DEVELOPMENT OF MULTIPLEX MPN-PCR TO DETECT MAJOR *VIBRIO* SPECIES IN RAW FISH AND SUSHI

SHARMIN QUAZI BONNY

THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY
(BIOLOGY & BIOCHEMISTRY)

INSTITUTE OF GRADUATE STUDIES
UNIVERSITY OF MALAYA
KUALA LUMPUR

2018

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: **Sharmin Quazi Bonny**

Matric No: **HGA 150020**

Name of Degree: **Master of Philosophy**

Title of Project Paper/Research Report/Dissertation/Thesis:

"**DEVELOPMENT OF MULTIPLEX MPN-PCR TO DETECT MAJOR *VIBRIO* SPECIES IN RAW FISH AND SUSHI**"

Field of Study: **Biology & Biochemistry**

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge, nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every right in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date: 19 March, 2018

Subscribed and solemnly declared before,

Witness's Signature Date:

Name:

Designation:

DEVELOPMENT OF MULTIPLEX MPN-PCR TO DETECT MAJOR *VIBRIO* SPECIES IN RAW FISH AND SUSHI

ABSTRACT

Fish and fish products, the second most important source of protein after meat, constitute an important part in the international trade and incessantly exposed to an extensive range of microorganisms present in the environment. Among them *Vibrio parahaemolyticus*, *Vibrio cholerae* and *Vibrio vulnificus* are three most notorious aquatic pathogens causing a wide range of diseases in humans. However, the occurrence of these three pathogenic Vibrios in Malaysian raw fishes and ready-to-eat (RTE) fish products namely, sushi has not been thoroughly investigated. The most-probable-number-PCR (MPN-PCR) assays had been reported to be highly sensitive for the identification of a single bacterial pathogen but they have not been applied for the enumeration of major Vibrios in a multiplex platform. Therefore, this study has coupled MPN method with Multiplex PCR to differentiate three major Vibrios in one single test. A total of 120 raw fishes were analyzed using optimized multiplex PCR, simplex MPN-PCR and the developed Multiplex MPN-PCR approaches in order to compare the sensitivity of these three methods through determining the occurrence of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus*. The developed multiplex MPN-PCR was applied to examine the occurrence of these target Vibrios in 70 sushi samples. The microbial loads of the target Vibrios in sushi were recorded in three different time and conditions to determine microbial survivability upon storage at room temperature and after the recommended 7 days of refrigeration period. The developed Multiplex MPN-PCR was found to be more sensitive compare to the Multiplex PCR. Besides, a rise in microbial load was recorded after leaving the sushi for 4-5 hrs. at the room temperature while the recommended 7-days refrigeration period reduced the bacterial growth but could not make the sushi entirely free of the pathogens. To conclude, combination of MPN and multiplex PCR assay was found significantly beneficial to achieve

quantitative detection of multiple species in a single assay platform. Furthermore, the high prevalence of target Vibrios in edible raw fishes (27-48%) and sushi (4-28%) indicate considerable risk of Vibrios onset in Malaysia due to consumption of undercooked or raw fish and contaminated sushi.

Keywords: *Vibrio* spp., Multiplex MPN-PCR, Seafood

PEMBANGUNAN MPN-PCR MULTIPLEKS UNTUK MENGESAN SPESIS *VIBRIO* UTAMA DI DALAM IKAN MENTAH DAN SUSHI

ABSTRAK

Ikan dan produk ikan adalah sumber protein kedua yang paling penting selepas daging. Ia merupakan bahagian yang penting dalam perdagangan antarabangsa dan sering terdedah kepada pelbagai mikroorganisma yang terdapat di persekitaran. Antaranya, *Vibrio parahaemolyticus*, *Vibrio cholerae* dan *Vibrio vulnificus* adalah tiga patogen akuatik terkenal menyebabkan pelbagai jenis penyakit terhadap manusia. Walau bagaimanapun, kehadiran ketiga-tiga patogen *Vibrio* ini di dalam produk ikan mentah Malaysia dan produk ikan segera (RTE) seperti sushi masih belum disiasat dengan teliti. Prosedur PCR bilangan-paling-mungkin (MPN-PCR) telah dilaporkan sebagai suatu kaedah yang paling sensitif untuk mengenal pasti patogen bakteria tunggal tetapi kaedah ini masih tidak digunakan untuk penghitungan Vibrio terutamanya dalam platform multipleks. Oleh itu, kajian ini telah menggabungkan kaedah MPN dengan PCR multipleks untuk membezakan tiga jenis *Vibrio* ini dalam satu platform kajian tunggal. Sejumlah 120 ekor ikan mentah telah dianalisis menggunakan kaedah PCR multipleks yang dioptimumkan, MPN-PCR simpleks dan MPN-PCR Multipleks bagi membandingkan tahap kesensitifan ketiga-tiga kaedah ini dengan menentukan kehadiran *V. parahaemolyticus*, *V. cholerae* dan *V. vulnificus* di dalam ikan mentah. Sebagai alternatif, kaedah MPN-PCR multipleks digunakan untuk menguji kehadiran Vibrio ini didalam 70 sampel sushi. Jumlah mikrob *Vibrio* sasaran di dalam sushi direkodkan pada tiga masa dan keadaan berbeza untuk mengenalpasti tahap ketahanan mikrob apabila disimpan dalam suhu bilik dan selepas tempoh pendinginan 7 hari dikenakan. MPN-PCR multipleks yang dibangunkan didapati lebih sensitif berbanding PCR multipleks. Selain itu, jumlah mikrob direkodkan meningkat apabila sushi dibiarkan selama 4-5 jam pada suhu bilik namun tempoh pendinginan yang dicadangkan selama 7 hari hanya mampu mengurangkan kadar pertumbuhan bakteria tetapi tidak menjamin sushi untuk bebas sepenuhnya daripada patogen. Kesimpulannya,

penggabungan teknik MPN dengan ujian PCR multipleks didapati sangat bermanfaat untuk mendapatkan pengesanan kuantitatif pelbagai spesis dalam satu platform esei tunggal. Tambahan pula, sasaran *Vibrio* yang tinggi dalam ikan mentah yang dimakan (27-48%) dan sushi (17.14%) menunjukkan risiko besar wabak *Vibrio* di Malaysia akibat pemakanan ikan kurang masak atau mentah dan sushi yang tercemar.

Kata kunci: *Vibrio* spesis, MPN-PCR multipleks, makanan laut

ACKNOWLEDGEMENTS

First and foremost, I would like to convey my gratitude to the almighty for his uncountable grace and mercy over me.

I would like to convey my sincere appreciation to my supervisor, Associate Professor Dr. Md. Eaqub Ali and Prof. Dr. Thong Kwai Lin, for their valuable guidance, substantial supervision and enormous support. Being their student is a privilege and life changing resolution for me.

I would like to extend my gratitude to my lab-mates for their love and cooperation. I enjoyed the time spent with them, and their backings helped me extensively to bring this project to a successful end.

I want to thank and acknowledge my husband for being my strong pillar for all through the period of my study here and I have no hesitation to accept that it might not be possible for me without having him there.

Last but not the least, I would like to express my heartiest indebtedness to my parents for their unconditional love, support and sacrifices. Their motivations and incentives encouraged me a lot to fulfil my degree requirements. Without them none of this would have been possible and therefore, I would like to dedicate my work to my parents.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percentage
℃	:	Degree Celsius
&	:	And
Spp.	:	Species
dNTP	:	Deoxynucleotide triphosphate
ddH2O	:	Double-distilled water
MgCl2	:	Magnesium Chloride
Nacl	:	Sodium chloride
Taq	:	*Thermus aquaticus*
µl	:	Microliter
mL	:	Milliliter
VP	:	*Vibrio parahaemolyticus*
VC	:	*Vibrio cholerae*
VV	:	*Vibrio vulnificus*
IAC	:	Internal Amplification Control
SPB	:	Salt Polymyxin Broth
TSB	:	Tryptic Soy Broth
TCBS	:	Thiosulfate-citrate-bile salts-sucrose
RTE	:	Ready to Eat
ASPW	:	Alkaline Saline Peptone Water
PBS	:	Phosphate Buffered Saline
VBNC		Viable but Nonculturable

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Background of the study

V. parahaemolyticus, V. cholerae and *V. vulnificus* are the three most notorious aquatic pathogens of genera *Vibrio.* It is comprised of over 100 different species and 12 are pathogenic to human and associated with a wide range of human diseases (Tantillo, Fontanarosa, Di Pinto & Musti, 2004; Özer et al., 2008). *V. parahaemolyticus* was first isolated in Japan in early 1950s following food poisoning outbreaks due to the consumption of sardine (Chen et al., 2010) and it is responsible for about 50% of the foodborne outbreaks in Southeast Asian regions with an upward trend in the recent decades (Martinez-Urtaza et al., 2016). While, *V. cholerae* which is the etiological agent of cholera is transmitted from their aquatic niche to humans through seafood or other environmentally related food or water sources (Senderovich, Izhaki, & Halpern, 2010; Dickinson, Lim, & Jiang, 2013). *V. vulnificus* that is known as "Flesh eating Bacteria" (Ralph & Currie, 2007) causes primary septicemia in human following ingestion of undercooked or raw seafood such as raw oysters with 50-60% fatality rate (Doyle & Buchanan, 2012).

The ubiquitous nature of *Vibrio* species in marine and estuarine environments makes it impossible to ensure that fish and fish products, the second most important source of protein after meat, is entirely free of these bacteria. Fishes have a rich source of essential nutrients along with high protein content required for supplementing both infant and adult diets and according to BBC News, global fish consumption per capita has made a record in 2016 (FAO, 2016). However, the concern arise as these fish are incessantly exposed to an extensive range of microorganisms present in the environment including the pathogenic Vibrios and thus become the carrier and reservoir

of these microbes. Hence, these microbes can be transmitted to humans through the food chain and handling (Boylan, 2011).

Raw fish products undergo various processing steps that inactivate the bacterium but consumption of raw or undercooked fish and cross-contamination of these bacteria in other food products can cause a serious health issue. While, the presence of pathogen in ready-to-eat fish products such as sushi-a cold cooked rice with raw or undercooked fish or seafoods as toppings- is more of a serious affair since these products do not receive any decontamination treatment or bactericidal step before consumption (Mercanoglu Taban & Halkman, 2011). Furthermore, the take-away meal, for instance sushi, is usually consumed at consumer's convenient which might refer to leaving the food at room temperature for few hours and this delay might allow the bacteria, present in the food to multiply in number and thus increase the health risk.

Besides, all of these three target bacteria can be found to persist in viable but non-culturable (VBNC) state, when exposed to adverse environmental condition including low-nutrient, low-temperature, preservatives, disinfectants and so on. Hence, these VBNC pathogens can introduce a serious food safety and public health crisis through incurring the capabilities of causing disease once resuscitated. However, conventional plate counting techniques fail to detect them and thus incorporation of other means of detection become more significant (Marian et al., 2012; Oliver,2000; Zhao, Zhong, Wei, Lin, & Ding, 2017). Herein, the MPN-PCR (Most probable number PCR) technique, estimating the density of viable organisms in a sample can be particularly useful in detection of low levels of microorganisms along with the VBNC pathogens curtailing the limitation of usual conventional method (Martín, Jofré, Garriga, Hugas, & Aymerich, 2004).

Recently, several MPN-PCR based methods have been proposed but they cannot identify and differentiate more than one bacterial species at a time (Elhadi, Radu, Chen, & Nishibuchi, 2004; Arslan & Özdemir, 2008; Luan et al., 2008; Lee et al., 2009; Ponniah et al., 2010). This study has undertaken this research gap by coupling multiplex PCR with MPN method which is capable of detecting more than one bacteria in a single assay. Moreover, considering the claim of having the MPN-PCR 100 times higher in sensitivity than the direct PCR by Luan et al. (2008), this study has aimed to compare the sensitivity of Multiplex PCR and Multiplex MPN-PCR.

1.2 Project rationale

Vibrio is one of the most notorious aquatic bacteria responsible for a wide range of diseases. The spectrum of illness can vary from gastroenteritis to 'primary sepsis' and necrotizing fasciitis. Fishes, hereby are acting as a carrier and reservoirs of major waterborne Vibrios such as *V. parahaemolyticus, V. cholerae* and *V. vulnificus* and might play a role in the global dissemination of these virulent pathogens (Halpern & Izhaki, 2017).

The potential routes of contamination include but not limited to consumption of raw or undercooked fish or fish product (i.e sushi), washing and placing the carrier fishes in the vicinity of other food products, inappropriate preservation conditions and the usage of the same utensils for washing the raw fishes and other foods (New, Ubong, Nur Hasria, Nur Fatihah, & Son, 2016). Moreover, contaminated ready-to-eat food stored at ambient temperature can reach up to the infectious dose within few hours and can cause the infection (Luan et al., 2008).

Controlling the processing conditions is emphasized during the production and storage of the raw food products, sushi, for instance, as they might contain pathogenic

bacteria. According to Food Authority of Canada (2013), freezing and storing seafood at -20°C (-4°F) or below for 7 days to prevent bacterial contamination.

Therefore, the rapid detection of Vibrios at the carrier level is required to halt their infection and outbreak through various routes. Likewise, the evaluation of the controlling measurements including the assessment of the recommended 7 days of refrigeration is essential to prevent the onset of bacterial infections. The identification of any routes of *Vibrio* transmission might play an important role in future biosafety assessments and controlling measures to safeguard human health and fish industries.

1.3 Problem statements

The conventional culture-based methods of identifying bacteria are laborious, time-consuming, and non-specific (Xu et al., 2017) and require further confirmation by additional assays, such as biochemical and immunological (FDA approved) techniques. Alternatively, PCR and Multiplex PCR are extensively used to substitute the conventional culture-based method through rapid identification of bacterial species and detection of virulence genes because of their inherent ability to amplify as low as single-copy nucleic acid targets into multiple copies even from complex matrices (Ali, Razzak, & Hamid, 2014). However, the quantification of the microbial load cannot be achieved using the PCR assay.

Nevertheless, to overcome these limitations, an alternative, convenient and improved method in the form of multiplex MPN-PCR for the discriminatory identification and quantification of three major live Vibrios in a single assay platform, has been provided to achieve the advantage of the sensitivity of MPN assay along with the specificity of PCR system in one platform. The method MPN-PCR is capable of quantifying the bacterial count and offer the advantage of increasing the sensitivity using a large

volume of inoculum in enrichment series and thus detection of the small number of bacteria through PCR become easier.

Since, the MPN-PCR has been claimed to be more sensitive previously and this developed multiplex method is the first ever documented observation of MPN-PCR in triplex format so, a comparison was administered in order to validate the developed multiplex MPN-PCR method in term of the already stablish Simplex MPN-PCR method. Besides, comparison of the sensitivity in respect to the qualitative data of multiplex PCR and multiplex MPN-PCR has been recorded.

1.4 Research Objectives

1.4.1 General Objective

The objective of the overall study is to develop a multiplex MPN-PCR method and validate in respect to the established simplex MPN-PCR method, to compare its sensitivity in terms of conventional multiplex PCR, to evaluate the occurrence of three major pathogenic Vibrios in raw fish and to assess the bacterial survivability in RTE sushi in terms of storage in different temperature.

1.4.2 Specific Objectives

1. To optimize and validate multiplex MPN-PCR for major Vibrios.
2. To enumerate the occurrence of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* in raw fish and RTE sushi.
3. To study the survivability of the target Vibrios in sushi at three different storage conditions.

1.5 Scope of Research

The Center of Disease Control (CDC (2013) reported that *Vibrio* infections was increased by 43% in 2013 from that of 2006-2008 because of the consumption or

exposure to contaminated food or water. Moreover, the abundance of *Vibrio* spices in raw fish and marine products, govern them as potential carrier and reservoir of these pathogens and among over 100 species, *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* are the most commonly encountered bacterial contaminants related to consumption of raw fish (Håstein et al., 2006; US Food & Drug Administrator, 2011).

The study has focused on two types of sample including raw fish and sushi. Regularly available finfishes in Malaysian market have been chosen to assess the occurrence of target bacterial pathogen that pose a risk to human health through direct ingestion, cross-contamination and handling exposure. Alternatively, industrially prepared RTE sushi, available in supermarkets have been chosen over the sushi from the sushi bars/restaurants because sushi prepared in restaurants involves consumption after a relatively short time unlike the commercially prepared RTE sushi. Therefore, the time and temperature profile from preparation, during storage until consumption is critical for both shelf life and food safety aspects of sushi prepared industrially.

To compare the sensitivity of the method, only qualitative data of multiplex PCR and multiplex MPN-PCR could be analyzed because multiplex PCR cannot quantify the microbial load while both qualitative and quantitative data of simplex MPN-PCR and multiplex MPN-PCR was documented and analyzed.

1.5.1.1 Thesis Organization

This thesis comprises of six chapters namely Introduction, Literature review, Material and methods, results, Discussion and Conclusion. The contents of each chapters are described below:

Chapter 1 (Introduction): This chapter describes the brief background of the study, project rationale, problem statement, objectives and scope of the present research. It

also includes the importance of this study and the prospects of the developed method in terms of the limitation of the previously reported methods.

Chapter 2 (Literature Review): This chapter reviews the literatures that include the characteristics of target bacteria and associated outbreaks, available detection methods and a general outline referring relevance of the sample types chosen for the study.

Chapter 3 (Materials and Methods): All materials and protocols including the list of samples have been subtly described in this chapter.

Chapter 4 (Results): Outcome of all the experiments have been illustrated in this chapter including DNA Extraction, optimization of the new method, comparison between the developed method and previously used methods in terms of the qualitative and quantitative data, and lastly, the validation of the developed method in determining the occurrence of target group of samples.

Chapter 5 (Discussion): This chapter includes the discussion on experimental findings, previously published studies and the limitations of the study.

Chapter 6 (Conclusion): Summary of the findings and scopes of future works have been included in this chapter.

CHAPTER 2: LITERATURE REVIEW

2.1 Characteristics of *Vibrio* species

Members of the *Vibrio* species belong to the family *Vibrionaciae* and genus *Vibrio.* They are non-enteric, fermentative, oxidase positive, motile, Gram-negative rods currently cause a huge burden on global health. The genus *Vibrio* includes over 100 species and they are common inhabitants of the natural marine and estuarine aquatic ecosystems (Özer et al., 2008). They have been frequently associated with many food poisoning outbreaks and they are considered as one of the most notorious pathogens related to food and water borne diseases (Paydar, Teh, & Thong, 2013). Most of the *Vibrio* species are halophilic and they require salt for their growth except a few namely, *V. cholerae* and *V. mimicus.* These bacteria are tolerant to alkaline pH, but sensitive to acidic pH.

Out of the 106 *Vibrio* species, at least 12 are reported pathogenic to humans and are associated with a wide range of human diseases (Khaira & Galanis, 2007). *V. parahaemolyticus*, *V. cholerae*, and *V. vulnificus* have been recognized as the three most notorious pathogens causing septicemia, cholera, wound infection, nausea, vomiting, bloody diarrhea, abdominal pain and fever in healthy and immune-compromised individuals either by consumption of contaminated seafood or by exposure to aquatic environments (Austin, 2010; Ottaviani et al., 2010; Paydar et al., 2013).

2.2 *Vibrio parahaemolyticus*

V. parahaemolyticus is considered as the major food-borne pathogen involving in bacterial seafood poisoning incidents in Asia (Fujikawa, Kimura, & Fujii, 2009). It has been classified as the major foodborne pathogen by the detection network of microbial foodborne illness in China (Wu, Wen, Ma, Ma, & Chen, 2014). Similarly, cases of foodborne illness caused by *V. parahaemolyticus* are common in Europe and the United

States specially, if the temperature 25ºC and above while it can be found in Southeast Asia all the year round (Zulkifli et al., 2009; Shen et al., 2009). In fact, the largest outbreak of *V. parahaemolyticus* gastroenteritis did occur in the USA in 1978 and triggered up to 1133 cases (Daniels et al., 2000). Recently, occurrence of *V. parahaemolyticus* in raw fish and seafood have been reported in Malaysia (Elhadi, Radu, Chen & Nishibuchi, 2004; Noorlis et al., 2011; Letchumanan, Yin, Lee, & Chan, 2015) by several researchers. The sustainable climate for the growth of *Vibrio* species intensified the probability of *V. parahaemolyticus* outbreaks in Malaysia (Elhadi et al., 2004). As demonstrated by recent surveillance data (Yeung & Boor, 2004; Tang et al., 2014; Ma et al., 2014; Elmahdi, DaSilva, & Parveen, 2016), the control of *V. parahaemolyticus* continues to be challenging worldwide.

