PREVALENCE AND CHARACTERISATION OF ANTIBIOTIC RESISTANCE OF *Vibrio parahaemolyticus* FROM SEAFOOD IN SELANGOR, MALAYSIA

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PREVALENCE AND CHARACTRISATION OF ANTIBIOTIC RESISTANCE OF *Vibrio parahaemolyticus* FROM SEAFOOD IN SELANGOR, MALAYSIA

ABSTRACT

Aquaculture industry has been professed as one of the fast-growing industries that serves a major source of seafood and revenue to many countries worldwide. Despite the nutritional benefits of seafood consumption, health risks linked to seafood consumption cannot be disregarded. Microbiological safety of seafood is of global concern recent years due to occurrence of seafood-borne cases and increase reports on antibiotic resistance among *Vibrio parahaemolyticus* isolated from seafood. The emergence of antimicrobial resistant *V. parahaemolyticus* poses treat to human health. In regard to increase reports on *V. parahaemolyticus* as a causative agent of seafood-borne illness, the study aimed to enumerate and characterise the antibiotic resistance profiles of *V. parahaemolyticus* isolated from seafood. A total of 770 seafood samples namely shrimp and shellfish were collected from both local wetmarket and supermarket in Selangor. The enumeration and identification using microbiological plating method on selective agar, thiosulphate citrate bile salt sucrose (TCBS) agar revealed that all seafood samples collected from wetmarket and supermarket sites were contaminated with *Vibrio* sp. The seafood samples analyzed had a microbial load of 2.29 log CFU/g to 6.63 log CFU/g. The *toxR*-PCR assay identified positive amplification of *toxR* gene in 50% (385/770) of the presumptive isolates. 32/385 (8.3%) isolates harboured the thermostable-related direct haemolysin (*trh*) gene and none with thermostable direct hemolysin (*tdh*) gene. The antibiotic susceptibility test revealed a total of 102 different types of antibiograms profiles among the *V. parahaemolyticus* isolates. The isolates were seen to be resistant to at least one type of antibiotic tested with MAR index ranged from 0 to 0.79. The chloramphenicol (*catA2*) gene was detected in 18/22 chloramphenicol-resistant isolates and 18/193 kanamycin-resistant isolate was positive for kanamycin *aphA-3* gene. Further analysis on the plasmid profiles of *V.*

parahaemolyticus isolates revealed 1-7 plasmids, with sizes ranging from 1.2kb to 10kb. There was no correlation seen between the plasmid profiles and antibiotic resistance patterns. Even within the isolates with same resistance profiles, the plasmid profiles were different and a few isolates even did not exhibit any plasmids. The isolates either demonstrated plasmidial or chromosomally mediated antibiotic resistance after plasmid curing assay. In conclusion, the results demonstrate that all the seafood samples collected are contaminated with *V. parahaemolyticus* regardless the sampling location and some of which carried the *trh*-gene which are potential to cause foodborne illness. The occurrence of multidrug resistance emphasizes the importance of study of antibiotic susceptibility of *V. parahaemolyticus*. Hence, constant monitoring of the prevalence and characterisation of resistance profiles of *V. parahaemolyticus* is needed to ensure food safety and human wellbeing.

Keywords: *Vibrio parahaemolyticus*, seafood, antibiotic resistance, plasmid curing, seafood safety.

PREVALENSI DAN KARAKTERISASI ANTIBIOTIK RESISTAN PROFIL *Vibrio parahaemolyticus* DARI MAKANAN LAUT DI SELANGOR, MALAYSIA

ABSTRAK

Industri akuakultur dikenali sebagai salah satu industri yang telah berkembang pesat dan berfungi sebagai sumber utama makanan laut serta sumber pendapatan kepada kebanyakan negara di serantau dunia. Walaupun makanan laut menpunyai pelbagai sumber nutrisi, risiko kesihatan yang dikaitkan dengan pengambilan makanan laut tidak boleh diabaikan. Semenjak kebelakang ini, isu keselamatan mikrobiologi makanan laut menjadi topik hangat yang dibingcangkan kerana peningkatan bilangan penyakit yang disebarkan melalui makanan laut dan juga peningkatan kes antibiotik resistan *Vibrio parahaemolyticus* yang disebarkan melalui makanan laut. Kemunculan antibiotik resistan *V. parahaemolyticus* memudaratkan kesihatan manusia. Oleh yang demikian, kajian ini bertujuan untuk mengkaji dan meneliti profil antibioitk resistan *V. parahaemolyticus* isolate dari makanan laut. Sebanyak 770 sampel makanan laut dikumpulkan dari pasar tempatan dan pasar raya di Selangor. Teknik enumeration konvensional dan identifikasi menggunakan, thiosulphate citrate bile salt sucrose (TCBS) agar menunjukkan bahawa makanan laut yang dikumpul dari kedua-dua lokasi tercemar dengan pathogen *Vibrio* sp. Pengujian gen *toxR* PCR yang digunakan berjaya mengenal pasti 385/770 (50%) *V. parahaemolyticus* dengan 32/385 (8.3%) isolate mempunyai thermostable-related direct haemolysin (*trh*) dan tiada *tdh* gen. Antibiotik susceptibility mendedahkan sejumlah 102 jenis profil antibiotik yang berlainan di kalangan isolate. *V. parahaemolyticus* isolate resistan pada sekurang-kurangnya satu jenis antibiotik yang diuji dengan indeks MAR di antara 0 hingga 0.79. Gen chloramphenicol (*catA2*) dikesan dalam 18/22 isolate resistan chloramphenicol dan 18/193 isolate resistan kanamycin positif untuk kanamycin *aphA-3* gen. Analisis lanjut mengenai profil plasmid isolate *V. parahaemolyticus* menunjukkan

1-7 plasmid, dengan saiz antara 1.2kb hingga 10kb. Tidak terdapat korelasi antara profil plasmid dan corak rintangan antibiotik. Isolate samada menunjukkan rintangan plasmidial atau kromosom yang ditengahi selepas plasmid curing. Walaupun dalam kaitan dengan profil rintangan yang sama, profil plasmid adalah berbeza dan beberapa isolate tidak mempamerkan sebarang plasmid. Kesimpulannya, hasil kajian ini menunjukkan bahawa makanan laut yang terkumpul tercemar dengan *V. parahaemolyticus* tanpa menghiraukan lokasi persampelan dan ada yang membawa *trh*-gen yang berpotensi menyebabkan penyakit kepada manusia. Pengesanan multidrug resistan diantara isolate menekankan pentingnya pengawasan menggunakan antibiotik. Oleh yang demikian, pemantauan berterusan dan pencirian antibiotik profil *V. parahaemolyticus* perlu dilakukan untuk memastikan keselamatan makanan dan kesejahteraan manusia.

Kata Kunci: *Vibrio parahaemolyticus*, makanan laut, antibiotik resistan, pemantauan, keselamatan makanan laut.

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LIST OF SYMBOLS AND ABBREVIATIONS

g	:	Gram
µg	:	Microgram
µL	:	Microliter
%	:	Percentage
AO	:	Acridine orange
APW	:	Alkaline peptone water
ANOVA	:	Analysis of variance
AST	:	Antibiotic susceptibility test
bp	:	Base-pair
CFU	:	Colony forming unit
EB	:	Ethidium bromide
MAR	:	Multiple antibiotic resistance
PCR	:	Polymerase chain reaction
rpm	:	Rotation per minute
NaCl	:	Sodium chloride
sp.	:	Species
trh	:	*tdh*-related hemolysin gene
tdh	:	Thermostable direct hemolysin gene
TCBS	:	Thiosulphate citrate bile salt agar
toxR	:	Toxin operon gene
TSA	:	Tryptic soy agar
TSB	:	Tryptic soy broth
w/v	:	Weight per volume

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CHAPTER 1: INTRODUCTION

"Food safety and foodborne pathogens" – are two main topics that are broadly been discussed by people worldwide and its implications to human health. How safe is our food that we are consuming? Typically, an individual hardly pay attention to food safety until the individual or their kin are infected by foodborne illness. Annually, millions of foodborne related infections have been reported around the world (Scallan *et al.,* 2011; Lund, 2015). World Health Organization (WHO) have estimated 1 in every 10 individuals worldwide get sick from foodborne illnesses, thus causing a minimum of 420,000 deaths cases yearly and result in the loss of 33 million healthy life years (WHO, 2017).

Seafood is regarded as nutritious part of a healthy diet containing vitamins, protein, minerals, and fatty accids that are essential for an individual (Iwamoto *et al.,* 2010). The worldwide consumption of seafood harvests has increased by more than two folds over the past 50 years, from 10kg in the 1960's to more than 20kg in the year 2014 (Wall *et al.,* 2016). Regardless of the nutritional benefits of seafood, health risks linked to seafood consumption cannot be ignored (Wang *et al.,* 2015b). Seafood including molluscs, finfish, fish, and crustaceans constitute a route of transmission for pathogenic microorganism to infect human (Iwamoto *et al.,* 2010; Wang *et al.,* 2015b). Of all the foodborne pathogen, *Vibrio parahaemolyticus* is frequently isolated from shellfish, oysters, shrimps, cockles, and fish (Abd-Elghany & Sallam, 2013).

V. parahaemolyticus, a member of the *Vibrionaceae* family naturally survives in the estuarine, marine and coastal environments (Zhang *et al.,* 2013; Ceccarelli *et al.,* 2013). Due to its nature of habitat, *V. parahaemolyticus* is able to accumulate in aquatic animals including shellfish at high levels (Tan *et al.,* 2017). The consumption of contaminated food or water with high levels of total and/or pathogenic *V. parahaemolyticus* could lead to gastroenteritis diseases (Zhang *et al.,* 2013; Daniel *et al.,* 2016). The infection is usually

manifested with symptoms of watery diarrhea, stomach pains, nausea, and fever (Daniel *et al.,* 2016). In rare cases, infections of *V. parahaemolyticus* can cause septicemia, which could cause an increase in the number of death cases (Zhang *et al.,* 2013). According to the data published by Centers for Disease Control and Prevention (CDC) in Foodborne Diseases Active Surveillance Network (FoodNet), and Morbidity and Mortality Weekly Report (MMWR), *V. parahaemolyticus* has accounted for approximately 34,664 incidents of domestically developed foodborne infection cases and known as the leading bacterium unlike to other *Vibrio* sp. in the United States (US) in 2016 (Scallan *et al.,* 2011; Huang *et al.,* 2016). The infection pathogenesis is initiated by pathogenic *V. parahaemolyticus* carrying the two major virulence genes, thermostable direct hemolysin (*tdh*) and/or TDH-related hemolysin (*trh*) (Letchumanan *et al.,* 2017). Notwithstanding most of the *V. parahaemolyticus* isolates from ecological areas are non-pathogenic, some isolates pose the virulence factors that are capable of causing foodborne gastroenteritis (Raghunath, 2015; Xie *et al.,* 2017).

In Malaysia, *V. parahaemolyticus* is found naturally disseminated in the marine coastal regions and is a causative agent that is responsible for gastroenteritis cases reported in Malaysia (Wall *et al.,* 2016). Occurrence of *V. parahaemolyticus* in shellfish have been reported in Malaysia (Zulkifli *et al.,* 2009; Tang *et al.,* 2014; Al-Othrubi *et al.,* 2014; Sahilah *et al.,* 2014; Malcolm *et al.,* 2015). Recently, the news on seafood from Malaysia been barred to European Union (EU) countries due to the existent of *V. parahaemolyticus* has raised the concerns of Malaysian consumers on food safety (Al-Othrubi *et al.,* 2014). Furthermore, the Food Safety News have reported on the ban of shrimp and prawns from Malaysia to the US by US Food and Drug Administration (FDA) due to possible antibiotic residues from nitrofuran and chloramphenicol. These evidences trigger the necessity for continuous monitoring and surveillance on Malaysian seafood to ensure food safety and consumer's protection (Nasreldin *et al.,* 2004).

The United Nation (UN) have projected that today's world population of 7 million will escalate to 9 billion by 2030 and to 10 billion by 2050 (Smith *et al.,* 2010). A rapid growth in population size with increasing demand of food globally results in stable development of the Asian aquafarming sector (Rico *et al.,* 2012). The over expansion and intensified aquafarming have resulted in marine animals to be vulnerable to bacteria contaiminations (Bondad-Reantaso *et al.,* 2005; Harikrishnan *et al.,* 2011). As a result, aquafarmers hinge on antimicrobials to avert, control and treat bacteria contaiminations in aquaculture farms (Cabello *et al.,* 2013). The Asia aquafarming regulatory body has permitted the use of gentamicin, nalidixic acid, oxytetracycline, tetracycline, quinolones, sulphonamides, trimethoprim, and trimethoprim-sulfamethoxazole in farms to control bacterial infections. Likewise, nitrofuron, chloramphenicol, and dimetridazole/metronidazole are no longer is use and been barred in many countries (Rico *et al.,* 2012; Yano *et al.,* 2014; Weese *et al.,* 2015). For instance, Dimeton – a type of sulfonamide, is an example of veterinary antibiotic that sold commercially to hatchery farmers. Yearly, virulent *Vibrio* sp. isolates are displaying there are many reports of intensifying numbers of virulent *Vibrio* sp. strains expressing resistance patterns to clinically used antibiotics (Letchumanan *et al.,* 2015b).

Today the worldwide community wellbeing and food safety is lurked by the prevalent cases of multidrug resistance (MDR) in bacteria (Wall *et al.,* 2016). In hospitals, most of the clinically prescribed antimicrobials are losing its efficacy in controlling and treating bacterial infections. The misuse of antimicrobials in aquafarming productions has given a rise in reports of resistant *V. parahaemolyticus*, subsequently instigating the community fear on health and soscioeconomic risk of *V. parahaemolyticus* (Vaseeharan *et al.,* 2005; Han *et al.,* 2007; Lesley *et al.,* 2011; Manjusha & Sarita, 2011; Noorlis *et al.,* 2011). Human and animals are infected by resistant pathogens via any kinds of food products for instance seafood. In addition, resistant genes are easily transferable from a bacterium to another in the environment by horizontal transfer causing plasmidial or chromosomal

mediated resistance, which the latter form is difficult to be controlled (Duran & Marshall, 2005; Guglielmetti *et al.,* 2009; Letchumanan *et al.,* 2015b).

In summary, there are potential risks of *V. parahaemolyticus* contamination in seafood from Malaysia as well as increased prevalent cases of resistant isolates towards many clinical antibiotics that straining the hospitalcare sector. By taking into consideration of past reports and possibility of severe infections, persistent study on *V. parahaemolyticus* antibiotic susceptibility is essential for epidemiology studies and provide a guide line for infection treatments in hospital. For this reason, this research study intended to investigate the prevalence and assess antibiotic susceptibility patterns of *V. parahaemolyticus* from shrimp and shellfish in Selangor, Malaysia. The study incorporated microbiological and molecular techniques to identify *V. parahaemolyticus* from seafood samples collected from wetmarket and supermarket. Further investigation was performed to characterise the antibiotic resistant patterns of the isolates by plasmid profiling and plasmid curing assay. There was no correlation observed between the number of plasmid profiles and antibiotic resistance of each isolates. The antibiotic resistance was plasmidial and chromosomally mediated, suggesting the concern of misappropriate use of antibiotics in aquaculture. This comprehensive information is essential in providing better understanding on the antibiotic resistant profiles of *V. parahaemolyticus* in Malaysia, therefore enables the regulatory bodies to formulate suitable management plans on the antibiotic application in Malaysia aquacutlture industry.

OBJECTIVES

General Objectives:

To enumerate, identify and study the prevalence and antibiotic resistant profile of *Vibrio parahaemolyticus* from shrimp and shellfish samples.

Specific Objectives:

1. To enumerate and isolate presumptive *Vibrio parahaemolyticus* from shrimp and shellfish samples using selective thiosulphate citrate bile salts agar (TCBS) and purification via tryptic soy agar (TSA).
2. To identify *Vibrio parahaemolyticus* at species level using *toxR*-PCR assay and detect virulence thermostable direct hemolysin (*tdh*) and TDH-related hemolysin (*trh*) genes using duplex PCR assay.
3. To determine the antimicrobial resistance profile of *Vibrio parahaemolyticus* isolates and detect antibiotic resistance genes using PCR assay.
4. To perform plasmid profiling and determine the antibiotic resistance mediation via plasmid curing of *Vibrio parahaemolyticus* isolates.

CHAPTER 2: LITERATURE REVIEW

2.1 Seafood

Seafood constitutes of proteins, long-chain omega-3, fatty acids, vitamins and essential minerals that are beneficial for human consumption (Feldhusen, 2000; Iwamoto *et al.,* 2010; Elbashir *et al.,* 2018). Furthermore, consumption of seafood decreases the risk of cardiovascular illnesses (Zarrazquin *et al.,* 2014), encompasses neural, enhance visual, and cognitive development amid gestation and infancy (Emmett *et al.,* 2013). Food and Agriculture Organization (FAO), the United Nations reported that there is a sharp increase in the demand and supply of seafood worldwide. The world per capita supply of seafood increased nearly 2-folds from 9.9 kg in the 1960s to 19.7 kg in 2013 and more than 20 kg in 2014 (Wall *et al.,* 2016). In Southeast Asia countries, the aquaculture production has increased rapidly in recent the 15 years. The total aquaculture production in Southeast Asia nearly tripled from less than 2 million tons in 1990 to more than 7 million tons in 2005 and the figure was likely to grow by 16 percent by 2015 (Hishamunda *et al.,* 2009). These figures demonstrate that there is a growing demand for seafood by consumers and continuous aquaculture production in the Southeast Asia countries.

In Malaysia, the aquaculture industry is primarily associated with its economic gains from supplying domestic and foreign demands, and as well as generating a steady income for farmers (Witus & Vun, 2016). Crustacean and molluscs are the two groups under the shellfish family (Lawley *et al.,* 2008). Crustaceans are characterized by segmented body structure, chitinous exoskeleton, and jointed limbs. Crabs, prawns, lobsters, and crayfish are among the types of sea life clustered under the crustacean group. Sea life animals with a calcareous shell for instance mussels, oysters, clams, and cockles are grouped as molluscs (Iwamoto *et al.,* 2010). Molluscs and crustaceans are a nutritious and excellent source of protein for humans, however, they can be a vehicle for foodborne pathogens to

cause illnesses (Iwamoto *et al.,* 2010). Former epidemiological studies have identified *V. parahaemolyticus* as a major cause of foodborne illness in Asia, South America, and the United States. *V. parahaemolyticus* has been frequently isolated from shellfish, oysters, clams, crabs, shrimps, and cockles, thus providing an excellent substrate for the survival of the pathogen in the aquatic environments (Fuenzalida *et al.,* 2006; Zarei *et al.,* 2012; Abd-Elghany & Sallam, 2013; Suffredini *et al.,* 2014b).

Generally, the microbial status of seafood depends on the type of seafood, the catchment location, environmental conditions, the natural occurrence of bacteria in water, method of catch and post-catch conditions (Feldhusen *et al.,* 2000, ICMSF, 2011). The microflora of crustaceans is usually found in their chitinous shell and intestines. Hence, the crustacean's shells are identified as the vehicle for *V. parahaemolyticus* transmission (ICMSF, 2006). *V. parahaemolyticus* utilize the chitin present on the surface of a marine organism as their primary energy source (Aunkham *et al.,* 2018).

Of all the marine organism, studies reported that bivalve molluscs are often associated with seafood-borne illness. During 2012 and 2013, the number of gastroenteritis cases after ingestion of shellfish in the United States was three-fold higher compared to the reported yearly mean cases from 2007 to 2011 (Newton *et al.,* 2014). Bivalve molluscs (cockles, clams, mussels) are found by seaside and estuaries environments. Their nature of habitat and filter-feeding habits enables them to accumulate microorganism including *Vibrio* species (Romalde *et al.,* 2014). As a filter feeder, they pump water into their gills, briskly filters and accumulate microorganisms including bacteria, viruses, and parasites that are naturally present in the marine environments (Rubini *et al.,* 2018). Bivalves are also easily contaminated with microbes derived from human sewage because of their sessile way of life in the estuarine environments. These factors highlight the presences of bivalve molluscs as a vehicle for microorganisms that may cause consumers health risks

if bivalve molluscs are eaten raw or undercooked (Rubini *et al.,* 2018). In summary, some seafood is inherently riskier than the others due to the influence of several biological such as their natural habitat, their feeding habits, harvesting season's postharvest preparation and processing of the products (Iwamoto *et al.,* 2010).

2.2 *Vibrio* - The Genus

Vibrionaceae family within the class of Gammaproteobacteria comprises of Gram-negative halophilic bacteria, straight or curved rod-shaped, highly motile with a single polar flagellum, ubiquitous, and indigenous in aquatic environments (Tison & Kelly, 1984, Tantillo *et al.,* 2004; Sawabe *et al.,* 2013; Yang & Defoirdt, 2015). As a facultative anaerobe, *Vibrio* sp. are capable of both fermentative and respiratory metabolism (Tison & Kelly, 1984). They oxidase-positive and utilize D-glucose as the main source of carbon and energy (Thompson *et al.,* 2004). Moreover, *Vibrio* sp. produce extracellular enzymes, gelatinase, amylase, chitinase, and DNase (Ripabelli *et al.,* 1999). Since *Vibrio* sp. are halophilic, they require sodium ion for survival and growth. Their ability to live in different salinity marine environments reflect the range of sodium ion concentrations required for bacterial growth (Tantillo *et al.,* 2004). *Vibrio* sp. is able to survive well in alkaline condition with pH value up to pH 9.0 and grow well in 2-3% sodium chloride (NaCl) (Igbinosa *et al.,* 2008).

Vibrio genus was first described by an Italian physician, Filippo Pacini in 1854. He discovered the first *Vibrio* species, *Vibrio cholera*, the causative agent of cholera while studying outbreaks of cholera disease in Florence (Thompson *et al.,* 2004). Subsequently, this strain was renamed as *Vibrio cholerae*, which is now the type of species of the genus. He further pointed out that cholerae is contagious but his discovery on *Vibrio* was ignored by the scientific community around the world (Thompson *et al.,* 2004). After nearly 30 years, Robert Koch successfully isolated *Vibrio* from pure culture in Calcutta, India. At

that time *Vibrio* epidemic was very active in Calcutta, India. Koch's discovery had created an important social consequence and regarded as a public health triumph (Lippi & Gotuzzo, 2014).

The *Vibrio* genus consists of 142 species that are marine originated and its taxonomy is continuously been revised due to the discovery and inclusion of new species (Summer *et al.,* 2001; Igbinosa *et al.,* 2008; Sawade *et al.,* 2013). *Vibrio* sp. infects any living being including animals and humans (Austin, 2010). It was reported that a few of the species from this genus have been identified and classified among the top 15 pathogens causing nearly 95% of the foodborne diseases, hospitalizations and even deaths in the United States (Batz *et al.,* 2012). Recently, the worldwide ocean warming and climate changes have caused emerges of *Vibrio* sp. including the foodborne pathogenic strains with several virulence factors in marine environments. This issue has been discussed by the European Food Safety Authority (EPSA) and warrants extra investigation and awareness (Vezzulli *et al.,* 2013).

There is a total of 12 *Vibrio* sp. that been identified as pathogenic to human (Pruzzo *et al.,* 2005). The common three *Vibrio* sp. are *Vibrio cholerae* and *Vibrio parahaemolyticus* – often associated with foodborne gastroenteritis (Igbinosa *et al.,* 2008; Robert-Pillot *et al.,* 2014), and *Vibrio vulnificus* - an agent of septicemia and wound infection that associated with exposure of seawater or ingesting of raw seafood (Heng *et al.,* 2017). Vibriosis occurs upon ingestion of seafood harvests that are contaminated and poorly seafood (Daniels *et al.,* 2000). In spite of the advanced manufacturing technologies, food safety is continuously challenged by reasons linked to variations in life, eating behaviors of consumers, production of food harvests and increased demand in the international trade (Newell *et al.,* 2010).

2.2.1 *Vibrio parahaemolyticus*

Vibrio parahaemolyticus, a free-living pathogen originated from estuarine, marine, or coastal surroundings worldwide and needs salinity for survival (Broberg *et al.,* 2011; Zhang & Orth, 2013; Ceccarelli *et al.,* 2013; Letchumanan *et al.,* 2014). It is commonly seen swimming freely and its motility conferred by a single polar flagellum attached to inert and animate surfaces including zooplankton, fish, shellfish or any other marine life (Gode-Potratz *et al.,* 2011). The history of *V. parahaemolyticus* started way back in 1950 when it was first identified by Tsunesaburi Fujino of the Research Institute of Microbial Diseases (RIMD), Osaka University from an acute gastroenteritis outbreak. The outbreak occurred in a southern suburb of Osaka, Japan due to consumption of 'shirasu', a type of dried sardine which resulted in 20 deaths and 272 infected patients (Fujino *et al.,* 1953; Shinoda, 2011). After several bacteriological testing and analysis, Fujino noticed that the isolated strain exhibited hemolytic features on blood agar plates and named the strain as *Pasteurella parahaemolytica*, assigning it to the genus *Pasteurella*. The progression in taxonomy and various scientific discoveries led to the reexamination of *Pasteurella parahaemolytica* by Fujino. He reported that the genus of the isolate should be *Vibrio* instead of *Pasteurella*. In 1963, Sakazaki investigated Fujino's isolates and confirmed it was the same species belonging to *Vibrio* genus and propose to name the isolate as *Vibrio parahaemolyticus* (Shinoda, 2011).

Since its discovery, *V. parahaemolyticus* have been identified as the prevalent cause of foodborne gastroenteritis in many continents (Daniels *et al.,* 2000). The distribution of *V. parahaemolyticus* has increased worldwide due to global climate change and rising ocean temperatures (O'Boyle & Boyd, 2014). In Asian countries, it is of concern that nearly half of the reported foodborne cases are associated with *V. parahaemolyticus* (Alam *et al.,* 2002; Bhuiyan *et al.,* 2002; Letchumanan *et al.,* 2015a). Recurrent

occurrences of *V. parahaemolyticus* cases have been reported in the United States and coastal European countries such as Spain, Italy and Norway (Caburlotto *et al.,* 2010; Scallan *et al.,* 2011; Ottaviani *et al.,* 2013). Generally, this gastroenteritis disease is established by bloody and watery diarrhea, stomach pains, vomiting, nausea, mild fever and chills. In intermittent cases, septicemia and wound infection have been observed in immunocompromised patients infected with *V. parahaemolyticus* (Fernando, 2011).

