UNIVERSITI PUTRA MALAYSIA

DETECTION OF TDH AND TRH VIRULENCE GENES, ANTIBIOTIC RESISTANCE AND GENETIC DIVERSITY OF VIBRIO SPP. ISOLATED FROM SHRIMP

SAIFEDDEN AYAD GARGOUTI

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By

SAIFEDDEN AYAD GARGOUTI

Thesis Submitted to the School of Gradute Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

August 2016

DEDICATION

I dedicate this piece of work to my beloved

Wife

For Her patience on, sacrifice and unlimited support

and to my children

Wahib, Dalal, and Osama

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

DETECTION OF *tdh* AND *trh* VIRULRNCE GENES, ANTIBIOTIC RESISTANCE AND GENETIC DIVERSITY OF *Vibrio* spp. ISOLATED FROM SHRIMP

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Chairman : Professor Son Radu, PhD
Faculty : Food Science and Technology

Vibrio parahaemolyticus and *Vibrio alginolyticus* are considered among gastrointestinal pathogens. *Vibrios* are a common causative agent of seafood-borne illness in tropical and subtropical countries including Malaysia, where the temperature is optimal for their growth. Although *Vibrio parahaemolyticus* and *Vibrio alginolyticus* not documented well, the majority of illness is self-limiting, treatment is not necessary. Multiplex PCR, Antibiotic resistance and RAPD-PCR were used to detect and characterize of 60 samples of shrimp purchased from the local supermarkets in Selangor, Malaysia were examined for pathogenic and non-pathogenic genes (*tdh*, *trh*, and *toxR* genes). A total of 56 (93.3%) out of 60 samples were found positive for *V. parahaemolyticus*. The density of screened samples ranged from 62 MPN/g to >110,000 MPN/g. About 50% of positive samples contained $>10^4$ MPN/g of *V. parahaemolyticus*. Multiplex polymerase chain reaction (PCR), was conducted to determine pathogenic and non-pathogenic genes, estimated that 93.33% of samples were positive for the *toxR* gene at band 368 bp. The significant result comes with 2 samples of *V. alginolyticus* were isolated on CHROM^{TM} *Vibrio* agar. Both samples were positive for the *tdh*, *trh*, and *toxR* genes at bands 251, 484, and 368 bp, respectively. The 16S ribosomal DNA sequence (1.5 kb) full-length was also performed. The result showed 99% similarity to *V. alginolyticus* strain ATCC 17749. Thirty isolates were tested for their susceptibility to 14 different antibiotics. *Vibrio* isolates were resistant to ampicillin (80%), amoxicillin–clavulanic acid and cefotaxime (50%), ceftazidime (46.7%), cefepime (33.3%). However, the isolates were highly susceptible to imipenem (100%), and piperacillin and gentamicin (96.7%). Approximately 55% of the isolates showed a multiple antibiotic resistance (MAR) index of >0.2, thereby indicating the high risk of sources where these isolates originated. Randomly amplified polymorphic DNA–PCR (RAPD-PCR) was performed on 30 samples to determine the genetic diversity among *V. parahaemolyticus* and *V. alginolyticus* isolates. Two primers, namely, OPAR-03 (5′-GTGAGGCGCA-3′) and OPAR-10 (5′-TGGGGCTGTC-3′), showed the most satisfactory results and were selected for this study. Primers OPAR03 and OPAR10 produced 2–10 and 2–8 bands, respectively, and their amplicon sizes ranged within 200–2000 bp. A total of 19 RAPD patterns reflected the high genetic diversity among the *V. parahaemolyticus* isolates

obtained from shrimp samples. The incidence of *Vibrio parahaemolyticus* could be expected and natural. This study is the first to report about the expression of *tdh* and *trh* toxic genes of *V. parahaemolyticus* in *V. alginolyticus*. RAPD-PCR analysis showed that *V. parahaemolyticus* and *Vibrio alginolyticus* are exhibited a high level of genetic diversity. The result stressed the importance of *Vibrio* to food safety and public health and an economic impact.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGESANAN GEN VIRULENS *tdh* DAN *trh*, DAYA TAHAN TERHADAP ANTIBIOTIK, DAN KEPELBAGAIAN GENETIK *Vibrio* spp. YANG DIASINGKAN DARIPADA UDANG