Although 30-37°C is the optimum temperature for the growth of *V. parahaemolyticus*, it can grow in a temperature range of 5-42°C. The cells are able to multiply very rapidly in a medium containing 3-5% NaCI. They can withstand up to 8% NaCl but exhibited sensitivity to 10% NaCl. Growth is found to be restricted at pH 5.0 or below. The optimum pH for growth is 7.8 to 8.6 within a range of 4.8 to 11. The cells are very sensitive to heating (pasteurization), drying and freezing. It can grow in the presence or absence of oxygen but grows optimally under aerobic conditions (Barlow et al., 2015). It enters into a dormant, viable but nonculturable stage when conditions are unfavorable for growth and reproduction (Suzita, Abdulamir, & Abu Bakar, 2009).

Ingestion of raw, undercooked seafood and contaminated food is the primary route of *V. parahaemolyticus* transmission (Letchumanan et al., 2015). The presence of predisposing factors, such as use of antacid medications, absence of gastric hydrochloric acid (achlorhydria), and partial or complete gastrectomy, increase the risk of illness (Elbashir et al., 2018). Thermostable direct hemolysin (TDH) and TDH-

related hemolysin (TRH) are referred as major virulence factors of this bacterium and are closely related to its pathogenicity. Several studies havereported that TDH as one of the major pathogenic factors of *V. parahaemolyticus* and prevalent in almost 95% of the clinical isolates (Wang et al., 2015).

2.3 *Vibrio cholerae*

V. cholerae was first discovered in 1884 by Robert Koch (Lippi & Gotuzzo, 2014). It is the etiological agent of cholera and transmitted from their aquatic niche to humans through seafood or other environmentally related food or water sources (Senderovich, Izhaki, & Halpern, 2010; Dickinson, Lim, & Jiang, 2013). Cross contamination of seafood and seafood products may take place during harvesting, handling, preparation, processing, transportation, and storage (Elbashir et al., 2018). Epidemic cholera is created by toxigenic strains of serotypes O1 and O139. Different virulent agents are associated with their pathogenicity; of them the cholera toxin (ctx) and toxin coregulated pilus (TCP) are the most important pathogenicity factors associated with epidemic cholera (Barzamini, Moghbeli, & Soleimani, 2015).

It is highly sensitive to heat and can be killed by heating at the right temperature. Improper heating or inappropriate length of cooking may not be able to kill the whole cells present in a food. The optimum temperature for its growth is between 30℃ and 37°C. It has a very rapid growth rate, in raw or cooked seafoods at its optimum temperature or even at room temperature. Rapid growth can be facilitated in alkaline foods. At 5-10°C it has a better survival in cooked foods. Addition of 1% sodium chloride (NaCl) can stimulate the growth of *V. cholerae*. However, this bacterium is capable of growing in a nutrient broth or agar without added NaCl. Although it can grow in the pH range of 5.9 to 9.6, the optimum pH for its growth is 7.6 (Martinez, Megli, & Taylor, 2010).

Recently, reports have been published regarding the occurrence of *V. cholerae* in raw fish and seafood in Malaysia (Elhadi et al., 2004; Suzita, Abu Bakar, Son, & Abdulamir, 2010) by several researchers. As demonstrated by recent surveillance data (Tang et al., 2014) , the control of *V. cholerae* continues to be challenging worldwide.

2.4 *Vibrio vulnificus*

V. vulnificus was first discovered in 1970 (Daniels, 2011). Consumption of contaminated seafood containing *V. vulnificus* can result in a severe, fulminant systemic infection. On average, 34 cases of *V. vulnificus* infection are reported annually by the U.S. Food and Drug Administration (Austin, 2010). However, in recent years this number has risen dramatically. According to Drake et al. (2007), about hundred cases of primary septicemia arise in United States every year. The CDC reported *Vibrio* infections increased by 78% between 1996 and 2006, and in 2005, 121 cases of *V. vulnificus* disease were confirmed (Scallan et al., 2011). Recently, occurrence of *V. vulnificus* in raw fish and seafood have been reported in Malaysia by several researchers (Elhadi et al., 2004*;* Elmahdi et al., 2016). It has been reported that food poisoning has the highest incidence rate of 36.17 per 100,000 populations in Malaysia with a mortality rate of 0.01 per 100,000 populations in the year of 2009 (Vengadesh, Son, & Yoke-Kqueen, 2012). Furthermore, the incidence of cholera have risen from the year 2008 to 2009 at 0.34 per 100,000 populations to 0.98 per 100,000 populations and with a mortality rate of 0.01 per 100,000 populations (WHO, 2016).

It is categorized as "Flesh eating Bacteria" (Ralph & Currie, 2007) and causes primary sepsis in certain high-risk populations, including patients with chronic liver disease, immunodeficiency, iron storage disorders, end-stage renal disease, and diabetes mellitus following ingestion of undercooked or raw seafood. The infection and fatality rate caused by *V. vulnificus* is extremely high (40-60%) among the people who have

liver and gastric problems and immune deficiencies (Horseman & Surani, 2011). It can also cause severe wound infections in the individuals who have skin lesions, pre-existing cuts and skin punctures. This organism possesses a wide array of virulence factors, including iron acquisition, capsular polysaccharide expression, expression of proteins involved in attachment and adhesion, the hemolysin encoded by the *vvh* (cytotoxin-hemolysin), zinc metalloprotease, and transmembrane regulatory protein (ToxR) may contribute in *V. vulnificus* infection (M. K. Jones & Oliver, 2009). Metalloprotease is a cardinal pathogenic factor and is assigned as *V. vulnificus* protease (VVP). It has a significant role in skin lesions produced by *V. vulnificus* infections (Miyoshi, 2006).

The cells grow optimally at 37°C with the temperature range from 8 to 43°C. The organism is selective regarding preferred habitat has including the water temperatures in excess of 18 °C and salinities between 15 to 25 parts per thousand (ppt). However, salinities at or greater than 30 ppt will substantially reduce the burden of *V. vulnificus* regardless of the water temperature (Horseman & Surani, 2011). The pH range for growth is 5 to 10, in which the optimum is 7.8. It is phenotypically very similar to *V. parahaemolyticus* (Jakšić, Uhitil, Petrak, Bažulić, & Gumhalter Karolyi, 2002). Similar to *V. parahaemolyticus*, *V. vulnificus* enters into a dormant, viable but nonculturable stage when conditions are unfavorable for growth and reproduction (Suzita et al., 2009). Cold-adaptive or protective proteins produced by *V. vulnificus* has been found to enhance survival and tolerance to cold and freezing temperatures. Iron plays a significant role in adaptation at cold temperature, since the removal of iron from the growth medium prior to cold adaptation reduced viability by 2-$\log_{10}$ CFU/mL (Bryan, Steffan, DePaola, Foster, & Bej, 1999). Fermentation of lactose and production of β-D-galactosidase are two most distinctive characteristics of *V. vulnificus* that can be used to distinguish it from the related *V. parahaemolyticus* (Drake et al., 2007).

2.5 Host Range and Transmissions of *Vibrio*

High occurrence of *Vibrio* species in marine and aquatic environments, leads to their presence in any food of freshwater origin, especially in temperate regions of the world. Some species develop relationship with aquatic animals and thus, they can have a wide variety of hosts among marine organisms including fish, shellfish, prawn, shrimp, oyster, squid etc. (Sujeewa, Norrakiah, & Laina, 2009). The abundance of *Vibrio* spices in raw fish and marine products, govern them as potential carrier and reservoir of these pathogens and this is how these species are associated to food safety issue.

2.5.1 Fish

Fishes continually exposed to an extensive range of microorganisms present in the environment and thus become the carrier and reservoir of these microbes. Finfish was found to be responsible for the highest number of foodborne-disease outbreaks in 2007, which was more than poultry and beef (Elbashir et al., 2018). Human infections and intoxications have been reported by several bacterial species including *Mycobacterium* spp. (Bhatty, Turner, & Chamberlain, 2000; Ucko & Colorni, 2005), *Streptococcus iniae* (Sun, Yan, Yen, Lee, & Lu, 2007), *Vibrio alginolyticus* (Feingold & Kumar, 2004), *Escherichia coli* (Makino et al., 2000), *Aeromonas* spp.(Chou, Tsai, Kau, Kau, & Hsu, 2004), *Salmonella* spp.(Heinitz, Ruble, Wagner, & Tatini, 2000), *Staphylococcus aureus* (Mus, Cetinkaya, & Celik, 2014), *Listeria monocytogenes* (Miettinen & Wirtanen, 2005), *Clostridium botulinum* (Lalitha & Surendran, 2002), *Campylobacter jejuni* (Lyhs, Hatakka, Maki-Petays, Hyytia, & Korkeala, 1998), and *Edwardsiella tarda* (Golub, Kim, & Krol, 2010) along with *V. parahaemolyticus* (Ralph & Currie, 2007), *V. cholerae* (Senderovich et al., 2010) and *V. vulnificus* (Depaola, Capers, & Alexander, 1994).

The abundance of *Vibrio* spices in raw fish and marine products, govern them as potential carrier and reservoir of these pathogens (US Food & Drug Administrator, 2011).

2.5.2 Sushi

Raw fish is quite popular in Japanese, Chinese and Thai cuisines and has grown quite popular all over the world and sushi is one of the most popular and widely consumed Japanese cuisine though a big population are skeptical about eating seafood entirely raw (Liang et al., 2016). Sushi was originally prepared in Southeast Asia as a method to preserve fish. It was formerly used for carps, that were caught, gutted, salted and preserved between two stones, and left to ferment for a period ranged from one to three years. However, lately, it is prepared with cold cooked rice acidified with vinegar, shaped into bite-sized pieces with different toppings including raw or undercooked fish or seafoods (Nawa, Hatz, & Blum, 2005). The distinct features of sushi are direct consumption of fresh raw fish or seafood without further cooking. Apart from buying sushi in restaurants or bars, industrially prepared RTE (ready-to-eat) sushi is quite popular.

The practice of eating Japanese food, sushi in particular, emerged in the large cities of USA on 1970s and gradually spread to the European capitals. By the end of the 20th century, sushi had acquired huge popularity in the Western culinary repertoire (De Silva & Yamao, 2006).

However, the Australia New Zealand Food Standards Code labelled “Sushi” as potentially hazardous food because raw fish is known to carry diverse pathogenic microorganisms and so consumption of raw fish incurs potential health risks (Barralet, Stafford, Towner, & Smith, 2004). Moreover, the preparation process of sushi involves minimal heat treatment and direct hand contact from sushi chefs that increases the risk

of improper food handling techniques (Tan, Haresh, Chai, Ghazali, & Son, 2008). Though maintaining the temperature is considered to reduce the possibility of bacterial contamination, several outbreaks including *Salmonella* outbreaks in Singapore (Barralet, Stafford, Towner, & Smith, 2004) , *Escherichia coli* outbreaks in Nevada (Jain et al., 2008), *Salmonella* outbreak in Australia (Thompson et al., 2017) has been found to be associated with sushi consumption. Likewise, several studies have reported the association between the bacterial infection and consumption of sushi (Diplock, 2003; Millard & Rockliff, 2003; Barralet et al., 2004; Atanassova, Reich, & Klein, 2008; Muscolino et al., 2014; Liang et al., 2016; Puah, Chua, & Tan, 2017) including *V. parahaemolyticus* (Kim, Lee, Lee, & Cho, 2012) and *V. cholerae* (Novotny, Dvorska, Lorencova, Beran, & Pavlik, 2004).

2.6 Etiology of *Vibrio* infections in humans

Etiology of the bacterial infections refer to direct and indirect transmission of the etiological agents that lead to outline the control measurements. The scopes of direct and indirect transmission are mentioned below:

(1) Through direct contact with infected fish while handling the water or other constituents of fish: Several report has been stated regarding the incidence if infections through direct contact including exposure to fish tank water (Lewis, Marsh, & von Reyn, 2003), through contact with wild fish (Novotny et al., 2004), after having injuries from fish, e.g. by thorns (Imberg, Potasman, Weissman, & Grupper, 2008) and through processing of infected fish in food industry (Notermans & Hoornstra, 2000),

(2) Through consumption of contaminated fish and fish products: Several findings have reported the consumption of raw fish (Noorlis et al., 2011), seafood (Butt, Aldridge, & Sanders, 2004; Hara-Kud et al., 2005; Su & Liu, 2007; Iwamoto, Ayers,

Mahon, & Swerdlow, 2010) or uncooked fish products (e.g. sushi) (Atanassova, Reich, & Klein, 2008; Liang et al., 2016) as the major route of *Vibrio* transmission.

(3) Through cross-contamination: The potential routes of cross contamination include but not limited to washing and placing the carrier fishes in the vicinity of other food products, inappropriate preservation conditions and the usage of the same utensils for washing the raw fishes and other foods (New, Ubong, Nur Hasria, Nur Fatihah, & Son, 2016)

Apart from these, the development of an infectious disease is markedly affected by internal factors such as the physiological status of consumer, particularly by immunosuppression and chronic diseases. For instance, people with liver disease, especially Hepatitis C and cirrhosis, are 80 times more likely to develop any infections and 200 times more likely to die from it (Centre of Disease Control , 1993)

2.7 Control Measures

The continuous rise in consumption of seafood over the last few decades increases concerns about seafood safety (Elbashir et al., 2018) and demands stringent controlling measures.

The following control measures should be taken to evade these infections and diseases. Consumption of raw, improper-cooked or post-heat contaminated seafoods should be strongly avoided, especially by susceptible individuals. To do a proper heat treatment for a suspected food the length and the temperature of the treatment should be taken into consideration to make sure about its efficiency to kill the pathogen. Raw or cooked seafood products should be refrigerated or freeze, and they must be consumed in a proper period of time. Harvesting seafood from contaminated water which is found to harbor *V. cholera* should be stopped. One problem that must be taken into consideration

is cross-contamination of the products, which it can be avoided by an appropriate way of sanitation and personal hygiene. Appropriate hygienic measures can prevent the spread of the causative bacteria. Finally, proper temperature for either cooking the products or storing them may drastically reduce the contamination of food products. Temperature abuse may lead to irreparable results, even if it is only for a short period of time (FDA, 2011).

2.7 Detection of *Vibrio* spp.

2.7.1 Sample collection

As previously stated Vibrios inhabit marine environments and are associated with aquatic animals including fish, shrimp, oyster, shellfish, squid, prawn, and other freshwater animals (Bonnin-jusserand, Copin, Bris, & Brauge, 2017). Therefore, fish, seafood and marine products are mostly used for isolation of *Vibrio* species. Samples should be immediately placed at 7° C to 10° C after isolation, and subsequent analysis should be done as early as possible. Rapid cooling might cause the injury in the bacterial cells as Vibrios are temperature sensitive and therefore, avoiding direct ice contact with the samples is suggested, in order to maximize the survival and existence of Vibrios. These bacteria are able to grow fast at ambient temperatures in seafood (Oliver, Pruzzo, Vezzulli, & Kaper, 2013). Though extreme high and low temperature may kill Vibrios and inhibit their recovery but they can survive well under mild refrigeration (FDA, 2011).

2.8 Traditional Culture-based Method

2.8.1 Culture Media

The first stage in traditional detection methods exploits the ability of *Vibrio* Spp. to grow rapidly at relatively high pH values. Media containing sodium chloride and with a pH of about 8.6, such as alkaline saline peptone water (ASPW), are used for enrichment

(Kaysner & Angelo DePaola, 2004). Thiosulfate-citrate-bile salts-sucrose (TCBS) have been widely used for selective isolation of Vibrios from food samples. On TCBS agar, *V. parahaemolyticus* colonies appear blue-green, *V. cholerae* colonies are smooth and yellow and *V. vulnificus* colonies are green or yellow. However, colonies on TCBS agar are sometimes difficult to distinguish visually from other bacterial colonies since they can be covered by a yellow color produced by sucrose-fermenting bacteria (Hara-Kudo et al., 2001).

To counter the relative inefficiency of TCBS medium, commercial chromogenic agar media such as CHROMagar *Vibrio* has been reported by researchers and found to be exhibited better results than TCBS agar in distinguishing between pathogenic *Vibrio* spp. and other species (Di Pinto, Terio, Novello, & Tantillo, 2011).

2.8.2 Biochemical Tests

A panel of biochemical tests comprise of oxidase test, Triple Sugar Iron (TSI) test, sulfur reduction – Indole – Motility (SIM) test, methyl red (MR) and Voges-Proskauer (VP) tests and salt tolerance test has been used to characterize the Vibrios (Kaysner & Angelo DePaola, 2004). The biochemical characteristics of three Vibrios have been summarized in Table 2-1.

Typical *V. parahaemolyticus* can grow in broth or agar media containing salt up to 8% while, the maximum NaCl tolerance of *V. vulnificus* is 5-6%. *V. cholerae* doesn't require salt in the media to grow but can tolerate up to 3% salt in the media (Kaysner & Angelo DePaola, 2004).The salt tolerance rates of different *Vibrio* spp. have been summarized in Table 2-2.

Table 2-1: Biochemical characteristics of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus*

		V. parahaemolyticus	*V. cholerae*	*V. vulnificus*
Acid From:	Lactose	-	-	+
	Sucrose	-	+	-
	D-Mannose	+	+	+
	D-Mannitol	+	+	V
	Arabinose	+	-	-
	D-Cellobiose	V	-	+
	VP	-	V	-
Sensitivity to:	Gelatinase	+	+	+
	Urease	V	-	-
Oxidase		-	+	-

V=Variable among strains

Table 2-2: Growth response of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* to various concentrations of NaCl

Salt Tolerance	*V. parahaemolyticus*	*V. cholerae*	*V. vulnificus*
0% Nacl	-	+	-
3% Nacl	+	+	+
6% Nacl	+	-	+
8% Nacl	+	-	-
10% Nacl	-	-	-

API-20E test kit based on the analytical profile index (API) is another method which is frequently used to identify the bacteria based on a variety of biochemical reactions. The API-20E test kit is specifically designed to examine the presence of the metabolites and wastes produced by viable bacteria indicating certain kinds of bacteria including Vibrios (Kaysner Charles A. and DePaola Angelo, 2004).

2.9 Most Probable Number (MPN) Method

The most probable number (MPN) assay is based on the dilution of a sample for an endpoint titration. Each dilution, which may or may not contain viable organisms, is inoculated into enrichment broths and the results obtained from the MPN enumeration reveal the amount of target viable organism present in the diluents, providing an estimating of the original undiluted concentration of bacteria in the sample (Gronewold & Wolpert, 2008). Thus, serial dilutions of several tubes are used to attain the estimation over a wide range of possible concentrations at each dilution. Finally, MPN tables or Microsoft excel tools are used to compute the correct combination of positive tubes that are turbid (FDA-BAM, 2003).

2.10 Molecular Methods to Identify and Characterize *Vibrio* spp.

Recent molecular advances in microbiology have greatly improved the detection of bacterial pathogens including *Vibrio* species in food and environmental samples (Bonnin-jusserand et al., 2017). Improvement and a downward trend in the cost of molecular detection methods have contributed to increased frequency of detection of pathogenic Vibrios where traditional culture-based detection methods have failed.

