Faculty of Resource Science and Technology

DEVELOPMENT OF A SYBR GREEN BASED REAL-TIME POLYMERASE CHAIN REACTION ASSAY FOR SPECIFIC DETECTION AND QUANTIFICATION OF *Vibrio parahaemolyticus* FROM SELECTED SEAFOOD

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(Biotechnology Resource)
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Declaration

I hereby declare that this thesis entitled "Development of a SYBR Green Based Real-time Polymerase Chain Reaction Assay for Specific Detection and Quantification of *Vibrio parahaemolyticus* from Selected Seafood" is the result of my own research work and effort. It has not been submitted anywhere for any award. Where other sources of information that have been used, they have been acknowledged.

Signature:

Name: Nur Quraitu' Aini Bt Tajudin

Date:

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LIST OF ABBREVIATIONS

%	Percentage
^{o}C	Degree celcius
G	Gram
µl	Microliter
Ml	Milliliter
nm Wavelength	Nanometer Wavelength
rpm	Revolutions per minute
CFU	Colony Forming Unit
DNA	Deoxyribonucleic acid
PCR	Polymerase Chain Reaction
RT-PCR	Real-Time Polymerase Chain Reaction
spp.	Species
V. parahaemolyticus	*Vibrio parahaemolyticus*
ATCC	American type culture collection
TCBS	Tiosulphate Citrate Bile Salt Sucrose
NaCl	Sodium Chloride
LB	Luria Bertani
APW	Alkaline Peptone Water

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Development of a SYBR Green Based Real-time Polymerase Chain Reaction Assay for Specific Detection and Quantification of *Vibrio parahaemolyticus* from Selected Seafood

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ABSTRACT

Vibrio parahaemolyticus is a foodborne pathogen and their human infection is regularly associated with the consumption of contaminated, raw or undercooked seafood. To date, many biochemical identification and confirmation are done to detect the presence of this pathogen, especially from seafood samples. However these procedure require 2 or more days to complete. Thus, in this study we describe the development of a rapid SYBR green based real-time PCR assay for the detection and enumeration of *V. parahaemolyticus* from seafood sample. The real-time PCR assay and the primers designed were highly specific, and only generated the desired amplicons with *V. parahaemolyticus* DNA samples against other bacteria and fungi species. Our assay was able to detect *V. parahaemolyticus* with high coefficient values in concentrations as low as 5.0 pg/µl DNA in pure genomic DNA solutions and 50 CFU/ml in spiked samples.

Key words: *Vibrio parahaemolyticus,* real-time polymerase chain reaction (RT-PCR), SYBR Green dye, seafood

Vibrio parahaemolyticus adalah sejenis patogen bawaan makanan dan jangkitannya kepada manusia kerap dikaitkan dengan pengambilan makanan laut yang tercemar, dan mentah. Sehingga kini, banyak pengenalan dan pengesahan biokimia dilakukan untuk mengesan kehadiran patogen ini, terutamanya daripada sampel makanan laut. Walau bagaimanapun, prosedur ini memerlukan tempoh masa 2 hari atau lebih. Oleh itu, dalam kajian ini kami menghasilkan penggunaan SYBR hijau berasaskan real-time asai PCR untuk mengesan dan mendapatkan kuantiti *V. parahaemolyticus* daripada sampel makanan laut. Kaedah real-time PCR dan primer yang direka adalah spesifik, dan hanya menjana amplicons yang diingini di dalam sampel DNA *V. parahaemolyticus* berbanding terhadap bakteria lain dan spesis kulat. Asai kami dapat mengesan *V. parahaemolyticus* di kepekatan serendah 5.0 pg/µl DNA dalam larutan DNA tulen dan 50 CFU/ml pada sampel penyuntikan.