Oleh

SAIFEDDEN AYAD GARGOUTI

Ogos 2016

Pengerusi : Profesor Son Radu, PhD
Fakulti : Sains dan Teknologi Makanan

Vibrio parahaemolyticus dan *Vibrio alginolyticus* dianggap tergolong sebagai patogen gastrousus. Bakteria jenis *Vibrio* adalah agen yang lazimnya menyebabkan penyakit bawaan makanan laut di negara-negara tropika dan subtropika termasuklah Malaysia, kerana suhu di tempat-tempat begini paling sesuai bagi pembiakannya. Walaupun *Vibrioparahaemolyticus* dan *Vibrioalginolyticus* tidak didokumenkan dengan baik, namun kebanyakan penyakit akan baik dengan sendiri dan tidak perlu dirawat. PCR (tindak balas berantai polimerase) multipleks, ketahanan antibiotik, dan PAPD-PCR digunakan untuk mengesan dan menggambarkan sifat 60 sampel udang yang dibeli di pasar raya tempatan di Selangor, Malaysia, yang telah diperiksa untuk mencari gen patogen dan bukan patogen (gen *tdh, trh,* dan *toxR*). Sejumlah 56 (93.3%) daripada 60 sampel itu didapati mengandungi *V. parahaemolyticus*. Ketumpatan sampel yang diuji itu berjulat antara 62 MPN/g hingga >110,000 MPN/g. Kira-kira 50% sampel yang positif itu mengandungi $>10^4$ MPN/g *V. parahaemolyticus*. Tindak balas rantai polimerase (PCR) multipleks, yang dilakukan untuk menentukan gen patogen dan bukan patogen, menganggarkan bahawa 93.33% sampel itu berhasil positif bagi gen *toxR* pada jalur 368 bp. Hasil yang nyata ini diperoleh daripada dua sampel *V. alginolyticus* yang diasingkan pada agar-agar CHROM^{TM} *Vibrio*. Kedua-dua sampel berhasil positif bagi gen *tdh, trh,* dan *toxR* masing-masing pada jalur 251, 484, dan 368 bp. Seluruh jujukan DNA ribosom 16S (1.5 kb) juga dilakukan. Hasilnya menunjukkan persamaan sebanyak 99% dengan *V. alginolyticus* jenis ATCC 17749. Tiga puluh sampel yang diasingkan telah diuji untuk menentukan kerentanan bakteria ini terhadap 14 jenis ubat antibiotik. Asingan *Vibrio* pula berdaya tahan terhadap ampicillin (80%), asid amoxicillin-clavulanic dan cefotaxime (50%), ceftazidime (46.7%), dan cefepime (33.3%). Walau bagaimanapun, asingan ini sangat rentan terhadap imipenem (100%), dan terhadap piperacillin dan gentamicin (96.7%). Kira-kira 55% asingan menunjukkan indeks daya tahan terhadap beberapa jenis ubat antibiotik (MAR) >0.2, dengan itu membayangkan risiko tinggi sumber asingan ini. DNA-PCR polimorf yang diperbesar secara rawak (RAPD-PCR) dilakukan pada 30 sampel untuk menentukan kepelbagaian genetik pada asingan *V. parahaemolyticus* dan *V. alginolyticus*. Dua bahan primer, iaitu OPAR-03 (5′-GTGAGGCGCA-3′) dan OPAR-10 (5′-TGGGGCTGTC-3′), memberikan hasil yang paling memuaskan dan telah dipilih untuk kajian ini. Bahan

primer OPAR03 dan OPAR10 masing-masing menghasilkan jalur 2-10 dan 2-8, dan saiz amplikonnya berjulat 200-2000 bp. Sejumlah 19 pola RAPD mencerminkan kepelbagaian genetik yang tinggi pada asingan *V. parahaemolyticus* yang diperoleh daripada sampel udang. Kadar kejadian *Vibrio parahaemolyticus* ini boleh dijangkakan dan berlaku secara semula jadi. Inilah kajian pertama yang melaporkan ekspresi gen toksik *tdh* dan *trh* bagi *V. parahaemolyticus* di dalam *V. alginolyticus.* Analisis RAPD-PCR mendapati bahawa *V. parahaemolyticus* dan *Vibrio alginolyticus* menunjukkan tahap kepelbagaian genetik yang tinggi. Hasilnya menegaskan betapa pentingnya *Vibrio* bagi keselamatan makanan dan kesihatan awam, dan juga kesannya dari segi ekonomi.

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I certify that a Thesis Examination Committee has met on 4 August 2016 to conduct the final examination of Saifedden Ayad Gargouti on his thesis entitled "Detection of *tdh* and *trh* Virulence Genes, Antibiotic Resistance and Genetic Diversity of *Vibrio* spp. Isolated from Shrimp" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

Members of the Thesis Examination Committee were as follows:

Anis Shobirin binti Meor Hussin, PhD
Associate Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Chairman)

Janna Ong binti Abdullah, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Sahilah Abd. Mutalib, PhD
Associate Professor
National University of Malaysia
Malaysia
(External Examiner)

ZULKARNAIN ZAINAL, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 28 September 2016

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Son Radu, PhD
Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Chairman)

Farinazleen Mohamad Ghazali, **PhD**
Associate Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Member)

Nor Khaizura Mahmud Ab Rashid, PhD
Senior Lecturer
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Member)

_____
BUJANG BIN KIM HUAT, PhD
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School of Graduate Studies
Universiti Putra Malaysia

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Signature: _____
Name of
Chairman of
Supervisory
Committee: Professor Dr. Son Radu

Signature: _____
Name of
Member of
Supervisory
Committee: Associate Professor Dr. Farinazleen Mohamad Ghazali

Signature: _____
Name of
Member of
Supervisory
Committee: Dr. Nor Khaizura Mahmud Ab Rashid