While the majority of the strains isolated from environmental sources are innocuous members of marine microbiota, only a small number of *V. parahaemolyticus* strains are capable of causing human illness and are often associated with foodborne gastroenteritis (Raghunath, 2015). The transmission of *V. parahaemolyticus* virulent strains is often related to eating raw or undercooked seafood (Raghunath, 2015). Pathogenic and non-pathogenic *V. parahaemolyticus* isolates are differentiated with the presences of virulent genes encoded proteins that are responsible for the pathogenesis in animals and humans. The most significant virulence factors of *V. parahaemolyticus* are the thermostable direct hemolysin (*tdh*) and/or *tdh*-related hemolysin (*trh*) (Xu *et al.,* 1994; Nishibuchi & Kaper 1995; Gutierrez *et al.,* 2013; Raghunath 2015).

2.2.2 Pandemic Strains of *Vibrio parahaemolyticus*

Pandemic strains of *V. parahaemolyticus* are genetically defined strains with specific alteration in *toxRS* region which is normally observed in serotypes O3:K6, O4:K68, O1:K25 and O1:KUT (Ramamurthy & Nair, 2014). Depending on the environmental conditions, *V. parahaemolyticus* is able to produce a capsule with a number of different somatic (O) and capsular (K) antigens. This feature of producing O antigens and K antigens are employed as a primary basis of *V. parahaemolyticus* strain classification (Nair *et al.,* 2007). *V. parahaemolyticus* could be categorized into 13 O-serogroups and 71 K-serogroups, which suggest 75 combination of O:K serotypes of *V. parahaemolyticus*

(Iguchi *et al.,* 1995; Chen *et al.,* 2012). The O3:K6 serotype has been identified as the most commonly serotypes involved in outbreaks.

The serotype O3:K6 was identified during an ongoing surveillance in 1996 at the Infectious Disease Hospital in Calcutta, West Bengal India (Ceccarelli *et al.,* 2013; Ramamurthy & Nair, 2014). After its emergence in Calcutta, genetically alike O3:K6 was identified and isolated from foodborne outbreaks from around the Asian countries including world including Vietnam, Bangladesh, Thailand, Japan, Laos, and Korea. In addition, the O3:K6 strains was also isolated from intermittent outbreaks in Chile, France, Mozambique, Peru, Russia, Spain, and the United States of America, thus leading to the conclusion that first *V. parahaemolyticus* pandemic has taken place and bringing this pathogen to the top as global public health issue (Daniels *et al.,* 2000; Hara-Kudo *et al.,* 2012; Ramamurthy & Nair, 2014).

Matsumoto and colleagues employed specific method to detect the new clones based on the difference in the nucleotide sequence of the *toxRS* region (Matsumoto *et al.,* 2000). The results exhibited the presences of strains almost identical from the O3:K6 clone, even though the strains belonged to different serotypes (Chowdhury *et al.,* 2000). The variations among the O3:K6 strains led to the classification of non-pandemic O3:K6 strains isolated in 1980-1990 in Asian countries including India, Taiwan, Japan, Thailand, and Bangladesh (Ceccarelli *et al.,* 2013). Up to now, there are 21 serotypes of *V. parahaemolyticus* that have been identified, the most common being O4:K68, O1:K25, O1:K41, and O1:KUT (untyped) (Nair *et al.,* 2007). The O4:K68, O1:K25, O1:K41, and O1:KUT strains are able to exhibited alike genetic makeup as O3:K6 strains such as the presences of *tdh* gene, *toxRS* gene and PFGE profiles. Based on these characteristics, it could be suggested that pandemic *V. parahaemolyticus* clones are able to express variant of surface traits (Qadri *et al.,* 2005).

Other serotypes have also involved in recent *V. parahaemolyticus* foodborne outbreaks including O6:K18 in Alaska after consumption of oysters (McLaughlin *et al.,* 2005), and in US Atlantic coast where serotypes O4:K12 and O4:K (unknown) was identified upon ingestion of contaminated shellfish and seafood. In Spain, 100 banquet guests were infected with *V. parahemolyticus* due to consumption of contaminated shrimp. Of the isolated strains, seven strains carried the both *tdh* and *trh* virulence factor and belonged to the O4:K12 and O4: KUT serotypes (Martinez-Urtaza *et al.,* 2016). The serotype O4: K8 was recently isolated from foodborne diarrheal cases in southern China (Li *et al.,* 2017).

The occurrence of filamentous phage *f237* in many O3:K6 isolates proposes a definite link amongst the phage and prevalent of O3:K6 serotype (Nasu *et al.,* 2000). The *V. parahaemolyticus* O3:K6 strains was too identified to have *orf8* located in the phage and encoding a putative adherence protein which could play an important role in increasing the virulence of O3:K6 isolates by being more adhesive to host intestinal cells (Ceccarelli *et al.,* 2013). The genetic traits have been used as markers in the identification of pandemic strains, however there are inconsistencies noted whereby pandemic O3:K6 strains with atypical profiles was isolated in Taiwan, Bangladesh, Japan and Thailand (Jones *et al.,* 2012).

2.3 Pathogenesis of *Vibrio parahaemolyticus*

V. parahaemolyticus is known to be the leading cause of seafood borne gastroenteritis worldwide. Although the pathogenesis mechanism of *V. parahaemolyticus* instigating gastroenteritis is not fully understood, most of the clinical isolates are identified to produce either the thermostable direct hemolysin (*tdh*) or TDH- related hemolysin (*trh*) genes (Zhang & Austin, 2005; Ceccarelli *et al.,* 2013). These two distinctive virulence gene factors are often utilized in polymerase chain reaction (PCR) assay to differentiate

virulent and non-virulent *V. parahaemolyticus*. Studies have reported that majority of virulent strains carry the *tdh* gene or *trh* gene, and some strains do carry both virulent genes (Roque *et al.,* 2009; Jones *et al.,* 2012). Besides the *tdh* and *trh* genes, there are other virulent factors for instance type III secretion systems that are associated with *V. parahaemolyticus* and responsible for colonisation, infection and damage to host cell (Letchumanan *et al.,* 2014).

2.3.1 Thermostable Direct Hemolysin (*tdh*)

The thermostable direct hemolysin (*tdh*) secreted by virulent *V. parahaemolyticus* strain is identified as an important virulence factor because *tdh* demonstrates completely in clinical isolates from many epidemiological studies (Wong *et al.,* 2000a). However, only less than 5% of the ecological strains produce *tdh* gene (Roque *et al.,* 2009). The composition of *tdh* revealed that it is made up of four soluble monomers, a central pore that allows the diffusion of small molecules and has a molecular weight of 46 kDa (Honda *et al.,* 1993; Bechlars *et al.,* 2013). This hemolysin gene is termed as thermostable-direct because it is stable even after heated at 100^{o}C for 10 minutes (Shimohata & Takahashi, 2010). As described by Sakurai *et al.* (1973), lecithin does not enhance the hemolytic activity and its action is directly on erythrocytes.

The Kanagawa phenomenon (KP) is commonly associated with *tdh* positive strains of *V. parahaemolyticus.* In KP phenomenon, positive *tdh* strain exhibits β-hemolytic activity on Wagatsuma blood agar, thus indicating the strain's ability to lyse human erythrocytes (Su & Liu, 2007; Nelapati *et al.,* 2012; Ham & Orth, 2012). KP test was widely used to identify pathogenic *V. parahaemolyticus* isolated from clinical and environment samples by plating the pathogenic strains on Wagatsuma blood agar. Nevertheless, the utilization of this conventional plating test has been reduced over time because the results cannot be reproduced and the precision of KP test results is influenced by pH levels, salinity and

type of erythrocytes utilized in the agar (Hongping *et al.,* 2011). This pore-forming toxin is responsible to cause a group of biological activities including hemolysis, enterotoxicity, cytotoxicity and cardiotoxicity (Kodama *et al.,* 2015).

The main targets of *tdh* activities are the epithelial and intestinal cells in host (Ghenem *et al.,* 2018). The effect on these cells are very important for biological functions such as diarrhea during an infection (Shimohata *et al.,* 2010). Traveler's diarrhea is caused by pathogenic *V. parahaemolyticus* with the presence of *tdh* that acts as pore-forming toxin perforates cellular membrane thus causing modification in ion flux in intestinal cells. This mechanism of action leads to the response of secretory effectors and diarrhea (Honda *et al.,* 1988; Takahashi *et al.,* 2000). *tdh1, tdh2, tdh3, tdh4* and *tdh5* are among the five-variations of *tdh* genes that have over 97% nucleotide sequence homology (Nishibuchi & Kaper, 1995; Nakaguchi *et al.,* 2004).

2.3.2 TDH-related Hemolysin (*trh*)

In 1985, KP-negative strains of *V. parahaemolyticus* was isolated from patients with traveler's diarrhea from gastroenteritis outbreak in the Republic of Maldives (Honda *et. al.,* 1988). These identified strains expressed hemolysin activity on usual blood agar plates but not on Wagatsuma's medium and termed as TDH-related hemolysin (*trh*) (Honda *et al.,* 1988). The *trh* gene is immunologically parallel to *tdh* gene, lysed erythrocytes with high sequence homology of 70% between *tdh* and *trh* genes (Raghunath, 2015), and displayed 67% amino acid similarity with *tdh* gene (Ohnishi *et al.,* 2011).

The *trh* gene product is heat liable when exposed to heat at 60^{o}C for 10 minutes. Takahashi and colleagues revealed that *trh* gene activates the Cl^- channels by inducing Ca^{2+} which results in diarrhea due to the increased Cl^- secretion and raised intracellular

calcium (Takashi *et al.,* 2000). Furthermore, a reduction of fluid accumulation in rabbit ileal loops was seen in a KP-negative *V. parahaemolyticus* with a truncated *trh*, suggesting the significance of *trh* as one of the hemolysins in *V. parahaemolyticus* pathogenesis (Xu *et al.,* 1994). The *trh* gene is found in the pathogenic island that contains urease encoding gene (Chen *et al.,* 2011). A strong association between urease production and presence of *trh* gene has been suggested for the KP-negative strains due to their close proximity on the chromosome (Iida *et al.,* 1997). In West Coast of United States, it was reported that 98% of the clinical *V. parahaemolyticus* were *trh*-positive and urease positive (Okuda *et al.,* 1997).

2.3.3 Other Virulence Factors

V. parahemolyticus has other virulence factors that aid in the pathogenesis of the bacteria. It is identified that *V. parahaemolyticus* expreses another toxin known as thermolabile express hemolysin (*tlh*). The *tlh* gene is often used as a species-specific marker during identification of *V. parahaemolyticus* (Zhao *et al.,* 2011). Furthermore, it was reported *tlh* gene was able to lyse human erythrocytes and the expression was strongly upregulated under the simulating conditions of host intestinal environmental (Gotoh *et al.,* 2010; Broberg *et al.,* 2011). Hence, *tlh* gene is said to have similar functional role as the *tdh* gene but the pathogenicity of *tlh* is yet to be explored futher (Zhao *et al.,* 2011; Wang *et al.,* 2015a). *V. parahaemolyticus* also attain type III secretion systems (T3SSs) as its virulence factor. T3SSs are needle-like bacterial machinery used to inject bacterial protein effectors directly into the membrane and cytoplasm of eukaryotic cells without encountering with the extracellular environment (Cornelis, 2006). T3SSs is made up of 20-30 proteins with a secretion apparatus consisting of a basal body that spans the inner and outer bacterial membranes, a needle that is polymerized and extended into extracellular space, and a translocon pore that is inserted into the eukaryotic

cell membrane (Izore *et al.,* 2011). Some secretion apparatus proteins have homology to flagella export proteins, with core transmembrane proteins showing the highest level of conservation (Marlovits & Stebbius, 2010). The common targets of T3SS effectors are the actin cytoskeleton, innate immune signalling, and autophagy. The system can be either up regulated or down regulated depending on the pathogens needs (Broberg *et al.,* 2011).

V. parahaemolyticus encodes two T3SSs: the cytotoxic T3SS1 and the enterotoxin T3SS2. T3SS1 is reported to be present in all *V. parahaemolyticus* and it is responsible for cytotoxicity, mouse lethality and possible induction of autophagy (Park *et al.,* 2004; Burdette *et al.,* 2009; Hiyoshi *et al.,* 2010). Where else, the T3SS2 is responsible for enterotoxicity and plays a role in the environmental fitness of strains (Hiyoshi *et al.,* 2010; Matz *et al.,* 2011). The T3SS are encoded on the genome island VPaI-7 that confers *V. parahaemolyticus* with a fitness advantage in the interaction with aquatic organisms (Matz *et al.,* 2011). The toxin secreted by *V. parahaemolyticus* T3SS are believed to have effects in the progression and severity of infection in humans (Ono *et al.,* 2006). The strains that possess this needle-like T3SSs has the advantage to inject bacterial protein effectors directly into the membrane and cytoplasm of host cells without encountering with the extracellular environment (Cornelis, 2006). In addition, the T3SS2 is suggested to be associated with *tdh*- and/or *trh*-positive *V. parahaemolyticus* strains (Raghunath, 2014). There are two distinct lineages of T3SS2 that have been described and showed associations of *tdh* with T3SSα and *trh* with T3SSβ (Park *et al.,* 2004; Noriea *et al.,* 2010). It could be suggested that *V. parahaemolyticus* strains with the *tdh* and/or *trh* genes and T3SSs system has better ability to launch virulence in human during infections.

2.4 Epidemiology of *Vibrio parahaemolyticus*

V. parahaemolyticus is largely present in the marine environments and often isolated from seafood (Odeyemi *et al.,* 2016). Since discovered in 1950s, *V. parahaemolyticus* has caused many foodborne outbreaks around the world including in Japan (Su & Liu, 2007; Aberoumand, 2010; Kubota *et al.,* 2011; Hara-Kudo *et al.,* 2003; 2012), in Taiwan (Yu *et al.,* 2013), in China since early 1990s (Li *et al.,* 2014), Bangladesh (Bhuiyan *et al.,* 2002), Laos (Matsumoto *et al.,* 2000), Hong Kong and Indonesia (Matsumoto *et al.,* 2000) (Figure 2.1). Despite the advances in hygiene, food treatment and food processing, this foodborne pathogen still represents a significant threat to human health worldwide.

2.4.1 Asia

V. parahaemolyticus was originally recognized as major source of seafood-associated disease in the Eastern Asia region. In 1951, this bacterium was isolated from a major outbreak that resulted in 272 infected cases and 20 deaths after consumption of 'shirasu', a type of semi dried sardine fish in Osaka, Japan (Daniels *et al.,* 2000; Aberoumand, 2010). Nearly 70% of the foodborne gastroenteritis cases in Japan is instigated by *V. parahaemolyticus* contamination. Japanese people are often exposed and infected by *V. parahaemolytucs* due to their habit of consuming raw or undercooked seafood (Alam *et al.,* 2002; Toyofuku, 2014). Toyofuku (2014) reported the trend of *V. parahaemolyticus* associated foodborne outbreaks and cases increased from 837 cases in 1993 to 12, 318 cases in 1998. However, the number of gastroenteritis cases decreased drastically to only 14 outbreaks in 1999 and 280 cases in 2009. The pandemic O3:K6 strain was identified as the primary cause of all the infection cases during 2000 to 2008. In addition, seafood has accounted for 28% of the total identified food associated with *V. parahaemolyticus* outbreaks. Sushi, sashimi, cooked/processed seafood products of molluscan shellfish, crabmeat, fish, squid and sea urchin are among the known seafood that often involved in

outbreaks (Toyofuku, 2014). In Japan, there was a drastic decrease in the number of *V. parahaemolyticus* foodborne cases from 1999-2000 due to the implementation of control measures to improve hygiene conditions in all seafood production sites (Hara-Kudo *et al.,* 2012).

Ever since 1990s, *V. parahaemolyticus* has been the major cause of foodborne diseases in China. There were 5770 reported foodborne cases from 1991-2001 and 31% of the cases were caused by *V. parahaemolyticus* (Liu *et al.,* 2004). The number of reported outbreaks decline to 322 cases between 2003 and 2008 (Wu *et al.,* 2014). Aquatic products including crustaceans was identified among the common food vehicle for *V. parahaemolyticus* to cause illness in China (Wu *et al.,* 2014). A study conducted by Li and colleagues found that *V. parahaemolyticus* was the main cause of acute diarrhea during 2007-2012 in southern coastal region of China, with the most prevalent serotype O3:K6 followed by O4:K8 and O3:K29 (Li *et al.,* 2014). In Taiwan, many foodborne gastroenteritis outbreaks were identified to be caused by *V. parahaemolyticus* (Su & Liu, 2007; Wong *et al.,* 2000b; Yu *et al.,* 2013).

In Southeast Asia region such as in Laos (Matsumoto *et al.,* 2000), Thailand, Indonesia and Cambodia, *V. parahaemolyticus* has been accounted for many foodborne outbreaks. *V. parahaemolyticus* outbreak was reported occurred in Kampung Speu, Cambodia which resulted in forty-nine cases of acute diarrhea (Vandy *et al.,* 2012). In the neighbouring country Thailand, pandemic O3:K6 serotype strains was reported to be accountable for most of the foodborne cases between 2006 and 2010 (Thongjun *et al.,* 2013). In addition, pathogenic *V. parahaemolyticus* was also isolated in Thailand, the main producer and exporter of cultured shrimp worldwide (Yano *et al.,* 2014). Occurrences of antimicrobial resistant *V. parahaemolyticus* has been reported to be isolated from white leg shrimp and black leg shrimp cultured at inland ponds in Thailand (Yano *et al.,* 2014).

In Malaysia, *V. parahaemolyticus* naturally occurs in the marine coastal region of Malaysia. It is widespread during the tropical marine surroundings in all seasons and cause foodborne gastroenteritis (Al-Othrubi *et al.,* 2014). In the early 1980s, a study revealed the incidence of *V. parahaemolyticus* in Malaysian shrimp processing industry. It is of interest to note that 21 different serotypes were isolated from Malaysian shrimp, with type 01:K38 and 01:K32 were predominated (Cann & Taylor, 1981). In addition, the presences of *V. parahaemolyticus* in exported frozen black tiger shrimp and rejection by the EU countries further affected the economic of Malaysia (Sani *et al.,* 2013). Similarly, *V. parahaemolyticus* was also reported to be isolated from cockles (Anadara granosa) at a harvesting area at Tanjong Karang, Kuala Selangor. The analysis revealed virulent *V. parahaemolyticus* isolates having the thermostable direct hemolysin (*tdh*) and TDH-related hemolysin (*trh*) genes (Bilung *et al.,* 2005). Virulent *V. parahaemolyticus* carrying *tdh* genes and *trh* genes was also identified from frozen shrimp in Malaysia, prompting a possible health risk for people consuming raw shrimp (Sujeewa *et al.,* 2009). In 2011, a study reported high occurrence of *Vibrio* sp. (98.6%) and *V. parahaemolyticus* (24%) in freshwater fish collected from hypermarket level. This outcome indicates a potential source of food safety to consumers in Malaysia (Noorlis *et al.,* 2011).

Paydar and colleagues reported prevalence of *V. parahaemolyticus* in the seafood samples from retail and hypermarkets in Malaysia. Out of the 43/150 *V. parahaemolyticus* isolates detected, six isolates were reported to have *trh* genes and another two contained the *tdh* genes (Paydar *et al.,* 2013). Nakaguchi (2013) performed a comparative study to detect the contaimination of *V. parahaemolyticus* in seafood marketed in Thailand, Vietnam, Malaysia, and Indonesia. Interestingly, the study's results revealed that all the four countries had a similar levels of *V. parahaemolyticus* contaimation in fish, shrimp, squid, crab, and shellfish. The study did not detect any virulent strains among the seafood samples from Malaysia (Nakaguchi, 2013). The findings in agreement with other reports

globaly that mentioned virulent genes, the *tdh* and *trh* are very low number (1-7%) among environmental and seafood samples (DePaola *et al.,* 2000; Wong *et al.,* 2000a; Lee *et al.,* 2001; Dileep *et al.,* 2003; Nordstrom & DePoala*,* 2003).

The food safety in Malaysia is further declining due to the occurences of antimicrobial resistant *V. parahaemolyticus* isolates in seafood (Sahilah *et al.,* 2014; Letchumanan *et al.,* 2015a; 2015b). In Terengganu, Malaysia, a study reported the detection of cefuroxime and ceftazidime-resistant *V. parahaemolyticus* isolates in shellfish samples (Sahilah *et al.,* 2014). In addition, ampicillin resistant profiles are often detected among seafood samples in Malaysia (Tanil *et al.,* 2005; Al-Othrubi *et al.,* 2014, Letchumanan *et al.,* 2015a). Elexson and colleagues reported in their study that all of the *V. parahaemolyticus* isolates from cultured seafood products were resistant to both penicillin and ampicillin (Elexson *et al.,* 2014). In a recent study, high level of penicillin and ampicillin resistant isolates were obtained from short mackerels in Malaysia (Tan *et al.,* 2017). The ampicillin resistance observed may be due to the misappropriation of this first-generation antibiotic for pathogen management in aquaculture, thus reducing the efficacy of ampicillin in the treatment of *Vibrio* infection (Sudha *et al.,* 2014). Hence, it is indeed vital to address and manage the antimicrobial resistance issue.

In India, *V. parahaemolyticus* has been detected and identified from both clinical and environmental samples. The first serotype O3:K6 *V. parahaemolyticus* was discovered in an on-going surveillance in Calcutta, India (Okuda *et al.,* 1997; Ceccarelli *et al.,* 2013; Ramamurthy & Nair, 2014). Subsequently, the serotype O3:K6 *V. parahaemolyticus* has turned into a widespread around Asia. In a clinical study, 178 *V. parahaemolyticus* strains was isolated from 13,607 diarrheal patients admitted in Infectious Diseases Hospital, Kolkata since 2001 to 2012 (Pazhani *et al.,* 2014). *V. parahaemolyticus* diarrheal cases were also detected from around the urban slums of Kolkata, India (Kanungo *et al.,* 2012).

Reyhanath and colleagues have reported the detection and isolation of antimicrobial resistant *V. parahaemolyticus* strains from a fishing land in South India (Reyhanath *et al.,* 2014). In Cochin, a study reported the isolation of *Vibrio* sp. including pathogenic and antibiotic resistant *V. parahaemolyticus* strains from seafood. The isolates exhibited resistance towards ampicillin and multidrug resistance was prevalent among the isolates (Sudha *et al.,* 2014). The prevalence of multidrug resistant *V. parahaemolyticus* isolates in the environment and clinical setting is of public health concern, thus require continuous monitoring and management.

2.4.2 Europe

In European countries, *V. parahaemolyticus* infections are seldom reported, unlike Asia and US countries where *V. parahaemolyticus* infections are commonly reported (Baker-Austin *et al.,* 2009). Nevertheless, there were several sporadic outbreaks reported over the last 20 years in countries such as France and Spain (Su & Liu 2007; Baker-Austin *et al.,* 2009). *V. parahaemolyticus* was isolated from the Baltic Sea, the North Sea, the Mediterranean Sea (Miwatani & Takeda*,* 1976), and the Black Sea (Aldova *et al.,* 1971). In 1978, studies were conducted in coastal waters of Guadeloupe and isolated *V. parahaemolyticus* from 53/100 water samples that was investigated (Papa, 1980). As years passed, numerous cases of *V. parahaemolyticus* gastroenteritis were detected and isolated in Spain, Greece, Britain, Turkey, Denmark, Yugoslavia, the Scandinavian areas, and Italy (Qadri *et al.,* 2005; Serracca *et al.,* 2011).

In 1989, *V. parahaemolyticus* accounted for 8 cases of acute gastroenteritis associated with the consumption of fish and shellfish in Spain (Molero *et al.,* 1989). In 1997, a major outbreak of *V. parahaemolyticus* involving 44 patients had occurred in France and it was associated with the consumption of shrimps imported from Asia (Robert-Pillot *et al.,* 2004). In 1999, the first large outbreak of *V. parahaemolyticus* occurred in Galicia, Spain.

This outbreak involved 64 illnesses and it was associated with the consumption of raw oysters (Lozano-Léon *et al.,* 2003). A more recent outbreak of *V. parahaemolyticus* was reported in Spain in 2004, whereby it involved 80 illnesses among the guests who attended weddings in a restaurant. The investigation revealed that the outbreak was caused by consumption of boiled crab prepared under unsanitary conditions (Martinez-Urtaza *et al.,* 2005). In 2004-2005, only 57 cases of *V. parahaemolyticus* infections was reported in United Kingdom and most of the infections were obtained through travel to endemic areas (Wagley *et al.,* 2008). In addition, serotype O3:K6 *V. parahaemolyticus* strains were isolated from patients of outbreak in Spain and patients of gastrointestinal infection in Italy (Martinez-Urtaza *et al.,* 2005; Ottaviani *et al.,* 2008; 2010).

2.4.3 United States (US)

In 1971, *V. parahaemolyticus* was first identified as an etiological food borne pathogen in Maryland, US after three outbreaks of 425 gastroenteritis cases associated with consumption of improperly cooked crabs (Molenda *et al.,* 1972). Ever since then, intermittent *V. parahemolyticus* outbreaks have been reported throughout the US coastal regions due to the consumption of raw shellfish or uncooked seafood. The Centers for Disease Control and Prevention (CDC) have reported about 40 outbreaks of *V. parahaemolyticus* infection from the year 1973 to 1998 (Daniels *et al.,* 2000). Four out of 40 outbreaks involved over 700 cases of diseases linked with consumption of raw oyster in the Gulf Coast, Pacific Northwest, and Atlantic Northeast regions between the years 1997 to 1998. During the summer of 1997, there were 209 (including one death) of *V. parahaemolyticus* infection cases reported involving raw oyster consumption in the Pacific Northwest (Oregon, Washington, California and British Columbia of Canada) (CDC, 1999). Two outbreaks of 43 cases in Washington and 416 cases in Texas in the 1998 were also associated with consumption of raw oyster (DePaola *et al.,* 2000). Another

small outbreak of eight cases of *V. parahaemolyticus* illnesses was reported in Connecticut, New Jersey, and New York between July and September in 1998 as a result of eating oysters and clams harvested at Long Island Sound of New York (CDC, 1999).

In summer 2004, 14 passengers on board a cruise ship in Alaska manifested gastroenteritis symptoms after ingestion raw oysters produced in Alaska (McLaughlin *et al.,* 2005). The O6:K18 isolates from the Alaskan outbreak were in distinguishable by PFGE from those isolated in the sporadic cases from Pacific Coast states over the previous decade. From July to October of 2004, 96 environmental samples were collected from 17 Alaskan oyster farms, and 32% samples were tested positive for *V. parahaemolyticus*. The most frequently occurring serotypes were O1:K9, O4:K63, and O6:K18 (Newton *et al.,* 2012). In summer 2006, there was an outbreak occurred involving 177 cases of *V. parahaemolyticus* associated with consumption of contaminated oysters harvested in Washington and British Columbia (CDC, 2006).