2.10.1 Conventional PCR

PCR, the most widely used DNA-based method, works through amplifying a specific target DNA sequence in a cyclic three steps process. It can detect up to a single pathogen present in the food sample (Mandal, Biswas, Choi, & Pal, 2011). It also contributes in characterization of the pathogenicity (Khoo et al., 2009), molecular sub typing (Borucki & Call, 2003; Modarressi & Thong, 2010) and antibiotic resistance (Yoke-Kqueen et al., 2008). Having invented 30 years ago, a wide number of Simplex (Aznar & Alarcón, 2002) and Multiplex (Khoo et al., 2009; Chen, Tang, Liu, Cai, & Bai, 2012) PCR assays detecting foodborne pathogens including *V. parahaemolyticus*

(Neogi et al., 2010; Wei, Zhao, Xian, Hussain, & Wu, 2014) *V. cholerae* (Neogi et al., 2010; Wei, Zhao, Xian, Hussain, & Wu, 2014) and *V. vulnificus* (Neogi et al., 2010; Wei, Zhao, Xian, Hussain, & Wu, 2014) have been reported by researchers.

2.10.2MPN-PCR

2.10.2.1 Simplex MPN-PCR

PCR method and MPN assay have been combined in to MPN-PCR, considering that quantitative measures would supplement and extend the qualitative analysis. Previously, MPN concept was developed to assess the microbial load of target organism based on probability. This approach is capable of increasing the sensitivity if a large (up to 25 ml) inoculum is used in an enrichment series (Mancusi & Trevisani, 2014). Considering these benefits, the combined method, MPN-PCR was developed to have the advantage of specificity of PCR system and sensitivity of MPN assay in one platform. Moreover, MPN-PCR can enumerate the presence of viable cell which cannot be achieved by conventional PCR or RT-PCR. A considerable amount of literature has been published stating the use of MPN-PCR in detection of *V. parahaemolyticus* (Tunung et al., 2011; Copin, Robert-Pillot, Malle, Quilici, & Gay, 2012),*V. cholerae* (Suzita et al., 2010) and *V. vulnificus* (Hara-Kud et al., 2005).

2.10.2.2 Multiplex MPN-PCR

So far MPN analysis followed by Multiplex PCR was reported by Zappelini et al.(2017) for the characterization of *S. Enteritidis* in raw sewage and by Sandra et al. (2012) for enumerating *B. cereus* and *B. thuringiensis* in ready-to-eat cooked rice in Malaysia. Though these studies contributed to elucidate the applicability of combining MPN and multiplex PCR methods, none of them validated the results by simplex MPN-PCR or any other methods. To the best of our knowledge, no study quantified these three major Vibrios in one assay platform coupling PCR and MPN techniques.

2.10.3 RT-PCR

Real time (RT)-PCR monitors the PCR products formation through determining the fluorescent signal produced by specific dual-labeled probes or intercalating dyes. This method, unlike conventional PCR, does not require post-amplification treatment of the samples, such as agarose gel electrophoresis, reducing the time of analysis (Zhao, Lin, Wang, & Oh, 2014). It offers specific sequence-based detection of target genes through rapid, simultaneous amplification and so is increasingly being applied in food microbiology. It could be an effective alternative to the conventional phenotypic assays in order to get reliable information about the aerobic bacterial count in less than 5 h (Gómez, Pagnon, Egea-Cortines, Artés, & Weiss, 2010).

Several fluorescent systems have been developed for RT-PCR and the most commonly used fluorescent systems include SYBR green (Fukushima et al., 2010; Kim, Lee, Lee, & Cho, 2012), TaqMan probes (Lee et al., 2009) and molecular beacons (Hu et al., 2014) facilitating in detection of foodborne pathogens (Chen et al., 2010; Suo, He, Tu, et al., 2010) including *V. parahaemolyticus* (Park, Jeon, Kim, Park, & Kim, 2013), *V. cholerae* (Koskela et al., 2009; Park et al., 2013) and *V. vulnificus* (Kim et al., 2008; Park et al., 2013). Though among them SYBR green is simple and less expensive, TaqMan probes and molecular beacons are sequence specific probes and so exhibit greater sensitivity (Law, Mutalib, Chan, & Lee, 2014). However, RT-PCR fails to distinguish between viable and non-viable cells and requires trained personnel to handle.

2.10.4 LAMP

LAMP or Loop-mediated Isothermal Amplification is a novel nucleic acid amplification method that provides a rapid, sensitive and specific detection of foodborne pathogens. It works under isothermal conditions and is considered to be

faster than the conventional PCR (Hara-Kudo et al., 2008). In this method, four primers comprising two inner primers and two outer primers are used to target six specific regions of the target DNA. LAMP was used to detect *stx*A2 gene in *Escherichia coli* O157:H7 in the field of foodborne pathogen detection, for the first time (Maruyama, Kenzaka, Yamaguchi, Tani, & Nasu, 2003). Following that, several studies has been reported regarding the use of this method in detection of different foodborne pathogens (Yamazaki, Ishibashi, Kawahara, & Inoue, 2008; Cao, Wu, Jian, & Lu, 2010) including *V. parahaemolyticus* (Chen & Ge, 2010; Wang, Shi, Su, Ye, & Zhong, 2013) , *V. cholerae* (Srisuk et al., 2010; Yamazaki, 2011) and *V. vulnificus* (Han & Ge, 2010; Han, Wang, & Ge, 2011) However, subtle designing of the specific and sensitive primer sets make the method complicated (Zhao et al., 2014).

2.10.5 DNA Microarray

Microarrays are made up of glass slides or chips coated with up to hundreds of chemically synthesized short sequences oligonucleotide probes. Though Microarrays were initially used for the study of gene expression, but recently, oligonucleotide DNA microarray has been widely used in the field of foodborne pathogen detection (Rasooly & Herold, 2008) including *V. parahaemolyticus* (Wang et al., 2011; Gonza, Krug, Nielsen, Santos, & Call, 2004; Panicker, Call, Krug, Bej, & Bej, 2004), *V. cholerae* (Panicker et al., 2004) and *V. vulnificus* (Gonza et al., 2004; Panicker, Call, Krug, Bej, & Bej, 2004). Li et al.(2006) had reported the detection of pathogenic *Shigella* and *E. coli* serotypes by this method for the first time. In general, this method is highly sensitive and allows simultaneous identification of multiple foodborne bacterial pathogens (Chiang et al., 2012; Park et al., 2014). However, this system requires specialized instruments and trained personnel in order to run the experiments.

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

3.1.1 Positive control

Bacterial cultures of known *V. parahaemolyticus V. cholerae* and *V. vulnificus* strains, were used to spike the fish and sushi samples and incorporate as positive controls in all phases of the assessment (Figure 3-1). The strains were taken from the stocks available in Biomedical Science Laboratory, Institute of Graduate studies (IPS), University of Malaya.

3.1.2 Internal Amplification Control (IAC)

A pair of primers targeting plasmid pQE-30 (Qiagen, Venlo, Limburg), displaying amplicon of 187 bp was used as a non-competitive internal amplification control (IAC) in this system. The plasmid and primers were taken from the stocks available in Biomedical Science Laboratory, Institute of Graduate studies (IPS), University of Malaya.

3.1.3 PCR Negative Control

PCR negative control that contained everything along with the water as a replacement of template DNA was used in each of the PCR conducted in this experiment.

3.1.4 Raw Fish and Sushi samples

A total of 120 samples of 50 fish varieties (Appendix A) were purchased from various wet markets (Pasar Borong and Pasar Malam in Petaling Jaya and Selangor), and supermarkets (Tesco, Giant, Aeon). Twelve fish samples were purchased directly from the delivery agent who supplied raw fish to the IPS canteen.

A total 70 sushi samples of seven types (Appendix B) were collected from three different super markets (Aeon Big, Village Grocer and Giant) of Selangor, Malaysia.

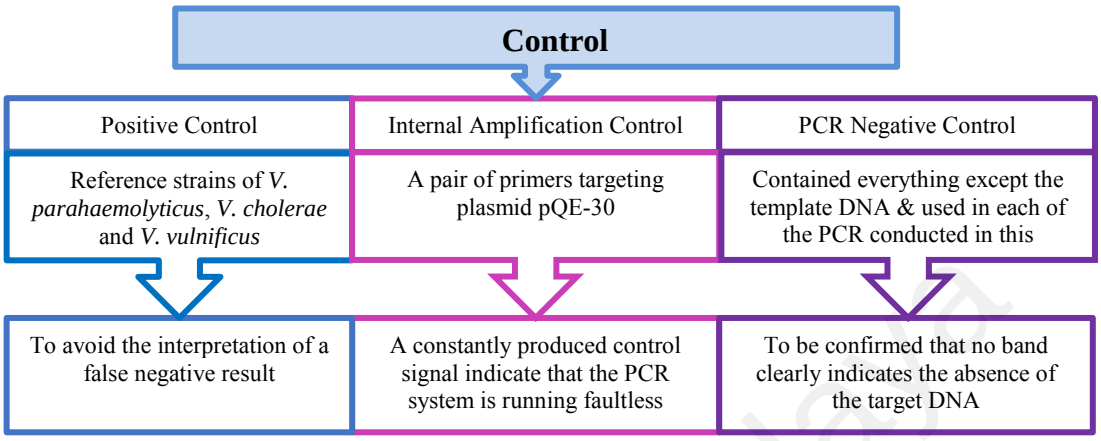

Figure 3-1: Control used in the experiment

3.1.5 Media for bacterial growth

3.1.5.1 Pre-enrichment media

(a) ***Tryptic Soy Broth (TSB) + 3% NaCl***

TSB (BactoTM, France)	3.0 g
Additional NaCl (AnalaR, Leicestershire, England)	3.0 g
Distilled water	100 mL

Tryptic soy broth (**TSB**) powder (3 g) and additional NaCl (3 g) was dissolved in 100 ml distilled water. The container was then heated for better mixing. Finally, the broth was autoclaved for 15 min at 121ºC.

3.1.5.2 Enrichment Media

(a) ***Salt Polymyxin Broth (SPB)***

SPB (Nissui, Japan)	3.3 g
Distilled water	100 mL

Salt Polymyxin Broth (**SPB**) powder (3.3 g) was dissolved in 100 ml distilled water. The container was then heated for better mixing. Finally, the broth was autoclaved for 15 min at 121ºC.

3.1.5.3 Non-selective media

(a) ***Luria-Bertani (LB) Broth***

Tryptone (Oxoid, Hampshire, England)	1.0 g
Yeast Extract (Oxoid, Hampshire, England)	0.5 g
NaCl (AnalaR, Leicestershire, England)	0.5 g
Distilled water	100 mL
Additional 3% NaCl (AnalaR, Leicestershire, England)	3.0 g

Tryptone (1 g), Yeast Extract (0.5 g) and NaCl (0.5 g) with an additional 3% NaCl was dissolved in 100 ml distilled water. The container was then heated for better mixing. Finally, the broth was autoclaved for 15 min at 121ºC.

(b) ***Luria-Bertani (LB)Agar***

LB broth powder (25 g) was dissolved into 1000 ml of water, heated and then autoclaved. It was cooled down into 45-50ºC in water bath and was dispensed into plates and tubes.

3.1.5.4 Primers

The primers used in the multiplex PCR targeting *pnt* A gene for *V. parahaemolyticus* and *V. cholerae* and *vvh* A gene for *V. vulnificus*. Primer sequences used in the multiplex PCR and the size of the expected amplicons are presented in Table 3.1.

3.1.5.5 Solutions and reagents for Agarose Gel Electrophoresis

(a) ***Tris-borate EDTA buffer*** **(1X)**

10X Ultra-Pure Grad Tris-borate EDTA buffer (Base Asia) were diluted up to 1X with distilled water to prepare the agarose gel.

(b) ***Agarose Gel***

Agarose Gel Powder (3 g) was dissolved in 150 ml 1X TBE buffer and heated to prepare a 2% gel.

Agarose powder (Oxoid, Hampshire, England)	3.00 g
1X TBE buffer	150 ml

(c) ***Florosafe DNA stain***

Florosafe DNA stain (Base Asia) has been used to stain the agarose gel in a ratio of 1:25 prior to pouring the semi liquid gel in the tray.

3.2 Methods

3.2.1 Sampling

The sampling was done according to Bacteriological Analytical Manual Standard Method (Kaysner & Angelo DePaola, 2004). Each sample was assigned to an identification code to maintain a database of the isolates.

3.2.1.1 Raw Fish

A total of 120 samples of 50 fish varieties (Appendix 1) were purchased from various wet markets (Pasar Borong and Pasar Malam in Petaling Jaya and Selangor), supermarkets (Tesco, Giant, Aeon) and direct purchase from delivery man who supplied raw fish to the canteen in IPS. Sampling was randomly carried out on 12 different dates between 8:00 am and 11:00 am. All of the samples were kept in the stomacher bag and

were transported ice-chilled to the laboratory and were immediately processed within an hour for subsequent analysis.

Twenty grams of meat with the skin from each fish was separated and processed using sterile blades, knives and foil paper in the laboratory. The separated sample was then kept in the stomacher bag (sterile), homogenized and divided into two portions (Figure 3-2). All the samples were handled and processed aseptically avoiding cross contamination. After homogenization, the samples were incubated and analyzed.

3.2.1.2 Sushi

A total 70 sushi samples of 7 types (Appendix 2) were collected from 3 different super markets (Aeon Big, Village Grocer and Giant) of Selangor, Malaysia between 11:00 am and 2:00 pm in 10 days. Out of the 70 samples, 30 were collected from Aeon Big while 20 samples were collected from both Village Grocer and Giant. All of the samples were kept in the stomacher bag and placed into insulation box with ice packs for transportation to the laboratory. The samples were transported from the market to the laboratory within 1 h and processed for subsequent analysis.

Each sample was equally divided in three portions inside the laminar flow using foil paper for subsequent analysis (Figure 3-2) with proper care avoiding chances of contamination. The first portion was analyzed within one hour, the second portion was stored in room temperature for 4-5 hours and the third portion has been refrigerated at -20 for 7 prior to analysis.

3.2.2 Pre-enrichment and Enrichment of the collected samples

All the samples (raw fish and sushi) were enriched in two steps because two-step enrichment is more effective than one step enrichment (Hara-Kudo et al., 2001). In the first step, ten grams of each sample was mixed with 90 ml of Tryptic Soy Broth (TSB;

BactoTM, France) supplemented with 3% sodium chloride (NaCl; Merck, Germany), homogenized and incubated at 37°C for 6 h prior to analysis.

In latter step, all of the samples were diluted from 10^{-1} to 10^{-7} in Salt Polymyxin Broth (SPB; Nissui, Japan). Next, all the tubes were incubated at 37°C for 14 to 16 h. After enrichment, the turbid diluted tubes were subjected to PCR and the concentrations (MPN/g) of viable microorganisms in the raw fish samples were calculated following computer-assisted Microbiological Methods & Bacteriological Analytical Manual (BAM) (US Food & Drug Administration, 2010).

3.2.3 Preparation of Controls

Three different controls (Positive control, Negative control and IAC) have been used in this study in order to avoid unwanted error.

Positive Control were prepared according to the method mentioned by Teh, Chua, Puthucheary, & Thong (2008) with some minor modification in terms of the percentage of NaCl. In brief, a single colony of *V. parahaemolyticus, V. cholerae* and *V. vulnificus* was inoculated in 1 mL of Luria Bertani (LB) broth, supplemented with 3.0% NaCl, and incubated overnight at 37 °C. An aliquot of 100 µl with concentration $>10^4$ from each of the overnight cultures of all three Vibrios were spiked in 5g of raw fish and fresh sushi samples and were kept at room temperature for 30 minutes.

Prior to use this spiked sample as a positive control in subsequent analysis, the presence of target Vibrios in the sample has been assessed. In this purpose, the spiked sample was homogenized with 45 mL of the alkaline peptone water (APW) and incubated overnight at 37°C. Next, 10-fold serial dilutions (10^{-1}-10^{-9}) was carried out. An aliquot of 100 µl of each dilution was boiled, centrifuged and the supernatant was used for direct PCR analysis.

Table 3-1: Primers used in Multiplex PCR

Target Bacteria	Target Gene	Primers		Amplicon Size	Reference
		Forward Primer	Reverse Primer		
V. parahaemolyticus	*pntA*	5'-AGCAAGTTTCGATGATGCTG-3'	5'-ACCAGCAACCAAAACTTTCGCT-3'	409	(Teh, Chua, & Thong, 2010)
V. cholerae	*pntA*	5'-CAGTAAAGAAACGACCAAACTC-3'	5'-TGCCAGTTTCGATGATGCCG-3'	338	(Teh et al., 2010)
V. vulnificus	*vvhA*	5′-TTCCAACTT CAAACCGAACTATGAC-3′	5′-ATTCCAGTCGATGCGAATACGTTG-3′	205	(Canigral, Moreno, Alonso, Ferru, & Gonza, 2010)

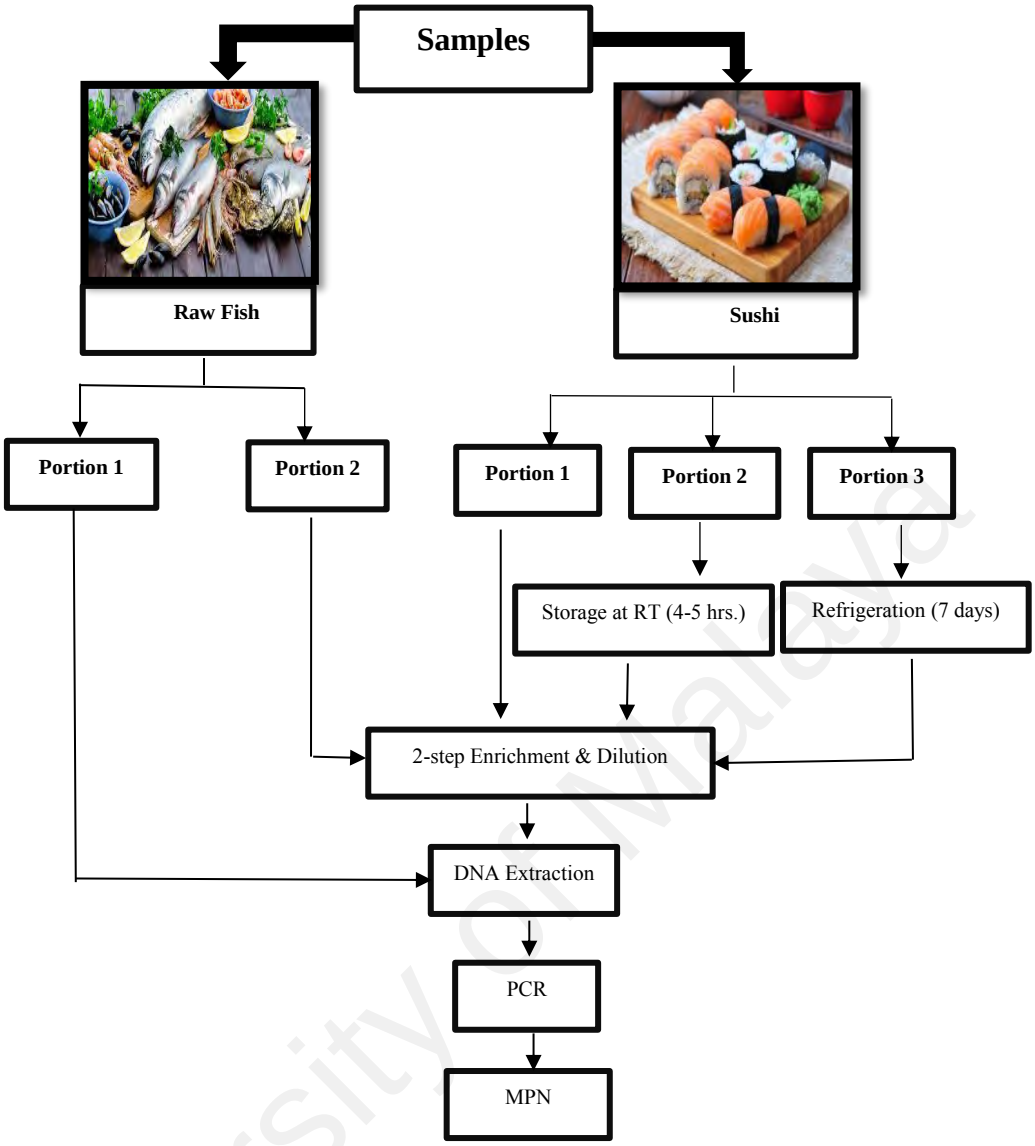

Figure 3-2: Overview of the experimental approach

For all PCR assays, an internal amplification control (IAC) was included. The IAC was originally developed and optimized by Thong, Tech, & Chua (2014). Based on the published work, the IAC was originally developed by cloning the plasmid pQE-30 and the primers target a 187 bp fragment of the plasmid (Thong et al., 2014). Both the primers and DNA for the IAC were provided by the Biomedical Science Laboratory, IPS.

