Faculty of Resource Science and Technology

RAPID DETECTION AND QUANTIFICATION OF *Vibrio parahaemolyticus* IN PRAWNS (*Penaeus monodon*) BY REAL-TIME PCR

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DECLARATION

I hereby declare that the study entitled "Rapid detection and quantification of *Vibrio parahaemolyticus* in prawns (*Penaeus monodon*) by Real-Time PCR" is my original work and that all the sources that I have quoted and referred to have been acknowledged by means of complete references. It has been submitted and shall not be submitted in any form to any institution or other university.

_____ _____

Student's signature Date

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

APW	Alkaline Peptone Water
bp	base pair
CFU	Cell Forming Unit
R^2	Correlation coefficient
Ct	Cycle Threshold
g	gram
LB	Luria Broth
µm	Micrometer
µl	Microliter
mm	Millimeter
mM	MilliMolar
mL	MilliLiter
PCR	Polymerase Chain Reaction
℃	Temperature
%	Percentage
s	Second
NaCl	Sodium Chloride
rpm	Round per minute
spp.	Species
TCBS	Thiosulphate Citrate Bile Salt Sucrose
V. parahaemolyticus	*Vibrio parahaemolyticus*

Detection and Quantification of *Vibrio parahaemolyticus* in prawns (*Penaeus monodon*) by Real-Time PCR

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ABSTRACT

Vibrio parahaemolyticus is one of the etiologic agents of human gastroenteritis associated with seafood consumption. *Vibrio parahaemolyticus* carries the hemolysin gene, *tlh* gene other than the virulence *tdh* and *trh* genes. This study is carried out to detect and quantify *Vibrio parahaemolyticus* in 25 shrimp samples by Real-Time PCR assay. In this study, Luria Broth (LB) used as enrichment media is added with 3% of NaCl. The isolation and detection of *Vibrio parahaemolyticus* was done on both Thiosulphate Citrate Bile salt Sucrose (TCBS) agar and CHROMAgar Vibrio (CV). Next, Polymerase Chain Reaction (PCR) was carried out to confirm the absence of *Vibrio parahaemolyticus* in the *Tilapia* fish (*Oreochromis niloticus*) as the spiked sample. Furthermore, PCR also carried out to test the specificity of *Vibrio parahaemolyticus tlh* gene primers. Thirteen shrimp samples showed positive occurrence of *Vibrio parahaemolyticus* from the Real-Time PCR assay.

Keywords: *Vibrio parahaemolyticus*; Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar; CHROMAgar Vibrio (CV); Polymerase Chain Reaction (PCR); Real-Time PCR.

ABSTRAK

Vibrio parahaemolyticus merupakan salah satu agen yang menyebabkan gastroenteritis di kalangan manusia berpunca daripada makanan laut. Spesis ini mengandungi gen hemolysin, iaitu gen *tlh* selain daripada gen toksigenik, *tdh* dan *trh* gen. Kajian ini dijalankan untuk mengenalpasti kewujudan *Vibrio parahaemolyticus* dan kuantiti yang terdapat dalam 25 sampel udang. Dalam kajian ini, Luria Broth (LB) digunakan sebagai media untuk memperkayakan jumlah bakteria dengan tambahan 3% NaCl manakala Thiosulphate Citrate Bile salt Sucrose (TCBS) agar dan CHROMagar Vibrio (CV) digunakan sebagai media selektif untuk mengenalpasti kehadiran *Vibrio parahaemolyticus*. Polymerase Chain Reaction (PCR) seterusnya digunakan untuk mengenalpasti ketidakhadiran *Vibrio parahaemolyticus* pada ikan tilapia (*Oreochromis niloticus*). Ikan tilapia digunakan sebagai sampel untuk mengisi kuantiti kandungan *Vibrio parahaemolyticus*. PCR juga digunakan untuk menguji spesifisiti primer gen *tlh*. Tiga belas sampel udang menunjukkan kehadiran *Vibrio parahaemolyticus* daripada Real-Time PCR assay.

Kata kunci: *Vibrio parahaemolyticus*, Thiosulphate Citrate Bile salt Sucrose (TCBS) agar, CHROMagar Vibrio (CV), Polymerase Chain Reaction (PCR), Real-Time PCR.