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LIST OF ABBREVIATIONS

APW	Alkaline Peptone Water
bp	Base pair
CDC	Center of Disease Control
cfu	Colony forming unit
CLISI	Clinical and Laboratory Standards Institution
CV	CHROM *Vibrio* agar
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
EDTA	Ethylene-diaminetera acetic acid
EU	European United
FDA	Food and Drug Administration
g	Gram
kb	Kilo base
KP	Kanagwana Phenomenon
L	Liter
MAR	Multiple Antibiotic Resistance
MgCl2	Magnesium Chloride
MH	Mueller Hinton agar
min	minute
mL	Milliliter
µL	Microliter
mM	Millimolar
µM	Micro molar
MPN	Most Probable Number
NaCl	Sodium Chloride
NCBI	National Center for Biotechnology Information

PCR	Polymerase Chain Reaction
RAPD	Random Amplification Polymorphic DNA
RPM	Revolution per minute spin speed
TBE	tris-borate EDTA buffer
TCBS	Thiosulfate Citrate Bile Salts Sucrose Agar
TDH	thermostable direct hemolysin
toxR	ToxRtransmembrane protein gene
TRH	TDH-related hemolysin
TSB	Tryptic Soy Broth
U	Unit
UV	Ultra Violet
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Food is a basic need of people to survive and maintain health. Governments and organizations have established rules to deal with the production, packaging, and distribution of safe food. Over the last few decades, numerous foodborne outbreaks related to the consumption of contaminated food in wide geographical areas worldwide have been reported. The epidemiology factors influencing of these outbreaks include proper hygiene during handling; supply and distribution of raw food and aquaculture; change in pathogen resistance; and variations in eating habits (Ramamurthy and Nair, 2007). Food safety is a scientific regulation that describes the preparation, handling, packaging, and storage of food. The system of food production, including food hygiene, food additive, antibiotics, and pesticide residue, should be monitored to prevent and avoid foodborne illness.

The aquaculture industry in South Asia countries are expansion regarding to demand globally. Shrimp is popular seafood consuming over the world. The shrimp has grown in farms, which cultured in tropical and subtropical environmental, where the temperature is optimal for growth *Vibrio parahaemolyticus* and *Vibrio alginolyticus*. In addition, the incidence of *Vibrios* outbreaks increasing within last decade according to Central Disease Control (Cdc, 2014). On the other hand, Antimicrobial therapies are widely used in aquaculture to treat various bacterial infections, which lead to the transmission of antimicrobial resistance between aquaculture and humans. The WHO recommended the reduction of antibiotic use in animal and fish farms (Allerberger, 2007). *Vibrio parahaemolyticus* and *Vibrio alginolyticus* has not been monitored well for antibiotic resistance, in contrast with other enteric pathogens.

The present study aims to contribute further understanding on the prevalence, pathogenicity, antimicrobial resistance, and genetic diversity of *V. parahaemolyticus* in Selangor, Malaysia.

1.2. Objectives

The objectives of this study are as follows:

1- To detect the virulence genes of *V. parahaemolyticus* by using multiplex PCR assay.
2- To characterize the antibiotic susceptibility of *V. parahaemolyticus* and other positive isolates.
3- To genotype *V. parahaemolyticus* and other positive isolates by using randomly amplified polymorphic DNA-PCR.

1.3. ..Literature Review

1.3.1 ..Introduction

A significantly increasing attention is given to the members of *Vibrio* genus because of their relation to mild-to-severe disease symptoms in humans. The most important pathogen that belongs to this genus is *V. cholerae* because it causes cholera. *V. parahaemolyticus* is another important species (Ramamurthy and Nair, 2007).

Foodborne and food spoilage is caused by the ability of several types and species of bacteria, yeast, and molds to grow in food (Ray, 2004). Seafood is an essential food source worldwide and an affordable source of protein and healthy benefits, particularly in developing countries. However, seafood has a high percentage of foodborne diseases (Karunasagar *et al*., 2004). The most important pathogens that cause foodborne diseases are *Campylobacter*, *Listeria*, *Salmonella*, *Shigella*, *Escherichia coli*, *Vibrio*, and *Yersinia*. Compared with the data from 2010 to 2012, the 2013 *Vibrio* infection is significantly higher at 32%, whereas *Salmonella* infection is lower. No significant changes in infection are observed with *Campylobacter*, *Listeria*, *Shigella*, *E. coli*, and *Yersinia* (Crim *et al*., 2014). In the family *Vibrionaceae*, *Vibrio* genus has 48 species, as shown in Fig. 1.1., which are classified into pathogenic (10) and non-pathogenic (38) species (Ramamurthy and Nair, 2007). *Vibrio* genus is a Gram-negative halophilic bacterium that possesses curved rods (0.5 µm × 1.0 µm). This bacterium is also motile and inhabits freshwater and marine environments. *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and *Vibrio alginolyticus* are the most important pathogenic species (Ray, 2004).