A PCR negative control was prepared separately during the PCR preparation in each experiment by replacing the template DNA with dH_20.

3.3 DNA Extraction

DNA was extracted from all turbid tubes using a boiled cell method (Lim and Thong (2009) with some modification to avoid inhibition in subsequent PCR analysis. In brief, an aliquot of 1 ml clear food homogenate was centrifuged in a 1.5 ml microfuge at 12,000 rpm for 3 min. The supernatant was discarded, and the pellet was re-suspended in 100 µl of 1X PBS buffer of pH: 7.4, followed by centrifugation at 12,000 rpm for 2 min. The washing step was repeated twice, and the pellet was re-suspended in 100 µl of sterile distilled water. The cell suspension was then boiled for 5 min, snapped cooled in ice for 10 min and re-centrifuged briefly at 13,400 rpm for 3 min. An aliquot of the supernatant (80 µl) which contained the crude DNA was transferred to a fresh sterile mic centrifuge tube and stored at -20°C until further use (Figure 3-3)

3.4 Optimization of Multiplex PCR

Previously published species-specific primers were used for the identification of *V. parahaemolyticus* (5'-AGCAAGTTTCGATGATGCTG-3'; 5'-ACCAGCAACCAAAACTTTCGCT-3') (Teh et al., 2010), *V. cholerae* (5'-CAGTAAAGAAACGACCAAACTC-3'; 5' -TGCCAGTTTCGATGATGCCG-3') (Teh et al., 2010) and *V. vulnificus* (5′-TTCCAACTTCAAACCGAACTATGAC-3′; 5′-ATTCCAGTCGATGCGAATACGTTG-3′) (Cañigral, Moreno, Alonso, González, & Ferrús, 2010). For *V. parahaemolyticus* (409 bp) and *V. cholerae* (338 bp) the primers targets were *pnt* A gene while it was *vvh* A gene for *V. vulnificus* (205 bp). Each PCR was repeated at least twice to determine reproducibility. The simplex PCR was performed in a 25 µl reaction mixture containing 5 µl of 5X PCR buffer, 2.5 µl of 25 mM MgCl2, 1.0 µl of 0.2 mM deoxynucleoside triphosphate mix, 1.0 µl of 0.5 µM of

each primer, 0.125 µl of 5U/ µl of *Taq* polymerase, 1.5 µl of DNA template (40 ng/uL) and 2.0 µl of IAC (Internal Amplification Control). The reaction mixtures were heated at 94°C for 4 min in the initial denaturation step, followed by 35 cycles of final denaturation at 94°C for 1 min. Primer annealing was at 60°C for 1 min, extension was at 72°C for 1 min and the final extension was performed at 72°C for 5 min. The PCR products were electrophoretically separated by running in 2% agarose gel in 0.5X Trisborate-EDTA buffer stained with Florosafe DNA stain (1st base Lab- oratories, Selangor, Malaysia) at 120 V for 60 min. The gel was visualized under a gel documentation system (AlphaImager HP, Alpha Innotech Corp., California, USA). A 50 bp DNA ladder (Promega, USA) was used as a molecular size marker. A negative control was made by replacing template DNA with the deionized water.

After optimizing the simplex PCR with IAC, duplex, triplex and tetraplex PCR were optimized step by step (Figure 4-1) for the detection of *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae* in one assay platform. Like simplex PCR, 25 µl reaction mixture was used having the same components but different amount of $MgCl_2$ (3.0 µl of 25 mM), *Taq* polymerase (0.130 µl of 5U/ µl), and primers (1.25 µl of 0.5 µM). The primer extension was at 74°C for 40 secs instead of 72°C for 1 min. The gel electrophoresis was set at 120 V for 80 min instead of 60 min.

3.5 Detection of target Vibrios in Raw Fish

Three methods including the multiplex PCR, simplex MPN-PCR and multiplex MPN-PCR were used to detect the microbial presence and to quantify and microbial load of the target Vibrios in raw fish samples (Figure 3-4).

In one hand, DNA was extracted from all the raw fish samples and those DNA were assessed using the optimized multiplex PCR to detect the presence of Vibrios and to compare the sensitivity of multiplex PCR with multiplex MPN-PCR.

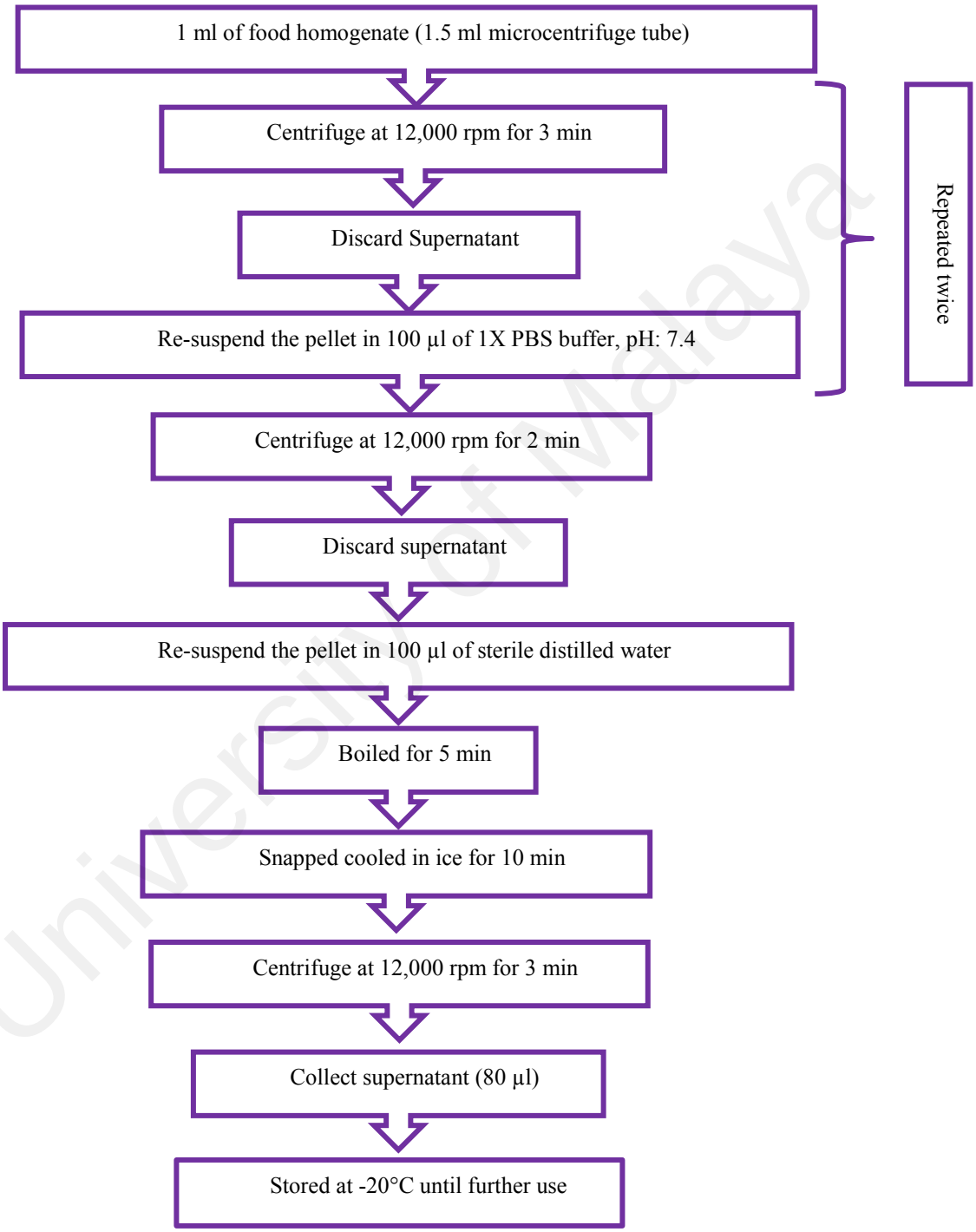

Figure 3-3: Steps in DNA extraction

In the other hand, all of the raw fish samples were enriched and diluted before the extraction of DNA. Extracted DNA of those diluents were then subjected for simplex and multiplex PCR simultaneously. Following that, MPN analysis was done for the detection and quantification of Vibrios.

3.6 Detection of target Vibrios in Sushi

All 70 sushi samples were divided in to three portions for discriminatory identification of Vibrios and for subsequent analysis (Figure 3-5).

The first portion of the sushi samples were assessed within the first hour of processing to check the microbial load in RTE sushi at the point of sale using the optimized multiplex MPN-PCR method.

The second portion of the samples were stored at room temperature for 4-5 hrs. to check the rise in microbial load if the sushi is consumed after several hours of buying and not stored in below 5 ℃.

The third portion of all the sushi samples were refrigerated at -20 ℃ for 7 days and microbial load were measured subsequently in order to observe if the recommended 7 days of refrigeration can prevent the microbial growth and ensure the food safety of sushi.

3.7 DNA sequencing

PCR products were submitted for the commercial sequencing service which includes the purification of PCR products using a PCR purification kit (1st Base, Selangor, Malaysia) and visualization of the purified samples in 1.5% agarose gel. BigDye® Terminator v3.1 cycle sequencing kit (Chang, Lin, Ren, Lin, & Shao, 2016) in an ABI PRISM 96-capillary 3730xl genetic analyzer (Applied Biosys-tems, USA) was used to

sequence the purified PCR products in both directions. Lately, the similarity between the reference sequence and the detected sequence has been assessed.

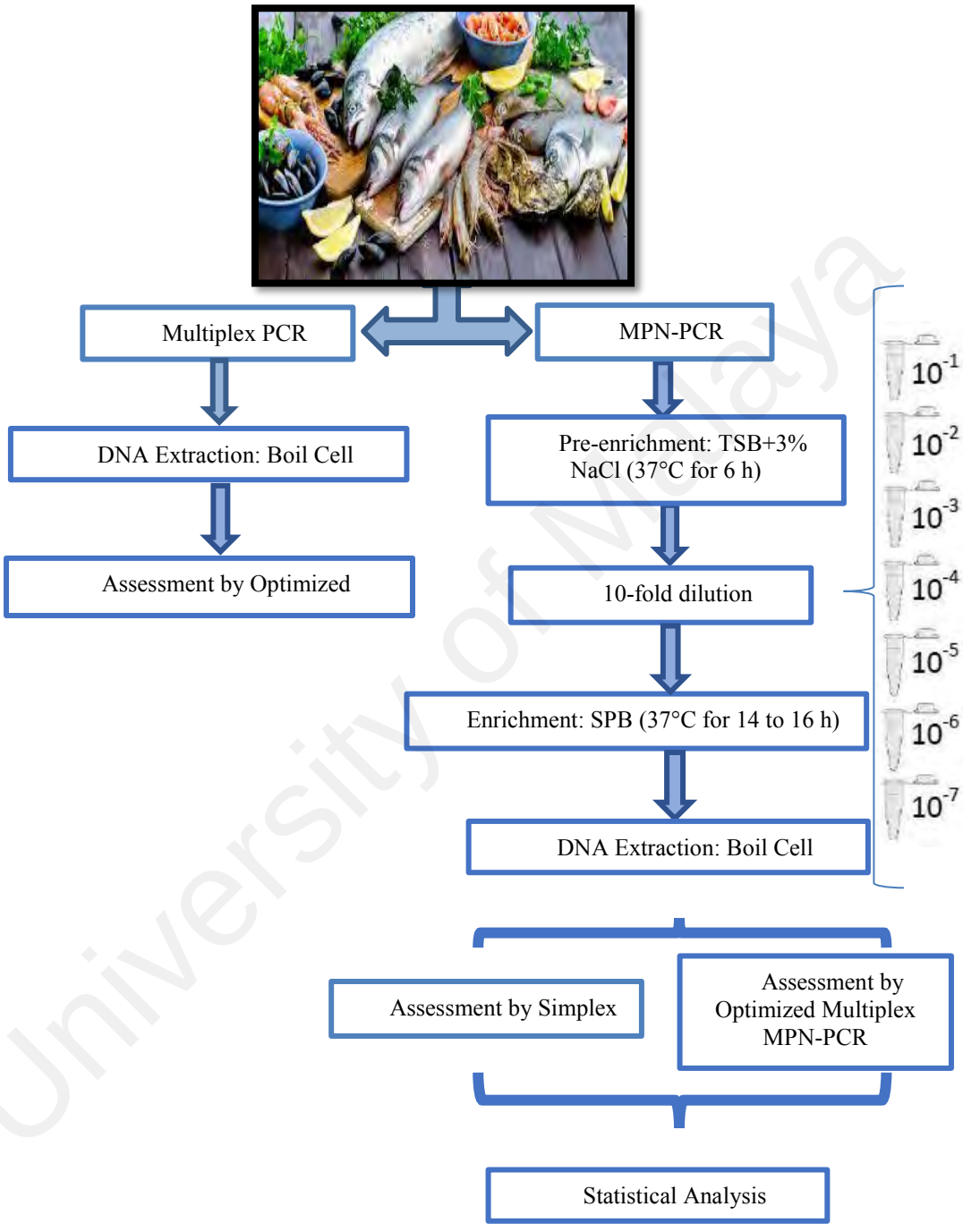

Figure 3-4: Steps to enumerate Vibrios in raw fish

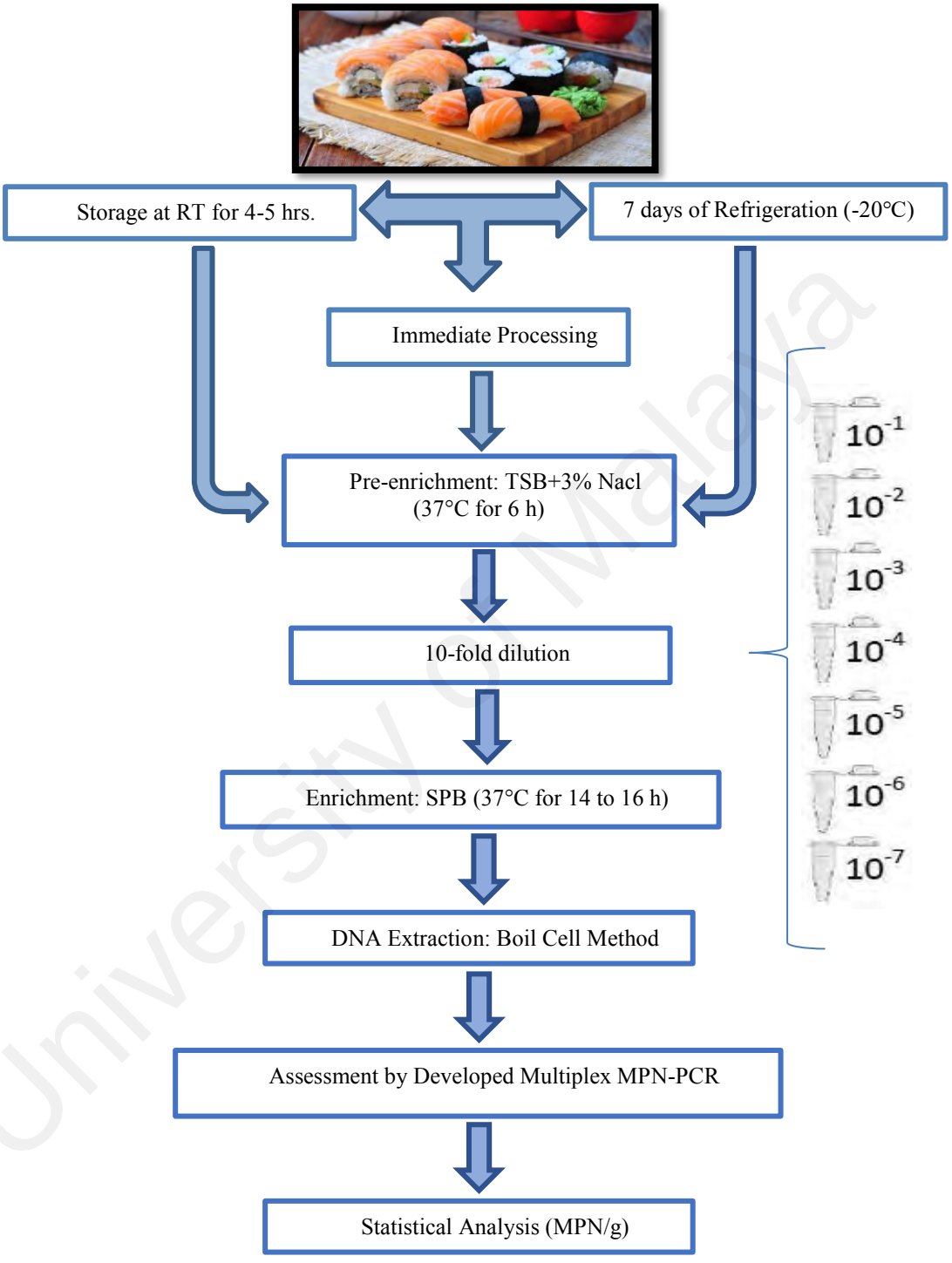

Figure 3-5: Steps to enumerate Vibrios in sushi

3.8 MPN Analysis

3.8.1 Dilution

Greater number (10^{-7}) of dilutions were set to ensure the presence of at least one negative PCR result because the result is considered as incomplete if all the tests are found positive (Jarvis, 2016).

3.8.2 Use of FDA-BAM Microsoft Excel Tool

MPN/g was calculated to estimate the microbial load of viable microorganisms in the raw fish and sushi samples following computer-assisted Microbiological Methods & Bacteriological Analytical Manual (BAM) (US Food & Drug Administration, 2010).

CHAPTER 4: RESULT

4.1 Optimization of Multiplex PCR

Species-specific PCR for *V. parahaemolyticus*, *V. cholerae*, and *V. vulnificus* along with IAC was optimized (Figure 4-1). Denaturation and primer annealing time was modified to minimize the occurrence of nonspecific bands (Thong et al., 2014). The use of an IAC facilitated the interpretation of reactions that showed a negative PCR result because a control signal is constantly produced by the IAC when there is no other target (Zijlstra & Van Hoof, 2006). Reference strains of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* were used as positive control in all phase of the experiment to avoid the interpretation of a false negative result. In contrast, the use of a negative control eliminated the anticipation of false positive results.

4.2 Detection of Vibrios in Raw Fish

The Vibrios have been detected through three different methods including multiplex PCR, simplex MPN-PCR and multiplex MPN-PCR.

Out of 120 samples, 41(34.2%), 22 (18.3%) and 27 (22.5%) samples were detected positive for *V. parahaemolyticus*, *V. cholerae*, and *V. vulnificus*, respectively by the optimized multiplex PCR.

The occurrence for *V. parahaemolyticus*, *V. cholerae*, and *V. vulnificus* were 48.33%, 27%, and 32.50% (Table 4-1) with microbial load of 0-2.43 $x10^6$, 0-2.16 $x10^6$, and 0-4.93 $x10^5$ MPN g^{-1}, respectively, in both simplex MPN-PCR and multiplex MPN-PCR (Appendix C-E).