Chapter 1

Introduction

Raw seafood is currently identified as the source of serious illnesses transmission. The main sources that can cause illness from food are bacteria, viruses and parasites (NSW Ministry of Health, 2012). The most occurrence of human diseases are probably foodborne infections and intoxication caused by *Salmonella*, pathogenic *E. coli*, *Campylobacter*, *Listeria*, *Shigella*, *Clostridium perfringens* and many more (Jones, 2003). Foodborne illness normally spread through improper cooked food, cross-contamination of food, spread from person-to-person and animal to human (NSW Ministry of Health, 2012). Examples of foodborne diseases caused by bacteria are Salmonellosis, Campylobacteriosis, Listeriosis, Perfringens, Staphylococcal poisoning and botulism. Those serious illnesses emergence have brought to worldwide concerned. Raw seafood, for example clams were believed to have pathogens that can cause food borne infection (Kingsley *et al*., 2002). Therefore, there is a need to detect and quantify the occurrence of pathogens in seafood to avoid the potential risk to human health.

Vibrio parahaemolyticus (*V. parahaemolyticus*) is from the genus Vibrio, one of the five genera assigned to *Vibrionaceae* family. *Vibrio* spp. is Gram-negative, facultative anaerobic motile curved-shaped with a single polar flagellum bacteria (Pujalte *et al*., 2010).

According to Michael *et al*. (1997), a special consideration in the taxanomy of *V. parahaemolyticus* is the ability of certain strains to produce a hemolysin termed

TDH (Thermostable Direct Hemolysin) or Kanagawa hemolysin, which is correlated with virulence in this species. The production of Kanagawa-positive (KP^+) strains of *V. parahaemolyticus*, are prime importance in human disease. Nevertheless, KP^+ strains constitute a very small percentage (typically <1%) of the *V. parahaemolyticus* strains found in aquatic environments and seafood (Michael *et al*., 1997). Thus the simple isolation of this species from the water or food does not indicate a health hazard.

Furthermore, *V. parahaemolyticus* is one of the etiologic agents of intestinal tract and extra intestinal infections in both animals and humans. It is a leading cause of human gastroenteritis associated with seafood consumption and an important seafood-borne pathogen throughout the world (Su & Liu, 2007). Moreover, gastroenteritis with *V. parahaemolyticus* is almost exclusively associated with seafood which is consumed raw, inadequately cooked, or cooked but recontaminated. (Michael *et al*., 1997). Healthy people are thought to be safe from the consumption of raw shellfishes, only those persons who are immunocompromised, which means suffer from HIV/AIDS and cancer, and those early age with liver disorder are at high risk (Daniels *et al*., 2000).

Vibrios are commonly found in brackish water or from marine and estuarine environment (Berlin *et al*., 1999). According to Michael *et al*. (1997), there is indication that vibrios are sensitive to cold and seafood are protected from vibrios at refrigeration temperature (Michael *et al*., 1997). Thus, it is essential that raw seafood be adequately refrigerated or iced to prevent bacterial growth.

Real-time PCR offers a rapid and quantitative analysis for the detection of food-borne pathogens. This project was done to detect and quantify the presence of *Vibrio parahaemolyticus* in prawns. SYBR Green-based Real-Time PCR assay was applied for the detection of *Vibrio parahaemolyticus* using target gene, *tlh* gene. Since no post-PCR detection procedures were required, Real-Time PCR procedure was much more rapid than conventional methods (Blackstone *et al*., 2003).

Objectives:

1. To detect the presence of *Vibrio parahaemolyticus* in prawns by SYBR Green-based Real-Time PCR assay targeting *tlh* gene.
2. To quantify the presence of *Vibrio parahaemolyticus* in prawns by SYBR Green-based Real-Time PCR.

Chapter 2

Literature Review

2.1 Vibrio genus

According to James and his coworkers (2005), the genus *Vibrio* consists of at least 28 species and *Vibrio spp.* that are often associated with aquatic environments and seafood are *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Vibrio alginolyticus* and *Vibrio cholerae*. These gram-negative straight or curved rods are members of the family *Vibrionaceae*. *Vibrio spp.* is 1.4 - 2.6 µm in length and 0.5 - 0.8 µm in diameter. Vibrios are commonly isolated during the warm weather months between April and October (James *et al*., 2005). Seasonality is most notable for *V. vulnificus* and *V. parahaemolyticus* infections, whereas those of some vibrios, for example *V. fluvialis*, occur throughout the year. There are several foodborne pathogens related to the *Vibrio* genus, which causes a spectrum of illnesses including cholera and milder form of gastroenteritis and occasional systemic infections (John & Deirdre, 2004).

