, iNtRON Biotechnology), 0.5 µl of *tlh* forward primer, 0.5 µl of *tlh* reverse primer, 0.5 µl of *tdh* forward primer, 0.5 µl of *tdh* reverse primer 0.5 µl of *trh* forward primer, 0.5 µl of *trh* reverse primer, 6 µl of nuclease-free water, and 1 µl of the DNA template.

The PCR amplified DNAs were separated in 1.5% (w/v) agarose gel with 0.5x Tris-Borate-EDTA (TBE) buffer containing nucleic acid staining solution (RedSafe, iNtRON Biotechnology, South Korea) at 120 V for 40 min and then visualized under the UV light.

Table 3 Primers sequence of species and pathogenic *V. parahaemolyticus*

Target gene	Primer sequences (5′ to 3′)	Product size (bp)
tlh	F : -aaa gcg gat tat gca gaa gca ctg- R : -gct act ttc tag cat ttt ctc tgc-	450
tdh	F : -gta aag gtc tct gac ttt tgg ac- R : -tgg aat aga acc ttc atc ttc acc-	269
trh	F : -ttg gct tcg ata ttt tca gta tct- R : -cat aac aaa cat atg ccc att tcc g-	500

(Jones et al., 2012)

Table 4 PCR condition for *V. parahaemolyticus* detection

Thermocycler conditions	Temperature (°C)	Time (min)
Initial denaturation	94	3
PCR cycle: -		
▫ Denaturation	94	1
▫ Primer annealing	58	1
▫ Primer extension	72	1
Final extension	72	5

(Jones et al., 2012)

3.1.6 Most Probable Number (MPN) enumeration

Three-tubes MPN table was used to enumerate concentrations of a micro-organism depending upon the positive pattern e.g. 3-3-2 corresponding to 1, 0.1, 0.01 g of shrimp sample in each tube. The concentrations of *V. parahaemolyticus* was enumerated for 2 levels. The first level was the concentration of total *V. parahaemolyticus* e.g. 3-3-2 positive pattern for *tlh* gene. The second level was that of pathogenic *V. parahaemolyticus* e.g. 2-2-1 positive pattern for *tdh* or *trh* genes (Figure 6).

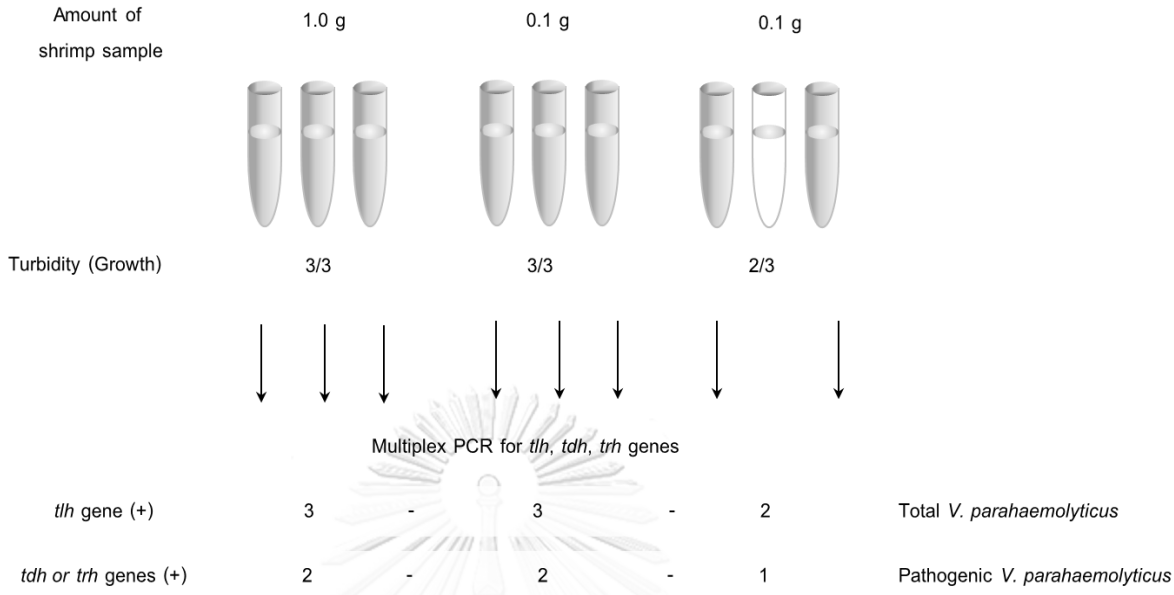

Figure 6 Example of 3-tubes MPN and multiplex PCR to enumerate total and pathogenic *V. parahaemolyticus*

3.2 Microbial risk assessment for pathogenic *V. parahaemolyticus* in white shrimps

Microbial risk assessment followed the Codex Alimentarius Commission Guidelines (CAC, 2001). Probability distribution of risk estimate of pathogenic *V. parahaemolyticus* from consuming shrimp was estimated by using Simulación 4.0 software Add-in on Microsoft® Excel.

3.2.1 Hazard identification

The risk profile in terms of the microbiology, epidemiology and clinical information of pathogenic *V. parahaemolyticus* along the food supply chain were obtained from scientific literatures.

3.2.2 Exposure assessment

The exposure assessment of pathogenic *V. parahaemolyticus* infection by white shrimp consumption was evaluated by using a modular approach (Figure 7).