Key words: *Vibrio parahaemolyticus,* real-time asai PCR (RT-PCR), SYBR Hijau, makanan laut

CHAPTER 1

INTRODUCTION

1.1 Introduction

Foodborne diseases are caused by agents that enter the body through ingestion of contaminated food. The main source of foodborne illness is through bacterial contamination, physical factors and chemical usage. These diseases are widespread and are growing public health problem that occurs worldwide. Besides being harmful, they also cause negative economical and social impacts. According to Center for Disease Control and Prevention (2011), it is estimated that annually approximately 128,000 Americans are hospitalized due to foodborne disease and 3,000 death are recorded due to the same cause. Meanwhile in 2007, WHO reported that up to 30% of the population in industrialised countries suffer from foodborne diseases each year. Thus, food safety is currently an increasing important public health issue and governments all over the world are intensifying their efforts to improve food safety.

There are more than 250 known foodborne diseases with bacteria as the main causing agent followed by viruses and parasites. In the Asian region, *Vibrio* spp. have been recognized as the leading cause of foodborne outbreaks in many countries including Malaysia (Tunung *et al*., 2010), Japan (Hara-Kudo *et al*., 2001), China (Luan *et al*., 2008) India (Gopal *et al*., 2005), Taiwan (Hara-Kudo *et al*., 2003) and Korea (Lee *et al*., 2008). Three *Vibrio* species, *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*, are the most important vibrios associated with human illness (Nishibuchi *et al*., 2005).

V. parahaemolyticus is widely present in brackish and marine waters, and infection to human is regularly associated with the consumption of contaminated, raw or undercooked seafood (Guoxiang *et al.,* 2009). *V. parahaemolyticus* was first isolated in Japan in 1950 and has since been recovered all around the world (Heinis *et al.,* 1977). Most recently, Tunung *et al.,* (2010), reported that vegetable samples collected in Selangor, Malaysia were positive for pathogenic species of *V. parahaemolyticus*. The pathogenesis of *V. parahaemolyticus* is based on the presence of several virulence factors, namely thermostable direct hemolysin (*tdh*) and tdh-related hemolysin gene (*trh*) (Kim *et al*., 2008). Symptoms of gastroenteritis caused by *V. parahaemolyticus* include watery diarrhea, abdominal cramps, vomiting, headache, chills, nausea and fever (Dadisman *et al.,*1972).

To date, many biochemical identification and confirmation procedures are done to detect and confirm the presence of this pathogen, especially from seafood samples. However, they require 2 or more days to complete. Standard PCR is also the method of choice, in which rapid detection of pathogens in biological materials can be achieved, however it does not allow for the quantitative analysis of template DNA or cell concentration (Gao *et al*., 2004). Therefore in this study, we describe the development of a rapid, easy and economical SYBR green-based real-time PCR assay for the detection and quantification of *V. parahaemolyticus* DNA particularly targetting the thermolabile hemolysin (*tl*) gene. Real-time PCR allows for more sensitive level of detection, less prone to contamination and increased number of samples that can be processed in a period of time when compared to conventional PCR (Cai *et al*., 2006). Thus, this would be useful for the surveillance of this pathogen and are the key issue in controlling food-borne infections.

1.2 Objectives

This study was undertaken with the following objectives:

I. To develop and design a species specific primer to detect and enumerate *V. parahaemolyticus*, targetting the thermolabile hemolysin (*tl*) gene.

II. To investigate the sensitivity and specificity of the designed primers developed for the detection and enumeration of *V. parahaemolyticus*.

III. To conduct real-time PCR for the detection and enumeration of *V. parahaemolyticus* from fish, prawn and cockle samples and validate the application of designed primers.

CHAPTER 2

LITERATURE REVIEW

2.1 The genus *Vibrio*

The term *Vibrio* is derived from the Latin word which means "to vibrate". The genus *Vibrio* belongs to the *Vibrionaceae* family and consists of approximately 34 recognized species (HPA, 2007). According to Broberg *et al*. (2011), vibrios are Gram negative, curved rods that are motile by means of a single polar flagellum. They are sensitive to low pH but can tolerate high pH between 9.0-9.6. They require salt to grow, and are thus associated with ocean-sourced seafood. Adeleye *et al*. (2010) reported that vibrios are one of the most common organisms in surface water of the world, and are present in both marine and freshwater habitats in associations with aquatic animals. These bacteria can cause sickness in three ways, wound infections, gastrointestinal illness and septicemia (blood infections) as most of them are able to secrete enterotoxins in water, foods and gastrointestinal tract (Nishibuchi and DePaola, 2005). Some species are pathogens of fish, eels and frogs as well as other vertebrates and invertebrates (Todar, 2005).