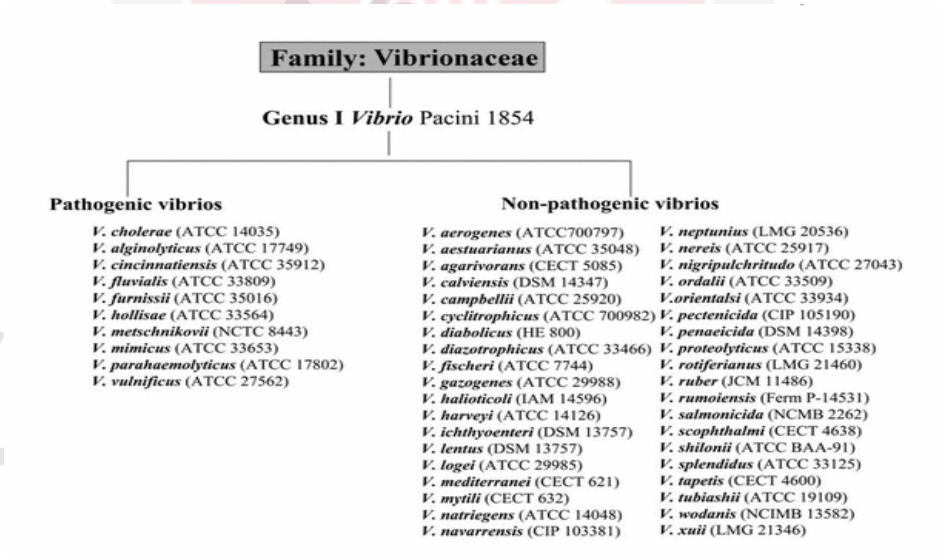

Figure 1.1: Classification and names under family *Vibrionaceae*. For each species, the strain type number is given in parentheses. Source (Ramamurthy and Nair, 2007).

Vibrio parahaemolyticus was discovered by Fujino after a shirasu food outbreak caused by consumption of half-dried sardines in Japan in 1950 (Shinoda, 2011). Since then, *V. parahaemolyticus* has been recognized a causative agent of gastroenteritis in estuarine areas worldwide. Various outbreaks of *V. parahaemolyticus* have also been reported in many countries (Qadri *et al*., 2005). *Vibrio* species are frequently associated with numerous food poisoning outbreaks, and they are important pathogens related to foodborne and waterborne diseases. Figure 1.2 shows the results of a report published in Morbidity and Mortality Weekly Report on the incidence and trend of infection with foodborne pathogens in the USA from 2006 to 2013 (Centers for Disease Control and Prevention, 2014). Currently, the population of *Vibrio* species shows an upward trend, particularly from 2010 to 2013. Furthermore, *V. parahaemolyticus* is a common cause of foodborne diseases in Asia and tropical region. In general, the outbreaks are small, but they occur frequently (Sumner, 2011).

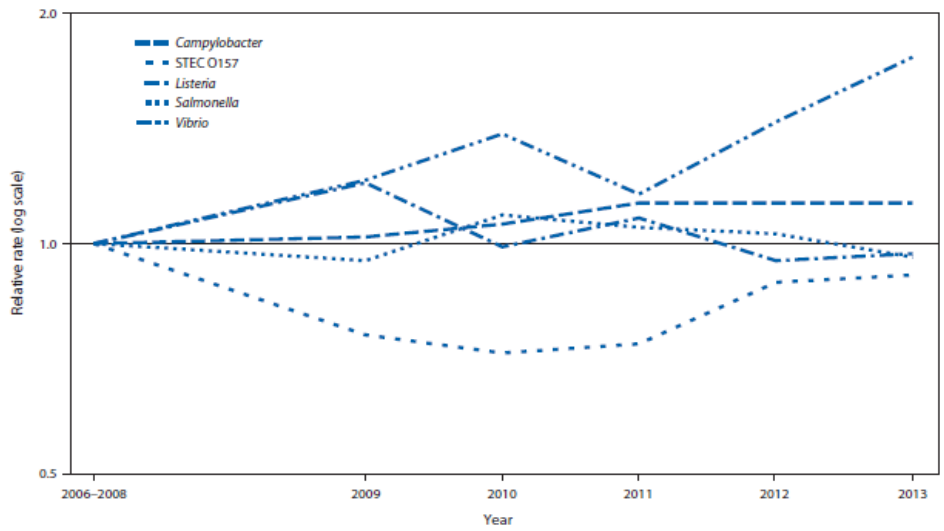

Figure 1.2: Relative rates of culture infected with *Campylobacter*, STEC o157, *Listeria*, *Salmonella*, and *Vibrio* from 2006 to 2013. Source (CDC, 2014).

V. parahaemolyticus is relatively common in Japan and accounts for 40%–70% of the total bacterial foodborne diseases. The high incidence is directly caused by raw seafood consumption. In the USA, its involvement in foodborne infection was first recognized in 1971 from a large outbreak associated with the consumption of steamed crabs contaminated with the pathogen (Ray, 2004). In the European Union (EU), the related chemical and microbiological hazards in crustaceans (prawn) rapidly increased from 1980 to 2010 (Table 1.1). The majority of the microbiological hazards linked to prawns, crabs, and lobsters with Asian origin are caused by *V. parahaemolyticus* and *V. cholerae* (Table 1.2). Malaysia has the highest EU alert because of microbiological hazard, which is mainly *V. parahaemolyticus* (Sumner, 2011).