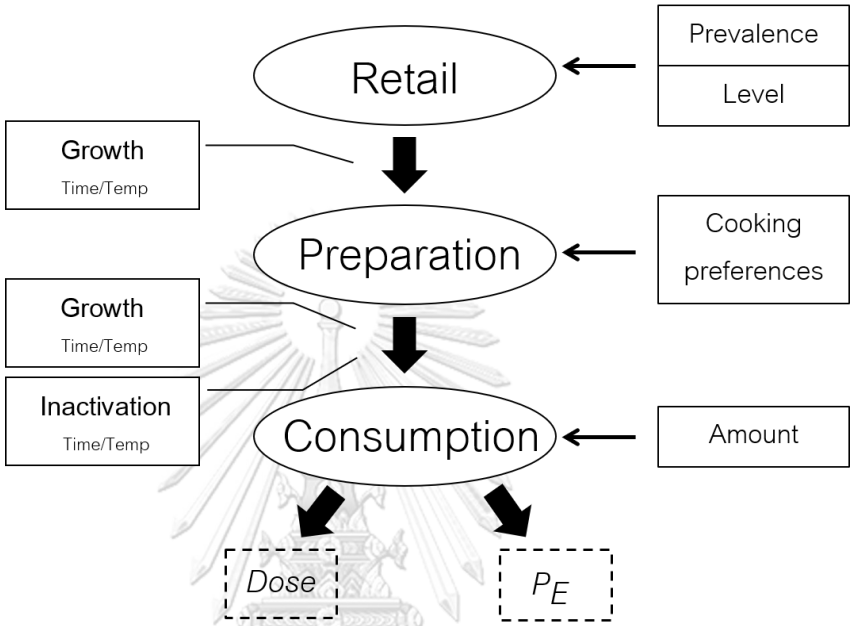

Figure 7 Conceptual framework of exposure assessment model in this study

3.2.2.1 Retail module

In this module, the prevalence of pathogenic *V. parahaemolyticus* in white shrimp (P_r) was described by Beta distribution of pathogenic *V. parahaemolyticus* positive samples in this study.

$$P_r \sim Beta(s+1, n-s+1) \quad (1)$$

where s is the number of positive samples and n is the sample size.

The levels of pathogenic *V. parahaemolyticus* in white shrimp (C_r) was assumed as triangular distribution.

$$C_r \sim triangular(min, most\ likely, max) \quad (2)$$

where min, most likely, max are minimum, most likely, and maximum concentrations of pathogenic *V. parahaemolyticus*

Table 5 Model input parameters in retail module

Variable	Description	Unit	Distribution/model	Reference
P_r	Prevalence of pathogenic *V. parahaemolyticus*	%	Beta(6+1, 648-6+1)	This study
C_r	Levels of pathogenic *V. parahaemolyticus*	MPN/g	triangular(0.3, 0.36, 0.92)	This study

3.2.2.2 Preparation module

This module evaluated the level of pathogenic *V. parahaemolyticus* at preparation (C_p) to the pathogenic levels at time of consumption (C_c).

The bacterial growth during retail to preparation step was evaluated by simple exponential model which is environment-independent model (Yamamoto et al., 2008). This study conducted under the assumption that there was no significant difference of growth rate between pathogenic and non-pathogenic strain of *V. parahaemolyticus* (Organization, 2011). Then, the level of pathogenic *V. parahaemolyticus* at the preparation (C_p) was calculated by

$$C_p = C_r e^{kt} \quad (3)$$

Where C_p = bacterial concentration at time t (MPN/g)

C_r = initial cell concentration (MPN/g)

k = the growth rate for a given temperature ($hour^{-1}$)

t = the time taken between retail and cooking stage (hour)

As a result of limited data collection, the time between retail to cooking stage was estimated based on a previous study (Yamamoto et al., 2008).

$$t \sim triangular(min, most\ likely, max) \quad (4)$$

where min, most likely, max are minimum, most likely, and maximum time between retail and cooking.

A thermal inactivation model was used to describe log reduction of pathogenic *V. parahaemolyticus* in shrimp during cooking. In this study, we assumed that shrimps were cooked at 60°C for 2 minutes, which is not significantly alter the texture and sensory quality of shrimp. Decimal reduction time (DRT) of *V. parahaemolyticus* at 60°C (D_{60}) was 0.71 min (FAO, 2003).

Therefore, the level of pathogenic *V. parahaemolyticus* after inactivating were calculated by

$$R = \frac{t_\Delta}{D_{60}} \quad (5)$$

Where R = log reduction of pathogenic *V. parahaemolyticus* (MPN/g)

t_Δ = inactivation (heating) time (min)

D_{60} = decimal reduction time of *V. parahaemolyticus* at 60 °C (min)

The concentration of pathogenic *V. parahaemolyticus* at the point of consumption (C_c) was estimated by

$$C_c = C_p - R \quad (6)$$

Table 6 Model input parameters in preparation module

Module	Variable	Description	Unit	Distribution/model	Reference
Growth	C_p	Level of pathogenic *V. parahaemolyticus* at preparation	MPN/g	$C_r e^{kt}$	(Yamamoto et al., 2008)
	C_r	Initial cell concentration	MPN/g	Triangular(0.3, 0.36, 0.92)	This study
	k	Growth rate for a given temperature	h^{-1}	Normal(0.236,0.390)	(Yamamoto et al., 2008)
	t	Time between retail and preparation stage	h	Triangular(0,1,max$t$)	(Yamamoto et al., 2008)
Thermal inactivation	D_{60}	Decimal reduction time of *V. parahaemolyticus* at 60 °C	min	0.71	(FAO, 2003)
	t_Δ	Inactivation (heating) time	min	2	
	R	Log reduction of pathogenic *V. parahaemolyticus*	Log MPN/g	t_Δ/D_{60}	

3.2.2.3 Consumption module

This module evaluated the final outcomes of exposure assessment by integrated between daily ingestion dose of pathogenic *V. parahaemolyticus* (*Dose*) and probabilities of exposure to this pathogen (P_E). Daily ingestion dose was calculated by:

$$Dose = C_c \times m \quad (7)$$

Where C_c is the levels of pathogenic *V. parahaemolyticus* at point of consumption (MPN/g), and *m* is daily shrimp consumption (g/person/day) which obtained from Thailand national survey.