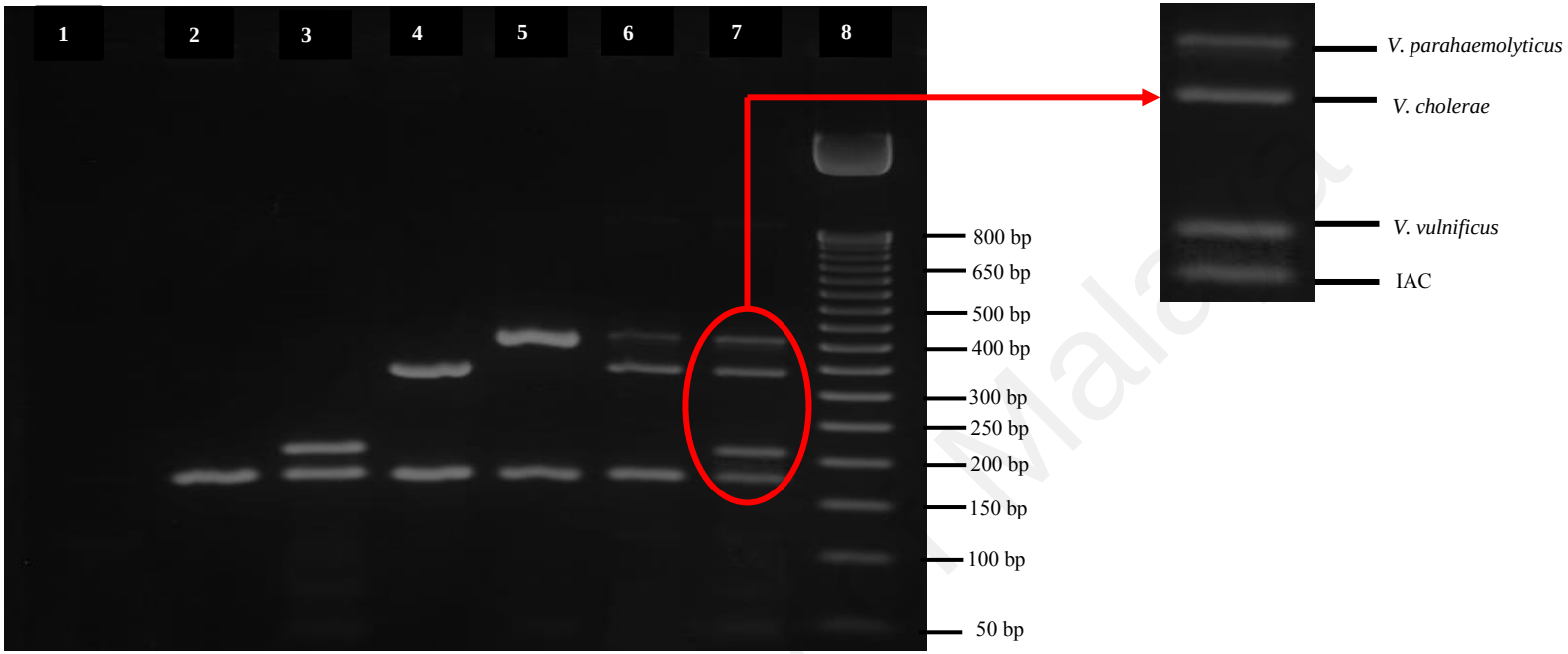

Figure 4-1: Multiplex-PCR for *V. parahaemolyticus* (409 bp), *V. cholerae* (338 bp) and *V. vulnificus* (205 bp).

The *pntA* gene was targeted for *V. cholerae* and *V. parahaemolyticus*, whereas *vvhA* gene was targeted for *V. vulnificus*. Shown are: *Lane* 1: Negative control; *Lane* 2: IAC (Internal Amplification Control); *Lane* 3: *V. vulnificus & IAC*; *Lane* 4: *V. cholerae* & IAC*; Lane* 5: *V. parahaemolyticus* & IAC; *Lane* 6: *V. cholerae*, *V. parahaemolyticus* & IAC. *Lane* 7: Optimized tetraplex PCR with three Vibrios and IAC; *Lane* 8: 50-bp DNA ladder

Table 4-1: Summary of *V. parahaemolyticus*, *V. cholera*, and *V. vulnificus* prevalence in raw fishes in Malaysian markets.

Types of PCR	n	*V. parahaemolyticus*		*V. cholerae*		*V. vulnificus*	
		PCR Positive	%	PCR Positive	%	PCR Positive	%
Simplex MPN-PCR	120	58	48.33	32	27	39	32.5
Multiplex MPN-PCR	120	58	48.33	32	27	39	32.5

4.2.1 Sensitivity of multiplex PCR and multiplex MPN-PCR

The data shows that the sensitivity of multiplex MPN-PCR was higher than the conventional multiplex PCR (Figure 4-2). MPN-PCR could detect 48.33%, 27% and 32.50% while multiplex MPN-PCR could detect 34.16%, 18.33% and 22.50% of *V. parahaemolyticus*, *V. cholera* and *V. vulnificus* respectively.

MPN based PCR methods offer several advantages over conventional PCR methods. Firstly, the sensitivity of MPN method can be increased using large volumes (practically up to 25 ml) of inoculum in enrichment series. Secondly, the PCR independently cannot determine the microbial load alike MPN based PCR methods (simple & multiplex).

4.2.2 The occurrence of Vibrios in raw fish (simplex MPN-PCR and multiplex MPN-PCR)

The occurrence of *V. parahaemolyticus*, *V. cholera*, and *V. vulnificus* in raw fish determined by the simplex PCR and multiplex MPN-PCR is shown in Figure 4-3, 4-4, 4-5. About 16, 7 and 2% counts of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* in the range ">5,000" MPN g^{-1}, clearly revealed that *V. parahaemolyticus* was the most prevalent among these three Vibrios in Malaysian raw fishes.

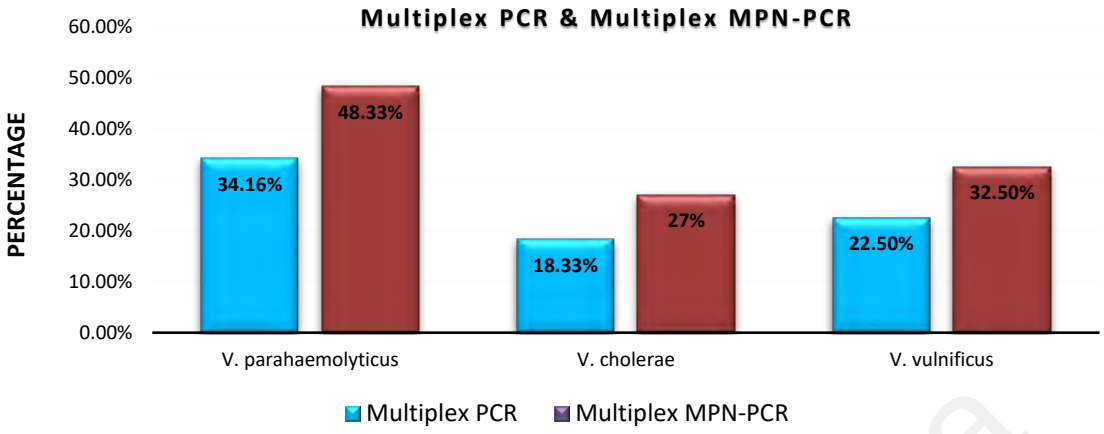

Figure 4-2: Comparison of the presence of three major Vibrios in raw fish using Multiplex PCR and Multiplex MPN-PCR

The MPN-PCR value (MPN g^{-1}) was placed over several ranges from 0 to >5,000 MPN g^{-1} (X-axis) and percentages (Y-axis) of each of the three Vibrios in each of those ranges were calculated; wherein "0" (X-axis) represented negative count and >5000 demonstrated highest number of bacterial counts. Thus, the percentage of Vibrios from the lowest (>0) to the highest (>5,000) MPN values reflected a greater risk of Vibrios' presence and subsequent infection threats. About 19, 2 and 9% counts of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus,* respectively is in the range ">5,000" MPN g^{-1}, clearly revealed that *V. parahaemolyticus* was the most prevalent among these three Vibrios in Malaysian raw fishes.

V. parahaemolyticus and *V. cholerae; V. parahaemolyticus* and *V. vulnificus;* and *V. cholerae* and *V. vulnificus*, were positive in 13 (10.83%), 17 (14.16%) and 2 (1.66%) samples out of 120 samples respectively. On the other hand, overall 8 (6.7%) samples were positive for all the three target *Vibrio* species (Figure 4-6).

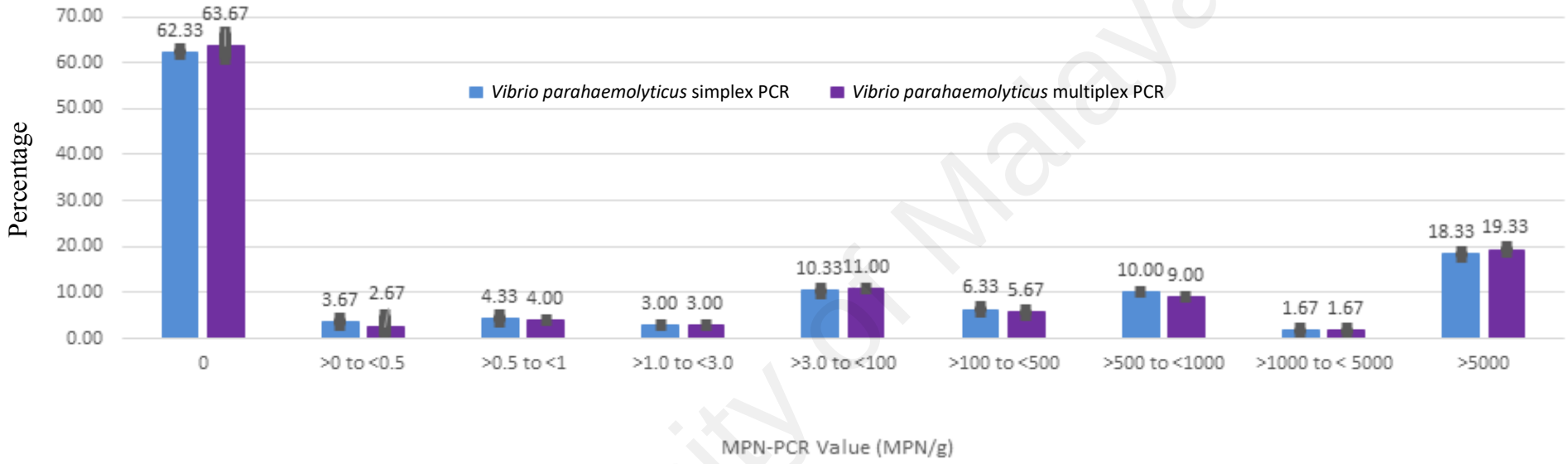

Figure 4-3: Distribution of MPN-PCR count of *V. parahaemolyticus*; in raw fishes: simplex and multiplex PCR. The mean values of all three trails for both simple and multiplex PCR are used.

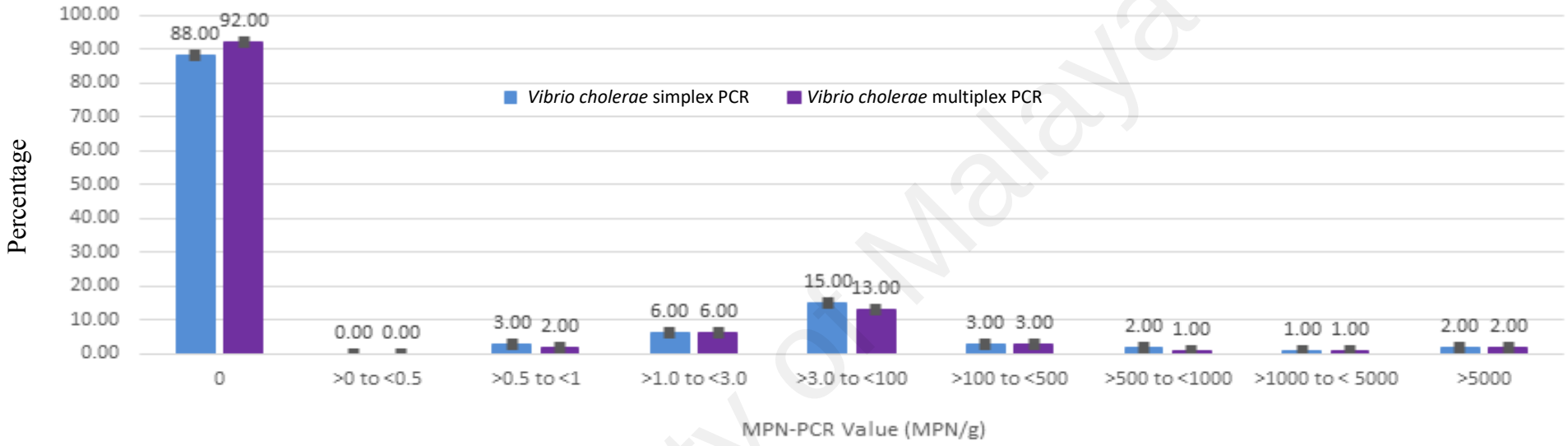

Figure 4-4: Distribution of MPN-PCR count of *V. cholera*; in raw fish: simplex and multiplex PCR. The mean values of all three trails for both simplex and multiplex PCR are used.

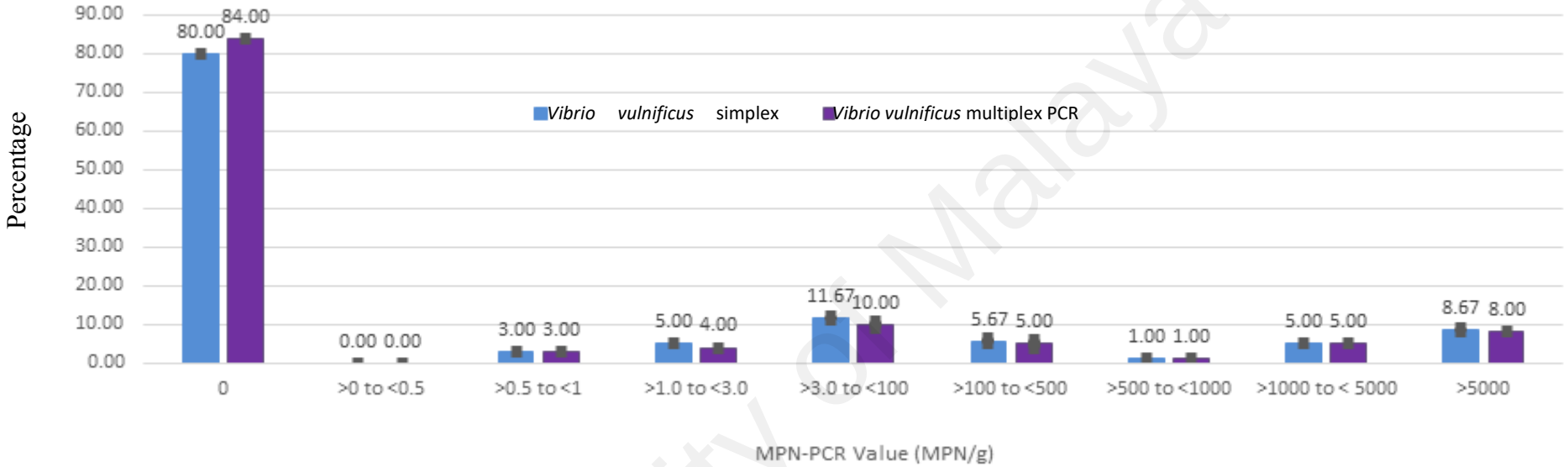

Figure 4-5: Distribution of MPN-PCR count of *V. vulnificus* in raw fishes: simplex and multiplex PCR. The mean values of all three trails for both simple and multiplex PCR are used.

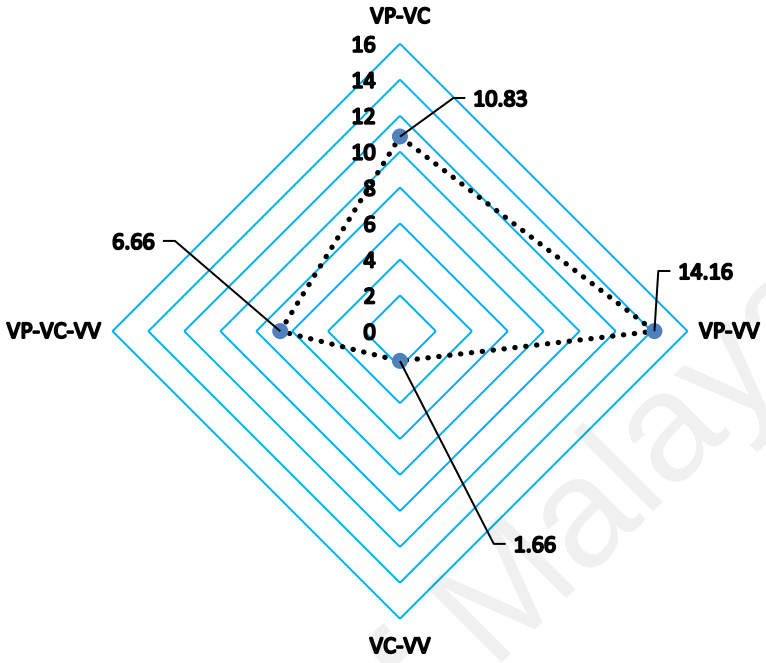

Figure 4-6: Relative abundance of the target Vibrios in the fish samples.

VP = *V. parahaemolyticus,* VC = *V. cholerae,* VV = *V. vulnificus*

4.3 Enumeration of target Vibrios in Sushi

Out of 70 samples, 16 (22.86%), 4 (5.71%) and 8 (11.43 %) samples were found to be positive for *V. parahaemolyticus, V. cholerae and V. vulnificus,* respectively in the original state of the sushi while 20 (28.57%), 4 (5.71%) and 9 (12.86%) samples positive for *V. parahaemolyticus, V. cholerae and V. vulnificus,* respectively following 4-5 hrs. storage at room temperature (25-27℃).In addition,7 (10%), 3 (4.29%) and 6 (8.57%) samples were found to contain *V. parahaemolyticus, V. cholerae* and *V. vulnificus,* respectively following 7 days of refrigeration at (-20℃) (Figure 4-7) (Appendix F).

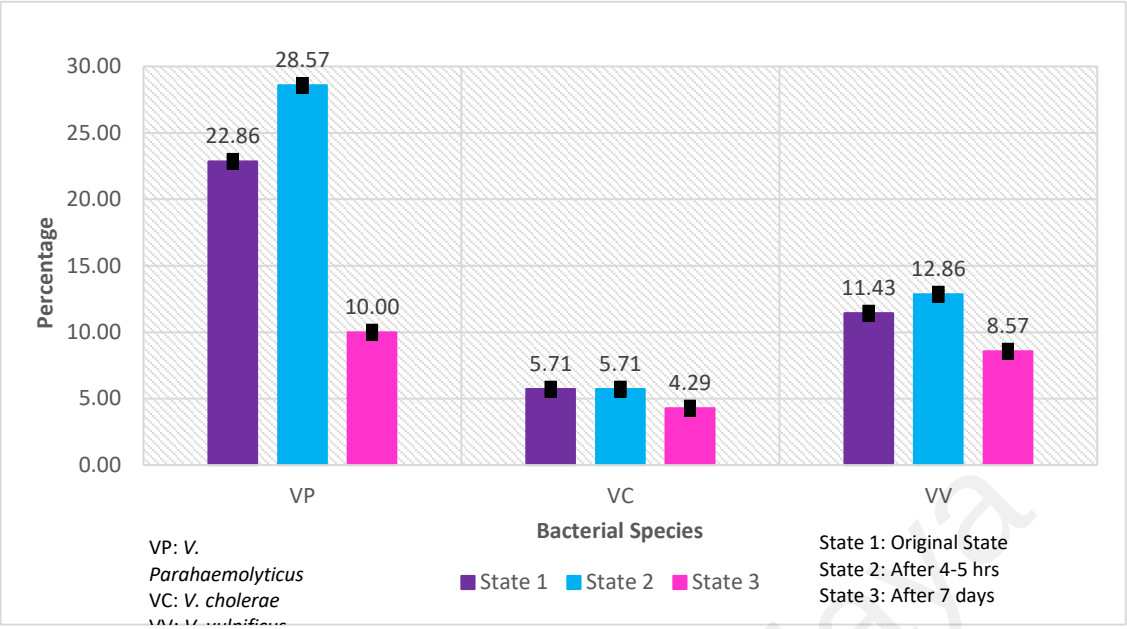

Figure 4-7: Percentage of the target Vibrios in the sushi samples.