Pandemic *V. parahaemolyticus* strains were also isolated in the United States. The O4:K12 serotype showed the highest prevalence among clinical *V. parahaemolyticus* isolates from the U.S. Pacific Coast between 1979 and 1995 (DePaola *et al.,* 2003). In 1998, another outbreak occurred involving 416 individuals from 13 states across US after consumption of raw oysters. From the patients stool samples, *V. parahaemolyticus* O3:K6 was isolated, which closely resembled the pandemic Asian O3:K6 isolates by PFGE (Danieals *et al.,* 2000a). Clinical isolates in the U.S., especially from the Pacific Northwest were also found to be encoded with *trh* gene (Paranjpye *et al.,* 2012). In addition, there was an increase in clinical isolates possessing either *tdh* gene, *trh* gene or both, and these severe cases required hospitalization (FAO/WHO, 2011). In summary, the prevalence of *V. parahaemolytius* in both clinical and environmental samples serious food safety concern in the US.

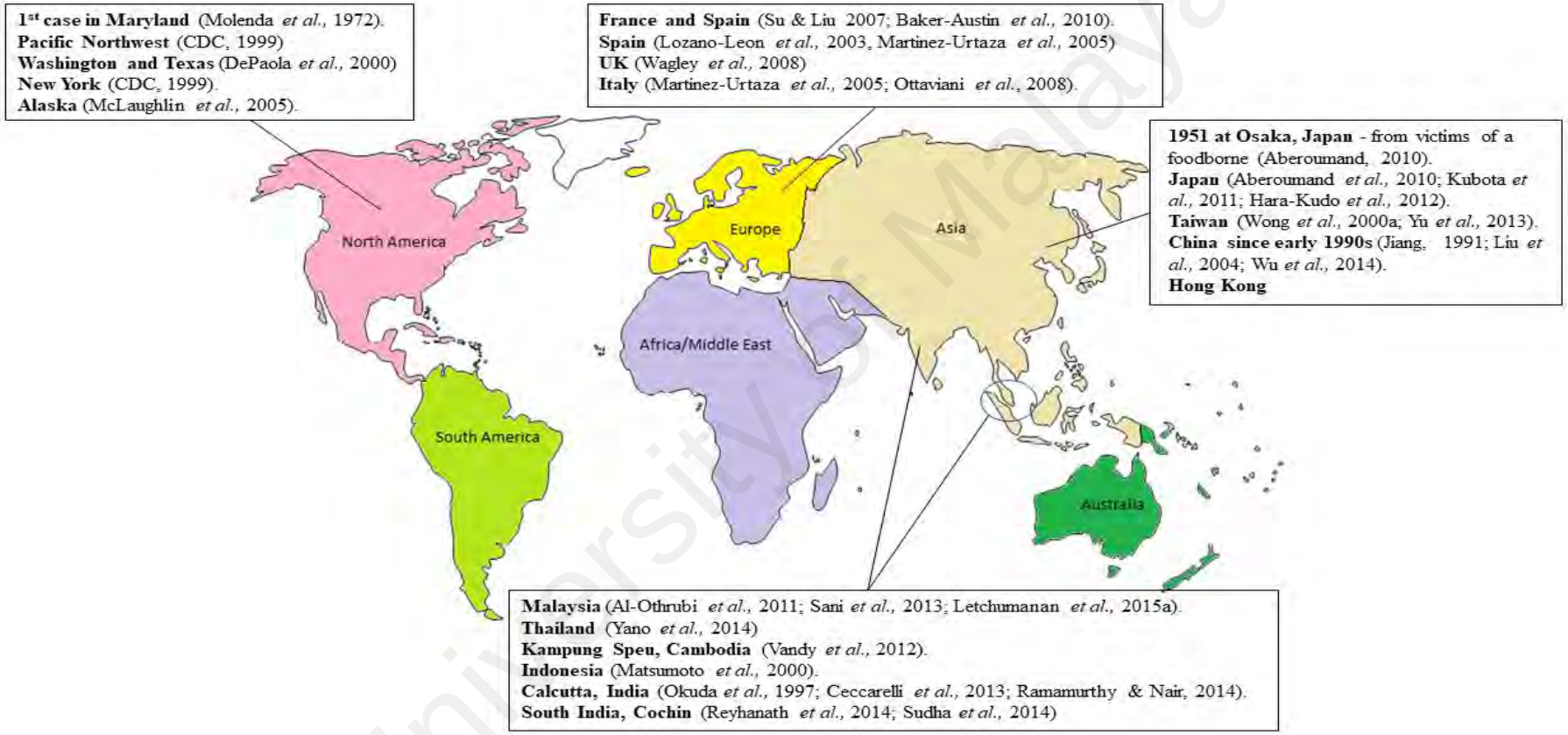

Figure 2.1: Illustration of *V. parahaemolyticus* epidemiology around the world. The first identified case was in Osaka, Japan in 1951 and ever since then the occurrence has spreaded to whole of Asia region, Australia, Europe, and the United States (US).

2.5 Ecology of Transmission

V. parahaemolyticus are naturally originated in a free-living state in the marine and estuarine environments. This bacterium is highly motile with a single polar flagellum and attaches itself to various seabed surfaces such as zooplankton (Johnson *et al.,* 2012; Ceccarelli *et al.,* 2013). The occurrence and dispersal of *V. parahaemolyticus* is influenced by many ecological factors including the water temperature, salt and oxygen concentrations, interaction with plankton, presences of sediment, organic matter in suspension and marine organisms. (Cabrera-Garcia *et al.,* 2004; Johnson *et al.,* 2012). Zooplanktons are not only the food source for *Vibrio* sp. but also acts an influential role in controlling the abundance of *V. parahaemolyticus* in aquatic environments (Kaneko & Colwell, 1978; Zimmerman *et al.,* 2007; DePaola *et al.,* 2003). *V. parahaemolyticus* often inhibits, multiplies, and binds in the gut of filter feeding animals such as clams, oysters, and mussels (Odeyemi *et al.,* 2016). Compared to other *Vibrio* sp. such as *V. vulnificus*, *V. parahaemolyticus* is able survive in a broad range of salinities levels in marine surroundings (Johnson *et al.,* 2012). *V. parahaemolyticus* is often isolated from aquatic environments with salinity of 5 to 25 ppt and water temperature above 15^{o}C during months of spring or summer (DePaola *et al.,* 2003). Furthermore, most of outbreaks caused by *V. parahaemolyticus* occur during the warmer months in moderate zones as the concentration of *V. parahaemolyticus* increases with increasing water temperature. This suggests that the water temperature may play an important factor in the epidemiology of *V. parahaemolyticus* infection. Current studies have reported that environmental factors which include interaction with other hosts play a huge effect in the evolution of certain pathogens (Wilson & Salyers, 2003). Therefore, pandemic strains that exhibit certain biological characteristics such as increased toxin production or having the capability to live within the natural environment could give better insights into the mechanisms underlying the emergence and spread of these strains (Wong *et al.,* 2000a).

2.6 Identification of *Vibrio parahaemolyticus*

There is a noticeable link between vibriosis and seafood consumption suggesting a major underlying food safety issue. This requires development of reliable and precise detection methods for pathogens in seafood products subsequently reducing the number of foodborne illness (Bisha *et al.,* 2012; Zhao *et al.,* 2014; Bonnin-Jusserand *et al.,* 2017). In a routine detection process, microbiological identification methods have been applied to detect the foodborne pathogen, however this method consumes time and inadequate to confirm the bacteria colony (Zhao *et al.,* 2014). Therefore, a rapid and precise molecular identification step is needed to confirm the identification of the bacteria (Nigro & Steward, 2015). The current microbiological identification methods and molecular techniques are discussed in the following sub-headings.

2.6.1 Microbiological Identification Method

The U.S. Food and Drug Administration (FDA) has described 3 main microbiological steps; enrichments, isolation and confirmation in order to detect *Vibrio* species (Kaysner & DePoala, 2004). Alkaline peptone water (APW) is used as the enrichment broth for all *Vibrio* sp. including *V. parahaemolyticus* (Kaysner & DePoala, 2004). APW has a pH level between pH8.5-9 and high concentration of NaCl which inhibits the growth of other bacteria (DePaola *et al.,* 2004). Preparation of this APW, 10.0g Peptone and 10.0g NaCl in 1000ml distilled water. The pH is adjusted to 8.5 ± 0.1 and autoclaved at 121^{o}C for 10 minutes. Besides APW, salt polymyxin broth (SPB), alternative protein source (APS) broth, salt colistin broth, glucose salt teepol broth and bile salt sodium taurocholate (ST broth) can be used as an enrichment broth for *Vibrio* sp. (Bisha *et al.,* 2012). Hara-Kudo and colleagues developed a procedure that consists of a non-selective enrichment step in salt trypticase soy broth followed by a selective enrichment step in SPB broth. SPB broth contains polymyxin B sulphate that inhibits the growth of gram-positive organisms. This

two-step enrichment method is found to be more effective to isolate *V. parahaemolyticus* compared to the one step enrichment in SPB broth (Hara-Kudo *et al.,* 2001).

The isolation and identification steps consist of plating enrichment broth on selective thiosulphate citrate bile salts sucrose agar (TCBS). TCBS agar is a selective differential medium that is widely used not only to isolate *V. cholerae* but all other pathogenic *Vibrios* except *V. holliseae* (Kobayashi *et al.,* 1963). This selective media consists of ox bile (0.8%), NaCl (1%) and alkaline pH 8.6 which suppresses the growth of other interfering gram-positive bacteria. The main advantage of TCBS media is its sucrose/bromothymol blue diagnostic system which differentiates sucrose-positive *Vibrios* such as *V. cholerae* from other *Vibrio* sp. colonies. Therefore, the growth of many unwanted bacteria is suppressed, while *Vibrio* species can grow and tentatively grouped by reading the effect of sucrose fermentation and formation of colony features. Tentative groupings of isolates can be made from colonial appearance on TCBS agar (Mrityunjoy *et al.,* 2013; Bisha *et al.,* 2012):

a) Sucrose fermenting with 2-3mm yellow colonies: *V. cholerae*.
b) Non-sucrose fermenting with green to bluish colonies: *V. parahaemolyticus*.
c) Non-sucrose fermenting colonies: *V. vulnificus* (green colony).
V. alginolyticus (yellow colony).

Based on literature, the results obtained from clinical evaluation have supported the claim for the relatively high efficiency of TCBS agar in the primary isolation of *Vibrio* species. Selective enrichment with alkaline peptone water (APW) and plating of the overnight enrichment culture onto thiosulphate citrate bile salts sucrose (TCBS) agar have been widely utilized for selective isolation of *V. parahaemolyticus* from seafood (Kirs *et al.,* 2011; Scharer *et al.,* 2011; Nakaguchi *et al.,* 2013; Paydar *et al.,* 2013; Robert-Pillot *et al.,* 2014; Yano *et al.,* 2014).

As year passed, researchers have noted from foodborne related studies that *V. parahaemolyticus* colonies on TCBS agar are difficult to distinguish physically from other bacterial colonies. Since TCBS is a universal media used for all *Vibrio* sp. isolation, a huge number of yellow colonies produced by sucrose-fermenting *Vibrio* sp. or green colonies will grow on this agar consequently making it difficult to effectively isolate and enumerate *V. parahaemolyticus* (Pinto *et al.,* 2011; Bisha *et al.,* 2012). To offset this issue, Hara-Kudo *et al.* (2001) developed a new enrichment procedure with a selective medium for the isolation of *V. parahaemolyticus* from seafood or environmental samples. The samples are cultured in selective salt polymyxin broth (SPB) broth and plated on a chromogenic CHROMagar *Vibrio* (CV) agar (CHROMagar Microbiology, France). CHROMagar *Vibrio* (CV) agar contains colorimetric substrates for *ß*-galactosidase and was developed specifically to distinguish ortho-nitrophenyl-*ß*-galactoside-positive *V. parahaemolyticus* from other closely associated *Vibrio* sp. (Bisha *et al.,* 2012). On this chromogenic medium, the mauve colour *V. parahaemolyticus* colonies are easily notable and differentiated from another *Vibrio* sp. The CHROMagar *Vibrio* (CV) agar is claimed to be specific and accurate identification of *V. parahaemolyticus* compared to TCBS agar (Su & Liu, 2007).

In addition, Wagatsuma agar was developed to identify virulent *V. parahaemolyticus* isolates and in the Kanagawa Phenomenon. This agar is made up of human or rabbit blood with NaCl, mannitol, crystal violet and K_2HPO_4. The main advantage of this agar is to assist in differentiation of *tdh* and non-*tdh* producing strains. *V. parahaemolyticus* strains which produce *tdh* gene will hemolyse a halo on this Wagatsuma agar (Nishibuchi *et al.,* 1995; Qadri *et al.,* 2005; Alipour *et al.,* 2014). The main disadvantage of this agar is that the agar cannot differentiate TDH-related hemolysin (*trh*) *V. parahaemolyticus* isolates from the non-pathogenic strains. The *trh* isolates will not exhibit hemolysis characteristic on Wagatsuma agar.

The enumeration of *V. parahaemolyticus* from seafood is important in the context of current FDA guidelines which indicate that shellfish should contain less than 10,000 *V. parahaemolyticus* cells per gram (Deepanjali *et al.,* 2005). In line with that, the most probable number (MPN) method was described by US Food and Drug Administration Bacterial Analytical Manual in detecting species in food samples. MPN is a conventional method that estimates the population density of viable microorganisms in a sample. This method is based upon the application of the theory of probability to the numbers of observed positive growth responses to a standard dilution series of sample inoculum placed into a set of replicate liquid broths (Sutton, 2010). The important perception of the MPN technique is to dilute the sample to an extent that inoculums in the tubes will contain viable organisms. Through replicates and dilution series, the results would be reasonably accurate in estimating the most probable number of cells in the sample. In addition, the nutrient broth used would support growth of organism and turn cloudy. This basic identification step of growth versus no growth provides useful information for low number of organisms (Sutton, 2010). This traditional enumeration method is usually employed during the identification process to identify and enumerate *V. cholerae* and *V. parahaemolyticus* (Nishibuchi, 2006).

Although the conventional detection method is useful in detecting and isolating *Vibrio* sp., the method has several major drawbacks. The amount of workload, materials, and the time needed to complete the whole identification process usually takes 7 to 10 days (Tunung *et al.,* 2011). In addition, traditional phenotypic assays differentiated by low sensitivity media fails to identify viable but non-culturable (VBNC) present in the samples and usually with odd phenotypic profiles (Lee *et al.,* 2014; Law *et al.,* 2015). Besides, the method suffers from the drawback that serotyping cannot differentiate all isolates that have originated from different regions or sources (Bhowmick *et al.,* 2009). The shortcomings of microbiological methods are overcome with the development of

highly sensitive and specific rapid molecular typing methods (Mandal *et al.,* 2011; 2012; Law *et al.,* 2015). Molecular typing method is very functional for epidemiological studies as it ables to provide genetic information of the strains, identify the virulent strains, and wide geographical and host distributions study of potential variants (Olive & Bean*,* 1999; Zhao *et al.,* 2014).

2.6.2 Molecular Typing Method

The conventional phenotyping and biochemical identification techniques of *V. parahaemolyticus* are complicated when the strains are isolated from seafood and aquatic environments (Nishibuchi *et al.,* 2006). As a result, Polymerase Chain Reaction (PCR) assay has become a popular molecular tool for identification of *V. parahaemoyticus* (Drake *et al.,* 2007). The genetic composition of *Vibrio* sp. is extremely variable thus the genes present inside a targeted strain of *Vibrio* can be used to distinguish the genus from other bacteria and are obvious candidates for the development of DNA based methods for identification of *Vibrio* sp. (Foley *et al.,* 2009). In fact, a number of researchers have studied pandemic isolates to carry bacteriophage sequences that non-pandemic strains do not, and they have exploited these differences to develop pandemic strain–specific detection methods (Bisha *et al.,* 2012). To increase the output and lessen the reagent costs, PCR primers can be multiplexed in a single reaction or tailored for the real-time PCR analysis to provide more rapid results (Grant *et al.,* 2006).

Polymerase Chain Reaction (PCR) is a method with high sensitivity and specificity for detection and identification of pathogenic bacteria from clinical, environmental or seafood samples (Nelapati *et al.,* 2012). The identification *V. parahaemolyticus* strains at the species level by targeting *toxR* gene was performed using PCR assay (Vimala *et al.,* 2010; Paydar *et al.,* 2013; Suffredini *et al.,* 2014b). The *toxR* gene is widely present in both pathogenic and non-pathogenic *V. parahaemolyticus* strains (Sujeewa *et al.,* 2009).

Croci and colleagues reported the detection of *toxR* gene by PCR assay confirmed the highest accuracy in identification of *V. parahaemolyticus* compared to other targeted genes such as *tlh* (Bej *et al.,* 1999) and *gyrB* (Venkateswaran *et al.,* 1998). The *toxR* gene is also able to precisely differentiate *V. parahaemolyticus* from closely related *V. alginolyticus*, thus it is a good molecular confirmatory gene for the identification of *V. parahaemolyticus* at species level after selection of colonies based on the morphology (Croci *et al.,* 2007).

Although *V. parahaemolyticus* often present and isolated from seafood, studies have reported that many of these isolates are non-pathogenic to human. Hence, the detection of two major virulence factor of *V. parahaemolyticus*, thermostable direct hemolysin (*tdh*) and TDH-related hemolysin (*trh*) gene is important to determine the risk posed to human health by the presence of this species in seafood. Duplex PCR assay targeting *tdh* and *trh* gene was performed on all *toxR* positive *V. parahaemolyticus* in this study. The primer sets used in this study to detect the two virulent genes were adapted from a widely used *tdh* and *trh* primers from Bej *et al.* (1999). This assay saves time and effort and decrease the number of reactions to be performed in order to assess the possible presence of pathogenic isolates (Elnifro *et al.,* 2000).

Alternatively, the thermolabile hemolysin (*tlh*) in *V. parahaemolyticus* is another gene that is used to develop a multiplex PCR procedure for simultaneous detection of total and virulent *V. parahaemolyticus* (Yi *et al.,* 2014) Although, the *tlh* gene is not considered a virulence factor of *V. parahaemolyticus*, the gene is reported to be a reliable marker for the bacteria (Su & Liu, 2007). Bej *et al.* (1999) reported a multiplex PCR protocol for amplification of *tlh, tdh* and *trh*, which could be employed for detecting total and virulent *V. parahaemolyticus* in shellfish. The outcome of results detected *tlh* gene in all 111 strains of *V. parahaemolyticus* isolated from clinical, seafood, environmental, and oyster

plants with sensitivity for detecting all three genes of at least 1–10 cells per gram of alkaline peptone water enriched sample homogenate.

Multiplex PCR assays have been very popular and are utilized to differentiate *V. parahaemolyticus*, *V. cholerae* and *V. alginolyticus* from each other (Di Pinto *et al.,* 2005; Wei *et al.,* 2014). Kaufman *et al.* (2004) used PCR on samples of oyster mantle fluid, rather than homogenized meat, and reported that *V. parahaemolyticus* levels in the mantle fluid were highly correlated to levels in oyster tissues with r=0.85. Many PCR assays have been employed to affect detection of the *tdh* or *trh* genes (Dileep *et al.,* 2003). In addition, real-time PCR has been used to detect total and pathogenic *V. parahaemolyticus* in seafood samples (Nordstrom *et al.,* 2007). Real-time PCR has the ability to process huge number of samples with speed and consistency in a single tube amplification targeting the gene (McKillip & Drake*.,* 2000). A multiplexed real-time PCR TaqMan assay was developed targeting four different genes – the *tdh* and *trh* genes (detection of pathogenic *V. parahaemolyticus*), ORF8 (detection of pandemic *V. parahaemolyticus* O3:K6) and *tlh* gene for the detection of total *V. parahaemolyticus*. This assay was proven to be successful in detecting total and pathogenic *V. parahaemolyticus*, with the pandemic O3:K6 serotype in shellfish (Ward & Bej, 2006). The real time PCR assay was effective in the detection of *tdh* gene (Blackstone *et al.,* 2003) and *tlh* gene (Kaufman *et al.,* 2004) in *V. parahaemolyticus* using specific primer sets and fluorogenic probes.

Current advancement in PCR technology has led to the development of loop mediated isothermal amplification (LAMP) based assays as an alternative to PCR (Notomi *et al.,* 2000). The main advantage of LAMP-based assays as compared to PCR is that during LAMP, nucleic acid amplification occurs at a single temperature, eliminating the need for thermal cyclers. Nemoto *et al.* (2009) utilized LAMP to detect *tdh*-positive isolates of *V. parahaemolyticus* targeting six regions of the *tdh* gene and compared the results to

PCR for detection of *tdh* and reverse passive latex agglutination for *tdh* detection. Another LAMP assay was developed for detection of *tlh* gene and tested both with pure *V. parahaemolyticus* cultures and artificially inoculated shrimp. The assay revealed all 143 pure *V. parahaemolyticus* culture were positive, while no LAMP product was detected from any of 33 non-*V. parahaemolyticus* or 56 non-*Vibrio* isolates (Yamazaki *et al.,* 2008). LAMP consistently identified 2.0 CFU per reaction, while PCR required approximately 10-fold more bacteria for detection. Yamazaki *et al.* (2010) followed up on their previous work by developing a LAMP assay to detect the *tdh* and *trh* genes in *V. parahaemolyticus* and related *Vibrio* sp. In addition, LAMP assays targeting *rpoD* and *toxR* genes of *V. parahaemolyticus* was developed and resulted in positive amplification in 78 *V. parahaemolyticus* strains (Nemoto *et al.,* 2011). The detection LAMP assay was sensitivity targeting *rpoD* and *toxR* was determined to be 3.7 and 450 CFU per test in pure culture. The *rpoD*-LAMP assay was combined with MPN method detection for detection of *V. parahaemolyticus* in spiked short-necked clams comparative to MPN method with a culture method using selective agar. The results showed higher sensitivity using the *rpoD*-LAMP method (Nemoto *et al.,* 2011).

The random amplified polymorphic DNA-PCR (RAPD-PCR) is another technique used for typing and differentiation of bacteria. This method is able to explore the genetic relationships between strains and microorganisms, plants or animal species (Oakey *et al.,* 1998). Wong *et al.* (2001) used and developed 3 different PCR methods namely RS-PCR, REP-PCR and ERIC-PCR to detect *V. parahaemolyticus* to avoid the use of random primers. The study concluded that REP-PCR was comparatively better than ERIC-PCR due to greater reproducibility. Another approach of detecting *V. parahaemloyticus* is through fluorescence in situ hybridisation, a method that employs fluorescently labelled short nucleotides to specifically hybridize targeted rRNA in whole permeabilized cells. Sawabe *et al.* (2009) employed a multi-probe approach (using designed probes VP437,

VP612 and VP1253); however, the assay was only species specific, which would only allow for employment of this method to detect total *V. parahaemolyticus*.

The emergence of a pandemic clone of *V. parahaemolyticus* and its widespread distribution has led to the development of specific method to detect such strains. A group-specific polymerase chain reaction (GS-PCR) based on the sequence variation in the *toxRS* operon was developed to differentiate between pandemic and non-pandemic strains (Matsumoto *et al.,* 2000). But other researchers claimed that the occurrence of GS-PCR positive *V. parahaemolyticus* strains do not belong to the pandemic clone group (Vongxay *et al.,* 2008). GS-PCR or *orf8*-PCR was developed as a diagnostic tool to identify the pandemic clone group (Nasu *et al.,* 2000).

PFGE of NotI digested genomic DNA is another good molecular tool to differentiate between pandemic and non-pandemic strains. This method is more appropriate then ribotyping using EcoRI, RAPD-PCR, GS-PCR and *orf8*-PCR because it produces many diverse patterns and groups the pandemic strains in closely related clusters (Yeung *et al.,* 2002). Many studies have utilized this method and validated the reproducibility and discriminatory nature of PFGE (Fakruddin *et al.,* 2013). PFGE is also able to produce results of the genetic diversity among strains which is important information that is not provided by GS-PCR or *orf8*-PCR. A few studies have stated that repetitive sequenced based PCR is found to be slightly more discriminatory compared to PFGE as it generates greater numbers of different patterns and was less likely to yield untypeable results caused by DNA degradation (Wong & Lin, 2001).

2.7 Antibiotic Resistance of *Vibrio parahaemolyticus*

The occurrence of multidrug-resistant (MDR) bacteria to clinically used antibiotics is a major health issue and a great challenge to the worldwide drug discovery programs (Alanis, 2005). Every year, more and more pathogenic *Vibrio* sp. have been reported to develop higher resistance towards most of the clinically used antibiotics. Drug resistance is an alarming issue worldwide and is spreading rapidly due to overuse, self-medication or the non-therapeutic use of antimicrobials (Slama *et al.,* 2005). Antibiotics and other chemotherapeutic agents are frequently utilized in aquaculture farms as feed additives or immersion baths to achieve either prophylaxis or therapy (Devi *et al.,* 2009; Manjusha & Sarita, 2011). The misuse of antibiotics in agriculture and aquaculture environments has caused the development of multidrug resistance in seafood pathogens such as *Vibrio* sp. (Sudha *et al.,* 2014).

Antibiotics are substances produced by living organisms such as fungi and soil bacteria, which are able to kill or inhibit microorganism's growth. There are also antibiotics obtained by chemical synthesis such as sulfa drugs (sulfamethoxazole), or by chemical modification of compounds of natural origin (Kummerer, 2009). Commonly antibiotics that are non-toxic to host are used as chemotherapeutic agents in the treatment regime for infectious diseases in humans, animals and plants.

Furthermore, antibiotics constitute quite a heterogeneous group of chemicals. Based on the chemical structures, antibiotics exerts effects on different structures or functions of the bacterial cell (Grenni *et al.,* 2018). Their actions of mechanism include inhibits of cell wall synthesis (e.g. β-lactams, cephalosporins, carbapenems, penicillin), inhibition of protein synthesis (aminoglycosides, tetracyclines, chloramphenicol, lincosamides and macrolides), damage of cell membrane function (e.g. polymixins), inhibition of the of nucleic acid synthesis (e.g. quinolones and rifampicin), and metabolic antagonis (e.g.

sulphonamides and trimethoprim) (Kummerer, 2009). A bacteria strain can be classified as resistant when it is able to survive in the presence of high antimicrobial agents' concentrations in comparison with phylogenetically related strain (Baggesen *et al.,* 2000).