An average daily shrimp consumption in Thailand was 28.06 g with a 97.5 percentile at 64 g (Standards, 2007-2010). The amount of shrimp consumption was assumed to be log-normal distribution. The mean and standard deviation of shrimp consumption were calculated as 3.219 and 0.479, respectively. The equation was showed below (8).

$$\mu = ln(mean, SD) \quad (8)$$

Finally, probability of exposure to pathogenic *V. parahaemolyticus* in shrimp (P_E) was calculated by:

$$P_E = P_r \times (1\text{-}e^{-Dose}) \quad (9)$$

Table 7 Model input parameters in consumption module

Variable	Description	Unit	Distribution/model	Reference
m	Amount of shrimp consumption of Thai people	g/person/day	lognormal(3.219,0.479)	(Standards, 2007-2010)
Dose	Number of ingested doses	MPN/ person/ day	C_c x m	
P_E	Probability of exposure to pathogenic *V. parahaemolyticus*		$P_r \times (1-e^{-Dose})$	

3.2.3 Hazard characterization

The probability of illness caused by pathogenic *V. parahaemolyticus* infections was calculated by using beta-Poisson dose-response model (FAO/WHO, 2011). The assumption of the model was that one single cell of *V. parahaemolyticus* can cause illness. To describe uncertainties parameters, bootstrap method was used follow by USFDA 2005 (FAO/WHO, 2011). The model was shown below (10).

$$P(d) = 1 - \left(1+\frac{D}{\beta}\right)^{\alpha} \quad (10)$$

where D is the number of pathogenic *V. parahaemolyticus* ingested by person daily (MPN/day), β was shape parameter, and α was infectivity parameter (Table 8).

Table 8 Model input parameters for hazard characterization

Variable	Description	Unit	Distribution/model	Reference
D	Number of ingested doses	MPN/day	C_c x m	
α	Infectivity parameter		8.59	(FAO/WHO, 2011)
β	Shape parameter		1.30×10^{7}	(FAO/WHO, 2011)

3.2.4 Risk characterization

The integration of the outputs of exposure assessment and hazard characterization provides the probability of adverse health effect to the population at risk. To estimate all possible annual risk from pathogenic *V. parahaemolyticus*, Latin Hypercube simulation model was set up to include uncertainty parameter. The risk estimate was calculated by the following equation.

$$P_I(D) = P_E \times P(d) \quad (11)$$

CHAPTER 4 RESULTS

4.1 Prevalence and levels of total V. parahaemolyticus contamination in white shrimp.

Prevalence and levels of total *V. parahaemolyticus* in shrimp in 6 provinces (Bangkok, Chiang Mai, Chon Buri, Kanchanaburi, Nakhon Ratchasima and Prachuap Khiri Khan) were shown in Table 9.

Table 9 Prevalence and level of total *V. parahaemolyticus* in 6 provinces

Provinces	Prevalence	Level of contamination (MPN/g)			
		≤10	>10-10^2	>10^2-10^3	>10^3
Bangkok	31.48%[a] (34/108)	70.59% (24/34)	8.82% (3/34)	2.94% (1/34)	17.65% (6/34)
Chiang Mai	72.22%[b] (78/108)	89.74% (70/78)	3.85% (3/78)	1.28% (1/78)	5.13% (4/78)
Chon Buri	77.77%[c] (84/108)	65.48% (55/84)	29.76% (25/84)	0% (0/84)	4.76% (4/84)
Kanchanaburi	76.85%[b] (83/108)	78.31% (65/83)	14.46% (12/83)	3.61% (3/83)	3.61% (3/83)
Nakhon Ratchasima	76.85%[b] (83/108)	74.7% (62/83)	14.46% (12/83)	2.41% (2/83)	8.43% (7/83)
Prachuap Khiri Khan	62.03%[b] (67/108)	76.12% (51/67)	14.93% (10/67)	4.48% (3/67)	4.48% (3/67)
Total	**66.20% (429/648)**	**76.22% (327/429)**	**15.15% (65/429)**	**2.33% (10/429)**	**6.29% (27/429)**

The same superscript indicated the non-significance differences ($p > 0.05$) while the different superscript indicated the significant difference ($p < 0.05$)

The mean prevalence of total *V. parahaemolyticus* in shrimps from both fresh and supermarket in 6 provinces was 66.2%, ranging from 31.48% to 77.77%. The highest prevalence of total *V. parahaemolyticus* was found in Chon Buri province (77.77%) while the lowest prevalence was found in Bangkok (31.48%). The majority of levels of total *V. parahaemolyticus* was found less than 10 MPN/gram. Compared among other provinces, prevalence of total *V. parahaemolyticus* in Bangkok was significantly two times less than others ($p < 0.001$). However, the high level of contamination (>10^3MPN/g) in Bangkok was two times higher than that in other provinces. There was a significant difference in this level of contamination between Bangkok and Chon Buri ($p < 0.05$), and Kanchanaburi ($p < 0.05$).

Prevalence of total *V. parahaemolyticus* in fresh market and supermarket were 67.13% (290/432) and 64.35% (139/216), respectively as shown in Table 10. No statistical difference was found between prevalence of total *V. parahaemolyticus* from fresh and supermarket ($p > 0.05$). Levels of total *V. parahaemolyticus* classified by market type was shown in Table 10. The high level of total *V. parahaemolyticus* contamination (>10^3MPN/g) was found in Fresh market from all provinces except Bangkok. Among 36% of total *V. parahaemolyticus* prevalence in Bangkok supermarket, almost three fourths of this prevalence were attributable to high level of contamination > 1,000 MPN/g.