2.2 *Vibrio parahaemolyticus*

Vibrio parahaemolyticus is an enteric pathogen that can cause gastroenteritis in human, usually after the ingestion of undercooked or mishandled seafood (DePaola *et al*., 1990). *V. parahaemolyticus* lives naturally in brackish saltwater worldwide (Johnson *et al*., 2010) and is present in higher concentrations during summer (Broberg *et al*., 2011). *V. parahaemolyticus* is a halophilic (salt-requiring), non-sporing, Gram-negative and facultatively anaerobic bacterium (Curtis *et al.*, 2003). Formerly, *V. parahaemolyticus* was known as *'Pasteurella parahaemolytica'*. This bacteria is an invasive organism affecting primarily the colon. They grow optimumly in 37 °C in which they can reach generation time up to 8 or 9 times in water and 12 to 18 minutes in seafood (Montville and Matthews, 2008).

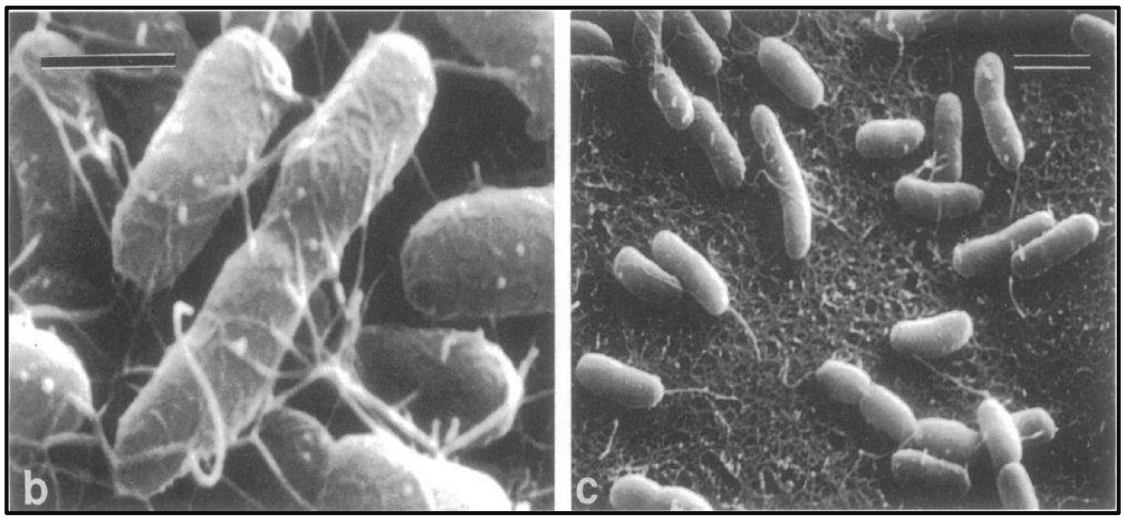

Figure 1: Scanning Electron Microscope Observation of *V. parahaemolyticus.*
Image source: Belas & Colwell, (1982)