Most of the genetic determinants that confer resistance to antibiotics are located on plasmids. The acquired antibiotic resistance in microorganisms is usually mediated by extrachromosomal plasmids and transferable to other bacteria in the environment through vertical gene transfer or horizontal gene transfer (Manjusha & Sarita, 2011). Horizontal gene transfer is very important in the evolution and transmission of resistance genes between species and includes the transfer of resistance genes from fecal bacteria to environmental bacteria (Baquero *et al.,* 2008). These extrachromosomal DNA sequences may be responsible for the emergence of resistance to multiple antibiotics (Schelz *et al.,* 2006). In recent years, the presences of antibiotic resistance genes detected in *Vibrio* species have increased and includes *β*-lactam and penicillin resistance genes *penA* and *blaTEM-1* (Srinivasan *et al.,* 2005; Zhang *et al.,* 2009), chloramphenicol resistance genes *cat*I, *cat*II, *cat*III, *cat*IV and *floR* (Dang *et al.,* 2007; Dang *et al.,* 2008) and tetracycline resistance genes *tet*A, *tet*B, *tet*C, *tet*D, *tet*E, *tet*G, *tet*H, *tet*J, *tet*Y, *tet*Z (Macauley *et al.,* 2007; Zhang *et al.,* 2009; Kim *et al.,* 2013).

It is reported that over 90% of the marine originated bacteria isolates display resistance towards more than one type of antibiotic. In addition, 20% of them exhibited resistance towards five types of antibiotics (Martinez *et al.,* 2003). The marine environments are more prone to antibiotics and antibiotic resistance genes contamination due to misuse of antibiotic agents in hospital or veterinary treatment, aquaculture and agriculture locations, and their successive release into wastewater treatment plant (Marti *et al.,* 2014). The elavated levels of antibiotic agents in the aquatic could play a role as a selective pressure contributing to the rise and distribution of resistant and pathogenic bacteria within the

same aquatic environment (Cabello, 2006). As well, bacteria in the enviroments are able to produce antimicrobial compunds, thus making them capable of acquiring or expressing antimicrobial resistant genes to protect themselves from the toxicity of antibiotics present in the environments (Baquero *et al.,* 2008). Therefore, the present aquatic bacterium may function as reservoirs for antibiotic resistance genes and plays a crucial role in the spread of antibiotic resistance in aquatic environments (Marti *et al.,* 2014).

Vibrio sp. is autochthonous in estuarine, coastal waters and sediments environments. They are known to be susceptible to almost all clinically used antibiotics (Oliver, 2006). Nevertheless, this observation is never same as before as antibiotic resistance has emerged and evolved in many bacterial genera, including *Vibrio* sp. resulted from overuse of antibiotic agents in aquaculture and agriculture settings. Antibiotics are utilized in the aquaculture as prophylactics to control infectious diseases in marine organisms (Manjusha & Sarita, 2011). In Malaysia, the aquaculture field has grown rapidly due to increase in consumer demand for aquatic products. Based on the Malaysia fisheries department, there is nearly 200% increase of fisheries products from both marine and freshwater aquaculture in 2010 as compared to the production in 2001 (Chowdhury & Yahya, 2012). Currently, the antibiotic susceptibility of *V. cholera* has been examined widely but only a few studies investigated the presences of antimicrobial resistance in *V. parahaemolyticus*, which is also been recognized as another major foodborne pathogen. (Akinbowale *et al.,* 2006; Han *et al.,* 2007; Baker-Austin *et al.,* 2009; Noorlis *et al.,* 2011; Shaw *et al.,* 2014). *V. parahaemolyticus* is usually display resistance towards ampicillin (Okuda *et al.,* 1997). There are reports showing that environmental *V. parahemolyticus* isolates exhibit increasing resistance phynotype towards aminoglycoside, tetracycline, trimethoprim/sulfamethoxazole and chloramphenicol (Han *et al.,* 2007). Furthermore, both extended-spectrum-β-lactam-resistant and fluoroquinolone-resistant strains of *V. parahaemolyticus* isolated from food samples were reported recently (Liu *et al.,* 2013;

Wong *et al.,* 2012). Any infections caused by multidrug resistant *V. parhaemolyticus* have revealed a negative impact with higher hospitalization cost, longer hospitalization time, treatment failure and higher fatality rate (Letchumanan *et al.,* 2015b). Hence, it is indeed important to address and manage the antimicrobial resistance issue.

2.8 Plasmid Profiling

Plasmid profiling is an early genotyping method applied for epidemiological studies of foodborne pathogens including *Vibrio* sp. (Molina-Aja *et al.,* 2002), *Salmonella* sp. (Nayak *et al.,* 2004), *V. parahaemolyticus* (Zulkifli *et al.,* 2009; Devi *et al.,* 2009), *E. coli* (Domingue *et al.,* 2003; Johnson *et al.,* 2007), *Campylobacter* (Stanley *et al.,* 1994), and Shigella (Gebre-Yohannes *et al.,* 1991; Liu *et al.,* 1995). Previous studies have reported that *Vibrio* sp. contain plasmids and there is a correlation between possessions of plasmid with antibiotic resistance (Molina-Aja *et al.,* 2002, Li *et al.,* 2003, Zulkifli *et al.,* 2009).

Plasmids are autonomous molecules which are present in bacteria cells as extra-chromosomal genomes providing great functionality in molecular science (Devi *et al.,* 2009). These circular DNA molecules are located in the bacterial cytoplasm and contain at least one origin of replication. Plasmids are known to confer a discriminatory benefit to their host isolate by carrying genes that encode antimicrobial resistance, resistance to heavy metals, and virulence (Meyer, 1988; Threlfall *et al.,* 1990).

It is well known that genetic determinants of antibiotic resistance are located in the plasmids. Resistance genes transmission can be acquired by bacteria in the environment through vertical gene transfer or horizontal gene transfers (HGT). Once introduced into a host cell, the plasmid can remain both integrated in the cell DNA or free in the cytoplasm. The plasmids encode functions not important for bacteria survival but provide significant advantages in particular conditions of growth or development; for instance; R-plasmids,

responsible for antibiotic resistance. Many plasmids accumulate a form of multiple resistance that thwarts the effect of several antibiotics, rendering any antibacterial therapy ineffective (Allen *et al.,* 2010).

The bacterial antimicrobial resistance profiles are identified to be associated with presences of large plasmids in a strain and the abilities of plasmid in conjugation (Silvester *et al.,* 2015). R plasmids that are in 30 kb are transferable, and the essential components of a conjugative plasmid make it big in size compared to other plasmids (Guiney & Landa 1989). Plasmid profiles of *Vibrio* sp. including *V. parahaemolyticus* have been reported in many studies involving both clinical and environmental samples (Kaufman *et al.,* 2002; Molina-Aja *et al.,* 2002; Urtaza *et al.,* 2004; Devi *et al.,* 2009; Lesley *et al.,* 2011), *V. ordalii* (Tiainen *et al.,* 1995), *V. vulnificus* (Radu *et al.,* 1998) and *V. salmonicida* (Sorum *et al.,* 1990), and *V. anguillarum* (Pedersen *et al.,* 1996). The existent of plasmids in *Vibrio* sp. isolated from polluted and pristine surroundings may be ecologically significant to ensure survival of these bacteria in the harsh surroundings (Zhang *et al.,* 2006). In Malaysia, plasmid profiling is being used as an epidemiological tool in the study of bacteria antibiotic resistance. Many reports have been cited to use of plasmid profiling as a phenotypic method to type environmental, food or clinical isolates in our country (Tanil *et al.,* 2005; Zulkifli *et al.,* 2009; Lesley *et al.,* 2011; Letchumanan *et al.,* 2015b; You *et al.,* 2016; Kurdial-Dulaimi *et al.,* 2016).

2.9 Plasmid Curing

Plasmid-mediated multidrug resistance is one of the most pressing problems in the treatment of infectious diseases. The use of plasmid-curing agents may serve as a possible way to eliminate the plasmid and reduce spreading of antibiotic resistance encoded by antibiotic resistance plasmids (R-plasmids) (Molnar *et al.,* 2003). Plasmid curing occurs naturally through cell division or by treating the cells with any chemical or physical

agents (Elias *et al.,* 2013). The inhibition of conjugational transfer of antibiotic resistance plasmid can be used to decrease the spread of antibiotic resistance plasmid in the environment. Inhibition of plasmid replication occurs in various stages and well demonstrated through the "rolling circle" model (replication, partition, and conjugal transfer). This could also be the theoretical basis for the elimination of bacterial virulence in the case of plasmid mediated pathogenicity and antibiotic resistance (Brussow, 2004).

Curing of plasmids from bacteria strains is a way to eliminate the bacteria plasmid and determine the antibiotic resistance mediation. There are several methods involving chemical and physical agents that have been developed to eliminate plasmids. Protocols for curing plasmids in *Vibrio* consist of chemical agents such as acridine orange (AO), ethidium bromide (EB) and sodium dodecyl sulphate (SDS), and physical agent (Liu *et al.,* 2012). Intercalating agents such as AO and EB have been successfully used in curing bacterial plasmids. The modes of action of intercalating agents are through preferential inhibition of plasmid replication.

Since the 1960s, AO has been used as curing agent and normally involves loss of the whole plasmid (Salisbury *et al.,* 1972; Costa *et al.,* 2014). In 1970s, Dastidar *et al.* (1977) reported on the efficiency of AO in eliminating R-plasmids in *V. cholerae* multidrug-resistant strains. Many other studies have demonstrated the usefulness of AO in clinical strains, animal or environmental isolates (Kamat *et al.,* 1992; Barman *et al.,* 2010; Reboucas *et al.,* 2011; Carvalho *et al.,* 2013; Costa *et al.,* 2014) although the conventional methods for curing plasmids by curing agents may induce mutations in the host chromosomal DNA (Liu *et al.,* 2012). EB with a formula molecule $C_{21}H_{20}N_3Br$ is an intercalating agent which resembles a DNA base pair. Due to its unique structure, EB can easily intercalate into DNA strand. Yano *et al.* (2014) demonstrated the successful use of EB to eliminate plasmids in antibiotic resistant *Vibrio* sp. isolated from shrimp cultured

in Thaniland inland ponds. Their results exhibited oxytetracycline resistance phenotype was eliminated through plasmid curing and the authors suggested that the resistance to oxytetracycline was related to R-plasmids (Yano *et al.,* 2014).

The efficacy of each plasmid curing agent discussed varies depending on the concentration and the organism being cured. EB is preferred by many researchers in comparative to AO because the latter is difficult to be disposed (Molina-Aja *et al.,* 2002). All these chemical curing agents are known to be harmful and cause health problems to human beings. Precaution steps should be followed strictly prior in handling with these curing agents during experiments. When compared with chemical curing agents, physical agent such as elevated growth temperature is least favored in *Vibrio* plasmid curing studies due to its low successful rate.

CHAPTER 3: METHODOLOGY

3.1 Sampling

The study mainly focused on two seafood categories; shrimp and shellfish. A total of 770 seafood samples comprising of red prawn (*Solenocera subnuda*), banana prawn (*Penaeus indicus*), mud crab (*Scylla serrate*), flower crab (*Portunus pelagicus*), carpet clam (*Paphia texile*), hard shell clam (*Meretrix meretrix*), and mud creeper (*Cerithidea obtuse*) was collected from three wet market and three supermarkets in Selangor (Table 3.1). The sampling was done weekly from January 2014 to June 2014. All the samples were kept in individual sterile sealed bags and transported to the laboratory in a cooler box. The samples were analyzed immediately thereafter.

3.2 Enumeration and Isolation of *Vibrio* sp. in Seafood Samples

The enumeration and isolation of *Vibrio sp.* was done according to Standard US Food and Drug Administration (FDA) protocol (Kaysner & DePaola, 2004) and FAO/WHO Risk Assessment of *Vibrio parahaemolyticus* in Seafood (FAO/WHO, 2011); this method was also previously reported by Zarei *et al.* (2012). Twenty-five grams of samples was homogenized for 60s in a stomacher (Bagmixer 400W, Interscience, St Nom, France) with 225 mL of alkaline peptone water with 2% (w/v) sodium chloride NaCl, pH 8.5, giving a first 10^{-1} dilution. Spread plate technique was employed for the enumeration of total presumptive *Vibrio* sp. in each seafood samples following the protocol described by Beneduce *et al.* (2010). 100 µL of each sample homogenate with appropriate dilution (1:10, 1:100 and 1:1000) was spread in duplicates onto thiosulphate citrate bile salt sucrose agar (TCBS) (HiMedia, India) and incubated at 37°C for 18 hours. The total colony count of *Vibrio* sp. in seafood samples (cfu/g) was calculated using the following formula:

$$\text{colony forming unit (cfu/g)} = \frac{\text{Average number of colony x dilution factor}}{\text{volume of homogenate inoculated (g)}}$$

For the isolation step, the homogenate was incubated at 37°C for 18 hours. A loopful of overnight culture was streaked onto selective media, thiosulfate citrate bile salt sucrose agar (TCBS) (HiMedia, India) and incubated at 37°C for 18 hours. After overnight incubation, one sucrose non-fermenting colony was picked from each sample agar plate. The presumptive *V. parahaemolyticus* colonies appeared in green or bluish green colour and measured about 3-5 mm in size (Chakraborty & Surendran, 2008). The colonies were purified by re-streaking onto typtic soy agar (TSA) (HiMedia, India) supplemented with 2% w/v sodium chloride (NaCl) (Vivantis, USA) and incubated at 37ºC for 18 hours. A loopful of pure colony was inoculated into semi-solid nutrient agar and tryptic soy broth (TSB) with 30% glycerol, incubated at 37°C for 18 hours and then stored until further analysis (Zarei *et al.,* 2012, Letchumanan *et al.,* 2015a).

3.3 DNA Extraction

Genomic DNA of presumptive *V. parahaemolyticus* isolates was extracted by direct boiled cell lysate method (Suzita et al., 2010; Vengadesh et al., 2012; Letchumanan *et al.,* 2015a). The isolates were revived in tryptic soy broth (TSB) (HiMedia, India) supplemented with 2% w/v sodium chloride (NaCl) (Vivantis, USA) and incubated in a shaker incubator at 220 rpm for 37°C for 18 hours. Overnight culture suspension was transferred into 1.5 mL microcentrifuge tube and centrifuged at 10,000 rpm for 5 minutes. The supernatant was carefully discarded, leaving the pellet dry as possible. The pellet was re-suspended in 1 mL of sterile ultrapure water, vortexed, boiled at 100°C for 7 minutes and then immediately cooled on ice for 5 minutes. Cell debris from the cell lysate was pelleted by centrifugation at 13,000 rpm for 1 minute. The supernatant was carefully transferred into 1.5 mL microcentrifuge tube and used as DNA templates for PCR assays.

Table 3.1: List of seafood samples examined in this study

		Wetmarket A	Wetmarket B	Wetmarket C	Supermarket A	Supermarket B	Supermarket C	Total
GPS Coordinate		3° 4' 11.054"N 101° 27' 8.584"E	3° 4' 55.732"N 101° 30' 32.081"E	3° 4' 28.227"N 101° 35' 15.423"E	3° 3' 39.254"N 101° 28' 19.839"E	3° 4' 24.421"N 101° 36' 24.695"E	3° 9' 2.019"N 101° 36' 53.927"E	
Seafood Category	**Samples**	**n**	**n**	**n**	**n**	**n**	**n**	
Shrimp (n=320)	Red prawn	27	27	26	27	27	26	160
	Banana prawn	27	27	26	27	27	26	160
Shellfish (n=450)	Mud crab	15	15	15	15	15	15	90
	Flower crab	15	15	15	15	15	15	90
	Carpet clam	15	15	15	15	15	15	90
	Hard shell clam	15	15	15	15	15	15	90
	Mud creeper	15	15	15	15	15	15	90
Total		129	129	127	129	129	127	770

n= Number of seafood samples purchased at each sampling site

3.4 Identification of *Vibrio parahaemolyticus* using *toxR*-PCR

Identification of the isolates at species level by targeting *toxR* gene was performed by a singleplex PCR assay (Kim *et al.,* 1999) (Table 3.2). The PCR assay was performed in final volume of 20 µL, containing 2 µL of DNA template, 10 µL of 2× Taq PLUS PCR Smart mix 1 (SolGent^{TM}, Korea), 6 µL of sterile ultrapure water and 1 µL of each primer (*toxR*-F and *toxR*-R) and completed using Kyratec SuperCycler: ThermalCycler (Kyratec, Australia) with the following cycling conditions: predenaturation at 95^{o}C for 4 minutes, 35 cycles of 94^{o}C for 1 minute, 68^{o}C for 1 minute and 72^{o}C for 30 seconds, and final elongation at 72^{o}C for 5 minutes. The PCR products were resolved by electrophoresis in 1.5% agarose gel and viewed under UV transilluminator with a gel documentation system (ChemiDocTM XRS, Bio-Rad, USA). *Vibrio parahaemolyticus* NBRC 12711 was used as positive control and *Vibrio vulnificus* NBRC 15645 was used as negative control.

3.5 Detection of Virulence Genes

Molecular identification of *V. parahaemolyticus* virulence genes – thermostable direct haemolysin (*tdh*) and thermostable-related direct haemolysin (*trh*) was performed by a duplex PCR assay (Bej *et al.,* 1999) (Table 3.2). The PCR amplification was performed in a final volume of 20 µL, containing 2 µL of DNA template, 10 µL of 2× Taq PLUS PCR Smart mix 1 (SolGent^{TM}, Korea), 4 µL of sterile ultrapure water and 1 µL of each primer and completed using Kyratec SuperCycler: ThermalCycler (Kyratec, Australia) with the following cycling conditions: pre-denaturation at 94^{o}C for 3 minutes, 30 cycles of 94^{o}C for 1 minute, 58^{o}C for 1 minute and 72^{o}C for 1 minute, and a final elongation at 72^{o}C for 5 minutes. The PCR products were resolved by electrophoresis in 1.5% agarose gel and viewed under UV transilluminator by gel documentation system (ChemiDocTM XRS, Bio-Rad, USA). *Vibrio parahaemolyticus* NBRC 12711 was used as positive control and *Vibrio vulnificus* NBRC 15645 was used as negative control.

Table 3.2: Primers used in PCR assay in this study

Primer Name	Target Amplified	Sequence (5' to 3')	Product Size (bp)	Reference
toxR- F	*Vibrio parahaemolyticus* specific gene	GTCTTCTGACGCAATCGTTG	368	Kim *et al.,* 1999
toxR-R		ATACGAGTGGTTGCTGTCATG		
tdh- F	Thermostable direct hemolysin gene	GTAAAGGTCTCTGACTTTTGGAC	269	Bej *et al.,* 1999
tdh-R		TGGAATAGAACCTTCATCTTCACC		
trh- F	*tdh*-related hemolysin gene	TTGGCTTCGATATTTTCAGTATCT	500	Bej *et al.,* 1999
trh-R		CATAACAAACATATGCCCATTTCCG		

3.6 Antibiotic Susceptibility Testing (AST)

The antibiotic susceptibility test was performed using fourteen antibiotics (Table 3.3). Kirby-Bauer disc diffusion method (Bauer *et al.,* 1966) was applied with the following antibiotics: ampicillin (AMP, 10ug), ampicillin (10µg), ampicillin/sulbactam (SAM, 30µg), amikacin (AK, 30µg), cefotaxime (CTX, 30µg), ceftazidime (CAZ, 30µg), chloramphenicol (C, 30µg), gentamicin (CN, 30µg), imipenem (IPM, 10µg), kanamycin (K, 30µg), levofloxacin (LEV, 5µg), nalidixic acid (NA, 30µg), oxytetracycline (OT, 30µg), suphamethoxazole/trimethoprim (SXT, 25µg), and tetracycline (TE, 30µg).

V. parahaemolyticus isolates was grown in tryptic soy broth (TSB) (HiMedia, India) 2% w/v sodium chloride (NaCl) (Vivantis, USA) at 37ºC for 18 hours under constant agitation, 200 rpm. Each suspension was then swabbed onto dry Mueller-Hinton agar (MHA) (HiMedia, India) supplemented with 2% (w/v) sodium chloride (NaCl) (Vivantis, USA) plate using a sterile cotton swab. Seven antimicrobial discs were dispensed onto each media plate using a disc dispenser (Oxoid, Basingstoke, UK) to ensure sufficiently separation from each other and avoid overlapping of inhibition zones. After incubation at 37ºC for 18 hours, the zone of inhibition was measured and interpreted based on Clinical and Laboratory Standards Institute (CLSI) M45-A2 (CLSI, 2010).

Table 3.3: Grouping of antimicrobial agents and their concentrations

Class of antibiotics	Antibiotics	Concentration (µg)
Penicillin	Ampicillin	10
	Ampicillin-sulbactam	30
Cephalosporin	Cefotaxime	30
	Ceftazidime	30
Carbapenem	Imipenem	10
Aminoglycosides	Amikacin	30
	Gentamicin	30
	Kanamycin	30
Tetracycline	Tetracycline	30
	Oxytetracycline	30
Quinolones	Nalidixic acid	30
	Levofloxacin	5
Folate pathway inhibitor	Sulfamethoxazole-trimethoprim	25
Phenicol	Chloramphenicol	30

3.8 Detection of Antibiotic Resistant Genes

V. parahaemolyticus isolates with resistance phenotype was screened for the presence of genes coding the resistance determinants. PCR assay was performed to detect genes associated with resistance to *β*-lactams (*blaTEM, blaSHV, blaOXA*), tetracyclines (*tetA, tetB, tetC, tetG*), chloramphenicol (*catA1, catA2, catA3, catB3*) and kanamycin (*aphA-3*) (Kim *et al.,* 2013) (Table 3.4). The PCR assay was performed in a final volume of 20 µL, containing 2 µL of DNA template, 10 µL of 2× *Taq PLUS* PCR Smart mix 1 (SolGent™, Korea), 6 µL of sterile distilled water and 1 µL of each primer and completed using Kyratec SuperCycler: ThermalCycler (Kyratec, Australia) by following conditions: initial denaturation at 95°C for 3 minutes, 35 cycles of 94°C for 30 seconds, 52°C for 1 minute and 72°C for 1.5 minute, and a final elongation at 72°C for 6 minutes. PCR amplification

products were resolved by electrophoresis in 1.5% agarose gel and viewed under UV transilluminator with a gel documentation system (ChemiDocTM XRS, Bio-Rad, USA).

3.9 Plasmid Profiling

V. parahaemolyticus isolates was revived in tryptic soy broth (TSB) (HiMedia, India) supplemented with 2% w/v sodium chloride (NaCl) (Vivantis, USA) and incubated at 37ºC, 200 rpm for 18 hours. The overnight culture was harvested and the cells' pellets collected were subjected to conventional plasmid extraction as described by Devi *et al.* (2009). The pellet cells were added with ice-cold 100ul alkaline lysis solution I (Glucose 50mM; Tris Cl 25mM; EDTA 10Mm) and vortexed vigorously. The mixture was then added with freshly prepared 200uL alkaline lysis solution II (NaOH 2N; SDS 2% w/v). The contents were mixed by vortexing rapidly after which 150ul ice-cold solution III (Potassium acetate 5M: 60ml; Glacial acetic acid 11.5ml; dissolved in 28.5m sterile distilled water) was added to it. The tube was closed and gently vortexed for 10 seconds to disperse solution III through the viscous bacterial lysate. Then the tubes were stored in ice for 5 minutes before being centrifuged at 12,000 rpm for 2 minutes at 4ºC. An equal volume of phenol-chloroform (1:1, w/v) was added to the supernatant in a fresh tube, by vortexing. The contents in the micro-centrifuge tube were centrifuged at 8,000 rpm for 3 minutes at 4ºC and the supernatant was transferred into a fresh tube. This was repeated with chloroform:isoamyl-alcohol (24:1, v/v) to remove the phenol.

The double stranded DNA was precipitated with 2 volumes of ethanol at room temperature, followed by vortexing before it was allowed to stand for 5 minutes at room temperature. The aliquot was centrifuged at 12,000 rpm for 12 minutes at 4ºC and the supernatant was removed by gentle aspiration. The pellet of double stranded DNA was rinsed with ethanol (1ml, 70% v/v) at 4ºC and centrifuged. The supernatant was removed leaving the pellet dry as possible. The pellet was air-dried before it was re-dissolved in

30ul ultrapure water. The plasmid was resolved by electrophoresis in 1.0% agarose gels and viewed under UV transilluminator with a gel documentation system (ChemiDocTM XRS, Bio-Rad, USA).

3.10 Plasmid Curing

The antibiotic resistance mediation of *V. parahaemolyticus* isolate was determined by plasmid curing method using two different intercalating agent, acridine orange (AO) and ethidium bromide (EB) (Letchumanan *et al.,* 2015a; 2015b). The *V. parahaemolyticus* isolates were revived in freshly prepared tryptic soy broth (TSB) supplemented with 0.2 mg/mL of each curing agent and incubated at 37°C for 18 hours under constant agitation. The treated culture was subjected to antibiotic susceptibility test as described in section 3.6 to re-examine the antibiotic resistance profiles. The phenotype results were compared with the antibiotic phenotype of non-treated isolate. PCR amplification was performed to screen the antibiotic resistance gene of the curing treated *V. parahaemolyticus* isolate. The treated culture was subjected to DNA extraction as described in section 3.3 and PCR assay as described in section 3.8. Finally, plasmid profiling as described in section 3.9 was performed with the treated culture in order to determine and compared the presences of plasmids before and after treatment.

3.11 Statistical Analysis

Data analysis was performed using IBM SPSS statistical analysis software version 20. Statistical analysis was done to determine whether there is any significant difference in between wetmarket and supermarket samples according to independent *t*-test, $p < 0.05$. One-way analysis of variance (ANOVA) followed by appropriate post-hoc test (Turkey) was performed to determine the significant differences between sampling location and the MAR index of resistant *V. parahaemolyticus* isolates with $p < 0.05$. In addition, One-way analysis of variance (ANOVA) followed by appropriate post-hoc test (Turkey) was performed to determine the significant differences between the type of samples and MAR index of resistant *V. parahaemolyticus* isolates. A difference was considered statistically significant when $p < 0.05$.

Futher multivariate analysis was done to determine the effects of resistant gene and seafood types (two types) to the MAR index. The significant diference of the MAR index between the resistant gene and the types of seafood sample were analyzed by using two-way ANOVA. The significance level was set at $p < 0.05$.