2.2 Vibrio parahaemolyticus

Vibrio parahaemolyticus (*V. parahaemolyticus*) is from the *Vibrionaceae* family. It is a Gram negative, halophilic and non-spore forming facultative anaerobe. Besides that, the organism is a curved rod-shaped bacteria and has polar flagellum which helps in locomotion. *V. parahaemolyticus* are 0.5 - 0.8 µm in width and 1.4 - 2.4 µm in length. It is an oxidase-positive facultative anaerobe that can ferment glucose without gas production (Butt, 2004).

V. parahaemolyticus virulent strains show the production of either the thermostable direct hemolysin (TDH) or the TDH-related hemolysin (TRH) (Nishibuchi and Kaper, 1995). Both virulent strains showed hemolytic properties encoded for the *tdh* and *trh* genes, respectively (Park *et al*., 2000).

Furthermore, *V. parahaemolyticus* is widely distributed in inshore marine waters. It has been found in seawater and sediments (Laurie, 2006). The temperature range for the growth of *V. parahaemolyticus* is 5℃ - 43℃, with an optimum temperature of 37 ℃ (Laurie, 2006). There is decline in the growth of *V. parahaemolyticus* when placed in chilled condition, between 0℃ - 5℃ of storage. The bacterium needs salt (NaCl) to grow. It can grow in salt concentration from 0.5% - 10%, with optimum salt concentration for growth is 3%.

Primary mode of transmission is through the ingestion of raw, uncooked, or contaminated shellfish, for example oysters and clams. It can still harbor *V. parahaemolyticus* in the cooked crustacean, if it has not been properly cooked, or recontamination occurred by contact with uncooked seafood (Yeung, 2004). In addition, *V. parahaemolyticus* infection, especially septicemia will occur with the exposure of open wounds to contaminated seawaters or shellfishes (Butt, 2004).

2.3 Prawns

According to Ron (2012), prawn come from the family of *Gammaridae* and classed as *Crustacea*. Besides, prawns mate several times per year. The size of shrimp can be up to 20 mm in length. Adult prawn is usually about 15 mm in length. Prawns commonly

feed on plant and animal materials that have settled to the bottom. Mostly they can be found in water less than 35 feet deep (Ron, 2012).

2.4 Environment isolates of *V. parahaemolyticus*

McLaughlin *et al*. (2005) carried out a research about the outbreak of *V. parahaemolyticus* gastroenteritis associated with Alaskan oysters (McLaughlin *et al*., 2005). They found occurrence of gastroenteritis on cruise ships and conducted active surveillance throughout Alaska to identify sources of *V. parahaemolyticus* and contributors to the outbreak (McLaughlin *et al*., 2005). On July, 2004, the Alaska Department of Environmental Conservation (DEC) notified several cases of gastroenteritis among 189 passengers on cruise ships. The diagnosis of laboratory confirmed *V. parahaemolyticus* gastroenteritis in a patient due to consumption of raw oysters served on board of the ship. Seventy percent (132/198) passengers were interviewed and the through the investigation, it had been found that 17% (22/189) met their case definition of the illness, where consumption of the seafood items served on board of ship was a significant predictor of the gastroenteritis illness.

According to Lozano *et al*. (2003), between August and September 1999, there were 64 cases of acute gastroenteritis associated with the consumption of live oysters from a typical outdoor street market in Galicia (northwest Spain). The presence of *V. parahaemolyticus* was revealed through the analysis of the stool samples of infected patients. Two strains isolated were used to study the antibiotic susceptibility, biochemical characteristics and the presence of virulence factors. Both isolates showed Kanagawa phenomenon positive and produced thermostable direct hemolysin.

These results showed the risk of illness associated with seafood consumption and the presence of *V. parahaemolyticus* in mollusks harvested in Europe.

2.5 Route of transmission

V. parahaemolyticus is a seafood-borne pathogen which can cause gastroenteritis in humans. Outbreaks of food poisoning related to *V. parahaemolyticus* are usually attributed to the consumption of contaminated seafood, especially raw seafood (Anita *et al*., 2012). Additionally, *Vibrio* spp. poisoning also has been found in cross contamination of cookware and utensils. *Vibrio* spp. also often been found in feces of animals and humans, and can be spread via unhygienic manner of food preparation.