2.3 Thermolabile hemolysin (*tl*) gene

Thermolabile hemolysin (*tl*) gene is also known as the lecithin-dependent haemolysin (*ldh*) gene (Zhang *et al*., 2005). These genes produce a heat labile protein. According to Zhang *et al*. (2005), this type of hemolysin is an exotoxin that attacks red blood cell membranes and causes cell rupture. Hemolysin is the most widely distributed toxin among pathogenic vibrios and exerts various roles in the infection process. Hemolysins ruptured the red blood cells and lead to the freeing up of the iron-binding proteins such as haemoglobin, transferrin and lactoferrin. In many cases, the destructive properties of haemolysin is not restricted to erythrocytes, but extends to other cell types including mast cells, neutrophils, and polymorphonuclear cells, thus, enhances virulence by causing tissue damage. The thermolabile hemolysin gene in *V. parahaemolyticu*s is species-specific, and can be used as a specific marker for PCR amplification detection of this pathogen. According to studies conducted by Taniguchi *et al*. (1985) and Rizvi *et al*. (2010), this gene was shown to be present in all the *V. parahaemolyticus* strains tested. However, the role of *tl* gene in the pathogenicity of *V. parahaemolyticus* is still unclear (Shinoda *et al.,* 1991).

2.4 Seafood

Seafood are important source of dietary protein. In addition to being nutritious they are needed for a healthy diet. However, there are health risks associated with their consumption. Pathogens can be introduced into estuaries from inadequately treated sewage, runoff from urban areas, medical waste and waste from pets and wildlife. Cockles and other filter feeding shellfish can concentrate bacteria such as vibrios that are naturally present in sea water, or other microbes that are present in human sewage

dumped into the sea (DePaola *et al*., 1990). Harmful microorganisms could also enter seafood processing chain due to inadequate process control, poor hygiene and sanitation in processing foods and incorrect handling or storage. Kvenberg (1991) classified bacteria pathogens associated with fish into two, the nonindigenous or indigenous bacteria pathogens. Vibrio species are those naturally living in the fish's habitat and are considered as indigenous bacteria.

2.5 Selective media for *V. parahaemolyticus* isolation

Plating of APW enrichment broth onto Thiosulphate-Citrate-Bile salt Sucrose (TCBS) agar has been widely used for selective pre-isolation of *V. parahaemolyticus* from foods (Di Pinto *et al.,* 2010). The culture is left overnight in 37 ^{o}C incubator for the *V. parahaemolyticus* to develop into smooth, green and about 2 to 3 mm in diameter of colonies (ISO, 2007). Another widely used selective medium for vibrios is CHROMagar. On this medium, *V. parahaemolyticus* will form mauve colonies (Di Pinto *et al*., 2010). This agar medium is more sensitive in identifying *V. parahaemolyticus* in food samples compared to TCBS (Canizalez-Roman, 2011). It contains substrates for beta-galactosidase which was developed specifically to differentiate *V. parahaemolyticus* from other bacteria by using a chromogenic substrate, instead of sugar fermentation in traditional growth media such as TCBS (Hara-Kudo *et al.,* 2001).

2.6 Alkaline peptone water (APW)

Alkaline peptone water is used for the enrichment of *Vibrio* species from food, water, feces and clinical studies. This medium has been recommended to increase the recovery of *Vibrio* species in fecal materials and other samples. Peptones provide nitrogen, vitamins, minerals and amino acids, meanwhile sodium chloride supplies essential electrolytes for transport and osmotic balance and encourages the growth of the vibrios. According to Blackstone *et al*. (2003), APW do not inhibit PCR reaction making it an ideal medium for direct PCR application. In 1983, Dupray and Cormier concluded that enrichment in APW yielded better *V. parahaemolyticus* recovery than enrichment in Glucose Salt Teepol Broth (GSTB). The ease of APW preparation also contributed to its widespread use.

2.7 Real – time polymerase chain reaction (RT-PCR)

Real-time polymerase chain reaction is a technique used to monitor progress of PCR reaction in real time. RT-PCR involves the use of fluorescent dyes or probes that label double-stranded DNA molecules (Ponchel *et al*., 2003). As the number of gene copies increases with each PCR cycle, the fluorescent signals becomes more intense (Wang and Levin, 2002). This method does not require post-PCR sample handling, thus avoiding potential PCR carryover contamination and thus saving time (Tyagi *et al*., 2009). RT-PCR can provide sensitive, species specific and quantitative detection of *V. parahaemolyticus* in seafood without the elaborate process of characterization of bacteria by conventional microbiological methods (Cai *et al*., 2006). Quantitative real time PCR is highly recommended to detect and enumerate bacteria, in highly complex microbial communities directly without conducting enrichment of samples to save time,