Table 1.1: Country of origin of prawns triggering alerts in the European Union (1980–2010).

Country	Number of Alerts	Main Cause
India	215	Nitrofurans
Bangladesh	161	Nitrofurans
China	135	Chloramphenicol, nitrofurans
Vietnam	123	Chloramphenicol, nitrofurans, *Vibrio*
France	120	Sulfite, cadmium (crabs)
Thailand	87	Nitrofurans
Malaysia	71	*Vibrio parahaemolyticus*
Indonesia	65	Nitrofurans
Brazil	62	Sulfite
Australia	39	Cadmium
Ecuador	39	*Vibrio*
Other	378	
Total	**1495**	

. Source(Sumner, 2011)

Table 1.2: Microbiological hazards from crustaceans triggering EU alert.

Hazard	Number of Alerts
B. cereus	1
Salmonella	6
S. aureus	1
Clostridium	1
V. parahaemolyticus	137
V. cholerae	98
Vibrio species	8
V. alginolyticus	5
V. vulnificus	4
V. fluvialis	1
V. mimicus	1
Total	**263**

. Source(Sumner, 2011)

Vibrio is a facultative anaerobic Gram-negative halophilic bacterium that is widely spread in marine and estuarine waters (Baffone *et al*., 2001). *V. parahaemolyticus* and *V. alginolyticus* are both straight rod in shape and motile via polar flagella. They both grow in 6% NaCl and on TCBS agar. The colony of *V. parahaemolyticus* and *V. alginolyticus* shows green and yellow color, respectively, as a result of sucrose fermentation. The three *Vibrio* species that are often associated with *V. parahaemolyticus* in the aquatic environment and seafood are *V. vulnificus*, *V. alginolyticus*, and *V. cholerae* (Jay *et al*., 2005).

TCBS agar is used as a selective medium to identify *V. parahaemolyticus*, *V. alginolyticus*, and *V. cholerae*. CV agar is also developed as a selective medium that detects *V. parahaemolyticus* colonies through a purple or violet color (Zulkifli *et al*., 2009b). Both of these selective media are accurate and reliable in detecting and differentiating *V. parahaemolyticus, V. cholerae*, and *V. alginolyticus* by color (Di Pinto *et al*., 2011). *V. parahaemolyticus* and *V. alginolyticus*, which are genetically more related than other *Vibrio* genus at 60%–70% (Fig 1.3.), from one close group based on the 16S rDNA (Ramamurthy & Nair, 2007).

V. parahaemolyticus infectious dose is within 2×10^5–3×10^7 cfu. The incubation period is about 15 h, and the disease may last for 2–3 days. Bacteria colonize and produce toxins, and the pathogenesis depends on the production of a set of toxins that cause cell damage, which results in pore formation and loss of fluids and electrolytes. Moreover, *V. parahaemolyticus* grows at a temperature range of 10 °C–44 °C but fails to grow at 4 °C. The low temperatures arrest multiplication and cause an initial rapid decline in the numbers of viable cells, although long survival occurs in refrigerated seafood.

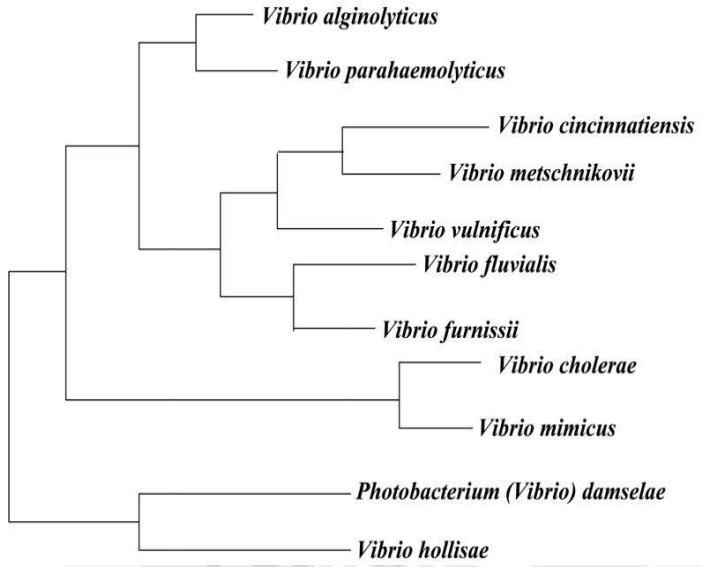

Figure 1.3: Schematic representation of phylogenetic relatedness of clinically significant Vibrio species based on 16S rDNA sequences. Source (Ramamurthy and Nair, 2007).