Table 10 Prevalence and level of total *V. parahaemolyticus* classified by market type

Provinces	Market type	Prevalence	Level of contamination (MPN/g)			
			≤10	>10-10^2	>10^2-10^3	>10^3
Bangkok	Fresh	29% (21/72)	81% (17/21)	10% (2/21)	0% (0/0)	10% (2/21)
	Supermarket	36% (13/36)	54% (7/13)	8% (1/13)	8% (1/13)	31% (4/13)
Chiang Mai	Fresh	76% (55/72)	85% (47/55)	5% (3/55)	2% (1/55)	7% (4/55)
	Supermarket	64% (23/36)	100% (23/23)	0% (0/0)	0% (0/0)	0% (0/0)
Chon Buri	Fresh	76% (55/72)	64% (35/55)	29% (16/55)	0% (0/0)	7% (4/55)
	Supermarket	81% (29/36)	69% (20/29)	31% (9/29)	0% (0/0)	0% (0/0)
Kanchanaburi	Fresh	69% (50/72)	76% (38/50)	14% (7/50)	4% (2/50)	6% (3/50)
	Supermarket	92% (33/36)	82% (27/33)	15% (5/33)	3% (1/33)	0% (0/0)
Nakhon Ratchasima	Fresh	75% (54/72)	72% (39/54)	15% (8/54)	4% (2/54)	9% (5/54)
	Supermarket	81% (29/36)	79% (23/29)	14% (4/29)	0% (0/0)	7% (2/29)
Prachuap Khiri Khan	Fresh	76% (55/72)	71% (39/55)	18% (10/55)	5% (3/55)	5% (3/55)
	Supermarket	33% (12/36)	100% (12/12)	0% (0/0)	0% (0/0)	0% (0/0)

4.2 Prevalence and levels of pathogenic *V. parahaemolyticus* contamination in white shrimp.

Among total *V. parahaemolyticus* isolates analyzed by multiplex PCR, percentages of pathogenic *V. parahaemolyticus* (*tdh* or *trh* genes) in 6 provinces nationwide were shown in Table 11. *V. parahaemolyticus* isolates from Bangkok, Chiang Mai, and Chon Buri were negative for *tdh* and *trh* genes while those from Kanchanaburi, Nakhon Ratchasima, and Prachuap Kiri Khan were 3, 2, and 2, respectively. Overall percentages of *tdh* and *tlh* gene were 0.42% and 0.17%, respectively.

Table 11 Percentages of *tdh, trh* gene isolates in 6 provinces

Province	No. isolates analyzed	*tdh (%)*	*trh (%)*
Bangkok	95	0 (0)	0(0)
Chiang Mai	215	0 (0)	0(0)
Chon Buri	252	0 (0)	0(0)
Kanchanaburi	221	3 (1.36)	0(0)
Nakhon Ratchasima	219	2 (0.91)	0(0)
Prachuap Khiri Khan	188	0 (0)	2 (1.06)
Total	**1,190**	**5 (0.42)**	**2 (0.17)**

As shown in the Table 12, prevalence of pathogenic *V. parahaemolyticus* was 1.40 % (6/429). All the pathogenic gene was found in Kanchanaburi, Nakhon Ratchasima and Prachuap Khiri Khan province with less than 10 MPN/g. Prevalence of *tdh* gene positive and *trh* gene positive were 0.93% (4/429) and 0.46% (2/429). No sample was positive for both virulence genes simultaneously.

The levels of pathogenic *V. parahaemolyticus* were range between 0.3 to 0.92 MPN/g with mean concentration and SD at 0.44 ± 0.23 MPN/g. Most of the virulence

gene was found in fresh market (5/6) while only one sample from supermarket was found positive to pathogenic gene. There was not a significant difference in the prevalence of pathogenic *V. parahaemolyticus* between fresh market and supermarket.

Table 12 Prevalence and level of pathogenic *V. parahaemolyticus* in provinces

Provinces	Prevalence (%)	Level of contamination (MPN/g)			
		≤10	>10-10^2	>10^2-10^3	>10^3
Bangkok	0 (0/34)	0	0	0	0
Chiang Mai	0 (0/78)	0	0	0	0
Chon Buri	0 (0/84)	0	0	0	0
Kanchanaburi	2.4 (2/83)	2	0	0	0
Nakhon Ratchasima	2.4 (2/83)	2	0	0	0
Prachuap Khiri Khan	3.0 (2/67)	2	0	0	0
Total	1.40 (6/429)	6	0	0	0

4.3 Quantitative microbial risk assessment of *V. parahaemolyticus* from shrimp consumption

4.3.1 Hazard identification

V. parahaemolyticus is a gram negative, facultative anaerobic, halophilic bacterium which habitats in a marine environment. It normally found in various seafoods such as fish, crab, shrimp, and shellfish. *V. parahaemolyticus* has been recognized as one of a major foodborne gastroenteritis worldwide. The pathogenic *V. parahaemolyticus* was described by carrying *tdh* or *trh* gene which can produce the toxins causing cytotoxic and hemolytic effects to the host cells (Raghunath, 2015).

The risk of *V. parahaemolyticus* infection were attributable to the consumption of raw, under cooked, or cross contaminated seafood after cooking. The infection can be occurred within 4-96 hours and the duration of illness can be ranged from 1 to 30 days (Daniels et al., 2000). The symptoms were presented with watery diarrhoea, abdominal pain, nausea, vomiting, fever, and headache and usually resolved within a few days under appropriate treatments. However, the infection can be more serious in some immunocompromised people or patients with chronic illnesses.

The levels of *V. parahaemolyticus* in seafood were highly related with the sea temperature. The lowest growth temperature for this pathogen in seafood was between 3 and 13°C depending on growth substrate and conditions. It has been reported that *V. parahaemolyticus* can grow rapidly in broth and seafood temperatures between 18 and 40°C (Yoon et al., 2008). According to this study, rapid growth of *V. parahaemolyticus* in live oysters after harvested and stored at 26°C was supported (Gooch et al., 2002). Additionally, Thomson and Thacker (1973) also found the rapid growth at 8°C which were in the range of household and retail refrigeration temperatures.