effort and cost (Abdul Amir *et al*., 2009). The progression of the PCR reaction may be monitored after each cycle rather than at the end, providing a much better quantification assay (Bankowski and Anderson, 2004). Cycle treshold (Ct) value is usually use for product quantification in RT-PCR. Ct value is the cycle number at which enough amplified product accumulated to yield a detectable fluorescent signals (Blackstone *et al*., 2003). Two strategies are commonly employed to quantify the results obtained by RT-PCR which are the standard curve method and the comparative threshold method (Raymaekers *et al*., 2009).

Figure 2 : Image of Real-time PCR thermocycler (Rotor gene 6000)

2.8 SYBR Green dye

SYBR Green is an asymmetrical cyanine dye used as a nucleic acid stain in molecular biology. It provides the simplest and most economical format for detecting and quantifying PCR products in real-time reactions (Ponchel *et al*., 2003). Since the dye binds to double-stranded DNA, there is no need to design a probe for any particular target being analyzed. The stain preferentially binds to double-stranded DNA and upon excitation emits light. As the product accumulates, fluorescence also increases (Rizvi *et al*., 2010). However there are also some disadvantages, in which the SYBR Green will bind to any double-stranded DNA in the reaction, including primer-dimers and other non-specific reaction products, which results in an overestimation of the target concentration (Ponchel *et al*., 2003). Hence, detection by SYBR Green requires extensive optimization and follow up assays such as melting curve analysis to validate the results.

CHAPTER 3

MATERIALS AND METHOD

3.1 Materials

The materials that were used in this study are listed below:

1. Fish, cockles and squid samples
2. PCR thermocycler (Eppendorf (R) Mastercycle Personal, USA)
3. Real-time PCR thermocycler (Rotor gene 6000, Corbett Research, Australia)
4. Alkaline peptone water (Merck, Germany)
5. Luria Bertani broth (Merck, Germany)
6. Thiosulphate-Citrate-Bile salt Sucrose (Merck, Germany)
7. CHROM agar *Vibrio*™ (CHROMagar 4, France)
8. Powersoil kit (MO BIO Laboratories Inc, US)
9. Polarizing microscope (Olympus BX51, USA)
10. Hemocytometer
11. Water bath
12. Distilled water
13. Ice
14. Stomacher bags
15. RT gene SYBR green PCR mastermix (Qiagen, USA)
16. MV2B primer :
    i) MV2B TLF : 5'- GTT GCA CTC GGT GAC AGC TTG-3'
    ii) MV2B TLR : 5'- AGT TTT GCG TAG GTT AAG TAC-3'

3.2 Primer designed

PCR primers were designed based on the thermolabile hemolysin gene region from several strains of *V. parahaemolyticus* which are VP-isolateSh06, VP-M36437.1, VP-AB012596.1, VP-AY289609.1 and VP-ATCC33846 sequences as retrieved from the GenBank (Figure 1). Blast analysis were used to checked the designed gene-specific primers for significant homology with other DNA sequences following sequence alignment with ClustalX2 algorithm. Two regions with single nucleotide polymorphisms that able to differentiate between *V. parahaemolyticus* and *Oceanimonas sp.* Be07 were identified and used to design the forward and reverse primers (Figure 1). The *tl* gene specific primer MV2B-TLF and MV2B-TLR amplify a 248 bp products. Primers were synthesized by IDT (Integrated DNA Technologies, Coralville, IA).