Table 3.4: List of antibiotic resistant genes tested in this study

Antibiotics	Target gene	Sequence 5'-3'	Amplicon size (bp)
Ampicillin	*blaSHV*-F	TTATCTCCCTGTTAGCCACC	796
	blaSHV-R	GATTTGCTGATTTCGCTCGG	
	blaOXA-F	ACCAGATTCAACTTTCAA	589
	blaOXA-R	TCTTGGCTTTTATGCTTG	
	blaTEM-F	ATAAAATTCTTGAAGAC	1073
	blaTEM-R	TTACCAATGCTTAATCA	
Chloramphenicol	*catA1*-F	CGCCTGATGAATGCTCATCCG	456
	catA1-R	CCTGCCACTCATCGCAGTAC	
	catA2-F	ATGAATTTTACCAGAATTGATCTGAA	639
	catA2-R	ATTTCAGTATGTTATCACACATCATCT	
	catA3-F	AAATTGGGTTCGCCGTGA	1863
	catA3-R	ATTTACTGTTACACAACTCTTGTAGCC	
	catB3-F	TCAAAGGCAAGCTGCTTTCTGAGC	566
	catB3-R	TATTAGACGAGCACAGCATGGGCA	
Kanamycin	*aphA-3*-F	GGGACCACCTATGATGTGGAACG	600
	aphA-3-R	CAGGCTTGATCCCCAGTAAGTC	
Tetracycline	*tetA*-F	GTAATTCTGAGCACTGTCGC	956
	tetA-R	CTGCCTGGACAACATTGCTT	
	tetB-F	ACGTTACTCGATGCCAT	1169
	tetB-R	AGCACTTGTCTCCTGTT	
	tetC-F	AACAATGCGCTCATCGT	1138
	tetC-R	GGAGGCAGACAAGGTAT	
	tetG-F	CCGGTCTTATGGGTGCTCTA	603
	tetG-R	CCAGAAGAACGAAGCCAGTC	

CHAPTER 4: RESULTS

4.1 Enumeration of *Vibrio* sp. in Seafood Samples

Seafood, edible greens and RTE (ready-to-eat) foodstuff are continuously challenged by the presences of *Vibrio* sp. which causes a food safety worry. People who eat uncooked meat or seafood are very prone to be infected with foodborne gastrointeristis (Wingstrand *et al.,* 2006; Rosec *et al.,* 2012; Omurtag *et al.,* 2013). In this study, the sampling selection was determined based on the high number of prevalence reports of *V. parahaemolyticus* in shrimp and shellfish (Khan *et al.,* 2002; Jun *et al.,* 2012; Abd-Elghany & Sallam, 2013; Zhang *et al.,* 2013; West *et al.,* 2013; Xu *et al.,* 2014; Yu *et al.,* 2016).

Prevalence and microbial loads of total *Vibrio* sp. in all the samples are summarized in Table 4.1. The seafood samples analyzed had a microbial load of 2.29 log CFU/g to 6.63 log CFU/g. A bacterial load of 5-7 log CFU/g is usually considered a necessary level to cause infections in humans. The total *Vibrio* sp. in wetmarket A carpet clam had the uppermost number of bacterial count of 6.63 log CFU/g, followed by wetmarket C of 6.50 log CFU/g and wetmarket B of 6.42 log CFU/g. The banana prawn and red prawn had a microbial load of 4.21 log CFU/g to 6.34 log CFU/g, with the samples from wetmarket having higher amount of *Vibrio* sp. loads compared to samples from supermarket. Of the shrimp samples analyzed, red prawn from wetmarket A had the uppermost total *Vibrio* sp. count of 6.34 log CFU/g and the banana prawn from supermarket C had the lowest total *Vibrio* sp. count of 4.21 log CFU/g. The wetmarket B banana prawn and red prawn had a microbial load of 5.04 log CFU/g, which is the lowest in comparative to wetmarket A and C.

The shellfish samples yielded a higher *Vibrio* sp counts compared to shrimp samples. Mudcrab samples from both sampling site surpassed 5 log CFU/g, which reveals that the contamination of *Vibrio* sp. in this sample is high regardless the sampling site. The carpet clams from all wetmarket sites surpassed 6 log CFU/g while supermarket B had the lowest microbial load of 3.03 log CFU/g. This pattern was reversed in mud creeper samples, with the supermarket site exhibiting higher *Vibrio* sp. loads. Swimming crab samples from 4/6 sampling site had a microbial load less than 3 log CFU/g, demonstrating the samples are least contaiminated with *Vibrio* sp.

Based on the colonial morphology on TCBS, all the seafood samples analyzed in this study were contaminated with *Vibrio* sp. A typical *V. parahaemolyticus* with bluish-green colony feature was observed on TCBS agar. Figure 4.1 illustrates the colony feature of *V. parahaemolyticus* NBRC 12711 (A) and presumptive *V. parahaemolyticus* isolate from seafood samples (B). Further, the selected presumptive *V. parahaemolyticus* colonies wa picked and purified on TSA agar (Figure 4.2).

4.2 Species Level Identification by *toxR*-PCR Assay

V. parahaemolyticus – a foodborne bacterium that is dispersed globally, yet its density in seafood and environmental samples varies relying on the time of year, sampling sites, type of samples, and the application of different analysis method (Martinez-Urtaza *et al.,* 2008; Zarei *et al.,* 2012). In this present study, 770 presumptive *V. parahaemolyticus* was isolated from 770 seafood samples that was analyzed. Upon purification of TSA agar, the isolates were screened at species level by *toxR*-PCR assay adapted from Kim *et al.* (1999) with slight modifications. The *toxR* regulatory gene (368bp) is well conserved in both pathogenic and non-pathogenic *V. parahaemolyticus* isolates (Sujeewa *et al.,* 2009).

The *toxR*-PCR product of reference strain *V. parahaemolyticus* NBRC 12711 was sequenced and was analysed using Bio Edit sequence alignment software. The consensus sequence generated from the alignment software was analysed by using NCBI BLAST to search for homology in GenBank database at http://blast.ncbi.nlm.nih.gov. The sequence showed an identity of 100% to corresponding of *Vibrio* sp. *toxR* gene (accession number AY527396.1) (Appendix A). This result affirms that the primer pairs specificity used to detect *toxR* gene and the PCR conditions employed were optimum for the *toxR* gene detection. The *toxR*-PCR assay yeilded positive amplification of *toxR* gene in 50% (385/770) of the presumptive isolates. Out of the 385 positive *V. parahemolyticus*, 24% (185/770) of the isolates was isolated from shrimp samples, and 26% (200/770) of the isolates was isolated from shellfish samples. Figure 4.3 illustrates a representative gel electrophoresis image demonstrating the positive amplification of *toxR*-PCR assay.

4.3 Detection of Virulence Genes

V. parahaemolyticus poses two major virulent gene, the thermostable direct hemolysin (*tdh*) or *tdh*-related hemolysin (*trh*) genes gene that are known be responsible for the pathogenesis and cause of infection. Hence, these two virulent genes are often utilized in PCR assay to differentiate virulent and non-virulent *V. parahaemolyticus*. In this study, a total of 385 *V. parahaemolyticus* isolates was identified and confirmed based on the *toxR*-PCR assay. All the isolates were screened for the virulent genes using a duplex PCR assay adapted from Bej *et al.* (1999) with minor modification. A total of 32/385 (8.3%) carried the *trh* gene and none of the isolates yielded *tdh*-positive amplification.

The PCR products of reference strain *V. parahaemolyticus* NBRC 12711 with *trh* gene were sequenced and analysed by Bio Edit sequence alignment software (Appendix B). The consensus sequence generated from the alignment software was analysed by using NCBI BLAST to search for the homology in GenBank database at http://blast.ncbi.

nlm.nih.gov. The sequence showed an identity of 99% to corresponding of *Vibrio* sp. *trh* gene (accession number LC271578.1) (Appendix B). The result affirms the primer pair specificity to detect *trh* gene and the PCR conditions employed were optimum for the *trh* gene detection.

Of the 32-*trh* psotive isolates, nineteen (10%) isolates were detected from shrimp samples and another thirteen (6.5%) were detected from shellfish samples. Figure 4.4 illustrates a representative gel electrophoresis image demonstrating the *trh*-positive by PCR assay. The results are in line with previous reports that revealed *V. parahaemolyticus* isolated from an ecology source are non-pathogenic since it does not have either virulence factors of thermostable direct hemolysin (*tdh*) or TDH-related hemolysin (*trh*), yet a small proportion of the ecology isolates had either of the virulence genes (Wong *et al.,* 2000b; Hervio-Heath *et al.,* 2002; Alam *et al.,* 2002; Velazquez-Roman *et al.,* 2014; Haley *et al.,* 2014).

Table 4.2 demonstrates the distribution of potentially virulent *V. parahaemolyticus* from both the wetmarket and supermarket samples. Majority of the virulent isolates was detected among the samples from wetmarket A and wetmarket B. Of the 15/32 (46.8%) *trh*-positive isolates from banana prawn, eleven isolates were isolated from wetmarket A samples where else, another four isolates were isolated from supermarket C samples. 13/32 (40.6%) *trh*-positive isolates from shellfish samples was collected from wetmarket B. Of these, six samples were mud crab, two samples were carpet clam, two samples were mud creeper and three samples were hard shell clam.

Table 4.1: The mean of total *Vibrio* counts (log CFU/g) of each seafood samples from respective sampling site

Samples	**Total *Vibrio* density (log CFU/g)**					
	Wetmarket A	Wetmarket B	Wetmarket C	Supermarket A	Supermarket B	Supermarket C
Banana Prawn	6.24 ± 0.48	5.04 ± 0.45	5.16 ± 0.38	4.36 ± 0.33	4.40 ± 0.45	4.21 ± 0.22
Red Prawn	6.34 ± 0.55	5.04 ± 0.40	5.19 ± 0.55	4.43 ± 0.35	4.35 ± 0.40	4.36 ± 0.45
Mud crab	5.53 ± 0.03	5.44 ± 0.12	5.39 ± 0.22	5.00 ± 0.20	5.33 ± 0.05	5.17 ± 0.48
Swimming crab	2.45 ± 0.21	4.52 ± 0.49	3.40 ± 0.40	2.29 ± 0.16	2.88 ± 1.24	2.59 ± 0.30
Hard shell clam	5.00 ± 0.69	4.90 ± 0.06	4.95 ± 0.13	4.13 ± 1.12	4.06 ± 0.12	4.00 ± 0.04
Carpet clam	6.63 ± 0.19	6.42 ± 0.04	6.50 ± 0.25	5.56 ± 0.47	3.03 ± 0.40	4.30 ± 0.43
Mud creeper	4.54 ± 0.25	5.23 ± 0.28	4.89 ± 0.55	5.86 ± 0.43	5.92 ± 0.19	5.90 ± 0.22
Total average of *Vibrio* count (log CFU/g)	5.25 ± 1.45	5.23 ± 0.60	5.07 ± 0.91	4.52 ± 1.17	4.28 ± 1.11	4.36 ± 1.03

Values = mean ± standard deviation

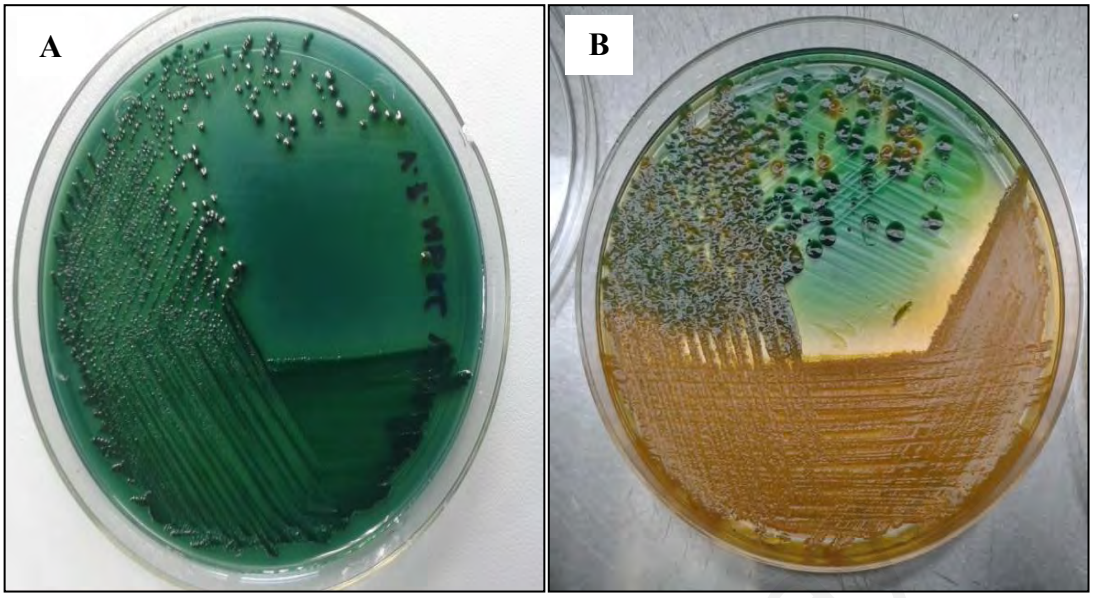

Figure 4.1: Colony morphology of *V. parahaemolyticus* reference strain NBRC 12711 (A) and presumptive *V. parahaemolyticus* isolate from seafood sample (B). The colonies appear to be round, 3-5mm in size, and green or bluish-green colour on TCBS agar.

Figure 4.2: The colony morphology of presumptive *V. parahaemolyticus* on tryptic soy agar (TSA). The colonies appear to be round and cream in colour on TSA agar.

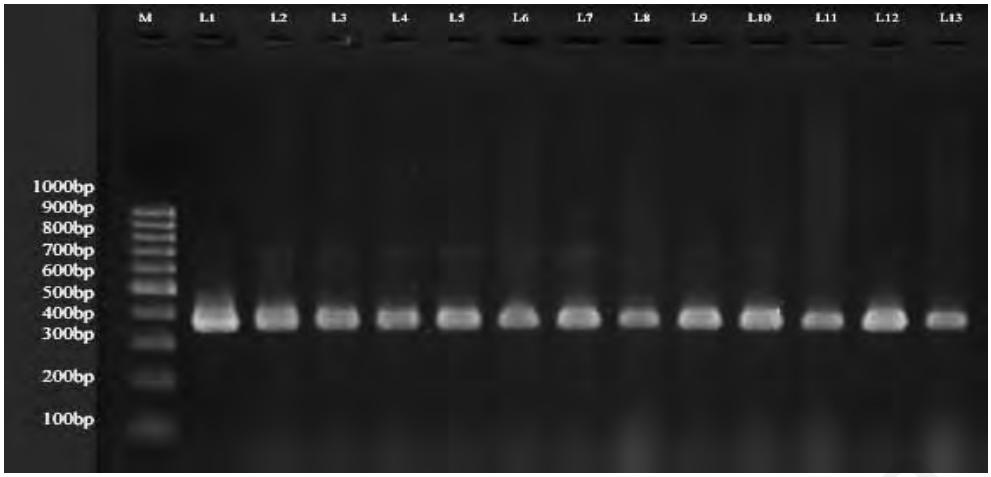

Figure 4.3: Representative gel electrophoresis of *toxR*-PCR. Lane M: DNA Ladder (100bp); L1: Positive control – *V. parahaemolyticus* NBRC 12711; L2-13 Presumptive *V. parahaemolyticus* isolates from seafood samples with positive amplification of *toxR* gene at 368bp.

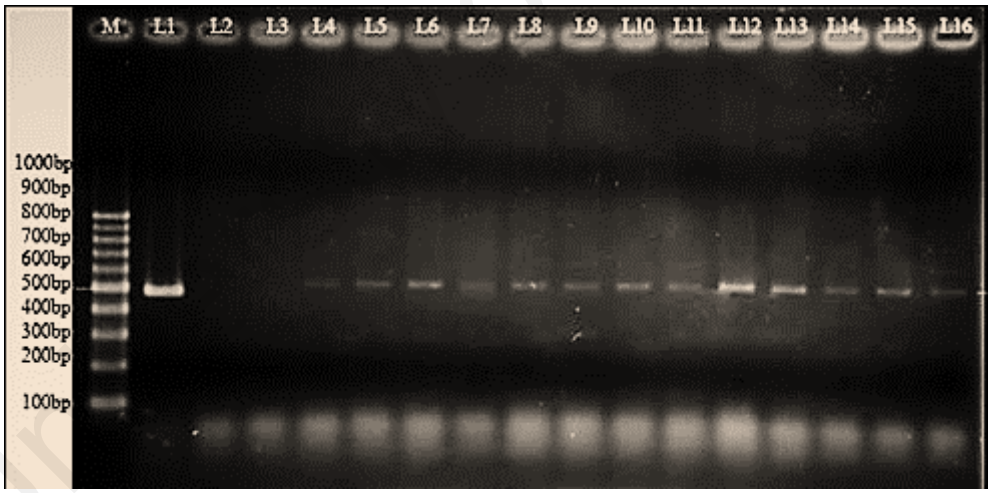

Figure 4.4: Representative gel electrophoresis image of *trh* gene by PCR assay. Lane M: DNA Ladder (100bp); L1: Positive control – *V. parahaemolyticus* NBRC 12711; L2-L3: Negative control – *V. vulnificus* NBRC 15645; L4-L16: *V. parahaemolyticus* isolates from seafood samples with positive amplification of *trh* gene at 500bp.

Table 4.2: List of *trh*-positive *V. parahaemolyticus* isolates

Isolates	Samples	Location	*toxR*-positive	*trh*-positive
VP93	Banana Prawn	Wetmarket A	+	+
VP94	Banana Prawn	Wetmarket A	+	+
VP95	Banana Prawn	Wetmarket A	+	+
VP96	Banana Prawn	Wetmarket A	+	+
VP97	Banana Prawn	Wetmarket A	+	+
VP98	Banana Prawn	Wetmarket A	+	+
VP99	Banana Prawn	Wetmarket A	+	+
VP100	Banana Prawn	Wetmarket A	+	+
VP101	Banana Prawn	Wetmarket A	+	+
VP102	Banana Prawn	Wetmarket A	+	+
VP103	Banana Prawn	Wetmarket A	+	+
SVP55	Mud crab	Wetmarket B	+	+
SVP56	Mud crab	Wetmarket B	+	+
SVP60	Mud crab	Wetmarket B	+	+
SVP61	Mud crab	Wetmarket B	+	+
SVP64	Mud crab	Wetmarket B	+	+
SVP66	Mud crab	Wetmarket B	+	+
SVP52	Carpet clam	Wetmarket B	+	+
SVP54	Carpet clam	Wetmarket B	+	+
SVP73	Mud creeper	Wetmarket B	+	+
SVP75	Mud creeper	Wetmarket B	+	+
SVP69	Hard shell clam	Wetmarket B	+	+
SVP70	Hard shell clam	Wetmarket B	+	+
SVP72	Hard shell clam	Wetmarket B	+	+
VP89	Red Prawn	Supermarket C	+	+
VP90	Red Prawn	Supermarket C	+	+
VP91	Red Prawn	Supermarket C	+	+
VP92	Red Prawn	Supermarket C	+	+
VP175	Banana Prawn	Supermarket C	+	+
VP176	Banana Prawn	Supermarket C	+	+
VP177	Banana Prawn	Supermarket C	+	+
VP178	Banana Prawn	Supermarket C	+	+

4.4 Antibiotic Susceptibility Testing (AST) and MAR Index

All 14 antibiotics used in this study are among the antibiotics recommended by Centre for Disease Control and Prevention (CDC) for the treatment of *Vibrio* sp. infections that includes tetracycline, folate pathway inhibitors (trimethoprim-sulfamethoxazole), third-generation cephalosporins (cefotaxime and ceftazidime), aminoglycosides (gentamicin and amikacin), and fluoroquinolones (ciprofloxacin and levofloxacin) (Daniels *et al.,* 2000; Shaw *et al.,* 2014). Some of these antibiotics are widely used in aquaculture industry as antimicrobial agents including oxytetracycline and chloramphenicol (*Dang et al.,* 2007). Figure 4.5 shows a multidrug resistant *V. parahaemolyticus* isolate displaying resistance phenotype on MHA agar supplemented with 2% NaCl.

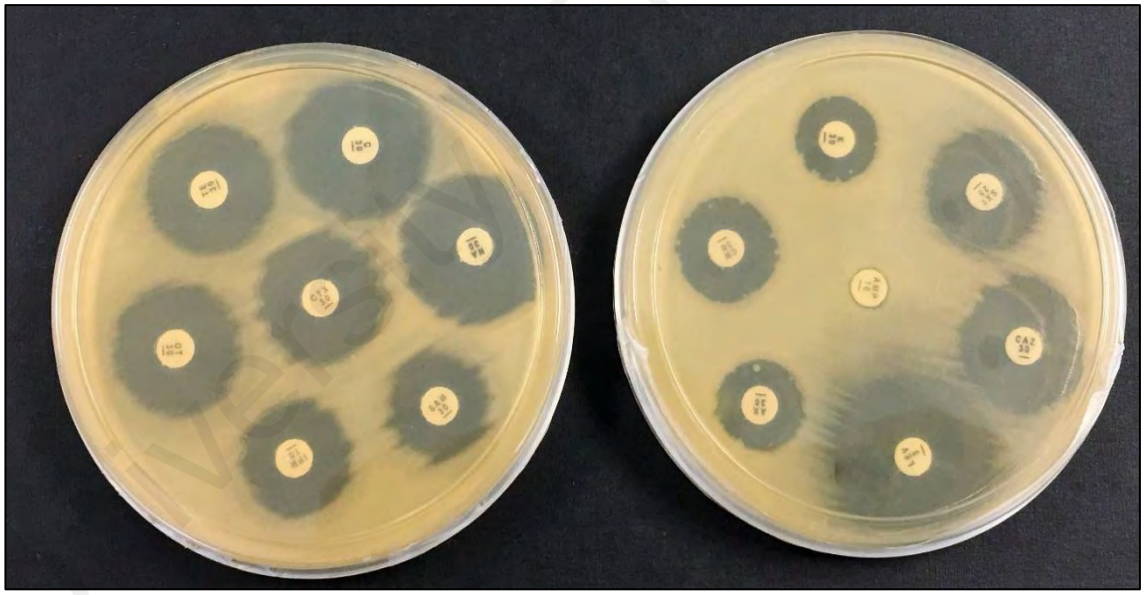

Figure 4.5: Bacterial lawn of a representative *V. parahaemolyticus* isolate on Mueller-Hinton agar (MHA) supplemented with 2% sodium chloride (NaCl). Antibiotic discs used on MHA plates: Ampicillin (AMP), Oxytetracycline (OT), Nalidixic acid (NA), Chloramphenicol (C), Cefotaxime (CTX), Sulfamethoxazole/Trimethoprim (SXT), Imipenem (IMP), Amikacin (AK), Ampicillin/Sulbactam (SAM), Levofloxacin (LEV), Ceftazidime (CAZ), Kanamycin (K), Gentamicin (CN), Tetracycline (TE).

The percentage of antibiotic resistant *V. parahaemolyticus* isolates isolated from shrimp and shellfish samples has been summarized in Table 4.3. Based on the results, the resistance rate of the 385 *V. parahaemolyticus* isolates was 85% to ampicillin, 66.8% to amikacin, and 50.1% to kanamycin. A notable resistance pattern could be observed to the third generation cephalosporins (cefotaxime 55.8% and ceftazidime 34%). In contrast, high susceptibility rate was observed to imipenem (94%), chloramphenicol (92.5%), tetracycline (83.1%), ampicillin-sulbactam (81%), levofloxacin (76.1%), trimethoprim-sulfamethoxazole (75.8%), nalidixic acid (73.85), and gentamicin (70.6%).

This study revealed a high percentage of susceptibility towards imipenem, however it should be noted that five of the isolates (VP71, SVP90, VP114, VP145, and VP146) exhibited resistance to imipenem. Although the resistance to imipenem is only 1.3% of the total isolates, yet it still warrants a concern on the use Carbapenems – a beta-lactams antibiotic group which is the last line of antimicrobial that given for microbial infections (Meletis, 2016). These five isolates had MAR index of 0.21 to 0.64, resistant to more than two different type of antibiotic tested. Imipenem resistance profiles was observed among isolates isolated from both shrimp and shellfish samples, demonstrating that the resistance occurred in different seafood samples regardless the habitat of marine organism. The VP114, VP145 and VP146 isolates was isolated from banana prawn samples where else, VP71 isolated from red prawn and SVP90 isolated from flower crab sample.

Interestingly, the 32 *trh*-positive *V. parahaemolyticus* exhibited resistance to more than two different type of antibiotic tested (Table 4.4). Of the thirty-two isolates, 30 *trh*-positive isolates were seen resistant to ampicillin. Isolate SVP54 demonstrated resistance to six different antibiotics tested including ampicillin, amikacin, ceftazidime, cefotaxime, kanamycin, and levofloxacin. The 32 *trh*-positive isolates had MAR index of 0.21 to 0.64, with 62.5% (20/32) isolates are resistance to three and more different types of antibiotics

tested. The presence of multiresistant *trh*-positive isolates in the marine environment may hamper clinical treatment if one gets infected with these strains. This emphasises the need for frequent monitoring of seafoods.

Table 4.3: The percentage of antibiotic resistant *V. parahaemolyticus* isolates isolated from shrimp and shellfish samples

Antibiotics	**Concentration (µg)**	**No. of resistant isolates (%)**	**No. of intermediate isolates (%)**	**No. of susceptible isolates (%)**
Ampicillin	10	327 (85)	29 (7.5)	29 (7.5)
Ampicillin-sulbactam	30	41 (10.6)	32 (8.3)	312 (81)
Cefotaxime	30	215 (55.8)	51 (13.2)	119 (30.9)
Ceftazidime	30	131 (34)	98 (25.5)	156 (40.5)
Imipenem	10	5 (1.3)	18 (4.7)	362 (94)
Amikacin	30	257 (66.8)	90 (23.4)	38 (9.9)
Gentamicin	30	28 (7.3)	85 (22.1)	272 (70.6)
Kanamycin	30	193 (50.1)	161 (41.8)	31 (8.1)
Tetracycline	30	57 (14.8)	8 (2.1)	320 (83.1)
Oxytetracycline	30	67 (17.4)	108 (28.1)	210 (54.5)
Nalidixic acid	30	38 (9.9)	63 (16.4)	284 (73.8)
Levofloxacin	5	31 (8.1)	61 (15.8)	293 (76.1)
Trimethoprim-sulfamethoxazole	25	18 (4.7)	75 (19.5)	292 (75.8)
Chloramphenicol	30	22 (5.7)	7 (1.8)	356 (92.5)

Table 4.4: Antibiotic resistant profile of *trh*-positive *V. parahaemolyticus* isolates

Isolates	Antibiogram	MAR Indes
SVP54	amp/ak/caz/ctx/k/lev	0.43
SVP55	amp/ak/caz/ctx/k	0.36
SVP56	amp/ak/caz/ctx/k	0.36
SVP70	amp/ak/caz/ctx/k	0.36
VP102	amp/ctx/ak/caz/k	0.36
VP103	amp/ctx/ak/caz/k	0.36
SVP61	amp/ak/caz/ctx	0.29
SVP66	amp/ak/caz/ctx	0.29
SVP69	amp/ak/caz/ctx	0.29
SVP72	amp/ak/caz/ctx	0.29
SVP75	amp/ak/ctx/k	0.29
VP90	amp/ctx/ak/caz	0.29
VP95	amp/ctx/ak/k	0.29
SVP73	ak/ctx/k	0.21
SVP64	amp/ak/ctx	0.21
SVP52	amp/ak/ctx	0.21
VP93	amp/ak/k	0.21
VP101	amp/ak/k	0.21
VP89	amp/ctx/ak	0.21
VP91	amp/ctx/ak	0.21
VP178	amp/ak	0.14
VP99	amp/ctx	0.14
VP175	amp/ctx	0.14
SVP60	AMP	0.07
VP92	amp	0.07
VP96	amp	0.07
VP97	amp	0.07
VP176	amp	0.07
VP177	amp	0.07
VP94	amp	0.07
VP100	amp	0.07
VP98		0.00

Ampicillin (AMP), Oxytetracycline (OT), Nalidixic acid (NA), Chloramphenicol (C), Cefotaxime (CTX), Sulfamethoxazole/Trimethoprim (SXT), Imipenem (IMP), Amikacin (AK), Ampicillin/Sulbactam (SAM), Levofloxacin (LEV), Ceftazidime (CAZ), Kanamycin (K), Gentamicin (CN), Tetracycline (TE).