The microbial load of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* were $9.79x10^{-1}$-$9.17x10^{2}$, $2.49x10^{1}$-$1.36x10^{2}$ $MPNg^{-1}$ and $2.49x10^{1}$-$4.22x10^{2}$ MPN g^{-1}, respectively in the initial state at the point of sale while $1.12x10^{0}$-$5.36x10^{3}$, $6.36x10^{1}$-$1.66x10^{2}$ $MPNg^{-1}$ and $1.95x10^{1}$-$5.36x10^{3}$ MPN g^{-1} respectively following 4-5 hrs. of storage and $7.36x10^{-1}$-$5.88x10^{1}$, $1.22x10^{0}$-$1.95x10^{1}$ $MPNg^{-1}$ and $9.79x10^{-1}$-$2.49x10^{1}$ MPN g^{-1} respectively after 7 days of refrigeration (Figure 4-8) in all three trails (Appendix G-I).

4.3.1 Survivability of Vibrios in Sushi

The variation in time and conditions have been included to ascertain the changes in bacterial growth upon storage at room temperature and the recommended 7-hour refrigeration period.

The distribution of microbial (*V. parahaemolyticus, V. cholerae* and *V. vulnificus)* loads in refrigerated sushi obtained by MPN-PCR is shown in Figure 4-9, Figure 4-10

and Figure 4-11, respectively. The MPN-PCR values (MPN/g) were placed over several ranges (Y-axis) in terms of log_{10} and the run-on time (X-axis) of each of the *Vibrio* were calculated. Different colors of the graphs represent the microbial load of different sushi samples in different conditions; wherein 1= At the point of sale; 2= After 4-5 hrs. of storage in RT and 3= After 7 days of refrigeration.

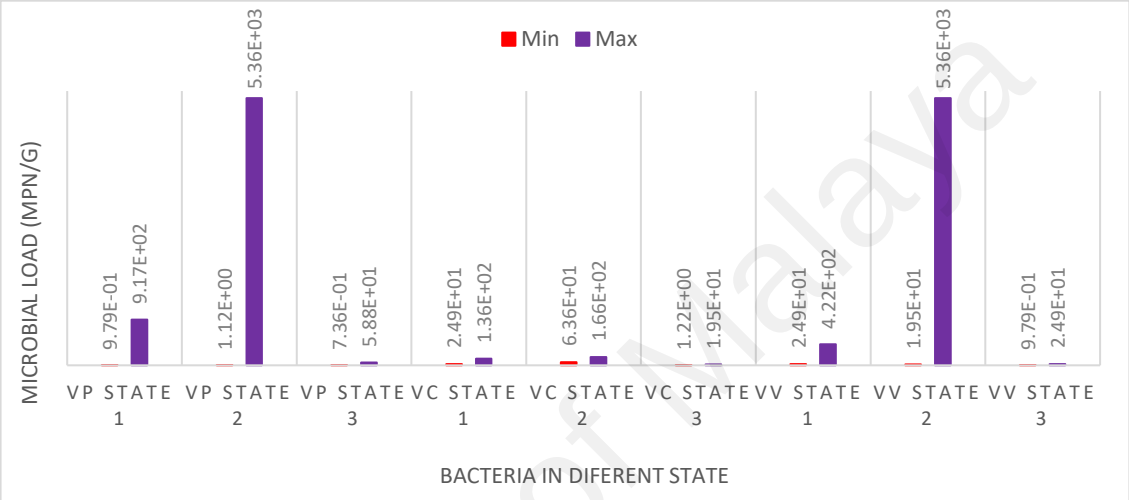

Figure 4-8: Microbial Load (Min-Max) of Vibrios in sushi.

The mean values of all three trails are used.

It can be speculated from the graph that the microbial load was increased after leaving the sushi for 4-5 hrs. at the room temperature and consumption of that sushi in that state might pose a health risk to the consumers while reduction in microbial load was observed after recommended 7-days refrigeration period, yet it could not make the sushi entirely pathogen free.

4.4 DNA sequencing

Species specific amplicons for Vibrios (*V. parahaemolyticus*, *V. cholerae* and *V. vulnificus*) were sequenced and 99% similarity was found with the reference strains which validate the amplified DNA fragments.

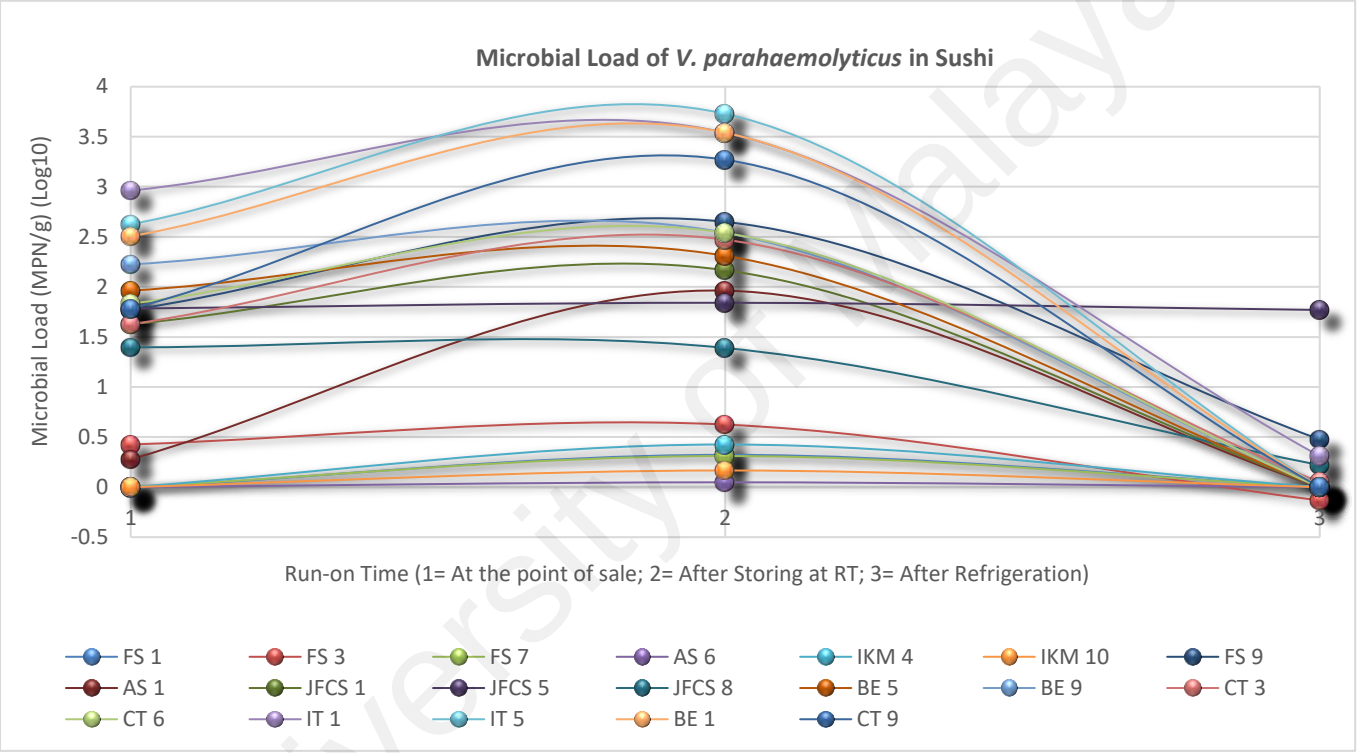

Figure 4-9: Survivability of *V. parahaemolyticus* at three different storage conditions in sushi. The mean values of all three trails are used.

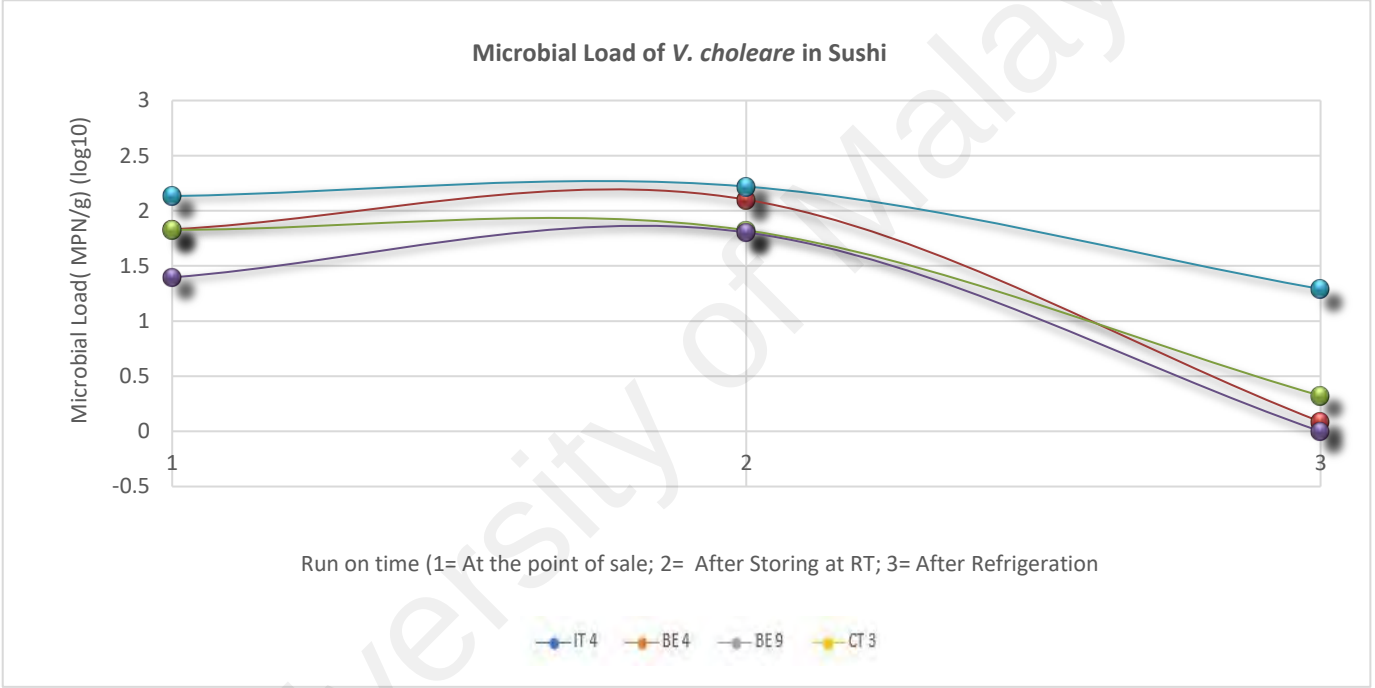

Figure 4-10: Survivability of *V. cholerae* at three different storage conditions in sushi. The mean values of all three trails are used.

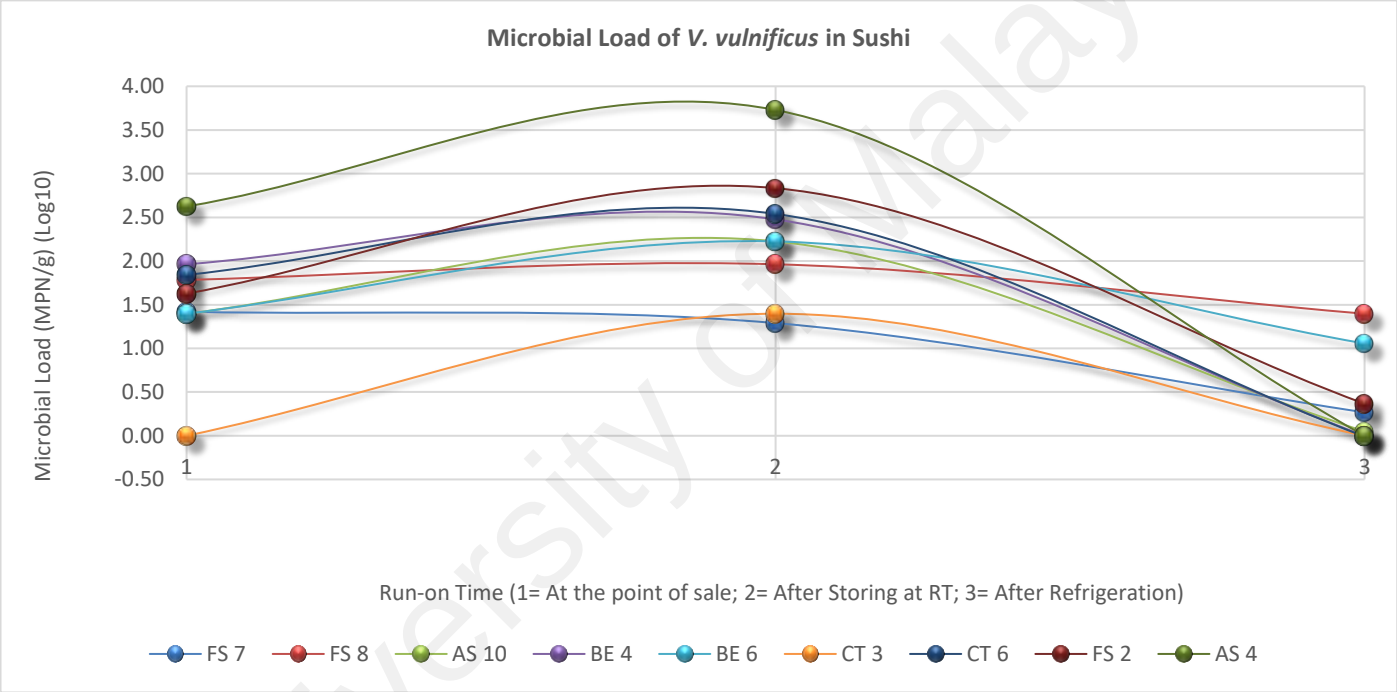

Figure 4-11: Survivability of *V. vulnificus* at three different storage conditions in sushi. The mean values of all three trails are used.

CHAPTER 5: DISCUSSION

5.1 DNA Extraction

Molecular techniques in bacteriology start with bacterial DNA extraction & purification and the attainment of the PCR mostly depends on the quality of the DNA. In this study, the direct boiled cell method was used in order to extract the crude DNA for subsequent analysis. Removing the cells from the growth media by centrifugation was required in the very first step of DNA extraction as the food homogenates (raw fish & sushi) were in the suspension. PBS was used to wash the pellet in order to wash away the impurities. The purpose of boiling the cells was to lyse bacterial cells and release the crude DNA into the water. The tube containing the samples was chilled in order to prevent renaturation of the double strand of DNA.

5.2 Selection of Biomarkers

The presence of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* was confirmed by amplifying specific regions of *pnt*A gene (409 bp for *V. parahaemolyticus*) (Teh et al., 2010) and (338 bp for *V. cholerae*) and a 205 bp region of *vvh*A gene for *V. vulnificus* (Canigral et al., 2010). The house-keeping gene *pnt*A that codes for transcription factor regulator was useful for the differential detection of *V. parahaemolyticus* and *V. cholerae* and was more specific (~100%) than those of *tox*R gene (Teh et al., 2010). On the other hand, *vvh*A gene that codes for a transmembrane transcription activator for hemolysis and cytolysis was an ideal target for *V. vulnificus* because the expression of the *vvh* (*vvh*A and *vvh*B) gene can be detected even after several months of VBNC stage (Saux, Hervio-Heath, Loaec, Colwell, & Pommepuy, 2002).

5.3 PCR Optimization

Species-specific primers for *V. parahaemolyticus*, *V. cholerae*, and *V. vulnificus* along with IAC was optimized (Figure 1). Denaturation and primer annealing time was modified to minimize the occurrence of nonspecific bands (Teh et al., 2010). The IAC was originally developed by Thong et al (2014) in which the authors cloned the plasmid pQE 30 into *E. coli.* These researchers also developed specific primers (propriety information) to target the clone fragment. The use of IAC helps to eliminate false negative results (Zijlstra & Van Hoof, 2006). Spiked samples with reference strains of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* were used as positive control in all the experiments to facilitate interpretation of the sample results. Sterile water was used as negative control in all PCR tests.

The primer specificity and melting temperature (Tm) is very crucial for the development of multiplex PCR because it requires all the primers to be selectively annealed with their respective targets under a single set of PCR conditions such as reaction volume, salts concentration as well as cycling and annealing temperatures (Ali, Razzak, & Hamid, 2014). Therefore, after optimizing the simplex MPN-PCR, duplex, triplex and tetraplex (3 Vibrios plus 1 IAC) MPN-PCRs were optimized step by step (Figure 1). To avoid any non-specific PCR products and primer dimers, primer extension temperature was increased up to 2 °C (Jofré et al., 2005).

5.4 Role of dilution factor in MPN analysis

For MPN analysis, the result is not considered as complete if all the tests gave positive outcomes (Jarvis, 2016). This issue can be resolved by setting a greater number of dilutions to ensure the presence of at least one negative PCR result. Hence, a greater number (10^{-7}) of dilutions than those (10^{-2}-10^{-3}) of previous studies were set (Tunung et

al., 2010; Marian et al., 2012) to ensure the presence of at least one negative PCR result because the result and MPN analysis was performed accordingly.

5.5 Detection and Quantification of Vibrios in raw fish

Recently, various molecular methods especially Polymerase Chain Reaction (PCR) assays have been extensively used for the rapid identification of bacterial species and detection of virulence genes (Wong, You, & Chen, 2012).

These methods include MPN, simplex PCR, multiplex PCR and MPN-PCR. These methods have been used by many researchers to detect and enumerate Vibrios in seafood. The advantages and disadvantages of the methods are tabulated in Table 5-1. Both the simplex (Blanco-Abad, Ansede-Bermejo, Rodriguez-Castro, & Martinez-Urtaza, 2009; Dalmasso et al., 2009; Li, Chiou, Chan, & Chen, 2016) and multiplex (Neogi, Chowdhury, Asakura, Hinenoya, Haldar, Saidi, & Kogure, 2010; Hossain, Kim, Kim, Kim, & Kong, 2013; Wei et al., 2014) PCR assays have been documented for the identification of various *Vibrio* species. Neogi et al. (2010) reported multiplex PCR assay to detect *V. parahaemolyticus* and *V. cholerae* by targeting *tox*R and *vvh*A genes, respectively and Hossain et al. (2013) discriminated *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* by mPCR involving *gro*EL gene. However, none of these assays estimated the microbial load of these Vibrios and differentiated the presence of live and dead cells.

Alam, Tomochika, Miyoshi, & Shinoda (2002), documented the use of MPN-PCR for the identification of *V. parahaemolyticus* for the first time and since then MPN-PCR technique has been used by several researchers for the enumeration of this bacteria in seafood samples (Luan et al., 2008); Vegetables (Tunung et al., 2010); raw fish (Noorlis et al., 2011).

Table 5-1: Occurrence of three major Vibrios in seafoods and raw fish detected by different methods.