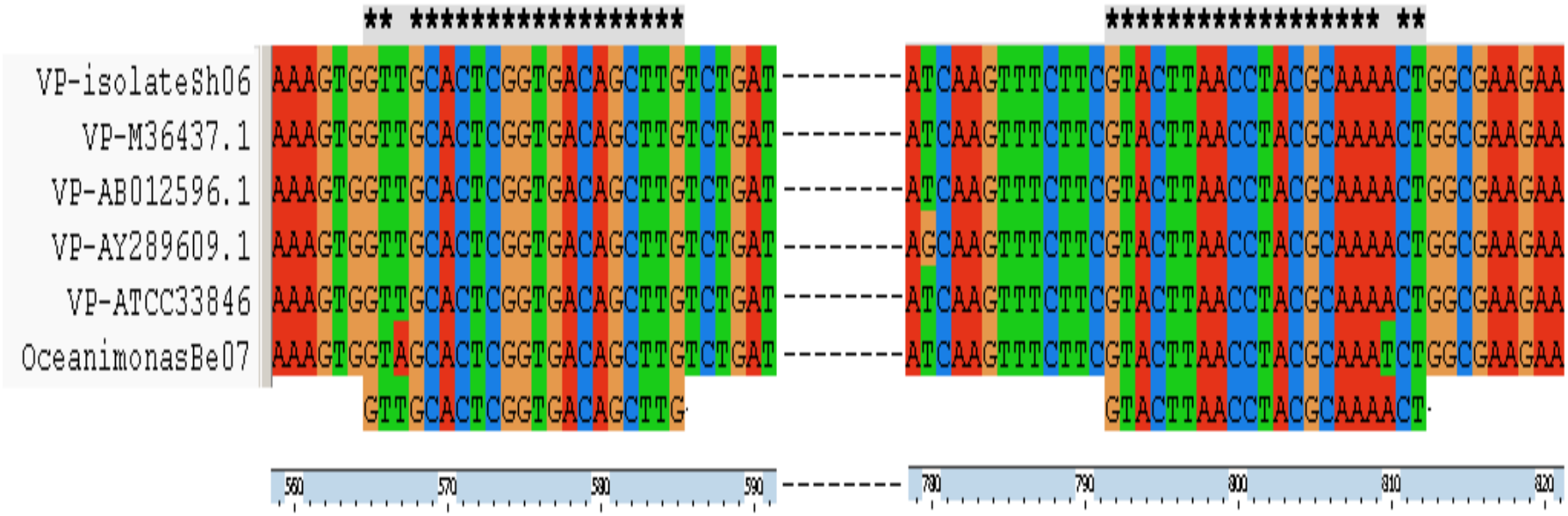

Figure 3 : Alignments of the *tl* gene regions used for primer design. The illustration demonstrates primer sequence which derived from aligned sequences of few strains of *V. parahaemolyticus* representatives. Nucleotide polymorphisms are marked with an absence of asterisk and gaps are indicated by dashes.

3.3 Isolation of *V. parahaemolyticus* positive strains

The isolation of *V. parahaemolyticus* was achieved by using two types of solid selective media namely Thiosulphate-Citrate-Bile salt Sucrose (TCBS; Merck, Germany) agar and CHROMagar (CHROMagar 4, France). After enrichment of fish flesh in alkaline peptone water (APW; Merck, Germany), 1 ml of the APW broth was transferred onto TCBS agar plates and was spread evenly. In addition, a loopful of the broth was also streaked onto another TCBS agar. The plates were sealed and incubated overnight in a 37 ^{o}C incubator. The suspected *V. parahaemolyticus* colonies which were indicated by smooth green and about 2 to 3 mm in diameter colonies were further picked and streaked onto CHROMagar. The plates were then incubated at 37 ^{o}C for another 24 hours. After incubation, the *V. parahaemolyticus* isolates on CHROMagar appeared as mauve colour colonies. Several colonies were picked and stored in Luria Bertani broth (LB; Merck, Germany) and Nutrient agar slant supplemented with 3% NaCl for further use.

3.4 DNA extraction

Bacterial DNA from overnight culture of *V. parahaemolyticus* in LB broth were extracted using Powersoil® DNA isolation kit (MO BIO laboratories, Inc; US) according to manufacturers instruction. All DNA concentrations were determined by absorbance at 260/280 nm using the NanoDrop system (Thermo-Fisher Scientific, Wilmington, DE). The DNA was immediately frozen at -20^{o}C to be later used for downstream applications.