Test results revealed 102 different antimicrobial resistances patterns as observed in the antibiograms (Table 4.5). A high percentage (68%) of isolates have a significant MAR index more than 0.2. The value of MAR index ranged from 0.00 to 0.79, with the highest MAR index attributed from two isolates respectively (VP152 from supermarket banana prawn and SVP129 from supermarket carpet clam) exhibiting resistance profile towards 11/14 antibiotics tested. Gwendelynne *et al.* (2005) stated that MAR indices higher that 0.2 could be due to contamination from high risk sources, thus leading to human health risk. A majority of *V. parahaemolyticus* isolates in this study had a MAR index of 0.36 (resistant to five different type of antibiotics tested). A total of 65/385 isolates exhibited MAR index of 0.07, indicating the isolates were resistant to at least 1/14 antibiotic tested.

Additional statistical investigation was done by comparing the MAR index between the wetmarket and supermarket samples. There was no significant difference between the wetmarket and supermarket samples according to independent *t*-test analysis (Appendix C). The wetmarket samples had a mean MAR index of 0.21 and the supermarket samples had a mean MAR index of 0.33. The *V. parahaemolyticus* isolates from supermarket A had the highest mean MAR index of 0.36 followed by supermarket B with mean MAR index of 0.35, supermarket C with mean MAR index of 0.29, and wetmarket C with mean MAR index of 0.26. The *V. parahaemolyticus* isolates from wetmarket B had a mean MAR index of 0.22 and the lowest mean MAR index was from wetmarket A of 0.16. According to the One-way ANOVA analysis, there was a significant effect, $p < 0.05$ between groups of sampling location on the MAR index of *V. parahaemolyticus* isolates (Appendix D).

According to Tukey's Post Hoc analysis, there was a significant difference in the mean MAR index ($p < 0.05$) of *V. parahaemolyticus* isolates between wetmarket A and all the other sampling locations except wetmarket B. There was a significant difference in the

mean MAR index between wetmarket B with supermarket A and supermarket B, p = 0.000 ($p < 0.05$). The mean MAR index of isolates from wetmarket C was significantly different with mean MAR index of isolates from wetmarket A (p = 0.003), supermarket A (p = 0.004), and supermarket B (p = 0.016). There was no significant difference in the mean MAR index of *V. parahaemolyticus* isolates between supermarket C and all the other sampling locations, except for supermarket C and wetmarket A, p = 0.000 ($p <$ 0.05). Figure 4.6 illustrates the comparison of mean MAR index of *V. parahaemolyticus* isolates from six different sampling sites.

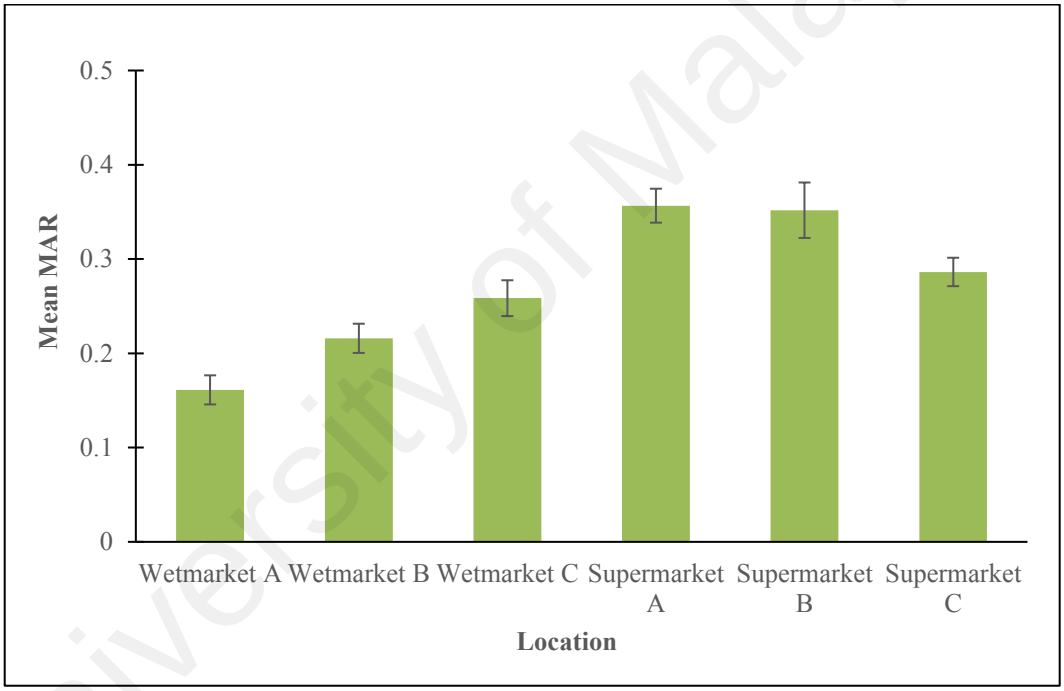

Figure 4.6: Comparison of the mean MAR index of *V. parahaemolyticus* isolates from six different sampling sites. Each bar represents mean MAR index of isolates from each sampling site. The vertical lines associated with the bars represent two times the standard error of the mean.

Based on the One-way ANOVA analysis, there was a significant effect, $p < 0.05$ between type of samples and MAR index of *V. parahaemolyticus* isolates (Appendix E). In line with Tukey's Post Hoc analysis, there was a significant difference in the mean MAR index ($p < 0.05$) of *V. parahaemolyticus* isolates between red prawn and all the other type of seafood. There was a significant difference in the mean MAR index between banana prawn with red prawn, $p = 0.000$ ($p < 0.05$). The MAR index of swimming crab sample was significantly different with MAR index of red prawn ($p = 0.000$) and carpet clam ($p = 0.041$) ($p < 0.05$). There was no significant difference in the mean MAR index of *V. parahaemolyticus* isolates between hard shell clam and all the other type of samples, except for red prawn, $p = 0.000$ ($p < 0.05$). Figure 4.7 illustrates the comparison of mean MAR index of *V. parahaemolyticus* isolates from different type of seafood.

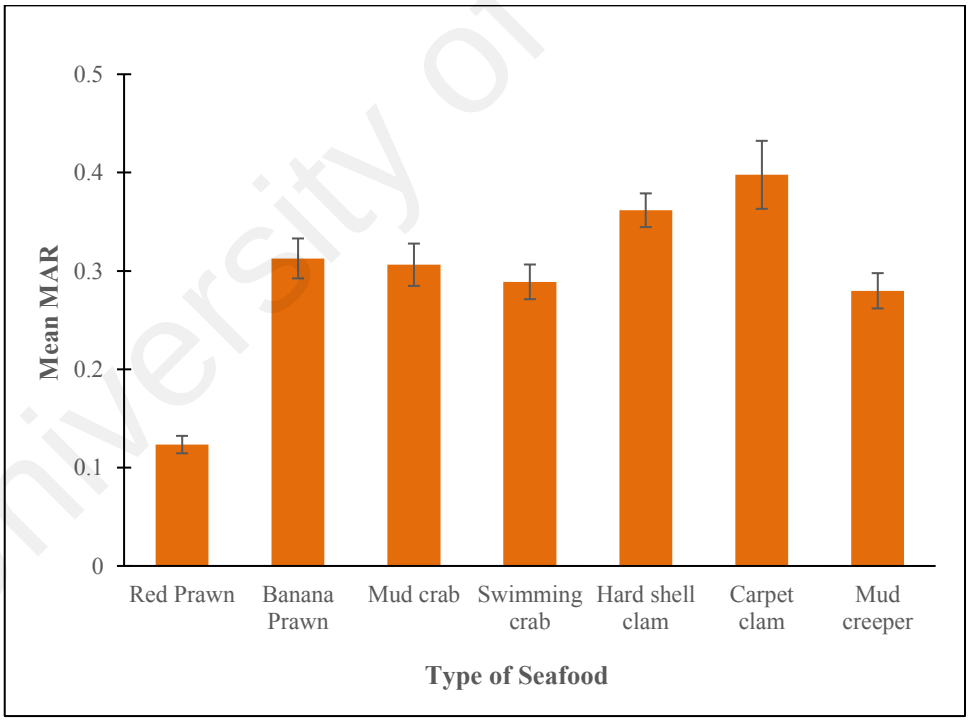

Figure 4.7: Comparison of the mean MAR index of *V. parahaemolyticus* isolates from different type of seafood. Each bar represents mean MAR index of isolates from type of seafood. The vertical lines associated with the bars represent two times the standard error of the mean.

Table 4.5: Antibiograms and multiple antimicrobial resistance (MAR) indices of 385 *V. parahaemolyticus* isolates

Antibiograms	Isolates	Total Antibiotic Resistance	MAR Index
AK/AMP/C/CAZ/CN/CTX/K/OT/SAM/SXT/TE	SVP129	11	0.79
AK/AMP/CAZ/CN/CTX/K/NA/OT/SAM/SXT/TE	VP152	11	0.79
AK/AMP/C/CAZ/CTX/K/OT/SAM/SXT/TE	SVP127	10	0.71
AK/AMP/CAZ/CN/CTX/K/LEV/ NA/OT/TE	VP134, VP135, VP139	10	0.71
AK/AMP/CAZ/CN/CTX/K/NA/OT/SXT/TE	VP158	10	0.71
AK/AMP/CAZ/CTX/CN/K/OT/SAM/SXT/TE	SVP128	10	0.71
AK/AMP/C/CAZ/CTX/IMP/K/OT/TE	SVP90	9	0.64
AK/AMP/C/CAZ/CTX/K/OT/TE	SVP126	9	0.64
AK/C/CAZ/CN/CTX/K/NA/OT/TE	VP160	9	0.64
AK/CAZ/CN/CTX/K/LEV/NA/OT/TE	VP136	9	0.64
AK/AMP/C/CAZ/CN/K/NA/ SXT	VP158	8	0.57
AK/AMP/CAZ/CN/CTX/K/LEV/ NA	VP165	8	0.57
AK/AMP/CAZ/CN/CTX/K/NA/ SAM	VP142	8	0.57
AK/AMP/CAZ/CTX/CN/K/LEV/SAM	SVP57, SVP68, SVP114	8	0.57
AK/AMP/CAZ/CTX/CN/K/OT/TE	VP153	8	0.57
AK/AMP/CTX/K/SAM/SXT/OT/TE	SVP131	8	0.57
AK/CAZ/CN/CTX/K/LEV/NA/SXT	VP174	8	0.57
AK/CAZ/CN/CTX/K/NA/OT/TE	VP162	8	0.57

Table 4.5, continued.

Antibiograms	Isolates	Total Antibiotic Resistance	MAR Index
AK/AMP/C/CAZ/CTX/OT/TE	SVP87, SVP92	7	0.50
AK/AMP/C/CTX/OT/SAM/TE	SVP91	7	0.50
AK/AMP/C/K/OT/TE	SVP143, SVP145, SVP146	7	0.50
AK/AMP/CAZ/CN/CTX/K/NA	VP163	7	0.5
AK/AMP/CAZ/CN/K/NA/OT	VP138	7	0.5
AK/AMP/CAZ/CTX/K/LEV/SAM	SVP58, SVP105	7	0.50
AK/AMP/CTX/K/LEV/SAM/NA	SVP96	7	0.50
AK/AMP/CTX/LEV/NA/OT/TE	VP137, VP167	7	0.5
AK/AMP/K/LEV/NA/OT/TE	VP130	7	0.5
AK/AMP/C/CTX/OT/TE	SVP89	6	0.43
AK/AMP/CAZ/CTX/K/LEV	SVP16, SVP31, SVP49, SVP54, SVP121, SVP150	6	0.43
AK/AMP/CAZ/CTX/K/NA	VP151	6	0.43
AK/AMP/CAZ/CTX/K/OT	SVP181, SVP182	6	0.43
AK/AMP/CAZ/CTX/K/SAM	VP157, SVP10, SVP67, SVP94, SVP112, SVP133, SVP141, SVP148, SVP155, SVP156, SVP191	6	0.43
AK/AMP/CAZ/CTX/K/SXT	SVP147	6	0.43
AK/AMP/CTX/K/IMP/OT	VP71	6	0.43
AK/AMP/CTX/K/LEV/NA	VP166, VP169	6	0.43

Table 4.5, continued.

Antibiograms	Isolates	Total Antibiotic Resistance	MAR Index
AK/AMP/CTX/K/LEV/SAM	SVP123	6	0.43
AK/CAZ/CN/CTX/K/NA	VP132, VP133, VP140, VP141, VP143	6	0.43
AK/CAZ/CTX/K/NA/SXT	VP161	6	0.43
AMP/CAZ/C/CTX/OT/TE	SVP88	6	0.43
AMP/CTX/NA/OT/SXT/TE	VP170	6	0.43
AMP/IPM/LEV/NA/OT/TE	VP145, VP146	6	0.43
AK/AMP/C/OT/TE	VP183	5	0.36
AK/AMP/CAZ/CTX/K	VP102, VP103, VP148, SVP14, SVP15, SVP23, SVP24, SVP25, SVP26, SVP27, SVP34, SVP35, SVP44, SVP55, SVP56, SVP69, SVP70, SVP71, SVP72, SVP83, SVP93, SVP98, SVP99, SVP102, SVP103, SVP104, SVP107, SVP113, SVP116, SVP118, SVP120, SVP132, SVP134, SVP136, SVP140, SVP142, SVP153, SVP154, SVP158, SVP183, SVP184, SVP189, SVP190, SVP192, SVP193, SVP195	5	0.36

Table 4.5, continued.

Antibiograms	Isolates	Total Antibiotic Resistance	MAR Index
AK/AMP/CAZ/CTX/OT	SVP171, SVP176	5	0.36
AK/AMP/CAZ/K/OT	SVP170	5	0.36
AK/AMP/CAZ/K/SAM	SVP18	5	0.36
AK/AMP/CAZ/OT/TE	SVP149	5	0.36
AK/AMP/CTX/K/SAM	SVP2, SVP4, SVP65, SVP81, SVP84, SVP117	5	0.36
AK/AMP/CTX/K/SXT	SVP19	5	0.36
AK/AMP/K/OT/TE	SVP130	5	0.36
AK/C/K/OT/TE	SVP115	5	0.36
AK/CAZ/CTX/K/LEV	SVP51	5	0.36
AMP/CAZ/CTX/K/OT	SVP172, SVP177	5	0.36
AMP/CAZ/CTX/K/SAM	SVP139	5	0.36
AMP/CN/K/SAM/TE	SVP161	5	0.36
AMP/CTX/K/SAM/TE	SVP165	5	0.36
AMP/CTX/NA/OT/TE	VP173	5	0.36
AMP/LEV/NA/OT/TE	VP131	5	0.36
CAZ/CTX/K/NA/OT	VP168	5	0.36
AK/AMP/CAZ/CTX	VP90, SVP47, SVP50, SVP61, SVP137	4	0.29
AK/AMP/CAZ/K	SVP22	4	0.29
AK/AMP/CN/K	SVP43	4	0.29

Table 4.5, continued.

Antibiograms	Isolates	Total Antibiotic Resistance	MAR Index
AK/AMP/CTX/K	VP73, VP77, VP82, VP95, VP104, VP117, VP149, VP154, VP156, SVP3, SVP13, SVP21, SVP30, SVP45, SVP46, SVP63, SVP66, SVP74, SVP75, SVP97, SVP111, SVP135, SVP144, SVP159, SVP178, SVP180, SVP185, SVP186, SVP187, SVP188, SVP194	4	0.29
AK/AMP/CTX/OT	SVP174	4	0.29
AK/AMP/K/CN	VP84	4	0.29
AK/AMP/K/SXT	VP119	4	0.29
AK/AMP/LEV/NA	VP171	4	0.29
AK/AMP/OT/TE	VP125, SVP53, SVP151	4	0.29
AK/CAZ/CTX/K	SVP80, SVP86, SVP196, SVP197, SVP198, SVP199, SVP200	4	0.29
AMP/CAZ/CTX/K	SVP179	4	0.29
AMP/CAZ/CTX/OT	SVP173	4	0.29
AMP/CTX/OT/TE	VP159	4	0.29
AMP/K/SAM/TE	SVP162	4	0.29
AMP/NA/OT/TE	VP129	4	0.29
C/OT/SXT/TE	SVP125	4	0.29
LEV/NA/OT/TE	VP144	4	0.29

Table 4.5, continued.

Antibiograms	Isolates	Total Antibiotic Resistance	MAR Index
AK/AMP/CAZ	SVP167, SVP168	3	0.21
AK/AMP/CTX	VP21, VP30, VP52, VP54, VP64, VP70, VP78, VP87, VP88, VP89, VP91, VP111, VP126, SVP52, SVP62, SVP64, SVP76, SVP101	3	0.21
AK/AMP/IMP	VP114	3	0.21
AK/AMP/K	VP55, VP93, VP101, VP110, VP118, VP123, VP128, VP150, SVP1, SVP37, SVP38, SVP39, SVP40, SVP41, SVP42, SVP95, SVP169	3	0.21
AK/AMP/SAM	SVP166	3	0.21
AK/CTX/K	SVP48, SVP73, SVP85, SVP119	3	0.21
AMP/C/CTX	VP182	3	0.21
AMP/C/OT	VP179, VP180, VP181	3	0.21
AMP/CTX/K	VP43, SVP48, SVP73, SVP85, SVP119	3	0.21
AMP/CTX/OT	SVP175	3	0.21
AMP/CTX/SAM	SVP9	3	0.21
AMP/LEV/NA	VP172	3	0.21
AMP/OT/TE	VP59, VP72, VP74, VP108, VP120, SVP124	3	0.21

Table 4.5, continued.

Antibiograms	Isolates	Total Antibiotic Resistance	MAR Index
AMP/SAM/TE	SVP163, SVP164	3	0.21
C/OT/TE	VP184	3	0.21
AK/AMP	VP31, VP34, VP41, VP45, VP46, VP57, VP60, VP61, VP63, VP66, VP79, VP83, VP85, VP107, VP109, VP112, VP113, VP121, VP124, VP178, SVP77, SVP152, SVP160	2	0.14
AK/CTX	VP42, VP44, SVP138	2	0.14
AMP/CTX	VP28, VP29, VP46, VP50, VP99, VP127, VP147, VP175, SVP36, SVP157	2	0.14
AMP/K	VP12	2	0.14
AMP/TE	VP2, VP105	2	0.14
CTX/OT	SVP33	2	0.14
OT/TE	VP5	2	0.14
AK	VP47, VP49, SVP122	1	0.07

Table 4.5, continued.

Antibiograms	Isolates	Total Antibiotic Resistance	MAR Index
AMP	VP6, VP7, VP8, VP9, VP10, VP11, VP16, VP18, VP19, VP20, VP22, VP25, VP27, VP32, VP33, VP35, VP37, VP38, VP48, VP51, VP53, VP58, VP62, VP67, VP68, VP69, VP75, VP76, VP80, VP81, VP86, VP92, VP94, VP96, VP97, VP100, VP106, VP115, VP116, VP122, VP155, VP164, VP176, VP177, SVP6, SVP7, SVP11, SVP12, SVP17, SVP20, SVP28, SVP32, SVP60, SVP100, SVP106, SVP108, SVP110	1	0.07
CTX	VP23, VP26, VP40, SVP79	1	0.07
NA	SVP29	1	0.07

Ampicillin (AMP), Oxytetracycline (OT), Nalidixic acid (NA), Chloramphenicol (C), Cefotaxime (CTX), Sulfamethoxazole/Trimethoprim (SXT), Imipenem (IMP), Amikacin (AK), Ampicillin/Sulbactam (SAM), Levofloxacin (LEV), Ceftazidime (CAZ), Kanamycin (K), Gentamicin (CN), Tetracycline (TE).

4.5 Antibiotic Resistance Determinants

The isolates exhibiting selected antibiotic resistant phenotype are screened by PCR analysis to detect the presences of resistant genes in the isolates. The study detected two genes namely the chloramphenicol (*catA2*) gene and kanamycin (*aphA-3*) gene by PCR assay (Table 4.6). The *catA2* was detected in 18/22 chloramphenicol resistant isolates (VP160, VP179, VP180, VP181, VP182, VP183, VP184, VP185, SVP87, SVP88, SVP89, SVP90, SVP92, SVP125, SVP126, SVP143, SVP145 and SVP146). The absence of *catA2* gene in *V. parahaemolyticus* isolates displayed that phenotypic resistance to chloramphenicol may possibly due to the presence of other resistance mechanism that conferred the resistance. The *catA2* gene encodes an enzyme called chloramphenicol acetyltransferase. This enzyme is responsible to inactivate chloramphenicol by attaching to chloramphenicol, hence prevents subsequent binding of chloramphenicol to ribosomes of the bacteria (Roberts & Schwarz, 2009). The sequence of the *catA2* gene detected in the *V. parahaemolyticus* isolates showed 97.0% homology to previously sequenced *catA2* gene of *Vibrio* sp. chloramphenicol resistance protein (catII) gene.

Table 4.6: The results comparing the number of isolates displaying phenotypic resistance and the number of isolates that carrying the resistance genes.

Antibiotic Resistant Genes	*V. parahaemolyticus* isolates displayed phenotypic resistance (n)	*V. parahaemolyticus* isolates carrying the resistance genes (n) (%)
Chloramphenicol		
catA2	22	18 (82)
Kanamycin		
aphA-3	193	18 (9.3)

In addition, the study also detected the kanamycin *aphA-3* gene in 18/193 kanamycin resistant isolates. Of the eighteen isolates, 15 kanamycin isolates (VP84, VP133, VP134, VP136, VP138, VP140, VP142, VP143, VP151, VP153, VP160, VP161, VP162, VP163, and VP174) isolated from shrimp samples and 3 isolates (SVP55, SVP56, SVP70) from shellfish samples. The discrepancies in the phenotype antibiotic resistance shown by the isolates that did not have the corresponding resistant genes tested could be due to the presence of other antibiotic resistant genes or resistant mechanisms that may confer the similar resistance to respective antibiotics (Martineau *et al.,* 2000). The study did not detect any *β*-lactam resistance genes (*blaSHV, blaOXA* and *blaTEM*) which are normally found as plasmid-encoded *β*-lactamase and tetracycline resistance (*tetA, tetB, tetC* and *tetG*) gene among the *V. parahaemolyticus* isolates.

A two-way ANOVA was performed to determine the effects of antibiotic resistant genes and seafood type to the MAR index. Histogram of residuals and Levene's test were used to evaluate the assumptions of normality and homogeneity of variance respectively. All the assumptions were met. The main effect of antibiotic resistant gene on the MAR index was statistically significant, F (1, 382) = 75.21, $p < 0.001$ (Table 4.7). There was a significant difference of MAR index between seafood sample with antibiotic resistant gene and those without the resistant gene. Seafood sample that carried antibiotic resistant gene (M = 0.45, SD = 0.14) had a significant higher MAR index than those sample without the resistant gene (M = 0.25, SD = 0.15). The partial eta-squared (η^2) for this effect was 0.165.

The main effect of seafood types on the MAR index was also statistically significant, F (1, 382) = 58.18, $p < 0.001$ (Table 4.7). There was a significant difference of MAR index between shrimp and shellfish sample. Shellfish sample (M = 0.31, SD = 0.14) had significant higher MAR index compared to shrimp sample (M = 0.21, SD = 0.17). The

partial eta-squared (η^2) for this effect was 0.132. There was no significant interaction been observed between antibiotic resistant gene and seafood types, F (1, 381) = 3.55, p = 0.060, partial η^2 = 0.009.

Table 4.7: The main effects of the antibiotic resistant gene and seafood types on the MAR index (N = 385).

Factors		Mean (SD)	*F* (df)	*p*-value	Effect size (partial η^2)
Antibiotic resistant gene	No gene	0.25 (0.15)	75.21 (1, 382)	<0.001	0.165
	With gene	0.45 (0.14)			
Seafood types	Shrimp	0.21 (0.17)	58.18 (1, 382)	<0.001	0.132
	Shellfish	0.31 (0.14)			

4.6 Plasmid Profiles of *V. parahaemolyticus* Isolates

Three hundred and eighty-five *V. parahaemolyticus* isolates were analyzed for the presence of plasmids. Only 338 *V. parahaemolyticus* isolates harboured 1-7 different plasmids (Figure 4.8) and could be categorized into 27 patterns based on the number and pattern of plasmid present (Table 4.8). The sizes of plasmids ranged from 1.2kb to above 10kb. As shown in Table 4.8, from 27 plasmid profiles, the profile that forms the largest group was the plasmid profile 1.3 that consisted of 1 band above 10kb size plasmid. A total of 95 isolates (24.7%) harboured plasmid profile 1.3. Additionally, in this profile, 22 isolates were from shellfish samples and 73 isolates were from shrimp samples. The isolates grouped in this plasmid profile were identified to be resistant to at least one typed of the antibiotic tested (as shown in Table 4.5). The isolate VP152 from supermarket banana prawn and isolate SVP129 from supermarket carpet clam which exhibited

resistance profile towards 11/14 antibiotics tested respectively were grouped under plasmid profile 1.3. The study findings correlate with previous studies that reported *V. parahaemolyticus* harboured plasmids (Kagiko *et al.,* 2001; Kaufman *et al.,* 2002; MolinaAja *et al.,* 2002; Manjusha *et al.,* 2005; Zulkifli *et al.,* 2009).

From the total 27 plasmid patterns, 8 patterns (30%) comprised of one isolate in each group. Within these 8 patterns, three isolates were identified to harbour six to seven plasmid profiles respectively. The isolate VP53 from wetmarket red prawn sample was only ampicillin resistant and harboured 6 plasmids profile size (1.2kb, 1.7kb, 2.5kb, 7kb, 10kb, above 10kb). Where else, isolate VP183 that was resistant towards 5/14 antibiotic tested (AK/AMP/C/OT/TE) and SVP61, a *trh*-positive isolate resistant towards 4/14 antibiotic tested (AMP/CTX/AK/CAZ) respectively harboured seven plasmids each. Overall, a total of 47/385 isolates (12%) did not express any plasmid profiles. The results demonstrated high discriminatory power of plasmid profiling conducted in this study.