4.3.2 Exposure assessment

In the exposure assessment step, probability of exposure (P_E) to pathogenic *V. parahaemolyticus* from consuming shrimp was calculated by prevalence and level of pathogenic *V. parahaemolyticus*.

4.3.2.1 Prevalence of pathogenic *V. parahaemolyticus*

Prevalence of pathogenic *V. parahaemolyticus* at retail stage (P_r) was described by Beta distribution, which was assumed to be constant from retail to consumption. After 20,000 iterations, mean prevalence of pathogenic *V. parahaemolyticus* was 1.07×10^{-2}. The probability distribution of P_E was shown in Figure 8.

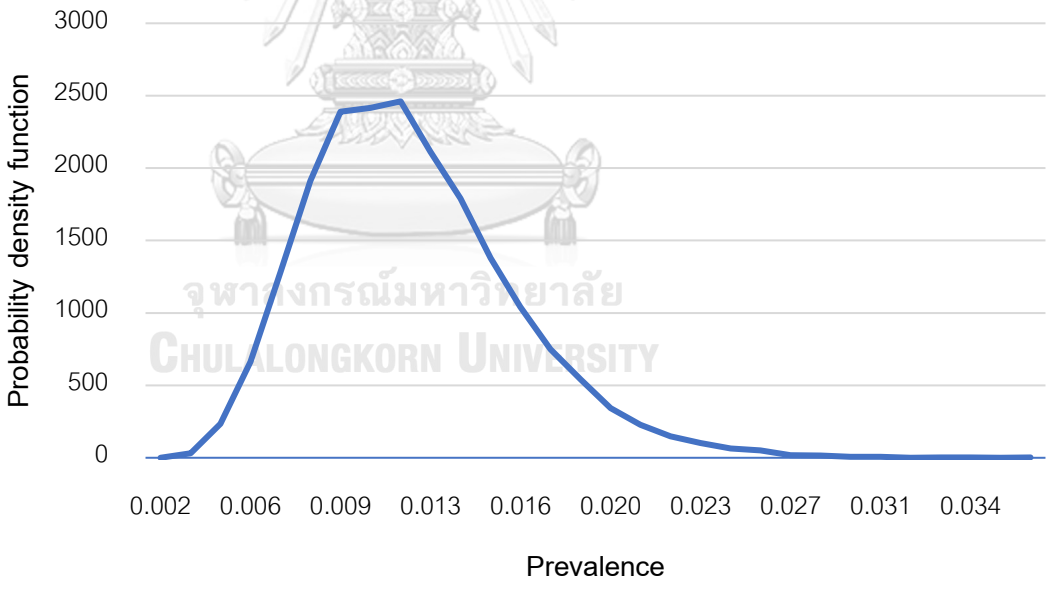

Figure 8 Probability distribution of prevalence of pathogenic *V. parahaemolyticus* in shrimp

4.3.2.2 Level of pathogenic *V. parahaemolyticus*

Unlike estimated prevalence, the level of pathogen was changed throughout the food chain. The initial concentration of pathogenic *V. parahaemolyticus* at retail stage (C_r) was explained by triangular distribution (Figure 9).

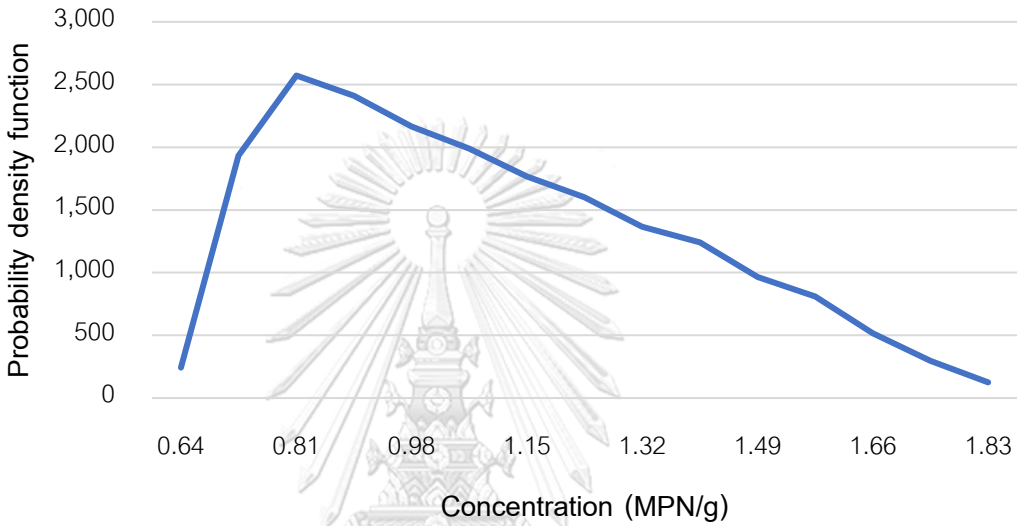

Figure 9 Probability distribution of level of pathogenic *V. parahaemolyticus* in shrimp at retail

The bacterial growth during retail to preparation stage was evaluated by exponential model which is environment-independent model (Figure 10).