Target Species		Carrier/Vector	Location	References
MPN **Advantage** • Sensitive • Can quantify • Can detect the presence of Live bacteria **Disadvantage** • Laborious, time-consuming, and complex process. • The specificity of using selective media is still questionable.	*V. parahaemolyticus*	Fresh Water Fish	Malaysia	(Noorlis et al., 2011)
		Seafood	Shanghai	(Zhang et al., 2017)
		Seafood	Japan	(Miwa, Norinaga, Tomohiro Nishio, Yono Arita, Fumihiko Kawamori, Takashi Masuda, 2003)
	V. cholerae	Seafood	Senegal	(Coly, Sow, Seydi, & Martinez-Urtaza, 2013)
	V. vulnificus	Shellfish	China	(Yano, Yokoyama, Satomi, Oikawa, & Chen, 2004)
		Seafood	Japan	(Hara-Kud et al., 2005)
Simplex PCR **Advantage** • Fast and robust method **Disadvantage** • Cannot quantify • Cannot differentiate between the	*V. parahaemolyticus*	Retail Shrimp	Malaysia	(Letchumanan et al., 2015)
		Seafood	India	(Dileep et al., 2003)
	V. cholerae	Seafood	India	(Saravanan, Sanath Kumar, Karunasagar, & Karunasagar, 2007)
		Seafood	Thailand	(Preeprem, Mittraparp-arthorn, Bhoopong, & Vuddhakul, 2014)

presence of live and dead bacteria	*V. vulnificus*	Seafood	Spain	(Canigral et al., 2010)
Multiplex PCR **Advantage** • Reduced cost and time since many species can be detected in a single assay **Disadvantage** • Cannot quantify • Cannot differentiate between the presence of live or dead bacteria	*V. parahaemolyticus*	Raw Oysters	Thailand	(Changchai & Saunjit, 2014)
		Seafood	Senegal	(Coly et al., 2013)
	V. cholerae	Seafood	Senegal	(Coly et al., 2013)
	V. vulnificus	Raw Oysters	Thailand	(Changchai & Saunjit, 2014)
		Raw Seafood and Sea water	Malaysia	(Paydar & Thong, 2013)
Simplex MPN-PCR **Advantage** • Capable of quantifying • Can specify the presence of live bacteria **Disadvantage** • Cannot detect more than one species in a single assay platform	*V. parahaemolyticus*	Cockle	Malaysia	(Bilung et al., 2005)
		Mollusk	Brazil	(Ramos et al., 2014)
		Seafood	Japan	(Miwa, Norinaga, Tomohiro Nishio, Yono Arita, Fumihiko Kawamori, Takashi Masuda, 2003)
	V. cholerae	Raw cockles	Malaysia	(Suzita et al., 2010)
	V. vulnificus	Marine Shrimp	China	(Ji et al., 2011)
		Mollusk	Brazil	(Ramos et al., 2014)
RT-PCR	*V. parahaemolyticus*	Seafood	France	(Robert-Pillot, Copin, Himber, Gay, & Quilici, 2014)

Advantage • Highly Sensitive • Capable of quantifying • Can detect the live bacteria **Disadvantage** • RNA is less stable in compare to DNA • Cannot be used in small-scale laboratory		Seafood	China	(Liu et al., 2012)
	V. cholerae	Seafood	France	(Robert-Pillot et al., 2014)
		Seafood	China	(Tang et al., 2017)
	V. vulnificus	Seafood, seawater and wastewater	Spain	(Canigral et al., 2010)
		Seafood	France	(Robert-Pillot et al., 2014)

Tunung et al. (2010) documented the presence of *V. parahaemolyticus* (<3 to >2,400 $MPNg^{-1}$) in 20.65% raw vegetables in Malaysian market but the value ">2400 $MPNg^{-1}$" indicated that all the PCR results were positive, and a greater number of dilution were needed. Noorlis et al. (2011) reported *V. parahaemolyticus* (0 to 1.1x107 $MPNg^{-1}$) in 24% of Malaysian raw fishes using *tox*R gene as a biomarker. Luan et al. (2008) reported that the MPN-PCR method is about 100 times more sensitive than the direct PCR, which is concurrent to my study.

Previously, Senderovich, Izhaki, & Halpern (2010) reported the presence of *V. cholerae* in 71% fishes using the conventional phenotypic method followed by confirmation by multiplex PCR and found fishes are reservoirs and vectors of *V. cholerae*. Traoré et al. (2014) also detected the presence of *V. cholerae* in 18 out of 318 (6%) fish and water samples using the phenotypic approaches. However, this rate of occurrences might be an underestimation because upon exposure to an unfavorable environment, Vibrios survive by entering VBNC state and conventional phenotypic methods cannot detect the presence of those pathogens. Several PCR-based methods have also been reported to detect *V. cholerae* in different water and food matrices (Norazah, Zainuldin, Kamel, Kamaliah, & Taha, 2001; Grim et al., 2010) Alternatively, Suzita et al. (2010) detected *V. cholerae* in 95% of raw cockles in Malaysia using a simplex MPN-PCR (<30 to > 24000 $MPNg^{-1}$).

Several reports have been published showing the occurrence and distribution of *V. vulnificus* in fish, shellfish, oyster, sediment and water (Givens, Bowers, Depaola, Hollibaugh, & Jones, 2014) and several PCR-based methods have been proposed to detect *V. vulnificus* in different sample matrices (Wong et al., 2012; Wei et al., 2014; Xu et al., 2017). DePaola, Capers, & Alexander (1994) determined *V. vulnificus* in fish

intestines using MPN-PCR from the U.S. Gulf Coast for the first time and since then many MPN-PCRs were documented for *V. vulnificus* identification. Randa, Polz, & Lim (2004) found that *V. vulnificus* exists in low number in the winter because they go into VBNC state at low temperature. Hara-Kudo et al. (2005) compared MPN-culture method with MPN-PCR method to enumerate *V. vulnificus* in seafood and found that MPN-PCR is more sensitive than that of MPN-culture method. They used two different temperatures (25℃ and 35℃) and found better enrichment at 35°C and the approach detected 65% of the samples by MPN-culture and more than 75% by MPN-PCR assay.

Recently, Machado & Bordalo (2016) assessed coastal waters of Guinea-Bissau to detect and quantify *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* targeting ISR 16S-23S rRNA, *tox*R gene, *vvh*A gene, *tdh* gene, *trh* gene and *ctx*A gene. Cantet et al. (2013) reported the presence of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* in coastal lagoons targeting *tox*R gene, ISR 16S-23S rRNA and *vvh*A gene, respectively. Nevertheless, none of these studies were capable of detecting the three Vibrios in a single assay platform involving multiplex MPN-PCR.

So far MPN analysis followed by Multiplex PCR was used for the characterization of *Salmonella enterica* serovar Enteritidis in raw sewage (Zappelini et al., 2017) and enumerating *B. cereus* and *B. thuringiensis* in ready-to-eat cooked rice in Malaysia (Sandra et al., 2012). Though these studies successfully applied MPN and multiplex PCR method to detect and enumerate the microbes concerned, the results were not validated by simplex MPN-PCR or any other methods. To the best of my knowledge, no study quantified these three major Vibrios in one assay platform coupling PCR and MPN techniques and reported the occurrence in raw fish.

5.6 Detection and Quantification of Vibrios in RTE sushi

Seafood contains pathogens in the natural polluted aquatic environments (Amagliani, Brandi, & Schiavano, 2012) and sushi is a type of ready-to-eat (RTE) seafood which is consumed without further cooking and so considered as high-risk food product. The time and temperature profile from preparation, during storage until consumption is critical for both shelf life and food safety aspects of sushi prepared industrially (Lorentzen, Wesmajervi Breiland, Cooper, & Herland, 2012).

The detection and survivability assessment of Vibrios in sushi in three different storage might play a significant role in assessing and ensuring the food safety. The first portions were analyzed within one hour of purchase to determine the occurrence of target bacteria at the state of sale. The second portions of the sushi samples were stored at room temperature (26-27°C) for 4-5 hrs. to assess the rise in microbial load because Vibrios can be doubled in number in less than 10 minutes under ideal conditions (Børresen, 2008) and the consumption of RTE food mostly depends on the consumers convenience. A considerable rise in microbial load was recorded in this phase of the study that might pose a bigger health risk to the consumers. Factors such as handling, processing and display along with the storage may influence the microbiological safety of these RTE food at the point of sale (Angelidis et al., 2006).

The third portion of the samples were refrigerated for 7 days at -20°C before analysis as a means of stopping bacterial multiplication. However, though a drastic incline of microbial load was recorded in the tested sushi samples, 10%, 4.29%, and 8.57% samples were found positive for *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* with a density of $7.36x10^{-1}$-$5.88x10^{1}$ MPN g^{-1}, $1.22x10^{0}$-$1.95x10^{1}$ MPNg^{-1} and $9.79x10^{-1}$-$2.49x10^{1}$ MPN g^{-1}, respectively. These results indicate that though the

microbial growth was reduced but refrigeration failed to make the sushi free of Vibrios. However, reduction in microbial load after refrigeration shows that at least 7 days of refrigeration at -20℃ with subsequent storage at below 5℃ till serving might be a considerable option to control the bacterial growth and make the sushi less prone to cause any bacterial infection over consumption. Report of a *Salmonella* outbreak due to the consumption of frozen, pre-packaged raw tuna showed that refrigerated RTE is no guarantee for safe food (Zuraw, 2015). According to Randa, Polz & Lim (2004), *V. vulnificus* exists in low numbers in winter because Vibrios are known go into VBNC state at low temperature and the microbial presence in the sushi samples even after 7 days of refrigeration could be associated with the similar survival strategy of bacteria.

Many studies have reported the presence of different bacteria in sushi. For example, Puah, Chua & Tan (2017) reported the presence of 26% *Staphylococcus aureus* (52/200) and 16% *Salmonella enterica* (32/200) in sushi and sashimi in Malaysia based on their phenotypic profiles and PCR analysis. In another study, Jain et al. (2008) reported an outbreak associated with sushi restaurants in Nevada in 2004 due to the possible presence of enterotoxigenic *Escherichia coli* (ETEC). Besides, Suppin, Rippel-Rachle, Schopf & Smulders (2004), found *Yersinia* spp. and *Listeria* spp. in 5% and 3% samples, respectively from the sushi samples in Vienna. Likewise, 53 people have been reported sickened with *Salmonella* Paratyphi B in an outbreak after consumption of sushi and this outbreak linked with frozen, pre-packaged raw tuna exhibits that freezing might slow down the growth of *Salmonella* but cannot ensure the killing of bacteria (Zuraw, 2015). In another report, 63.93% samples were classified as unsatisfactory due to the high levels of Enterobacteriaceae, *S. aureus*, B. *cereus* and yeasts out of sixty one samples collected from 23 restaurants in Portugal (Miguéis,

Santos, Saraiva, & Esteves, 2015). Hoel, Mehli, Bruheim, Vadstein, & Jakobsen (2015), reported the presence of potentially pathogenic *Aeromonas* spp. in sushi sold in Norway and emphasized on the importance of proper temperature control and the initial quality of the raw materials used in preparation to ensure stable quality and safety of this popular food. However, survivability of these vibrios at different storage conditions has not been found to be reported earlier.

5.7 Validation of PCR products

A properly designed and optimized species-specific PCR assay is usually conclusive to species detection but authentication of the amplicons by sequence analysis greatly increase the reliability of the PCR assay.

Therefore, species specific amplicons for Vibrios (*V. parahaemolyticus*, *V. cholerae* and *V. vulnificus*) were sequenced and 99% similarity was found with the NCBI reference strains.

5.8 Advantage of the developed method over other molecular methods

Vibrios enter into VBNC state to withstand unfavorable environment. Conventional phenotypic methods cannot detect the presence of such organisms. To explain subtly, environmental stresses drastically limit the usual metabolic activity of most bacteria and, so they do not grow in conventional culture medium. However, these dormant state bacteria retained their multiplication ability when environmental stressors are taken off and adequate enrichment is administered. Therefore, they are called viable but not culturable (VBNC) cells (Zhao, Zhong, Wei, Lin, & Ding, 2017). The MPN-PCR approach overcomes this limitation by supporting the detection of VBNC bacteria in two ways: (1) the two-stage enrichment approach resuscitates the metabolic activity and

stimulates the bacterial growth and (2) unlike the traditional culture-based method, PCR can detect the presence of the DNA under all phases of bacterial growth.

Detection of live bacterial cell of multiple species in one test cannot be achieved by multiplex PCR. In contrast, though RT-PCR (Reverse transcriptase PCR) can offer us all these advantages in a package, the RNA used in RT-PCR is less stable than the DNA and requires immediate refrigeration (-80°C). Therefore, RT-PCR appears to be less acceptable in compare to the developed method for the mobile or small-scale laboratory. However, bacterial enumeration using the developed multiplex MPN-PCR demands extensive laboratory effort including repeated dilution of the sample with subsequent DNA Extraction and PCR. Further development to shorten the long dilution series and DNA extraction steps might widen the use of the method.

5.9 Significance of the study

The safety of seafood is becoming an increasingly significant public health issue due to the global rise in the number of people vulnerable to disease, coupled with the customary tendency of the world population to consume raw or undercooked fish and fish products.

The multiplex MPN-PCR described here is a useful and meaningful contribution to science because this will allow the simultaneous detection and enumeration of three major Vibrios in fishes and ready to eat seafood. This approach also allows a shorter time for food analysis. Moreover, this study sheds some light on the possibilities related to improper implementation of Food Safety Management Systems including Hazard Analysis and Critical Control Point (HACCP), to ensure safe intake of the popular seafood sushi.

This innovation will ease the enumeration of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* in the settings of a small-scale laboratory or any fish or food industries. To ensure seafood safety the presence of food–borne pathogens such as pathogenic *Vibrio* species such as *V. parahaemolyticus, V. cholerae* and *V. vulnificus,* should be more strictly monitored and controlled.

5.10 Limitation of the study

The samples (raw fish and sushi) of this study were not collected from the primary sources. The samples were purchased from supermarkets and other places. The microbiological quality, in particular the presence of *Vibrio* species would have been picked up along the handling process. Hence, the occurrence of Vibrios in the samples might be attributed to many other aspects, for example, the contaminated ice used for storage and handlers. The results obtained need to be interpreted with caution.

In case of discrepancy of the results from simplex PCR and multiplex MPN-PCR, a sample was deemed as positive if either test gave a positive amplification.

For enumeration of microbial loads, there were some differences (especially values with <1 MPN/g) between simplex and multiplex MPN-PCR. Such discrepancy can make interpretation difficult.

CHAPTER 6: CONCLUSION

A multiplex MPN-PCR method was optimized and validated for screening of three most virulent Vibrios in the laboratory. Incorporation of MPN techniques with multiplex PCR assay is an advantage because it allows quantitative detection of multiple species in a single assay platform.

The occurrence of Vibrios in raw fish was determined by using three methods including multiplex PCR, simplex MPN-PCR and multiplex MPN-PCR. The microbial load of Vibrios in sushi was enumerated with the optimized multiplex MPN-PCR. *V. parahaemolyticus*, *V. cholera* and *V. vulnificus* were observed in 43.33%, 27%, and 32.5% raw fish samples, respectively with both simplex and multiplex MPN-PCR assay. For sushi samples (at the point of sale), the occurrence of *V. parahaemolyticus*, *V. cholera* and *V. vulnificus* were 22.86%, 5.71% and 11.43%, respectively. MPN-PCR could detect 9.17%, 8.67% and 10% more of *V. parahaemolyticus*, *V. cholera* and *V. vulnificus*, respectively in the food samples as compared to the lower percentages using the conventional multiplex PCR.

Lastly, the survivability of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* in RTE sushi at different storage condition was assessed. The increased in microbial load was noted after 4 - 5 hrs. of storage at room temperature while reduction in bacterial load was observed after 7 days of refrigeration at -20℃.

REFERENCES

Alarcon, R. A. B. (2002). On the specificity of PCR detection of *Listeria monocytogenes* in food: a comparison of published primers. *Systematic and applied microbiology*, *25*(1), 109.

Ali, M. E., Razzak, M. A., & Hamid, S. B. A. (2014). Multiplex PCR in species authentication: probability and prospects: A Review. *Food Analytical Methods*, *7*(10), 1933–1949.

Alves, J. C., Lima de Paiva, E., Milani, R. F., Bearzoti, E., Morgano, M. A., & Diego Quintaes, K. (2017). Risk estimation to human health caused by the mercury content of sushi and sashimi sold in Japanese restaurants in Brazil. *Journal of Environmental Science and Health - Part B Pesticides, Food Contaminants, and Agricultural Wastes*, *52*(6), 418–424.

Amagliani, G., Brandi, G., & Schiavano, G. F. (2012). Incidence and role of *Salmonella* in seafood safety. *Food Research International*, *45*(2), 780-788.

Angelidis, A. S., Chronis, E. N., Papageorgiou, D. K., Kazakis, I. I., Arsenoglou, K. C., & Stathopoulos, G. A. (2006). Non-lactic acid, contaminating microbial flora in ready-to-eat foods: A potential food-quality index. *Food Microbiology*, *23*(1), 95–100.

Atanassova, V., Reich, F., & Klein, G. N. (2008). Microbiological quality of sushi from sushi bars and retailers. *Journal of Food Protection*, *71*(4), 860–864.

Austin, B. (2010). Vibrios as causal agents of zoonoses. *Veterinary microbiology*, *140*(3-4), 310-317.

Barlow, S. M., Boobis, A. R., Bridges, J., Cockburn, A., Dekant, W., Hepburn, P., ... & Bánáti, D. (2015). The role of hazard-and risk-based approaches in ensuring food safety. *Trends in Food Science & Technology*, *46*(2), 176-188.

Barralet, J., Stafford, R., Towner, C., & Smith, P. (2004). Outbreak of *Salmonella* Singapore associated with eating sushi. *Communicable diseases intelligence quarterly report*, *28*(4), 527.

Barzamini, B., Moghbeli, M., & Soleimani, N. A. (2015). Rapid Identification of *Vibrio cholerae* Bacteria using LAMP and PCR methods in Water and Wastewater, *12*(4), 569–575.

Bhatty, M. A., Turner, D. P. J., & Chamberlain, S. T. (2000). *Mycobacterium marinum* hand infection: case reports and review of literature. *British Journal of Plastic Surgery*, *53*(2), 161–165.

Bilung, L. M., Radu, S., Bahaman, A. R., Rahim, R. A., Napis, S., Ling, M. W. C. V., … Nishibuchi, M. (2005). Detection of *Vibrio parahaemolyticus* in cockle

(Anadara granosa) by PCR. *FEMS Microbiology Letters*, *252*(1), 85–88.

Blanco-Abad, V., Ansede-Bermejo, J., Rodriguez-Castro, A., & Martinez-Urtaza, J. (2009). Evaluation of different procedures for the optimized detection of *Vibrio parahaemolyticus* in mussels and environmental samples. *International Journal of Food Microbiology*, *129*(3), 229–236.

Bonnin-Jusserand, M., Copin, S., Le Bris, C., Brauge, T., Gay, M., Brisabois, A., ... & Midelet-Bourdin, G. (2017). Vibrio species involved in seafood-borne outbreaks (Vibrio cholerae, V. parahaemolyticus and V. vulnificus): Review of microbiological versus recent molecular detection methods in seafood products. *Critical reviews in food science and nutrition*, 1-14. https://doi.org/10.1080/10408398.2017.1384715

Børresen, T. (Ed.). (2008). *Improving seafood products for the consumer*. Elsevier.

Borucki, M. K., & Call, D. R. (2003). *Listeria monocytogenes* serotype identification by PCR. *Journal of Clinical Microbiology*, *41*(12), 5537–5540.

Boylan, S. (2011). Zoonoses associated with fish. *Veterinary Clinics: Exotic Animal Practice*, *14*(3), 427-438.

Bryan, P. J., Steffan, R. J., DePaola, A., Foster, J. W., & Bej, A. K. (1999). Adaptive response to cold temperatures in *Vibrio vulnificus*. *Current microbiology*, *38*(3), 168-175.

Butt, A., Aldridge, K. and Sanders, C. (2004). Infections related to the ingestion of seafood Part I: viral and bacterial infections. *The Lancet Infectious Diseases*, *4*(4), 201-212.