Table 5.2 shows an interesting relationship between the antibiotic resistance and plasmid profiles of the 32 *trh*-positive *V. parahaemolyticus* isolates. 21/32 *trh*-positive isolate contained 1-7 plasmids, where else another 11 isolates did not exhibit any plasmid profiles. All the *trh*-positive isolates were resistant to at least one type of antibiotic tested in study except isolate VP98 that was not resistant to any antibiotic and did not harbour any plasmid profile.

4.7 Plasmid Curing of *V. parahaemolyticus* Isolates

In this study, two different intercalating agents – acridine orange (AO) and ethidium bromide (EB) were used to determine the antibiotic resistance mediation. The plasmid curing revealed that both intercalating agents AO and EB produced same curing profiles of isolate and the results is demonstrated in Figure 4.9.

All 338 *V. parahaemolyticus* isolates that harbour 1-7 different plasmid ranging of size 1.2kb to above 10kb in size lost their plasmids upon being subjected to curing agents. In Figure 4.8, it could be observed that 327 *V. parahaemolyticus* isolates that were resistant towards ampicillin before plasmid curing showed the same phenotype resistance after plasmid curing. Similar resistance pattern could be observed in a group of 57 tetracycline resistant isolates. The plasmid curing results revealed that 51/57 isolates (89%) were still resistant towards tetracycline. This suggests that the resistance phenotype to ampicillin and tetracycline expressed by the isolates could be chromosomally mediated. All the ampicillin/sulbactam resistant strains lost their plasmid after the curing assay and subsequently were susceptible to ampicillin/sulbactam suggesting that the resistance was plasmid mediated. The antibiotic resistant profiles of OT/C/CTX/SXT/AK/CAZ/K/CN presented after plasmid curing had lower number of resistant isolates towards respective antibiotic. These results demonstrate that the phenotype resistance observed could be both plasmid and chromosomal mediated.

Prior to plasmid curing, the chloramphenicol (*catA2*) gene was detected in 18/22 chloramphenicol-resistance *V. parahaemolyticus* isolates. The curing results revealed six of the isolates had the gene present in their plasmid where else another twelve isolates showed possibility of chromosomal-mediated since these isolates exhibited positive *catA2* gene amplification and demonstrated phenotypic resistance to chloramphenicol on the disc diffusion test after plasmid curing. The result of plasmid curing revealed that 18/193 kanamycin-resistant isolates were potentially chromosomal-mediated since the isolates exhibit positive amplification with *aphA-3* gene and demonstrated phenotypic resistance to kanamycin on the disc diffusion test after plasmid curing.

With reference to the 32 *trh*-positive *V. parahaemolyticus* isolates (Table 4.9), the antibiotic resistance profile of the 21 plasmid containing isolates changed after curing while the remaining 10 were unchanged. All 20/21 were ampicillin resistant initially, and after curing, the isolates (SVP61, SVP54, SVP75, SVP69, SVP72, VP89, VP90, VP91, VP92, VP93, VP94, VP95, VP99, VP101, VP102, VP102, VP103, VP1175, VP176, VP177, VP178) remained resistant to ampicillin and cefotaxime, and became susceptible to the other antibiotics tested. One isolate (SVP73) became susceptible to all antibiotic resistant after plasmid curing, suggesting the resistance phenotype observed was plasmid mediated. This suggests that while antibiotic resistance is mediated by both plasmid and chromosomes in pathogenic *V. parahaemolyticus* isolates, in plasmid containing strains aside from ampicillin and cefotaxime resistance, most of the remaining resistance phenotypes are plasmid mediated.

SVP129 isolate contained one plasmid profile with size more than 10kb and expressed antibiotics resistance towards 11/14 antibiotics tested. After plasmid curing, SVP129 isolate lost its plasmid and changed its antibiotic resistance phenotype. SVP129 isolate remained resistant to 5/14 antibiotic tested namely ampicillin, oxytetracycline, chloramphenicol, tetracycline and sulfamethoxazole/trimethoprim. The isolate showed intermediate resistance to amikacin, ceftazidime, cefotaxime and kanamycin, while it was susceptible to gentamycin and ampicillin/sulbactam after plasmid curing assay.

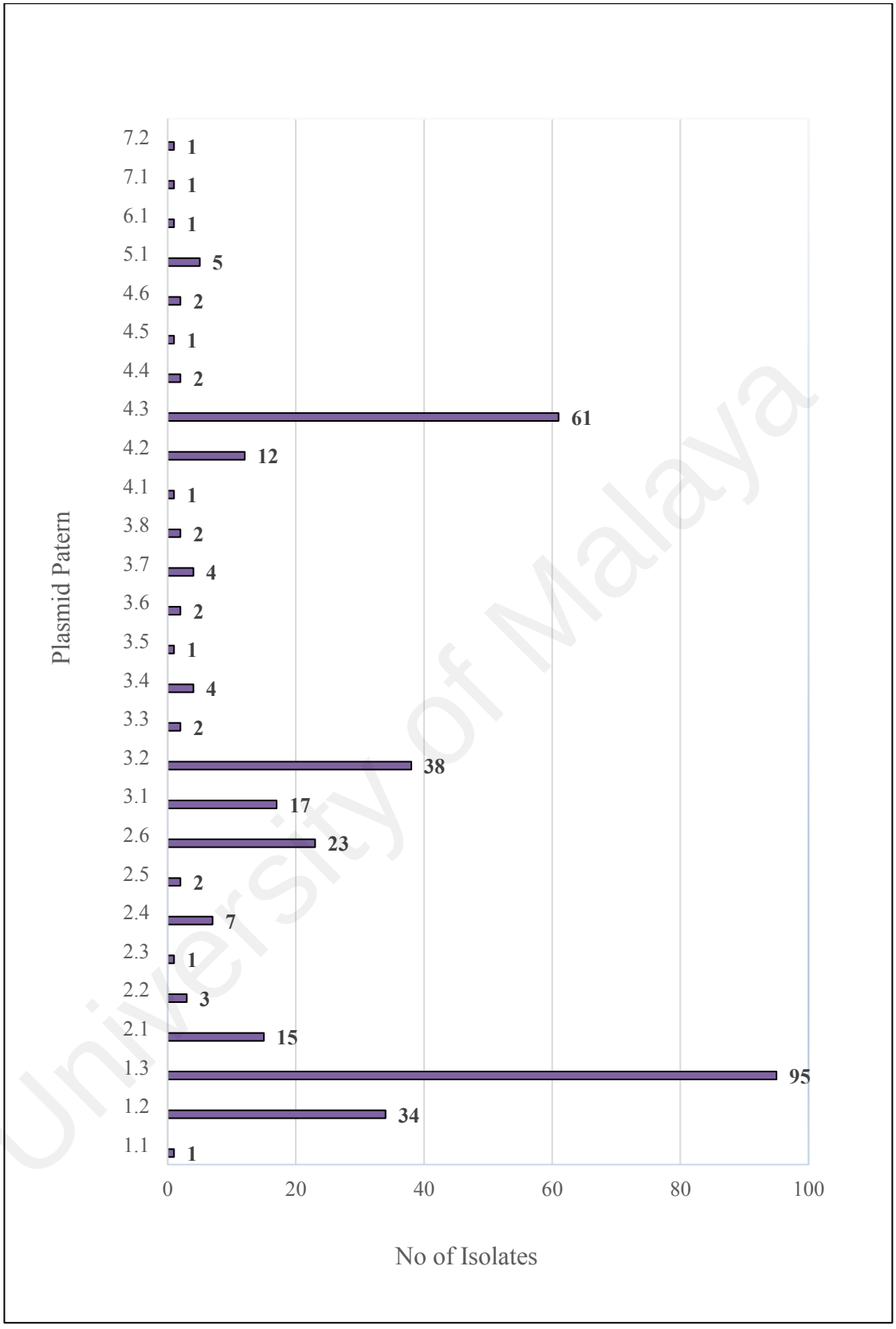

Figure 4.8: Bar Chart on plasmid profile of 385 *V. parahaemolyticus* isolates. The Y-axis represents different type of plasmid pattern while the X-axis represents the number of isolates that possess the particular pattern.

Table 4.8: Plasmid profile of 385 *V. parahaemolyticus* isolates.

No of Plasmid	Plasmid Pattern	Plasmid size (kb)	No of Isolates	Isolate Description
1	1.1	1.75	1	SVP69
	1.2	10	34	VP59, VP64, VP66, VP70, VP80, VP81, VP82, VP83, VP84, VP85, VP118, VP119, VP120, VP123, VP124, VP153, VP154, VP155, VP156, VP157, VP158, VP159, SVP54, SVP62, SVP63, SVP65, SVP67, SVP68, SVP79, SVP80, SVP81, SVP83, SVP84
	1.3	above 10	95	VP7,VP8, VP9, VP13, VP14, VP15, VP16, VP18, VP19, VP20, VP23, VP24, VP25, VP26, VP27, VP29, VP30, VP31, VP32, VP33, VP36, VP37, VP47, VP48, VP49, VP50, VP51, VP52, VP71, VP72, VP73, VP74, VP77, VP78, VP79, VP87, VP88, VP89, VP90, VP91, VP92, VP105, VP125, VP126, VP127, VP128, VP129, VP130, VP131, VP133, VP134, VP135, VP136, VP137, VP138, VP142, VP143, VP144, VP145, VP146, VP147, VP152, VP160, VP161, VP162, VP163, VP164, VP165, VP166, VP167, VP168, SVP9, SVP10, SVP13, SVP18, SVP29, SVP36, SVP45, SVP57, SVP58, SVP59, SVP71, SVP73, SVP74, SVP75, SVP76, SVP77, SVP85, SVP86, SVP103, SVP104, SVP105, SVP107, SVP129
2	2.1	1.2, 1.7	15	VP121, VP122, VP148, VP149, VP150, VP151, VP169, VP170, VP171, VP172, VP173, VP174, VP175, VP176, VP177
	2.2	1.2, 2	3	SVP14, SVP15, SVP16
	2.3	2.5, above 10	1	SVP72
	2.4	4,10	7	VP60, VP61, VP62, VP93, VP94, VP95, VP99
	2.5	10, above 10	2	VP45, VP46
	2.6	two above 10	23	VP10, VP35, VP107, VP108, VP109, VP110, VP111, VP112, VP113, VP114, VP115, VP116, VP117, SVP19, SVP21, SVP42, SVP146, SVP147, SVP196, SVP197, SVP198, SVP199, SVP200

Table 4.8, continued.

No of Plasmid	Plasmid Pattern	Plasmid size (kb)	No of Isolates	Isolate Description
3	3.1	1, 1.7, above 10	17	VP,67, VP68, VP132, VP139, VP140, VP141, SVP30, SVP31, SVP33, SVP43, SVP44, SVP46, SVP47, SVP49, SVP50, SVP51, SVP53
	3.2	1.2, 2, above 10	38	VP101, VP102, VP103, VP104, VP178, VP180, VP181, VP284, VP185, SVP1, SVP2, SVP3, SVP4, SVP22, SVP23, SVP24, SVP25, SVP26, SVP27, SVP150, SVP151, SVP153, SVP154, SVP155, SVP156, SVP161, SVP162, SVP163, SVP164, SVP165, SVP166, SVP167, SVP168, SVP169, SVP170, SVP171, SVP172
	3.3	2, 3, 10	2	VP58, SVP91
	3.4	2, 10, above 10	4	VP21, VP22, VP42, VP43
	3.5	2, 5.2, above 10	1	VP28
	3.6	4, 10, above 10	2	VP41, VP44
	3.7	7, 10, above 10	4	VP54, VP55, SVP87, SVP88
	3.8	10, two above 10	2	VP56, SVP89
4	4.1	1.2, 1.7, 4, above 10	1	VP182
	4.2	1.2, 1.7, 10, above 10	12	SVP111, SVP112, SVP117, SVP118, SVP119, SVP144, SVP145, SVP148, SVP149, SVP157, SVP158, SVP159

Table 4.8, continued.

No of Plasmid	Plasmid Pattern	Plasmid size (kb)	No of Isolates	Isolate Description
	4.3	1.2, 1.7, two above 10	61	SVP34, SVP35, SVP38, SVP39, SVP40, SVP41, SVP48, SVP92, SVP94, SVP95, SVP96, SVP97, SVP98, SVP99, SVP101, SVP102, SVP120, SVP121, SVP123, SVP124, SVP125, SVP126, SVP127, SVP128, SVP130, SVP131, SVP132, SVP133, SVP134, SVP135, SVP136,SVP137, SVP138, SVP139, SVP140, SVP141, SVP142, SVP143, SVP144, SVP174, SVP175, SVP176, SVP177, SVP178, SVP179, SVP180, SVP181, SVP182, SVP183, SVP184, SVP185, SVP186, SVP187, SVP188, SVP189, SVP190, SVP191, SVP192, SVP193, SVP194, SVP195
	4.4	2, 3, 10, above 10	2	VP57, SVP90
	4.5	2, 4, 6.2, above 10	1	VP63
	4.6	5.2, 10, two above 10	2	VP11, VP12
5	5.1	1.2, 1.7, 2.5, two above 10	5	SVP113, SVP114, SVP115, SVP116, SVP173
6	6.1	1.2, 1.7, 2.5, 7, 10, above 10	1	VP53
7	7.1	1.2, 1.7, 2, 10, three above 10	1	VP183
	7.2	1.2, 1.75, 3, 3.1, 4, two above 10	1	SVP61

Table 4.9: List of *trh*-positive *V. parahaemolyticus* isolates description before and after plasmid curing.

Isolates	Samples	Location	*toxR*-positive	*trh*-positive	Before plasmid curing			After plasmid curing	
					Antibiotic resistance pattern	No of plasmid	Plasmid size (kb)	Antibiotic resistance pattern	No of plasmids
SVP55	Mud crab	Wetmarket B	+	+	AK/AMP/CAZ/CTX/K	None		AK/AMP/CAZ/CTX/K	
SVP56	Mud crab	Wetmarket B	+	+	AK/AMP/CAZ/CTX/K	None		AK/AMP/CAZ/CTX/K	
SVP60	Mud crab	Wetmarket B	+	+	AMP	None		AMP	
SVP61	Mud crab	Wetmarket B	+	+	AK/AMP/CAZ/CTX	7	1.2, 1.75, 3,3.1, 4, two above 10	AMP	Lost
SVP64	Mud crab	Wetmarket B	+	+	AK/AMP/CTX	None		AK/AMP/CTX	
SVP66	Mud crab	Wetmarket B	+	+	AK/AMP/CAZ/CTX	None		AK/AMP/CAZ/CTX	
SVP52	Carpet clam	Wetmarket B	+	+	AK/AMP/CTX	None		AK/AMP/CTX	
SVP54	Carpet clam	Wetmarket B	+	+	AK/AMP/CAZ/CTX/K/LEV	1	above 10	AMP	Lost
SVP73	Mud creeper	Wetmarket B	+	+	AK/CTX/K	1	above 10	All susceptible	Lost

Table 4.9, continued.

Isolates	Samples	Location	*toxR*-positive	*trh*-positive	Before plasmid curing			After plasmid curing	
					Antibiotic resistance pattern	No of plasmid	Plasmid size (kb)	Antibiotic resistance pattern	No of plasmids
SVP75	Mud creeper	Wetmarket B	+	+	AK/AMP/CTX/K	1	above 10	AMP	Lost
SVP69	Hard shell clam	Wetmarket B	+	+	AK/AMP/CAZ/CTX	1	1.75	AMP	Lost
SVP70	Hard shell clam	Wetmarket B	+	+	AK/AMP/CAZ/CTX/K	None		AK/AMP/CAZ/CTX/K	
SVP72	Hard shell clam	Wetmarket B	+	+	AK/AMP/CAZ/CTX	2	2.5, one above 10	AMP	Lost
VP89	Red prawn	Supermarket C	+	+	AK/AMP/CTX	1	above 10	AMP/CTX	Lost
VP90	Red prawn	Supermarket C	+	+	AK/AMP/CTX/CAZ	1	above 10	AMP	Lost
VP91	Red prawn	Supermarket C	+	+	AK/AMP/CTX	1	above 10	AMP/CTX	Lost
VP92	Red prawn	Supermarket C	+	+	AMP	1	above 10	AMP	Lost
VP93	Banana prawn	Wetmarket A	+	+	AK/AMP/K	2	4, 10	AMP	Lost
VP94	Banana prawn	Wetmarket A	+	+	AMP	2	4, 10	AMP	Lost

Table 4.9, continued.

Isolates	Samples	Location	*toxR*-positive	*trh*-positive	Before plasmid curing			After plasmid curing	
					Antibiotic resistance pattern	No of plasmid	Plasmid size (kb)	Antibiotic resistance pattern	No of plasmids
VP95	Banana prawn	Wetmarket A	+	+	AK/AMP/CTX/K	2	4, 10	AMP/CTX	Lost
VP96	Banana prawn	Wetmarket A	+	+	AMP	None		AMP	
VP97	Banana prawn	Wetmarket A	+	+	AMP	None		AMP	
VP98	Banana prawn	Wetmarket A	+	+		None			
VP99	Banana prawn	Wetmarket A	+	+	AMP/CTX	2	4,10	AMP/CTX	Lost
VP100	Banana prawn	Wetmarket A	+	+	AMP	None		AMP	
VP101	Banana prawn	Wetmarket A	+	+	AK/AMP/K	3	1.2, 2, above 10	AMP	Lost
VP102	Banana prawn	Wetmarket A	+	+	AK/AMP/CAZ/CTX/K	3	1.2, 2, above 10	AMP/CTX	Lost
VP103	Banana prawn	Wetmarket A	+	+	AK/AMP/CAZ/CTX/K	3	1.2, 2, above 10	AMP/CTX	Lost

Table 4.9, continued.

Isolates	Samples	Location	*toxR*-positive	*trh*-positive	Before plasmid curing			After plasmid curing	
					Antibiotic resistance pattern	No of plasmid	Plasmid size (kb)	Antibiotic resistance pattern	No of plasmids
VP175	Banana prawn	Supermarket C	+	+	AMP/CTX	2	1.2, 1.7	AMP/CTX	Lost
VP176	Banana prawn	Supermarket C	+	+	AMP	2	1.2, 1.7	AMP	Lost
VP177	Banana prawn	Supermarket C	+	+	AMP	2	1.2, 1.7	AMP	Lost
VP178	Banana prawn	Supermarket C	+	+	AK/AMP	3	1.2, 1.7, above 10	AMP	Lost

Ampicillin (AMP), Oxytetracycline (OT), Nalidixic acid (NA), Chloramphenicol (C), Cefotaxime (CTX), Sulfamethoxazole/Trimethoprim (SXT), Imipenem (IMP), Amikacin (AK), Ampicillin/Sulbactam (SAM), Levofloxacin (LEV), Ceftazidime (CAZ), Kanamycin (K), Gentamicin (CN), Tetracycline (TE).

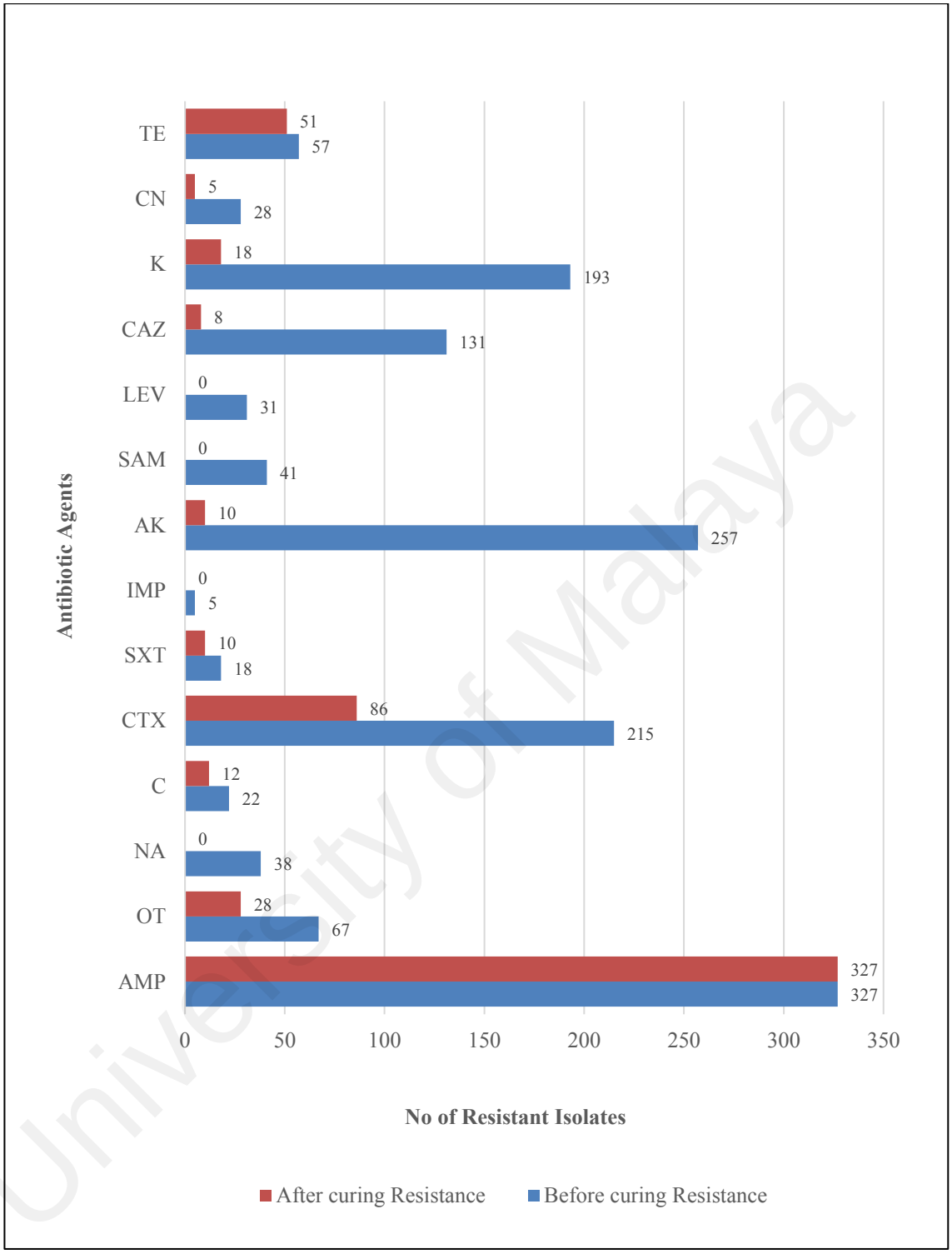

Figure 4.9: Bar Chart on antibiotic resistance profiles of *V. parahaemolyticus* before and after plasmid curing. The Y-axis represents different type of antibiotic agents while the X-axis represents the number of resistant isolates towards the antibiotic agents. Ampicillin (AMP), Oxytetracycline (OT), Nalidixic acid (NA), Chloramphenicol (C), Cefotaxime (CTX), Sulfamethoxazole/Trimethoprim (SXT), Imipenem (IMP), Amikacin (AK), Ampicillin/Sulbactam (SAM), Levofloxacin (LEV), Ceftazidime (CAZ), Kanamycin (K), Gentamicin (CN), Tetracycline (TE).

CHAPTER 5: DISCUSSION

Vibrio sp. is a pathogenic bacterium that causes harm to human and found in aquatic and sea surroundings. Among the *Vibrio* sp., *V. cholerae* and *V. parahaemolyticus* have been known as causative agent of foodborne illnesses and outbreaks linked with eating of raw or undercooked seafood (Letchumanan *et al.,* 2014). There are many outbreaks of gastroenteritis that were caused by *V. parahaemolyticus* in several countries and regions of the world including Japan, India, Malaysia, Thailand, Taiwan, and USA (Abd-Elghany & Sallam, 2013). This study aimed to evaluate the presences of *V. parahaemolyticus* and characterise antibiotic resistant profiles of *V. parahaemolyticus* from shrimp and shellfish samples in order to obtain beneficial data for future biosafety assessment.

5.1 Enumeration of *Vibrio* sp. in Seafood Samples

The sampling selection of this study was based on the high number of reported cases of *V. parahaemolyticus* associated with shrimp and shellfish (Zhang *et al.,* 2013; West *et al.,* 2013; Xu *et al.,* 2014; Yu *et al.,* 2016). Shrimp and shellfish have a close relationship with their individual habitat in the aquatic environments. The filter feeder characteristics of a shellfish enables them to filter larger volvume of seawater to attain food (Yu *et al.,* 2016). During filter-feeding process, shellfish have a habit of collecting and keeping bacteria resultant from sewage pollution (Lees, 2000) or aquatic environments. The pathogens take this oppurnity to utilize the shellfish as vehicle to transmit foodborne illness. Likewise, shrimp hatcheries are very prone to *Vibrio* sp. infections during the cultivation process thus leading to early mortality syndrome (EMS) among the shrimps (Zorriehzahra & Banaederakhshan, 2015). *V. parahaemolyticus* was identified as one of the causative agents of EMS infection and subsequently could possibly transmit vibriosis to humans via shrimps. Therefore, these mentioned characteristics of shrimps and shellfish makes them a suitable sampling candidate for this study.

This study detected and isolated *V. parahaemolyticus* from 770 samples comprising of shrimp and shellfish. The samples were collected from three wetmarkets and three supermarkets in Selangor, Malaysia. The sample size of this study is relatively higher to those reported studies in Malaysia and Asian region (Malcolm *et al.,* 2015; Xie *et al.,* 2015; Yu *et al.,* 2016; Tan *et al.,* 2017). Microbiological method using thiosuphate citrate bile salt agar (TCBS) was employed to enumerate and isolate *V. parahaemolyticus* from seafood samples. Based on the colony feature seen during the enumeration step, all the seafood samples exhibited the presences of *Vibrio* sp. The seafood samples analyzed had a microbial load of 2.29 log CFU/g to 6.63 log CFU/g. The wetmarket samples had a higher mean total *Vibrio* sp. count compared to the supermarket samples. The variance in the occurrence of *Vibrio* sp. in seafood samples from each sampling locations might be instigated by environmental origin of sources from which the seafood was collected, post-harvest methods, and good sanitation practice through handling, conveyance, and storage of the marine crops.