Under optimal conditions, the generation time of *V. parahaemolyticus* in the exponential phase is 9–13 min (Bhunia, 2008). *V. parahaemolyticus* also causes acute gastroenteritis characterized by a headache, fever, abdominal cramp, nausea, vomiting, and severe diarrhea (Shimohata & Takahashi, 2010). In 2011, 853 *Vibrio* infections (excluding toxigenic *V. cholerae* O1 and O319) were reported. *V. parahaemolyticus*, *V. alginolyticus*, and *V. vulnificus* exhibited the highest number of cases at 39%, 18%, and 11%, respectively (CDC, 2014). *Vibrio* species are generally causative pathogens for both humans and marine animals; they cause systemic infections, gastroenteritis, and skin lesions (Grimes *et al*., 2012). *V. parahaemolyticus* strains are grouped into pathogenic and non-pathogenic ones based on their ability to cause hemolysis on blood agar media (Wagatsuma agar); this production of hemolysis is known as the Kanagawa phenomenon (KP). The hemolysin ability is called the thermostable direct hemolysin (TDH), which is encoded by *tdh* genes (Kaper, 1995). Identification of *Vibrio* species can be performed based on colony appearance on selective media, such as thiosulfate-citrate-bile salts-sucrose (TCBS) and CHROM^{TM} *Vibrio* (CV) agar (Di Pinto *et al.*, 2011). Some pathogenic strains carry a thermostable-related hemolysin (TRH) that is encoded by *trh* genes, which are almost 65% identical to *tdh* genes (Nishibuchi *et al*., 1989). Both the *tdh* and *trh* genes are considered virulence factors of pathogenic *V.*

parahaemolyticus strain (Park *et al*., 2004). *Vibrio* species also express various hemolysis toxins. Some of these toxins are similar, but not necessarily identical (Zhang and Austin, 2005).

V. alginolyticus is another species that is widely spread geographically in an aquaculture environment. Since 1973, this species is recognized as a pathogenic agent of gastroenteritis, wound infection, and septicemia in humans, as well as skin ulcer in marine animals (Baffone *et al*., 2001; Qian *et al*., 2007). *V. alginolyticus* is a pathogen of both humans and marine animals and commonly associated with an ear infection, wound infection, septicemia, and gastroenteritis in humans, as well as in marine animal-related skin ulcer (Pruzzo *et al*., 2005; Qian *et al*., 2007). Commonly, gastroenteritis treatment uses oral rehydration in mild cases, although antibiotics may help cases associated with severe diarrhea, wound infection, or septicemia (Daniels *et al*., 2000). Additionally, *V. alginolyticus* is a causative agent of gastroenteritis, wound infection, and septicemia in humans, as well as skin ulcer in marine animals (Qian *et al*., 2007). Recently, reports on isolated pathogenic and non-pathogenic genes (*trh* and *toxR*) from *V. alginolyticus* are increasing in countries worldwide; in 2005, *toxR* genes are detected in China (Chang *et al*., 2012; Xie *et al*., 2005). In 2006, *trh* genes in *V. alginolyticus* were first reported in the USA (Gonzalez-Escalona *et al.*, 2006) and Tunisia (Abdallah *et al*., 2011; Ben Kahla-Nakbi *et al*., 2007). In 2012, a strain of *V. alginolyticus* carrying the *trh* genes was reported in Morocco (Mustapha *et al.*, 2012) and Norway (Ellingsen *et al.*, 2013). Nevertheless, the detection of these virulence genes, or one of them, in mixed culture does not typically reveal that pathogenic *V. parahaemolyticus* is present (Gonzalez-Escalona *et al*., 2006; Sabir, 2013).

1.3.2 Most probable number (MPN) method

In general, the MPN method is a useful tool for estimating quantity of cell microorganisms. The US Food and Drug Administration "Bacterial Analytical Manual" (BAM) ("FDA: Bacteriological Analytical Manual," 2001) describes that the sample was prepared in 10-fold dilution series, and about 1 mL of each dilution is inoculated in triplicate broth tubes for incubation. Following incubation, all tubes were examined for turbidity, and the growth in tubes is compared against a table of such value, that is, the table established by the US FDA (Sutton, 2010). However, the MPN method is labor-intensive and time-consuming.

The MPN technique has been developed and combined with a polymerase chain reaction (PCR) assay for the detection of a particular gene of the target organism (Miwa *et al*., 2003). The MPN-PCR method is a suitable, useful, and rapid analysis tool that does not require expensive material. Even a small number of microorganism cells are present in the initial homogenates (Copin *et al*., 2012). Many researchers have conducted the MPN-PCR and proven its success in laboratory analysis for food samples (Gomez-Gil & Roque, 2006; Hara-Kudo *et al*., 2001).

1.3.3 Detection of virulence genes of *V. parahaemolyticus* by using multiplex PCR assay

Foodborne infection needs accurate and rapid methods to detect the pathogen. Molecular researchers have been developing a method for the detection, identification,

and characterization of pathogens, such as the detection of a specific gene or virulence marker via PCR assay. This assay can be used to detect one gene (simplex PCR) or two to five genes (multiplex PCR) in a signal reaction (Ramamurthy & Nair, 2007). PCR assay is based on amplifying a specific sequence of DNA. The PCR mixture is loaded into agarose gel, and the amplicon is separated through electrophoresis based on amplicon size. This protocol targets a specific gene by using a useful marker to identify pathogen (Maurer, 2006). Genomic sequences provide the *Vibrio* species the ability to carry two circular chromosomes of unequal size. The agarose contains ethidium bromide (0.5µg/ml) and dye to visualize the DNA in the gel under UV lights.