2.6 Virulence properties of *V. parahaemolyticus*

The most potential virulence of *V. parahaemolyticus* is the Kanagawa reaction, with almost all virulent strains being positive (K^+) and most avirulent strains being negative (K^-) (James *et al*., 2005). Moreover, K^+ strains produce a thermostable direct hemolysin (TDH) while K^- produces a heat-labile hemolysin, and some strains produce both (James *et al*., 2005). Some *V. parahaemolyticus* strains contain virulence factor of thermostable-related hemolysin (TRH).

2.7 Real-Time PCR detection and quantification of *V. parahaemolyticus*

Real-Time Polymerase Chain Reaction (Real-Time PCR) is a technique used to observe the progress of a PCR reaction in real time and the quantification of small amount of PCR products. Real-Time PCR is particularly related to the detection of fluorescent produced by the reporter molecule. As the PCR product accumulates, the fluorescent emission increases as the reaction proceed. In a research done by Panicker

and Bej, (2004), they had used SYBR Green I dye for Real-Time PCR for the detection of *Vibrio vulnificus* in shellfish and Gulf of Mexico water. The study resulted in the expected reaction conditions of the primers used and the cycling parameters. The amplification of the *vvh* primers was confirmed by the melting temperature analysis for all *V. vulnificus* strains. Moreover, in one of the research of Panicker and Bej (2005), they had developed a Taqman-based Real-Time PCR assay, which can detect high specificity and sensitivity towards *V. vulnificus* in oysters.

According to Dharmaraj (2011), there are currently four different chemistries for Real-Time PCR, namely Taqman, Molecular Beacons, Scorpions and SYBR Green. SYBR Green is a flurogenic dye that can emits strong fluorescent signal when binds to double-stranded DNA. Nevertheless, one of the disadvantages of SYBR Green is it will also binds to any double-stranded DNA in the reaction, including primer-dimers (Dharmaraj, 2011).

According to Cai *et al*. (2006), the quantitative detection of *V. parahaemolyticus* in seafood was carried out by using Taqman PCR assay based on the gyrase B gene (gyrB) sequence of *V. parahaemolyticus*. Twenty-seven *V. parahaemolyticus* strains and 10 strains of other species were used in the study and this indicated that the Real-Time PCR test was highly specific. The sensitivity test was approximately a single CFU per PCR in pure culture and 6 - 8 CFU per PCR in spiked raw oyster. Total of 300 seafood samples from eastern China were used in the analysis and 78 (26%) of these samples gained positive result for *V. parahaemolyticus* using conventional culture method and 97 (32.3%) using Real-Time PCR assay. The

results showed that the seafood harvested during harvest season in eastern China is commonly contaminated with *V. parahaemolyticus*.

Chapter 3

Materials and Methods

3.1 Samples collection

A total of 25 samples of prawn (*Penaeus monodon*) were purchased randomly from stalls at Stutong wet market and 7th Mile wet market. Sampling was carried out within December 2011 until April 2012. All samples were kept in polystyrene box before brought to the laboratory for further processing.

3.2 Sample preparation and enrichment of bacterial sample

The prawn samples were enriched in Alkaline Peptone Water (APW) to enhance the growth of *V. parahaemolyticus.* Next, 500 µl of the bacterial sample was added into 10 mL of Luria Broth. The enrichment of the bacterial sample was incubated in an incubator-shaker at 37 ℃ overnight.

3.3 Genomic DNA preparation

A total of 25 g of the prawn samples were homogenized using stomacher bag with 225 ml of Alkaline Peptone Water (Kumar *et al.*, 2009). After that, 1 mL of each of the prawns' homogenate was aliquoted into Eppendorf tubes in triplicate. The homogenates were centrifuged at 800 rpm for 1 minute. Next, the supernatant obtained was transferred into a new Eppendorf tube for each sample. The supernatant was subjected to centrifugation at 12000 rpm for 3 minutes. Furthermore, the supernatant was removed and the pellet obtained was added with 500 µl sterile

distilled water for each sample. The mixture was boiled at 100 ℃ for 10 minutes. Next, it was immerged in -20 ℃ for 10 minutes and recentrifuged at 13000 rpm for 3 minutes. Lastly, the supernatant was kept as template for Real-Time PCR assay (Noorlis *et al*., 2011).

3.4 Selective isolation and detection of *V. parahaemolyticus*

The overnight enriched *V. parahaemolyticus* was streaked onto TCBS agar and incubated overnight at 37 ℃ incubator. Subsequently, the growth and the morphology of the colonies were observed. Furthermore, green colonies obtained were streaked onto Chromagar^{TM} Vibrio, incubated overnight at 37 ℃ for further confirmation of the bacteria species. Light purple colonies were observed from the streaked Chromagar^{TM} *Vibrio* (Di Pinto *et al*., 2011).