There was limited access to the origin of each seafood sample, hence the sampling site were observed and inspected. The atmosphere at both sampling sites are different and this probably the cause of higher *Vibrio* sp counts in wetmarket seafood samples. The wetmarket site are more prone to contamination because the seafood was managed in a poor sterile manner by the vendors comparative to vendors in supermarkets. At the wetmarket locations, the seafood is usually displayed on ice rack which is left at room temperature and eventually melts allowing a favaourable condition for pathogens on the seafood. The atmoshepre is in reverse at the supermarket sites where the seafood is on ice rack and at air-conditional temperature. The variance of surrounding temperature is the causative factor for the high microbial load of *V. parahaemolyticus* in wetmarket samples. In order to lower the risk and levels of *Vibrio* sp. in fish, the food must be placed in cold temperature throughout the delivery and storage (Elhadi *et al.,* 2004). Our results are in

agreement with studies around India and China. In a similar study design, high occurrence of pathogenic *Vibrio* sp. detected in shellfish gathered from roadside stalls in comparison to markets in Cochin, India (Sudha *et al.,* 2014). Likewise, in China, there was a study reported 59.7% of *V. parahaemolyticus* isolated from retail shellfish samples (Zhao *et al.,* 2011). These findings demonstrate that seafood samples are commonly contaiminated with *Vibrio* sp. regardless the sampling locations weather its wetmarket or supermarkets.

This study results are in line agreement with previous reports that stated the occurrence of *V. parahaemolyticus* in supermarket is maybe due to improper handling, lack of hygiene, cross contamination or difference in storage temperature during delivery (Yang *et al.,* 2008; Tunung *et al.,* 2010; Sudha *et al.,* 2014). *V. parahaemolyticus* cells multiple rapidly with increase in ambient temperature, hence non-refrigerated post-harvest storage may possible cause cosumers to the exposure risk of pathogenic *V. parahaemolyticus* (Sudha *et al.,* 2014). However, *V. parahaemolyticus* cell viability can be reduced if the seafood is maintained on ice (Su & Liu, 2007). Therefore, in effort to reduce the risk of *V. parahaemolyticus* in seafood, retailers should be well educated on seafood handling techniques, storage temperature conditions, and proper hygiene.

5.2 Occurrence of *V. parahaemolyticus* in Seafood Samples

In order to evaluate the definite hazard to human wellbeing caused by the incidence of *V. parahaemolyticus* in seafood, it is essential to identify all the pathogenic strains by virulence factors identification via PCR assay. 770 presumptive *V. parahaemolyticus* isolate were isolated from all the seafood samples. Although the microbiological method found all the samples contaiminated with *Vibrio* sp., only 50% (385/770) of isolates were confirmed to be *V. parahaemolyticus* based on *toxR* assay and only 10% (32/385) were found to be pathogenic (*trh*-positive).

The *trh* gene is known as a virulence genes of *V. parahaemolyticus* and has alike features of *tdh* gene in the *V. parahaemolyticus* pathogenicity of infections (Nelapati *et al.,* 2012). This study's findings are in line with previous report from Malaysia that detected 12% (6/50) isolates to harbor *trh* gene in the food samples (Paydar *et al.,* 2013). Likewise, another study by Al-Othrubi *et al.* (2014) in Malaysia reported detected eight *tdh*-positive isolate and twenty-six *trh*-positive isolates from shrimp and cockle samples studied. Similar foodborne studies in USA and Japan have also reported only 3% pathogenic *V. parahaemolyticus* isolated from seafood and environments (Mahmoud *et al.,* 2006; Abd-Elghany & Sallam, 2013). Normally, only 1-2% of environmental isolates are identified to harbour the either virulence *tdh* and/or *trh* genes (Wong *et al.,* 2000b; Hervio-Heath *et al.,* 2002; Alam *et al.,* 2002; Velazquez-Roman *et al.,* 2012; Haley *et al.,* 2014). The variation in the occurrence of pathogenic *V. parahaemolyticus* between the studies may be associated with differences in sampling techniques, sample sources, and the detection techniques employed. The environmental factors including interaction with other hosts play a huge effect in the evolution of certain pathogen in the environment (Wilson & Salyers ,2003). Overall, the level of virulent isolates in the study is low (8.3%) and not in worrying state, however, the results should not be overlooked. A continuous surveillance should be carried on to manage the occurrence of *V. parahaemolyticus* in the environment and ensure food safety.

5.3 Antibiotic Susceptibility Test

The continuous monitoring of antibiotic resistance in *V. parahaemolyticus* isolates is needed due to the prevalent cases of antibiotic resistance reported in several countries worlwide (Al-Othrubi *et al.,* 2011; Zhang *et al.,* 2012; Kim *et al.,* 2013; Pazhani *et al.,* 2014; Sudha *et al.,* 2014; Chikwendu *et al.,* 2014; Reyhanath & Kutty, 2014; Shrestha *et al.,* 2015; Khan *et al.,* 2015). Antibiotics are often employed in aquafarming as food

additives or directly in water to attain any control or prevention of bacterial infections (Devi *et al.,* 2009; Manjusha & Sarita, 2011; 2012). Nevertheless, the misuse of these permitted antibiotics in the agriculture and aquaculture environments has instigated the increasing number of multidrug resistant among foodborne pathogens including *Vibrio* sp. and as well as distribution of resistant genetic factors inside the microbial populace (Tendencia & Rena, 2002; Reboucas *et al.,* 2011; Sudha *et al.,* 2014).

The study's susceptibility test placed the 1st generation antibiotic – ampicillin at the top of the *V. parahaemolyticus* resistance scope (85%) (Table 4.3). This finding is in agreement with previous reports from India, Indonesia, Korea and Malaysia that reported prevalent of ampicillin resistant *V. parahaemolyticus* strains isolated from seafood samples (Okuda *et al.,* 1997; Lesmana et al., 2001; Han *et al.,* 2007; Devi et al., 2009; Zulkifli *et al.,* 2009; Melo *et al.,* 2011; Oh *et al.,* 2011; Al-Othrubi *et al.,* 2014; Letchumanan *et al.,* 2015a). The 1st generation antibiotics including ampicillin has a very low efficacy in treatment of infections due to the misuse of these antibiotics in aquaculture and agriculture which in turn led to a low susceptibility rate (Sudha *et al.,* 2014). These findings signify that ampicillin is ineffective for the treatment and control of *Vibrio* sp. infections. The occurrence of high ampicillin resistance rate in the environment is still of great concern since the resistance phenotype seen could be chromosomally mediated in the bacteria thus require appropriate management method to control the resistance phenotype (Lee *et al.,* 2018).

Interestingly, multidrug resistance pattern was observed among the 32 *trh*-positive *V. parahaemolyticus*. These pathogenic isolates were seen resistant to aminoglycosides, 3rd generation cephalosporins, and quinolone. The *V. parahaemolyticus* isolates showed high resistance rate towards the 3rd generation cephalosporins – cefotaxime (55.8%) and ceftazidime (34%) in this study. These findings are in line agreement with Sahilah *et al.*

(2014) in Terengganu, Malaysia, who reported ceftazidime and cefuroxime resistant *V. parahaemolyticus* isolates from shellfish. In the neighboring country, Korea, another similar study reported high percentage (70%-80%) of *V. parahaemolyticus* isolates from Korean seafood to be resistant to the 3rd generation cephalosporin, cefotaxime and ceftazidime (Jun *et al.,* 2012). In contrast, a study from the US reported low percentage of cefotaxime resistant *V. parahaemolyticus* isolates isolated from food (Shaw *et al.,* 2014). The discrepancies in the resistance rate of *V. parahaemolyticus* to 3rd generation cephalosporin may be due to different sample type, geographical variations, or difference in methodology test applied.

Reassuring, the isolates in this study was still susceptible to some antibiotics tested including imipenem (94%) (Table 4.3). Nevertheless, there was five isolates (SVP90, VP71, VP114, VP145, VP146) from this study exhibited resistance towards imipenem. The discovery of imipenem resistant isolates raises concern as carbapenems are the most potent *β*-lactam antibiotic and usually administrated in treatment of any serious bacterial infections (Meletis, 2016). The results are in agreement with previous reported findings on the isolation of carbapenem-resistant *Vibrio* sp. from environmental samples. Walsh and colleagues reported carbapenem resistant *V. cholerae* isolated from drinking water and seepage in New Dehli, India and further analysis revealed that the carbapenem gene bla_{NDM-1} was found in the chromosome of *V. cholerae* isolate (Walsh *et al.,* 2011). The occurrence of carbapenem resistance was also detected in a *V. cholerae* 01 El Tor Ogawa strain isolated from fecal specimen of a 2-year-old child in Puducherry, India. Another study reported an increasing trend of carbapenem resistance among *V. cholerae* 01 or 0139 isolates between 1986 to 2012 in southwest China (Gu *et al.,* 2014). Recently, Bier and colleagues reported the isolation of four carbapenem resistant *V. cholerae* from different locations of the German coast line. These four isolates were not only resistant to carbapenem but also exhibited resistance to cefoxitin, aztreonam, and amiopenicillin

(Bier *et al.,* 2015). In addition, there have been reports on the emergence and spread of carbapenem-resistant *Enterobacteriaceae* (CRE) in the United States (US) (Guh *et al.,* 2014; Vasoo *et al.,* 2015). Any infections with carbapenem resistant bacteria may cause higher mortality rates compared to those infections caused by carbapenem-susceptible bacteria. The wide incidence of carbapenem resistant *Vibrio* sp. is an important emerging threat to public health, thus requires proper management action to limit the spread of this organism.

Of the 102 different antibiograms observed in this study (Table 4.5), seventy-six different resistance profiles had a significant MAR value more than 0.2. Collectively, these profiles were expressed by 68% of the *V. parahaemolyticus* isolates and resistant to 3 to 11 types of antibiotics tested. This result is in agreement with a study by Elexson *et al.* (2014) in Malaysia that reported 97.2% of *V. parahaemolyticus* isolates studied had MAR index > 0.2, however a lower percentage (14.5%) with MAR index more than 0.2 was reported by Tang *et al.* (2014). The vast difference in the MAR index of the *V. parahaemolyticus* isolates seen in past and present findings from Malaysia are contributed by the resistant level reliant on the origin of collected samples (Khan *et al.,* 2007; Tunung *et al.,* 2012). Lesley *et al.* (2011) proposed that the alteration in topographical sites could have difference discerning pressures for the antimicrobial resistant levels.

In this study, the sampling location and MAR index of *V. parahaemolyticus* isolates was not significantly different. The result shows that the *V. parahaemolyticus* isolates from wetmarkets and supermarkets is contaiminated with antibiotics from the aquatic fields. Our results are in corresponding to several other reports that discovered soaring proportion of *V. parahaemolyticus* isolated from seafood are resistant to more than one antibiotic tested (Zulkifli *et al.,* 2009; Lesley *et al.,* 2011; Manjusha & Sarita, 2011; Noorlis *et al.,* 2011). According to the One-way ANOVA analysis results, the groups of

sampling location and the MAR index of *V. parahaemolyticus* isolates was significantly different at $p < 0.05$. The isolates from the supermarket sampling sites had a greater mean MAR index compared to the isolates from wetmarket sampling sites. This situation could be attributed to the geographical change in seafood samples that been sold in supermarket, thus causing a MAR resistance profile. In addition, it could be suggested that seafood samples could have derived from alike ecological situations depending on the exposure of antibiotic or cross contamination could have happened thru the post-harvest, resulting in the isolates to have similar MAR index.

5.4 Antibiotic Resistance Determinants

The present study identified the association between the antibiotic resistance phenotypes of *V. parahaemolyticus* isolates and the presences of antibiotic resistance genes. A total of 12 resistant genes (Table 4.6) were amplified to detect ampicillin, tetracycline, chloramphenicol and kanamycin resistant isolates by PCR. The selection of antibiotic resistant genes was based on the use of the selected antibiotic in the aquaculture settings and reports of occurence of these genes in the environments. Chloramphenicols are still often been used in the aquaculture settings thus causing the rising number of bacteria exhibiting the resistance phenotype. The Food Safety News, 2016 also reported the ban on shrimp and prawns from Malaysia to the US due to residue of chloramphenicols is these seafood. Similarly, kanamycin, a type of aminoglycosides are among the antibiotics used in hospital treatment and misuse of this antibiotic causes the residue to flow to river or stream by sewage treatment plants and spread of the resistant genes among bacteria. These choosen gene primers were also utilized in studies to detect resistance in *V. parahaemolyticus* as well as from past studies detecting resistance in bacteria of different genera for example *Pseudomonas*, *Acinetobacter*, *Stenotrophomonas*, *Bacillus*, *Campylobacter*, *Klebsiella* and *Lactobacillus* (Gibreel *et al.,* 2004; Soge *et al.,* 2006; Kim *et al.,* 2013). Hence, screening of

these genes are important to understand the selected antibiotic efficacy in treatment of infection.

This study, detected *catA2* gene (chloramphenicol) and *aphA-3* (kanamycin) among the resistant isolates and verifies these isolates are encoded with the respective resistance genes. The *catA2* gene encodes an enzyme called chloramphenicol acetyltransferase and functions to deactivate chloramphenicol by conferring to chloramphenicol, thus stops the following binding of chloramphenicol to ribosomes of the bacteria (Roberts & Schwarz, 2009). The sequence of the *catA2* gene detected in *V. parahaemolyticus* isolates showed 97.0% homology to previously sequenced *catA2* gene of *Vibrio* sp. chloramphenicol resistance protein (catII) gene. The inconsistencies in the phenotype antibiotic resistance shown by the isolates did not have the corresponding resistance genes tested could be due to the occurrence of additional antibiotic resistance genes or resistance mechanisms that could result in similar resistance to a particular antibiotic (Martineau *et al.,* 2000). These types of inconsistency have been extensively discussed in several studies that reported amplified resistant genes are not conferred within the resistant *Vibrio* sp. strains (Raissy *et al.,* 2012; Chiwendu *et al.,* 2014; Jiang *et al.,* 2014).

The present study did not identify any *β*-lactam resistance genes (*blaSHV, blaOXA* and *blaTEM*) which are generally originated as plasmid-encoded *β*-lactamase and tetracycline resistance (*tetA, tetB, tetC* and *tetG*) gene among the *V. parahaemolyticus* isolates. Since the ampicillin-resistant genes are diversified, the contrary results of the tested *β*-lactamase genes as well as tetracycline genes may well due to carrying of different encoding genes by the ampicillin and tetracycline resistant isolates in the present study. For example, a class A extended spectrum *β*-lactamase gene, bla_{PER-1}, which is mostly linked with Gram-negative clinical pathogens for instance *Pseudomonas aeruginosa* (Qing *et al.,* 2014) was also detected among *V. parahaemolyticus* (Wong *et al.,* 2012; Liu *et al.,* 2013). It was

reported in a study that ampicillin resistance of *V. parahaemolyticus* was not conferred by the *bla* gene but was mediated by an efflux system (Pazhani *et al.,* 2014). Similarly, it was reported that the incidence of tetracycline genes *tetM* and *tetS* in *Vibrio* sp. from seawater in Japan and Korea may be a critical reservoir of tetracycline resistance genes in the sea atmosphere in that country (Kim *et al.,* 2004).

Antibiotic resistant infection has become challenging to treat with existing antibiotics, leading to infections triggering higher morbidity and mortality, imposing huge cost on our society (Finley *et al.,* 2013). Reducing and improving the use of antibiotics in the aquatic environment can reduce resistance and allow the antibiotic to resurface eventually as an effective therapy (Barbosa & Levy, 2000). In summary, the agreement of correlation between the genotype and phenotype of *V. parahaemolyticus* isolates on chloramphenicol and kanamycin resistance were established, which shows the resistance genes examined has a vital part in the resistance seen within the isolates. Nevertheless, there is evident discrepancies among the antimicrobial resistance phenotypes and genotypes in the other *V. parahaemolyticus* isolates of this study. Henceforth, additional resistant genes should be used for screening in future in order to obtain a comprehensive data.

5.5 Plasmid Profiles and Plasmid Curing of *V. parahaemolyticus*

The plasmid profiling revealed that 338 *V. parahaemolyticus* isolates had 1-7 different plasmid with size ranging from 1.2kb to above 10kb. As observed in Table 4.8, isolate SVP129 and VP152 were resistant to 11/14 antibiotics tested and only had one plasmid above 10kb in size. In contrast, isolate VP53 was resistant to only ampcillin but carried 6 plasmids (1.2kb, 1.7kb, 2.5kb, 7kb, 10kb, above 10kb). Interestingly, of the 32 *trh*-positive *V. parahaemolyticus* isolates, 21/32 *trh*-positive isolate contained 1-7 plasmids (Table 4.9), while another 11 isolates did not exhibit any plasmid profiles.

When compared the antibiotic resistance patterns and plasmid profiles, there was no correlation observed. Even within the isolates with same resistance profiles, the plasmid profiles were different and a few isolates even did not exhibit any plasmids, which was similar to findings by Lajnef *et al*. (2012). Hence, it could be concluded that the antibiotic resistance is not been influenced by the number of plasmids acquired by the isolates. The exposure of antibiotic in environment causes the bacteria to display a multidrug resistant characteristic. In some strains, the resistance observed could be plasmid mediated, and in some are chromosomally coded. Further research could be done to confirm the origin of antibiotic resistance among the isolates.

In this study, two different plasmid curing agent, acridine orange (AO) and ethidium bromide (EB), both are intercalating agents. Two different intercalating agents were used because to observed the efficacy of each agent. Intercalating agents such as AO and EB have been successfully used many studies of curing bacterial plasmids (Salisbury *et al.,* 1972; Kamat *et al.,* 1992; Barman *et al.,* 2010; Reboucas *et al.,* 2011; Carvalho *et al.,* 2013; Yano *et al.,* 2014; Costa *et al.,* 2014). Intercalating agents will inhibit the plasmid replication and hinder the process. Both the intercalating agents of this study produced a similar curing profiles for each isolate (Figure 4.8).

The present findings are in corresponding to previous findings that described plasmid lost within *Vibrio* sp. isolates after been treated with 0.2mg/ml acridine orange (AO) and the isolates resistance patterns was altered (Molina-Aja *et al.,* 2002; Barman *et al.,* 2010; Reboucas *et al.,* 2011; Carvalho *et al.,* 2013; Costa *et al.,* 2014). A study by Reboucas *et al.* (2011) in Brazil, described the curing of multidrug resistant *Vibrio* isolates originated from marine shrimp by AO curing agent and summarized that ampicillin resistance strains were plasmid mediated. In contrary to previous evident findings, a report discovered the resistance observed in their isolates were chromosomally mediated after AO treatment

(Costa *et al.,* 2014). Similary, another study reported the alteration in antibiotic resistance patterns and loss of plasmid among *Vibrio* sp. isolates when treated with 0.3mg/mL EB. In that study 79% of the *Vibrio* sp. isolates loss their plasmid profiles but showed phenotype resistance pattern to amoxicillin, ampicillin, furazolidone and tetracycline after curing assay, which indicate the resistance may be chromosomally borne (Manjusha & Sarita, 2011).

In conclusion, the current study provides an overview on the seafood contamination levels in Selangor, Malaysia and the distribution of *V. parahaemolyticus* in shrimp and shellfish samples. The shrimp and shellfish samples analysed were contaminated with *V. parahaemolyticus* regardless their sampling locations. The mean total *Vibrio* sp. counts gives an estimation on the occurrence of *Vibrio* sp. present in seafood samples, which the data would be useful for microbiological risk assessment and monitoring of the seafood products in Selangor, Malaysia. Although the presences of *trh*-positive isolates are low in number in the present study, consumers should always be caution during the processing of these seafood to avoid any possibility of infections with *V. parahaemolyticus*. In spite of the presences of plasmids among the isolates, there were no correlation between the number of plasmid and antibiotic resistance. Hence, the antibiotic resistance exhibited by *V. parahaemolyticus* isolates may be due to mismanagement of antibiotic in aquaculture and healthcare sectors. Hence, these results would provide a baseline information on the severity of resistance among *V. parahaemolyticus* in seafood and may allow management personnels to overcome this problem with proper management strategies.

CHAPTER 6: CONCLUSION AND FUTURE RECOMMENDATION

Foodborne disease is a predominant global health threat to developed and developing countries (Scallan *et al.,* 2011). Generally, the symptoms are minor or self-limiting upon ingestion of contaiminated food products. However due to rising number of people get affected yearly, the disease constrains the economic and healthcare system. Anually, one in 10 individuals experience foodborne infection, 420,000 death cases that results in the loss of 33 million healthy life years (DALYs) (WHO, 2017). While not all gastroenteritis is caused by foodborne, and not all foodborne diseases is gastroenteritis, food represents a critical vehicle for pathogens of substantial public health significance.

There are various factors contributing for foodborne diseases to remain as a global public health challenge. Although many foodborne diseases have been controlled with proper management methods, new threats do continuously emerge. The changes among microorganisms lead to emerges of new pathogens, increased antibiotic resistant strains in the environment, and alteration in pathogen's virulence. In addition, people in many countries eat food prepared outside their homes which potentially exposing themselves to the risks of poor hygiene in retail foodservice surroundings. In many situations, foodborne diseases go unregonized, underreported, unreported, or not investigated at all (WHO, 2017). All these challenges involve a constant monitoring of foodborne pathogens and management food safety to ensure human wellbeing.

In line with the current food safety situation, this study exhibits the importantance of understanding the occurrence and detection of antibiotic resistant *V. parahaemolyticus* isolates from seafood in Selangor, Malaysia. All the four objectives of this study have been successfully achieved. The occurrence of *Vibrio* sp. in all seafood samples collected from wetmarkets and supermarkets in Selangor, Malaysia signifies a potential threat to consumers. The wetmarket samples had a higher load of *Vibrio* sp. count compared to the

supermarket samples. Further analysis and molecular identification revealed that 385/770 *V. parahaemolyticus* isolates with 32 (8.3%) of the isolates carried the *trh* gene. The antibiotic susceptibility test revealed a total of 102 different type of antibiograms profiles among the isolates (Table 4.5). The isolates were seen to be resistant to at least one type of antibiotic tested with MAR index ranged from 0 to 0.79. In addition, the study detected chloramphenicol resistance gene (*catA2*) in 18/22 chloramphenicol resistant isolates and kanamycin gene (*aphA-3*) in 18/193 kanamycin resistant isolates. The plasmid profiles as well as the antibiotic resistance mediation of each isolate was determined by plasmid profiling and plasmid curing assay. The 32 *trh+ V. parahaemolyticus* isolates was seen to carry plasmids and their antibiotic resistance was either in the plasmid or chromosomal.

The occurrence of pathogenic *V. parahaemolyoticus* in the seafood samples studied indicate a possible high risk of gastroenteristis if the seafood was eaten raw, undercooked, or improperly cooked. The *trh*-gene is a hemolysin gene which is responsible to trigger cl^- channels resultant change of ion flux throughout an infection (Takahashi *et al.,* 2000). Further, the detection of multidrug resistant pathogenic isolates is an alarming condition that requires attention by the healthcare personnel and public. These multidrug resistant isolates emerge due to misuse of antibiotics in human medicine, aquaculture, agriculture, and veterinary (Rao *et al.,* 2015). The modern medicine is always threatened by antibiotic susceptibility of bacteria. This effects the soccioeonomic development by damaging the healthcare sector and effects the national economies, tourism, and trade (WHO, 2017).

There was no correlation observed between the antibiotic resistance and the plasmid profiles. Yet, the antibiotic resistance mediation was studied via the plasmid curing assay. In some isolates, the resistance was plasmid mediated, while others were chromosomally borne resistance. The information derived from the curing assay is useful for public health personnels to understand better on the antibiotic resistance pattern of *V. parahaemolyticus*

in shrimp and shellfish from Selangor, Malaysia. In summary, the results resulting from plasmid curing assay is fast, cost saving, provides fundamental knowledge, and influence an effective antibiotic management policy in the aquaculture sectory. With this valuable knowledge, farmers could alternate the antimicrobial used in their farms occasionally which will allow the bacteria to lose its resistance to a specific antibiotic (Letchumanan *et al.,* 2015b).

Although the study successfully applied all the methods to isoate *V. parahaemolyticus*, there were certain drawbacks and limitation that could be improved in order to obtain better research study results. The use thiosulphate citrate bile salt agar (TCBS) agar in the isolation and identification step was unable to yield high number of *V. parahaemolyticus* isolates based on colony morphology. Chromogenic CHROMagar *Vibrio* (CV) agar could be used instead of TCBS to increase the number of *V. parahaemolyticus* isolates. The specificity and accurance of CHROMagar *Vibrio* (CV) agar in the isolation of *V. parahaemolyticus* from environmental samples is higher compared to TCBS agar (Su & Liu, 2007; Canizalez-Roman *et al.,* 2011).

Due to time constrain and funding limitation, the present study was only able to screen a limited antibiotic resistant gene namely associated with *β*-lactams (*blaTEM, blaSHV, blaOXA*), tetracyclines (*tetA, tetB, tetC, tetG*), chloramphenicols (*catA1, catA2, catA3, catB3*) and kanamycin (*aphA-3*) by PCR assay. Hence, future studies may include a wider panel of antibiotic resistant genes (such as *blaPER, blaPSE, blaCTX-M, blaOXA-10, blaOXA-48, tetA, tetB, tetM, tetO, tetS, tetG, catP, catS, catB, catQ, catB1, catB2, catB3, catB7*) to screen antibiotic resistant *V. parahaemolyticus* isolates. These results would be more inclusive and provide a clearer understanding on the resistance genes responsible for the phenotype seen in the isolates.

Aquatic products are one of the main reservoirs for pathogenic and multidrug resistant *V. parahaemolyticus*. Therefore, there is an urgent prerequisite for the expansion of non-antibiotic technique to manage multidrug resistance (MDR) among pathogens due to declining efficacy of antibiotics and deficiency of new antibiotic in development pipeline (Rice, 2008; Freire-Moran *et al.,* 2011; Carlet *et al.,* 2011). Hence, further research can be done using bacteriophages and exploring the usefulness of it the management of *V. parahaemolyticus*. Phages are approved and recognized by the US regulatory bodies as a potential bio-control agent to control and prevent pathogens including *Vibrio* sp (Vinod *et al.,* 2006; Karunasagar *et al.,* 2007; Bren *et al.,* 2007; Coffey *et al.,* 2010; Hagens & Loessner, 2010; Jassim *et al.,* 2014). In addition, it can be utilized in the agriculture and aquaculture industries instead of antibiotics to control bacterial infections that occur in the farms. This will sooner or later reduce the dependency towards antibiotics that leads to resistant genes profile in the environment (Golkar *et al.,* 2014).

Future studies could research deeper into how the pathogenesis of *V. parahaemolyticus* occurs and the relationship of bile sensing activation by *V. parahemolyticus*. There are many studies reported that *V. parahaemolyticus* is able to withstand the human bile in the human gastrointestinal system, then utilize bile as a signaling cue to launch its virulence (Letchumanan *et al.,* 2017). This information is significant to all healthcare personnels to understand the mechanism of *V. parahemolyticus* infections and able to decide the best treatment for the infection. In summary, the investigation and prevention of foodborne illnesses comprises a multi-disciplinary task that require involvement of all the regulatory bodies, healthcare personnel, and members of public. Awarness campaigns or talks thru a joint effort of healthcare bodies and food safety researchers could educate the public especially to those involved in aquaculture and agriculture sector on the proper usage of antibiotics and guarantee food safety thru out the whole food industry from production to consumption.

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