V. parahaemolyticus genome encodes 4,832 genes (Makino *et al*., 2003; Okada *et al*., 2005). Almost all clinical isolates of *V. parahaemolyticus* strains show hemolytic activity on Wagatsuma's agar, and such activity is called KP. This reaction considers a marker of virulence strain (Kaper, 1995). Hemolysin leading to KP is named as TDH and TRH. TDH and TRH, which are encoded by *tdh* and *trh*, respectively, are both considered an important virulent factor in the pathogenicity of *V. parahaemolyticus* (Hond *et al*., 1988; Honda and Iida, 1993; Nishibuchi *et al*., 1992). The presence of *tdh* and/or *trh* genes is used to differentiate between pathogenic and non-pathogenic *V. parahaemolyticus* strains (Kaper, 1995).

toxR is another important toxin gene. This gene is considered a global regulator of *V. parahaemolyticus* and other various genes of *Vibrio* species (Lin *et al*., 1993). The Vp-*toxR* operon influences the expression of *tdh* genes and is considered an essential regulator in *V. parahaemolyticus* (Kaper, 1995). Additionally, *Vibrio* species express various hemolysis toxins. Some of these toxins are similar, but not necessarily identical (Zhang & Austin, 2005). Vp-*toxR* genes are functionally and structurally similar to *V. cholerae toxR* genes. *V. parahaemolyticus toxR* and *toxS* genes have identical homologies of 52% and 62% to the *toxR* and *toxS* of *V. cholerae*, respectively (Kaper, 1995). *toxR* gene, a specific PCR target, is used to identify *V. parahaemolyticus* by Kim (1999). The identification of *V. parahaemolyticus* via targeting specific genes is an efficient and reliable technique (Croci *et al*., 2007). A simple and rapid method to detect and diagnose *V. parahaemolyticus* is through the use of a PCR assay, which is considered a successful approach (Bej *et al*., 1999; Tada *et al*., 1992).

1.3.4 Detection *V. alginolyticus* carrying the toxin genes of *V. parahaemolyticus*

Vibrio species express various hemolysis toxins. Some of these toxins are similar, but not necessarily identical (Zhang & Austin, 2005). *V. alginolyticus* strains carry virulence genes derived from other *Vibrio* species. During the investigation of the *V. parahaemolyticus* outbreak of 2004 in the USA, researchers isolated a *V. alginolyticus* that possesses and expresses a *trh* gene with 98% homology to the *trh2* gene of *V. parahaemolyticus* (González-Escalona *et al*., 2006). In Morocco, *V. alginolyticus* carrying *trh* gene was also isolated (Sabir, 2013). *V. alginolyticus* can be a reservoir of many virulence genes of other *Vibrio* species in the marine environment (Xie *et al*. 2005). *tdh* and *trh* genes are considered the major pathogenic factors of *V. parahaemolyticus* (Kaper, 1995). To differentiate between pathogenic and non-pathogenic strains, *V. parahaemolyticus* is examined for the presence of *tdh* and/or *trh* genes (Kaper, 1995).

1.3.5 Determination of antibiotic resistance

According to the WHO, antibiotic resistance is the resistance of a microorganism to an antimicrobial drug, which is originally an effective treatment for an infection caused by such microorganism, and its ability to survive when exposed to antimicrobes.
WHO's 2014 report on global surveillance of antimicrobial resistance showed that antibiotic resistance will become unpredictable in the future, and the world is heading to an era when common infection and minor injuries lead to death. Comprehensive reports over the last decade revealed that antimicrobial resistance has been increasing among many bacterial pathogen species, which is a consequence of the extensive misuse of antimicrobial drugs (Son *et al*., 1997). Antimicrobial therapies are widely used in aquaculture to treat various bacterial infections, which lead to the transmission of antimicrobial resistance between aquaculture and humans. Sulfonamide, tetracycline, amoxicillin, trimethoprim–sulfadimethoxine, and quinolone are used worldwide in aquaculture. Antimicrobial is no longer recommended as first-line therapy because of increasing antimicrobial resistance globally. The WHO recommended the reduction of antibiotic use in animal and fish farms (Allerberger, 2007).

Antibiotics are generally classified according to structure and function. The mechanism of antibiotic action operates via inhibiting the synthesis of cell wall material, DNA, RNA, ribosome, and protein. Structurally, antibiotics are divided into major groups, such as β-lactamase, glycopeptide, aminoglycoside, macrolide, tetracycline, quinolone, and phenicol (Ritschel, 1968). Antimicrobial resistance is a complex mechanism, such as the bacteria can increase the resistance via mutation or DNA transfer (Livermore, 2003; Son *et al*., 1997). Genetic resistance has been increasing among Gram-negative bacteria through resistance plasmid transfer between same and different species (Harris, 1964).