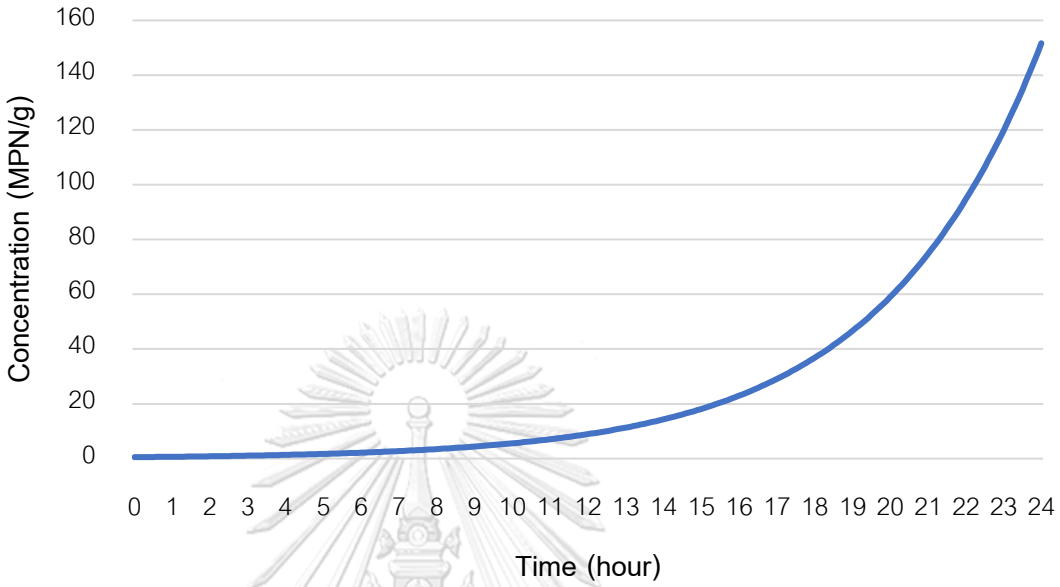

Figure 10 Growth model of pathogenic *V. parahaemolyticus* in shrimp during retail to preparation

After cooking step, it was found that heat could inactivate all *V. parahaemolyticus* in shrimp. So, the level of contaminated at preparation (*Cp*) step was used to assess risk from raw shrimp consumption instead. The contamination level in each step was shown in Table 13.

Table 13 Number of pathogenic *V. parahaemolyticus* in each stage

Stage	Mean level of contamination (MPN/g)
Retail (*Cr*)	5.28×10^{-1}
Preparation (*Cp*)	1.36×10^{6}
Consumption (*Cc*)	1.36×10^{6}

The estimated daily exposure dose of *V. parahaemolyticus* was calculated by daily shrimp consumption (*m*) and level of contamination (*Cc*). Then, probability of exposure of pathogenic *V. parahaemolyticus* (*Pe*) was calculated and shown in Table 4.6. Mean probability of exposure was 1.03×10^{-2}.

4.3.3 Hazard characterization

Probability of illness caused by pathogenic *V. parahaemolyticus* infections was described by Beta-Poisson dose-response model (Figure 11). Probability of illness was ranged from 2.58×10^{-13} to 1.00

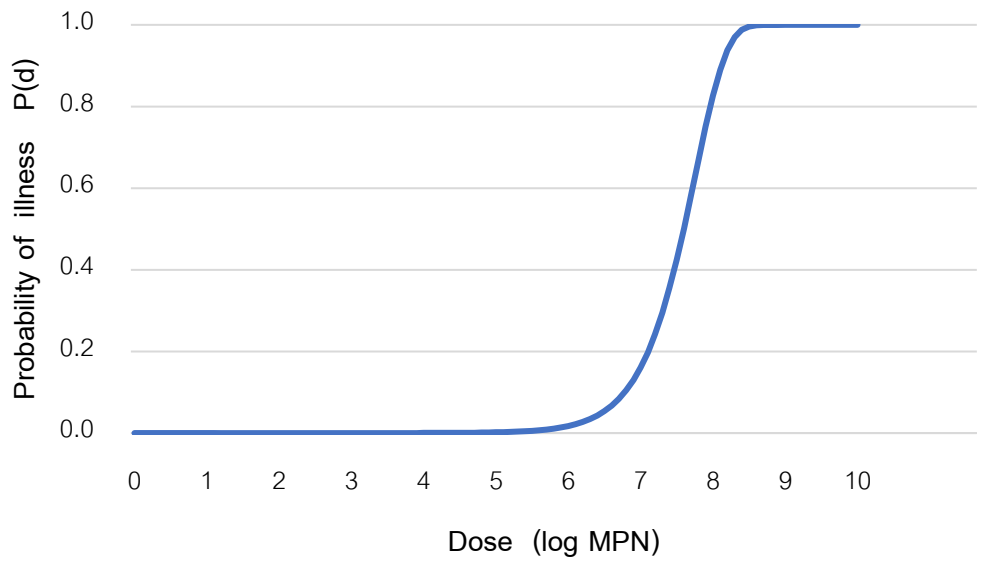

Figure 11 Beta-Poisson dose-response relationship for pathogenic *V. parahaemolyticus*

4.3.4 Risk characterization

Estimated daily risk from pathogenic *V. parahaemolyticus* infection by raw shrimp consumption was 1.02×10^{-4} and estimated annual cases was 3,711 cases per 100,000 populations.

Table 14 Variables summary for calculate the risk of *V. parahaemolyticus* infection

Variables	Minimum	5th Percentile	Mean	95th Percentile	Maximum
Pr	0.1×10^{-2}	0.5×10^{-2}	1.07×10^{-2}	1.8×10^{-2}	3.6×10^{-2}
Cr	3.0×10^{-1}	3.4×10^{-1}	5.3×10^{-1}	7.9×10^{-1}	9.2×10^{-1}
Cc	3.7×10^{-9}	5.2×10^{-2}	13.6×10^{5}	2.4×10^{8}	20.1×10^{9}
Dose	3.9×10^{-7}	1.19	33.4×10^{6}	6.3×10^{9}	5.4×10^{11}
Pe	4.6×10^{-9}	4.1×10^{-3}	1.0×10^{-2}	1.8×10^{-2}	3.6×10^{-2}
Pd	2.6×10^{-13}	7.9×10^{-7}	9.3×10^{-3}	5.6×10^{-3}	1.00
Pi(D)	1.2×10^{-21}	5.5×10^{-9}	1.0×10^{-4}	1.6×10^{-3}	2.2×10^{-2}

A sensitivity analysis results, using Spearman's rank correlation, suggested that the risk estimate was greatly influenced by the probability of illness (r = 0.94) followed by time between retail to consumption (r = 0.22), concentration at consumption (r = 0.16), and dose (r = 0.15) (Figure 12).