3.5 Polymerase Chain Reaction

The MV2B-*tl* primer, targeting hemolysin *tlh* gene of *V. parahaemolyticus*, was used in the Polymerase Chain Reaction (PCR). These primer was also tested for specificity with *Saccharomyces cerevisiae*, *Escherichia coli* and other bacterial strains. The PCR technique used was based on Zulkifli *et al.* (2009) with minor modification. Each PCR was performed in a total reaction volume of 25 µl containing 4 µl of 10X PCR buffer, 1 µL of 10 mM/200 µL dNTPs mix (RBC Bioscience, Taiwan), 3 µL of 50 mM $MgCl_2$, 1 µL of 5 µ/µL of *Taq* polymerase (Recombinant); (Fermentas, Canada), 1 µl each of both M1 and M2 forward and reverse primers, 12 µl of sterile distilled water and 2 µl of DNA template. For negative control, the PCR mixture was added

with sterile distilled water. The PCR amplification was performed on a thermocycler (Eppendorf^{R} Mastercycle Personal). The PCR cycling parameters were as following:

Table 3.5: PCR cycling parameter

Steps	Temperature / Time	
Pre-denaturation	96 ℃ / 5 minutes	
Denaturation	94 ℃ / 1 minute	35 cycles
Annealing	63 ℃ / 2 minutes	
Extension	72 ℃ / 2 minutes	
Final Extension	72 ℃ / 7 minutes	

3.6 Agarose Gel Electrophoresis

A 5 µl aliquot of each amplification product was analyzed using agarose gel electrophoresis on 1.3% agarose gel cast and run in 1X TBE buffer at 110 volts for 1 hour. A 100 bp-marker was included in the gel. The gel was stained with Ethidium Bromide for 15 minutes and de-stained for another 15 minutes using distilled water. The gel was visualized using transmitted ultraviolet illumination and photographed using gel documentation system (AlphaDigiDoc RT) (Pradeep, 2006).

3.7 Sensitivity test using pure culture for standard curve

3.7.1 Dilution and hemocytometer

V. parahaemolyticus from both TCBS agar and Chromagar™ Vibrio were subjected to grow in 10 mL of Luria Broth with addition of 3% NaCl. The mixture was incubated in the incubator shaker at 37 ℃ for 24 hours. After that, the overnight culture was serially diluted (10-fold) in saline solution. Next, only the 10^{-1} diluted mixture was used for hemocytometer counting. Furthermore, the 10-fold serially diluted mixtures were subjected to cell boil according to Noorlis *et al*. (2009). SYBR-Green based Real-Time PCR was performed at 8-fold dilution to generate a standard curve. (Cai *et al*., 2005).

3.7.2 Spiking of pure culture into raw fish

Tilapia fish (*Oreochromis niloticus*) was sliced into pieces of flesh and rinsed by using distilled water. Next, *Tilapia* fish was subjected to PCR in order to confirm that it is free from *V. parahaemolyticus* by testing with specific primers. The PCR procedure was done according to Zulkifli *et al*. (2009). Next, a total of 2 g of flesh was added into 19 mL of distilled water (10-fold). After that, 1 mL of serially diluted culture was introduced into homogenate mix of distilled water and allowed to enrich for 30 minutes. Spiking was done from serially diluted culture from 10^{-1} to 10^{-7}. The spiking samples were subjected to cell boil extraction according to Noorlis *et al*. (2011) and the supernatants obtained were used for Real-Time PCR assay.

3.7.3 SYBR Green-based Real-Time PCR and cycling parameter

Mv2B-tl primers were used to develop the Real-Time PCR assay. These primers have the amplicon size of 248 bp and it was targeting on specific *V. parahaemolyticus*-tlh gene. In addition, this primer has the melting temperature of 85.6 ℃. Real-Time PCR assay was performed by using 12.5 µl of 2 X RT genes SYBR Green PCR Master Mix, 2 µl of each forward and reverse primer, 3.5 µl of distilled water and 5 µl of DNA template. The PCR cycling parameters was as followed: Initial denaturation of the DNA template follows by 40 cycles of amplification of the DNA template. The cycles consist of denaturation at 95 ℃ for 15s, and primers annealing at 60 ℃ for 10s (Panicker *et al*., 2004). The optic graphs of melting curve and quantitation curve were obtained for the spiked samples and prawn samples.