Foodborne infection is commonly associated with gastroenteritis. *Vibrio* species are one of the foodborne pathogens that cause gastroenteritis. Infection is characterized by diarrhea, fever, abdominal cramp, and headache, and the illness is commonly self-limited (Di Pinto *et al*., 2008). In severe cases, the use of antibiotics, such as tetracycline, ciprofloxacin, and cephalosporin, is recommended (Al-othrubi *et al*., 2014; Zulkifli *et al*., 2009a). *V. parahaemolyticus* is considered highly susceptible to many types of antibiotics (Ottaviani *et al*., 2013). The disk diffusion method, which is established by Kirby–Bauer, is a useful and accurate protocol used to monitor drug resistance (Bauer *et al*., 1966) and recommended by the Clinical and Laboratory Standards Institute (CLSI, 2010).

Since 1978, studies have reported *Vibrio* resistance to ampicillin in the range of 40%–90% (Sudha *et al*., 2014). Ampicillin has low-efficiency treatment of *Vibrio* infection, as a result of excessive use. However, other reports showed a resistance average of 80%–90% (Al-othrubi *et al*., 2014; Han *et al*., 2007; Letchumanan *et al.*, 2014; Shaw *et al*., 2014; Yu *et al*., 2016).

Third- and fourth-generation cephalosporin (ceftazidime, cefotaxime, and cefepime) were also tested. *Vibrio* species have high sensitivity to ceftazidime, cefotaxime, and cefepime (Al-othrubi *et al*., 2014; Liu *et al*., 2013; Yu *et al*., 2016). These results are contradictory with other reports, which determined an intermediate resistance average

of 3%–46% (Elmahdi *et al*., 2016; Letchumanan *et al*., 2015a; Noorlis *et al*., 2011; Shaw *et al*., 2014; You *et al*., 2016; Zavala-Norzagaray *et al*., 2015).
Furthermore, *V. parahaemolyticus* isolates were detected in Korea in 2012 to have high resistance to cefotaxime and ceftazidime at 70%–80% (Jun *et al*., 2012). The contravention of reports is related to the differences in geography and test methodology. However, *Vibrio* reported high susceptibility to other types of antibiotics, such as piperacillin and gentamicin. Aminoglycosides (imipenem and meropenem), tetracycline, and quinolones (ciprofloxacin, levofloxacin, and ofloxacin) are also reported (Han *et al*., 2007; Shaw *et al*., 2014; Sudha *et al*., 2014; Yu *et al*., 2016).

Multiple antibiotic resistance (MAR) becomes a serious global impact because of antibiotic pollution in aquaculture and agriculture. The MAR index of the isolates is defined as a/b where "a" represents the number of antibiotics to which the particular isolate is resistant, and "b" is the number of antibiotics to which the isolate is exposed (Krumperman, 1983). The MAR index value (0.20) is differentiated between low and high risks; when the MAR value is >0.20, the sample has high-risk for source contamination (Tanil *et al*., 2005). *V. parahaemolyticus* isolates from Selangor, Malaysia determines the high frequency of MAR around Malaysia (A. Noorlis *et al*., 2011; Tanil *et al*., 2005; You *et al*., 2016; Zulkifli *et al*., 2009a). *V. parahaemolyticus* antibiotic susceptibility profile pattern and MARs have large variation among results. The discrepancies are related to test methodology and geographical variations (Letchumanan *et al*., 2015b).

1.3.6 Randomly amplified polymorphic DNA-PCR (RAPD-PCR)

Biochemical and morphological culture techniques in the microbiological laboratory are traditionally used to identify and classify microorganisms. These methods are challenging, time-consuming, and often confusing because of the high diversity of microorganisms (Sudheesh *et al*., 2002). Furthermore, the people and goods are moving rapidly worldwide, and a rapid technique to detect and diagnose the outbreak is needed (Son *et al*., 1998). The similarity between some subspecies of microorganisms causes the difficulty in identification. Thus, an accurate and rapid technique should be established to define and differentiate organisms.

Over the last three decades, researchers have developed a molecular typing technique to detect and identify pathogenic and non-pathogenic microbes. All these techniques are based on DNA, which is a distinct structure. A broad range of molecular technique is available because of the rapid development in the field of molecular biology. PCR is a rapid and highly specific technique for detection of *V. parahaemolyticus* in various food products and environmental samples (Robert-Pillot *et al*., 2002). RAPD-PCR is a PCR technique that uses arbitrary primers to detect changes in the DNA sequence and used for molecular epidemiology typing (Williams *et al.*, 1990).

RAPD-PCR is also a sensitive technique for detecting small differences in genome structure via employing short oligonucleotide primers (8–10 nucleotides) that target unspecified genomic sequence "random amplification" (Welsh & McClelland, 1990; Williams *et al*., 1990). In this technique, knowledge of the DNA sequence of the target gene is not required. RAPD-PCR can generate various fingerprint patterns with a small amount of template DNA (Leal *et al*., 2004). RAPD-PCR technique has been successfully used in studying *V. parahaemolyticus* and other *Vibrio* species (Austin *et*

al., 1995; Sadok *et al*., 2013; Wong & Lin, 2001; Zulkifli *et al*., 2009b). RAPD assay also helps in the observation of homogeneity or heterogeneity of *Vibrio* species in seafood and is a useful tool for tracking outbreaks (Son *et al*., 1998).

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