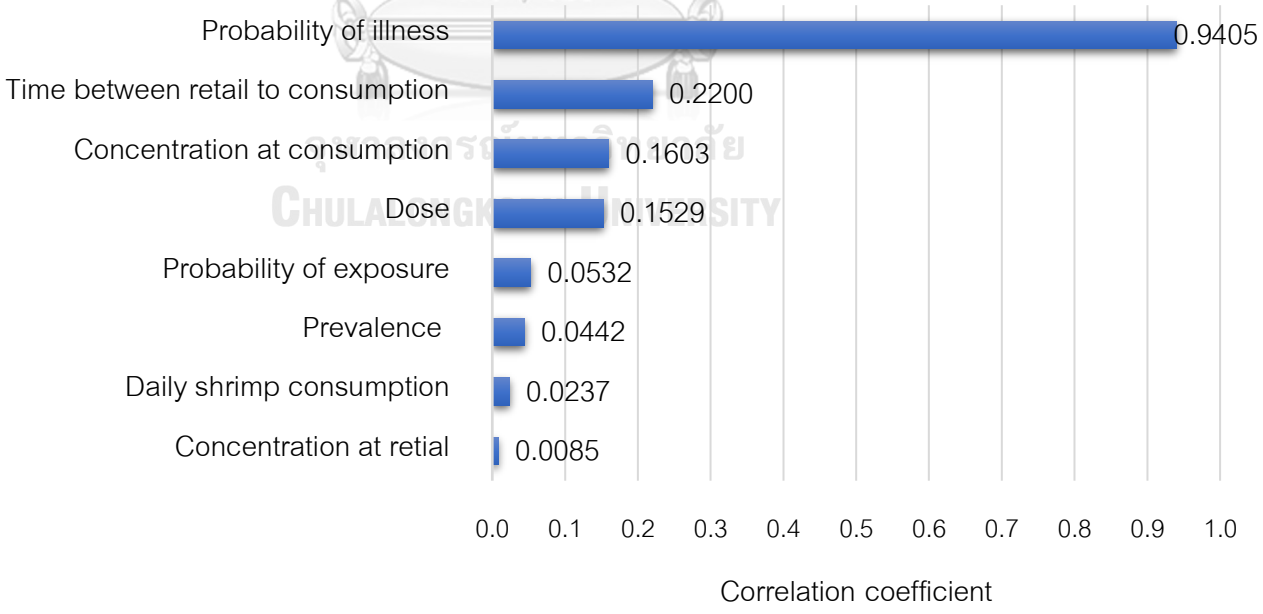

Figure 12 Sensitivity analysis of *V. parahaemolyticus* infection by raw shrimp consumption.

CHAPTER 5 DISCUSSION AND CONCLUSION

This study attempted to determine the prevalence and levels of both total and pathogenic *V. parahaemolyticus* contaminated in white shrimp in two retail types across 6 provinces in Thailand using MPN technique. In this study, among 648 samples of shrimp, 429 (66%) samples were contaminated with *V. parahaemolyticus*. This finding was in line with a study in China, which found the prevalence of *V. parahaemolyticus* in fresh shrimp as high as 70.3% by the same enumeration method (Xu et al., 2014). Another study found 75.8% of prevalence in shrimp products from an Asian country (Wong et al., 1999). Contrary to the result from middle east country, the prevalence of this bacterium in shrimp from Iran was only 39.3% (Rahimi et al., 2010). Compared with other seafoods, the prevalence of *V. parahaemolyticus* in shellfish in Atlantic and Gulf Coast was somewhat high as 61.4% (Cook et al., 2002). However, the prevalence of *V. parahaemolyticus* in shrimp in this study was higher than those of *V. parahaemolyticus* in mussel from Italy and in fish from China at 24.3% (Ottaviani et al., 2005) and 24% (Noorlis et al., 2011), respectively. Level of *V. parahaemolyticus* contaminated in shrimp in this study was found less than 100 MPN/g constituting 91% of samples. The result was compatible with some studies indicating that bacterial densities were less than 100 MPN/g with 95.1% prevalence (Xu et al., 2014).

U.S. Food and Drug Administration (FDA) reported that the maximum level of *V. parahaemolyticus* in seafood which less than 10,000 CFU/g was considered safe for consumption with minimal cooking (Klein and Lovell, 2017). Comparable to the finding of this study, the level of *V. parahaemolyticus* was mostly lower than 100 MPN/g. Since the enumerated of *V. parahaemolyticus* level in MPN method are greater than CFU around 25%, the level of contamination in the study is still lower than the safe limit as suggested by U.S.FDA.

Another objective of this study is to determine probabilities of illness of pathogenic *V. parahaemolyticus* from white shrimp consumption. Since *tdh* and *trh* genes were used to identified pathogenicity of *V. parahaemolyticus*, this research did classify pathogenic *V. parahaemolyticus* by detecting and enumerating only *tdh* or *trh* genes. The result revealed that among 429 *V. parahaemolyticus* positive samples, the prevalence of total pathogenic *V. parahaemolyticus* was 1.4% with mean level of contamination of only 0.44 ± 0.23 MPN/g. The result of this study was in line with other researchers demonstrating that only 1 – 5% of environmental samples were pathogenic *V. parahaemolyticus* strains (Thompson Jr and Vanderzant, 1976; Nishibuchi and Kaper, 1995); (Hervio-Heath et al., 2002); (Robert-Pillot et al., 2004). This finding also consistent with a previous study, they found only 2% positive for virulent *V. parahaemolyticus* carrying only *trh* gene (Xu et al., 2014). However, pathogenic gene percentage in this study was lower than a study in Thailand reporting pathogenic gene percentage at 5.6% in molluscan shellfish (Vuddhakul et al., 2006). Cook (2002) and DePaola (2003) also reported that the prevalence of *tdh* positive *V. parahaemolyticus* were 6% and 12.8%, respectively.

Although the virulence *V. parahaemolyticus,* carrying *tdh* and *trh* gene, was found in low prevalence in seafood, a study revealed that non-pathogenic strains can turn virulence by obtaining *tdh* gene from pathogenic strains (Hossain et al., 2013). So, the quantification of total *V. parahaemolyticus* cannot be discounted.

The risk assessment of this study indicated that likelihood of a person to get sick by consuming raw shrimp contaminated with pathogenic *V. parahaemolyticus* was 1.02×10^{-4} per year. Estimated annual cases was 3,711 cases per 100,000 population. Comparable to the result of Yamamoto (2008), the individual risk from bloody clams' consumption in southern Thailand was 5.6×10^{-4} per year.

Risk estimates from raw shrimp consumption in this study was higher than some other countries. In Malaysia, estimated annual cases from consuming of cooked black tiger shrimps and mackerel was 1.3×10^{-3} (Sani et al., 2013) and 6.53×10^{-3} (Tan et al., 2019) per 100,000 population. In Taiwan, risk of *V. parahaemolyticus* infection was conducted in raw oysters, the study found incidence rate at 0.28 case per 100,000 population (Huang et al., 2018). However, risk estimates from cooked shrimp consumption from the risk assessment model was negligible (less than 10^{-6}) since no pathogenic *V. parahaemolyticus* could survive the heat treatment process.

The annual morbidity rates of food poisoning in Thailand during 2017 to 2018 was 167.11 and 182.44 per 100,000 people (BOE, 2018). Among this morbidity rates, *V. parahaemolyticus* infection was around 0.1 to 0.2% of food poisoning. Therefore, mean morbidity rates of *V. parahaemolyticus* infection was ranged between 17 to 183 cases per 100,000 people. Compared with the result of this study, the risk estimated was very high than the reported by Bureau of Epidemiology. The reason might be from the fact that the illness can be self-limiting. The patients with mild symptoms may not be diagnosed by physician or they did not visit the hospital leading to underestimate of annual risk report.

This study also found that the proper cooked shrimp at 60°C for 2 minute can extremely reduce risk from *V. parahaemolyticus* which consistent with another study on Malaysians, noted that cooked temperature significantly reduced the level of pathogen (Sani et al., 2013). This information emphasized the importance of good handling and cooking time to prevent the infection from this harmful pathogen.

The difference between prevalence and levels of both total and pathogenic *V. parahaemolyticus* among various studies could be originated from the differences of detection method, season, or location of samples. A study suggested that using PCR

directly from enrichment broth could be more sensitive to detect the virulence gene in environment samples than conventional method (Dileep et al., 2003). Nordstrom (2007) has developed multiplex real-time PCR to detect total and pathogenic *V. parahaemolyticus.* Applying this method, the prevalence of *tlh*, *tdh*, and *trh* gene detected in oyster with this method went up to 44%, 44%, and 52%, respectively. In 2015, the study reveals that using modified method of MPN-immunomagnetic separation-LAMP assay altogether can be more useful for detection and quantification of *tdh* positive *V. parahaemolyticus*. In terms of seasonality and geographical location of samples, a study pointed out that seafoods from tropical area have high percentage of *V. parahaemolyticus* with 20 – 70% which could be the effect of high temperature of sea harvesting seafood samples (Zulkifli et al., 2009).

It should be noted that this study was lacking in some accurate data such as the storage temperature of shrimp at retail, time from retail to preparation, cooking preference, and factors affecting the bacterial growth. To increase the accuracy of risk assessment, suitable growth and thermal inactivation models using correct values of variables in such models are highly recommended. Moreover, the highly sensitivity of detection method should be concerned. Besides, the samples collected areas may not represent the true prevalence of pathogenic *V. parahaemolyticus* in Thailand therefore a wider area of sample collection covering more provinces was advised for the future study.

In conclusion, the prevalence and levels of total and pathogenic *V. parahaemolyticus* contaminated in shrimp at retail were estimated. The result showed moderate prevalence (66%) with levels of contaminated less than 10 MPN/g (76%) while prevalence (1.4%) and contaminated level (0.44 ± 0.23 MPN/g) of pathogenic *V. parahaemolyticus* were low. Although low levels of contaminated pathogen, detection of the pathogenic strains considered a health hazard. The risk assessment of

V. parahaemolyticus by consumption raw shrimp was evaluated in Thailand. The expected value of risk estimated was 1.02×10^{-4} equivalent to 3,711 cases per 100,000 population per year. The sensitivity analysis results showed that the transportation time from retail to consumption, the concentration of the pathogen at consumption, and ingested dose had greatly impact to the risk estimated.

The results of the study suggested that shrimp cooking at 60°C for at least 2 minutes is save from *V. parahaemolyticus* infection. However, if people desired to consume raw shrimp, reducing the transportation time from retail, well washing shrimp and decreasing the amount of raw shrimp consumption would be considered to minimize this risk. Moreover, the risk management measures in Thailand should be focusing on the transportation from harvest to retail and storage temperature at retail to reduce the initial level of *V. parahaemolyticus*.

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