Cañigral, I., Moreno, Y., Alonso, J. L., González, A., & Ferrús, M. A. (2010). Detection of *Vibrio vulnificus* in seafood, seawater and wastewater samples from a Mediterranean coastal area. *Microbiological Research*, *165*(8), 657–664.

Cantet, F., Hervio-Heath, D., Caro, A., Le Mennec, C., Monteil, C., Quéméré, C., … Monfort, P. (2013). Quantification of *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio cholerae* in French Mediterranean coastal lagoons. *Research in Microbiology*, *164*(8), 867–874.

Centers for Disease Control and Prevention (CDC. (1993). *Vibrio vulnificus* infections associated with raw oyster consumption--Florida, 1981-1992. *Morbidity and Mortality Weekly Report*, *42*(21), 405.

Chang, C. H., Lin, H. Y., Ren, Q., Lin, Y. S., & Shao, K. T. (2016). DNA barcode identification of fish products in Taiwan: Government-commissioned authentication cases. *Food Control*, *66*, 38–43.

Changchai, N., & Saunjit, S. (2014). Occurrence of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in retail raw oysters from the eastern coast of Thailand. *The Southeast*

Asian Journal of Tropical Medicine and Public Health, *45*(3), 662–669.

Chen, J., Tang, J., Liu, J., Cai, Z., & Bai, X. (2012). Development and evaluation of a multiplex PCR for simultaneous detection of five foodborne pathogens. *Journal of Applied Microbiology*, *112*(4), 823–830.

Chen, J., Zhang, L., Paoli, G. C., Shi, C., Tu, S. I., & Shi, X. (2010). A real-time PCR method for the detection of *Salmonella enterica* from food using a target sequence identified by comparative genomic analysis. *International Journal of Food Microbiology*, *137*(2–3), 168–174.

Chen, S., & Ge, B. (2010). Development of a toxR-based loop-mediated isothermal amplification assay for detecting *Vibrio parahaemolyticus*. *BMC microbiology*, *10*(1), 41.

Chen, Y., Liu, X. M., Yan, J. I. W., Li, X. G., Mei, L. L., Ma, Q. F., & Ma, Y. (2010). Foodborne pathogens in retail oysters in south China. *Biomedical and Environmental Sciences*, *23*(1), 32–36.

Chiang, Y. C., Tsen, H. Y., Chen, H. Y., Chang, Y. H., Lin, C. K., Chen, C. Y., & Pai, W. Y. (2012). Multiplex PCR and a chromogenic DNA macroarray for the detection of *Listeria monocytogens*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Enterobacter sakazakii*, *Escherichia coli* O157:H7, *Vibrio parahaemolyticus*, *Salmonella* spp. and *Pseudomonas fluoresc*. *Journal of Microbiological Methods*, *88*(1), 110–116.

Chou, S.Y., Tsai, C.C., Kau, S.C., Kau, H.C., & Hsu, W.M. (2004). Aeromonas hydrophila orbital cellulitis in a patient with myelodysplastic syndrome. *Journal of the Chinese Medical Association: JCMA*, *67*(1), 51–3.

Coly, I., Sow, A. G., Seydi, M., & Martinez-Urtaza, J. (2013). *Vibrio cholerae* and *Vibrio parahaemolyticus* detected in seafood products from Senegal. *Foodborne Pathogens and Disease*, *10*(12), 1050–8.

Copin, S., Robert-Pillot, A., Malle, P., Quilici, M. L., & Gay, M. (2012). Evaluation of most-probable-number–PCR method with internal amplification control for the counting of total and pathogenic *Vibrio parahaemolyticus* in frozen shrimps. *Journal of food protection*, *75*(1), 150-153.

Dalmasso, A., La Neve, F., Suffredini, E., Croci, L., Serracca, L., Bottero, M. T., & Civera, T. (2009). Development of a PCR assay targeting the *rpoA* gene for the screening of *Vibrio* genus. *Food Analytical Methods*, *2*(4), 317–324.

Daniels, N. A. (2011). *Vibrio vulnificus* oysters: Pearls and perils. *Clinical Infectious Diseases*, *52*(6), 788–792.

Daniels, N. A., MacKinnon, L., Bishop, R., Altekruse, S., Ray, B., Hammond, R. M., … Slutsker, L. (2000). *Vibrio parahaemolyticus* infections in the United States, 1973-1998. *The Journal of Infectious Diseases*, *181*(5), 1661–6.

DePaola, A. N. G. E. L. O., Capers, G. M., & Alexander, D. (1994). Densities of *Vibrio vulnificus* in the intestines of fish from the US Gulf Coast. *Applied and environmental microbiology*, *60*(3), 984-988.

Di Pinto, A., Terio, V., Novello, L., & Tantillo, G. (2011). Comparison between thiosulphate-citrate-bile salt sucrose (TCBS) agar and CHROMagar Vibrio for isolating *Vibrio parahaemolyticus*. *Food Control*, *22*(1), 124–127.

Dickinson, G., Lim, K.-Y., & Jiang, S. C. (2013). Quantitative microbial risk assessment of pathogenic vibrios in marine recreational waters of Southern California. *Applied and Environmental Microbiology*, *79*(1), 294–302.

Dileep, V., Kumar, H. S., Kumar, Y., Nishibuchi, M., Karunasagar, I., & Karunasagar, I. (2003). Application of polymerase chain reaction for detection of *Vibrio parahaemolyticus* associated with tropical seafoods and coastal environment. *Letters in Applied Microbiology*, *36*(6), 423–427.

Doyle, M. P., & Buchanan, R. L. (Eds.). (2012). *Food microbiology: Fundamentals and Frontiers*. American Society for Microbiology Press.

Drake, S. L., Depaola, A., & Jaykus, L. A. (2007). An overview of *Vibrio vulnificus* and *Vibrio parahaemolyticus*. *Comprehensive Reviews in Food Science and Food Safety*, *6*(4), 120–144.

Elbashir, S., Parveen, S., Schwarz, J., Rippen, T., Jahncke, M., & DePaola, A. (2018). Seafood pathogens and information on antimicrobial resistance: A review. *Food Microbiology*, *70*, 85–93.

Elhadi, N., Radu, S., Chen, C.H., & Nishibuchi, M. (2004). Prevalence of potentially pathogenic *Vibrio* species in the seafood marketed in Malaysia. *Journal of Food Protection*, *67*(7), 1469–75.

Elmahdi, S., DaSilva, L. and Parveen, S. (2016). Antibiotic resistance of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in various countries: A review. *Food Microbiology*, 57, 128-134.

FAO. (2016). *The state of world fisheries and aquaculture*. *The State of World Fisheries and Aquaculture, 2000* (Vol. 3). Food & Agriculture Org.

FDA. (2011). Fish and Fishery Products Hazards and Controls Guidance. *Fish and Fishery Products Hazard and Control Guidance 4*, (4), 1–401.

Feingold, M. H., & Kumar, M. L. (2004). Otitis media associated with *Vibrio alginolyticus* in a child with pressure-equalizing tubes. *Pediatric Infectious Disease Journal*, *23*(5), 475–476.

Fujikawa, H., Kimura, B. and Fujii, T. (2009). Development of a predictive program for *Vibrio parahaemolyticus* Growth under various environmental conditions. *Biocontrol Science*, *14*(3), 127-131.

Fukushima, H., Kawase, J., Etoh, Y., Sugama, K., Yashiro, S., Iida, N. and Yamaguchi, K. (2010). Simultaneous Screening of 24 Target Genes of Foodborne Pathogens in 35 Foodborne Outbreaks Using Multiplex Real-Time SYBR Green PCR Analysis. *International Journal of Microbiology*, 2010, 1-18. https://doi.org/ 10.1155/2010/864817

Golub, V., Kim, A. C., & Krol, V. (2010). Surgical wound infection, tuboovarian abscess, and sepsis caused by *Edwardsiella tarda*: Case reports and literature review. *Infection*, *38*(6), 487–489.

Gómez, P., Pagnon, M., Egea-Cortines, M., Artés, F., & Weiss, J. (2010). A fast molecular nondestructive protocol for evaluating aerobic bacterial load on fresh-cut lettuce. *Food Science and Technology International*, *16*(5), 409–415.

González, S. F., Krug, M. J., Nielsen, M. E., Santos, Y., & Call, D. R. (2004). Simultaneous detection of marine fish pathogens by using multiplex PCR and a DNA microarray. *Journal of Clinical Microbiology*, *42*(4), 1414-1419.

Grim, C., Jaiani, E., Whitehouse, C., Janelidze, N., Kokashvili, T., Tediashvili, M., Colwell, R. and Huq, A. (2009). Detection of toxigenic *Vibrio cholerae* O1 in freshwater lakes of the former Soviet Republic of Georgia. *Environmental Microbiology Reports*, *2*(1), 2-6.

Gronewold, A. and Wolpert, R. (2008). Modeling the relationship between most probable number (MPN) and colony-forming unit (CFU) estimates of fecal coliform concentration. *Water Research*, *42*(13), 3327-3334.

Halpern, M., & Izhaki, I. (2017). Fish as Hosts of *Vibrio cholerae*. *Frontiers in microbiology*, *8*, 282. https://doi.org/10.3389/fmicb.2017.00282

Han, F., & Ge, B. (2010). Quantitative detection of *Vibrio vulnificus* in raw oysters by real-time loop-mediated isothermal amplification. *International Journal of Food Microbiology*, *142*(1–2), 60–66.

Han, F., Wang, F., & Ge, B. (2011). Detecting potentially virulent *Vibrio vulnificus* strains in raw oysters by quantitative loop-mediated isothermal amplification. *Applied and Environmental Microbiology*, *77*(8), 2589–2595.

Hara-Kud, Y., Miwa, N., Yamasaki, S., Yatsuyanagi, J., Iwade, Y., Takahash, H., & Miyasaka, J. (2005). Quantitative detection of *Vibrio vulnificus* in Seafood. *Journal of the Japanese Association for Infectious Diseases*, *79*(12), 931-936.

Hara-Kudo, Y., Konishi, N., Ohtsuka, K., Hiramatsu, R., Tanaka, H., Konuma, H., & Takatori, K. (2008). Detection of Verotoxigenic *Escherichia coli* O157 and O26 in food by plating methods and LAMP method: A collaborative study. *International Journal of Food Microbiology*, *122*(1–2), 156–161.

Hara-Kudo, Y., Nishina, T., Nakagawa, H., Konuma, H., Hasegawa, J., & Kumagai, S. (2001). Improved method for detection of *Vibrio parahaemolyticus* in seafood.

Applied and Environmental Microbiology, *67*(12), 5819.

Håstein, T., Hjeltnes, B., Lillehaug, a, Utne Skåre, J., Berntssen, M., & Lundebye, a K. (2006). Food safety hazards that occur during the production stage: challenges for fish farming and the fishing industry. *Revue Scientifique et Technique (International Office of Epizootics)*, *25*(2), 607–625.

Heinitz, M. L., Ruble, R. D., Wagner, D. E., & Tatini, S. R. (2000). Incidence of *Salmonella* in fish and seafood. *Journal of Food Protection*, *63*(5), 579–92.

Hoel, S., Mehli, L., Bruheim, T., Vadstein, O., & Jakobsen, A. N. (2015). Assessment of microbiological quality of retail fresh sushi from selected sources in Norway. *Journal of Food Protection*, *78*(5), 977–82.

Horseman, M. and Surani, S. (2011). A comprehensive review of *Vibrio vulnificus*: an important cause of severe sepsis and skin and soft-tissue infection. *International Journal of Infectious Diseases*, *15*(3), 157-166.

Hossain, M. T., Kim, E. Y., Kim, Y. R., Kim, D. G., & Kong, I. S. (2013). Development of a groEL gene-based species-specific multiplex polymerase chain reaction assay for simultaneous detection of *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*. *Journal of Applied Microbiology*, *114*(2), 448–456.

Hu, Q., Lyu, D., Shi, X., Jiang, Y., Lin, Y., Li, Y., … Li, Q. (2014). A Modified Molecular Beacons–Based Multiplex Real-Time PCR Assay for Simultaneous Detection of Eight Foodborne Pathogens in a Single Reaction and Its Application. *Foodborne Pathogens and Disease*, *11*(3), 207–214.

Imberg, R., Potasman, I., Weissman, Y., & Grupper, M. (2008). Hand infections following penetrating fish fins or bones injuries (FFBI): A large, hospital based, retrospective study. *Infection*, *36*(6), 565–569.

Iwamoto, M., Ayers, T., Mahon, B. and Swerdlow, D. (2010). Epidemiology of Seafood-Associated Infections in the United States. *Clinical Microbiology Reviews*, *23*(2), 399-411.

Jahangir Alam, M., Tomochika, K. I., Miyoshi, S. I., & Shinoda, S. (2002). Environmental investigation of potentially pathogenic *Vibrio parahaemolyticus* in the Seto-Inland Sea, Japan. *FEMS Microbiology Letters*, *208*(1), 83–87.

Jain, S., Chen, L., Dechet, A., Hertz, A. T., Brus, D. L., Hanley, K., … Ram, P. K. (2008). An Outbreak of Enterotoxigenic *Escherichia coli* Associated with Sushi Restaurants in Nevada, 2004. *Clinical Infectious Diseases*, *47*(1), 1–7.

Jakšić, S., Uhitil, S., Petrak, T., Bažulić, D., & Gumhalter Karolyi, L. (2002). Occurrence of *Vibrio* spp. in sea fish, shrimps and bivalve molluscs harvested from Adriatic sea. *Food Control*, *13*(8), 491–493.

Jarvis, B. (2016). *Statistical aspects of the microbiological examination of foods*. Academic Press.

Ji, H., Chen, Y., Guo, Y., Liu, X., Wen, J., & Liu, H. (2011). Occurrence and characteristics of *Vibrio vulnificus* in retail marine shrimp in China. *Food Control*, *22*(12), 1935–1940.

Jofré, A., Martin, B., Garriga, M., Hugas, M., Pla, M., Rodríguez-Lázaro, D., & Aymerich, T. (2005). Simultaneous detection of *Listeria monocytogenes* and *Salmonella* by multiplex PCR in cooked ham. *Food Microbiology*, *22*(1), 109–115.

Jones, M. K., & Oliver, J. D. (2009). *Vibrio vulnificus*: disease and pathogenesis. *Infection and immunity*, *77*(5), 1723-1733.

Kaysner, C. A., & Angelo DePaola, J. (2004). Bacteriological Analytical Manual Chapter 9: Vibrio. *Administration, U.S. Food and Drug*, (40), 1–15.

Khaira, G., & Galanis, E. (2007). Descriptive epidemiology of *Vibrio parahaemolyticus* and other *Vibrio* species infections in British Columbia: 2001-2006. *Canada Communicable Disease Report*, *33*(11), 12–22.

Khoo, C. H., Cheah, Y. K., Lee, L. H., Sim, J. H., Salleh, N. A., Sidik, S. M., … Sukardi, S. (2009). Virulotyping of *Salmonella enterica* subsp. enterica isolated from indigenous vegetables and poultry meat in Malaysia using multiplex-PCR. *Antonie van Leeuwenhoek, 96*(4), 441–457.

Kim, D. G., Ahn, S. H., Kim, L. H., Park, K. J., Hong, Y. K., & Kong, I. S. (2008). Application of the rpoS gene for species-specific detection of *Vibrio vulnificus* by real-time PCR. *Journal of microbiology and biotechnology*, *18*(11), 1841-1847.

Kim, H.J., Lee, H.J., Lee, K.H., & Cho, J.C. (2012). Simultaneous detection of pathogenic *Vibrio* species using multiplex real-time PCR. *Food Control*, *23*(2), 491–498.

Koskela, K. A., Matero, P., Blatny, J. M., Fykse, E. M., Olsen, J. S., Nuotio, L. O., & Nikkari, S. (2009). A multiplatform real-time polymerase chain reaction detection assay for Vibrio cholerae. *Diagnostic Microbiology and Infectious Disease*, *65*(3), 339–344.

Lalitha, K. V., & Surendran, P. K. (2002). Occurrence of *Clostridium botulinum* in fresh and cured fish in retail trade in Cochin (India). *International Journal of Food Microbiology*, *72*(1–2), 169–174.

Law, J. W. F., Mutalib, N. S. A., Chan, K. G., & Lee, L. H. (2014). Rapid methods for the detection of foodborne bacterial pathogens: Principles, applications, advantages and limitations. *Frontiers in Microbiology*, *5*(12), 1–19.

Lee, S. H., Jung, B. Y., Rayamahji, N., Lee, H. S., Jeon, W. J., Choi, K. S., … Yoo, H. S. (2009). A multiplex real-time PCR for differential detection and quantification

of *Salmonella* spp., *Salmonella enterica* serovar Typhimurium and Enteritidis in meats. *Journal of Veterinary Science*, *10*(1), 43–51.

Letchumanan, V., Yin, W. F., Lee, L. H., & Chan, K. G. (2015). Prevalence and antimicrobial susceptibility of *Vibrio parahaemolyticus* isolated from retail shrimps in Malaysia. *Frontiers in Microbiology*, *6*(1), 1–11.

Lewis, F. M., Marsh, B. J., & von Reyn, C. F. (2003). Fish tank exposure and cutaneous infections due to *Mycobacterium marinum*: tuberculin skin testing, treatment, and prevention. *Clinical Infectious Diseases*, *37*(3), 390-397.

Li, R., Chiou, J., Chan, E. W. C., & Chen, S. (2016). A novel PCR-based approach for accurate identification of Vibrio parahaemolyticus. *Frontiers in Microbiology*, *7*(1). https://doi.org/10.3389/fmicb.2016.00044

Li, Y., Liu, D., Cao, B., Han, W., Liu, Y., Liu, F., … Wang, L. (2006). Development of a serotype-specific DNA microarray for identification of some *Shigella* and pathogenic *Escherichia coli* strains. *Journal of Clinical Microbiology*, *44*(12), 4376–4383.

Liang, W. L., Pan, Y. L., Cheng, H. Le, Li, T. C., Yu, P. H. F., & Chan, S. W. (2016). The microbiological quality of take-away raw salmon finger sushi sold in Hong Kong. *Food Control*, *69*, 45–50.

Lippi, D., & Gotuzzo, E. (2014). The greatest steps towards the discovery of *Vibrio cholerae*. *Clinical Microbiology & Infection*, *20*(3), 191-195.

Liu, B., He, X., Chen, W., Yu, S., Shi, C., Zhou, X., … Shi, X. (2012). Development of a real time PCR assay for rapid detection of *Vibrio parahaemolyticus* from seafood. *Protein & Cell*, *3*(3), 204–212.

Lorentzen, G., Wesmajervi Breiland, M., Cooper, M. and Herland, H. (2012). Viability of *Listeria monocytogenes* in an experimental model of nigiri sushi of halibut (Hippoglossus hippoglossus) and salmon (Salmo salar). *Food Control*, *25*(1), 245-248.

Luan, X., Chen, J., Liu, Y., Li, Y., Jia, J., Liu, R. and Zhang, X. (2008). Rapid quantitative detection of *Vibrio parahaemolyticus* in Seafood by MPN-PCR. *Current Microbiology*, *57*(3), 218-221.

Lyhs, U., Hatakka, M., Maki-Petays, N., Hyytia, E., & Korkeala, H. (1998). Microbiological quality of Finnish vacuum-packaged fishery products at retail level. *Archiv Fur Lebensmittelhygiene*, *49*(6), 146–150.

Ma, C., Deng, X., Ke, C., He, D., Liang, Z., Li, W., … Cui, Z. (2014). Epidemiology and etiology characteristics of foodborne outbreaks caused by *Vibrio parahaemolyticus* during 2008-2010 in Guangdong province, China. *Foodborne Pathogens and Disease*, *11*(1), 21–9.

Machado, A., & Bordalo, A. A. (2016). Detection and quantification of *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus* in Coastal Waters of Guinea-Bissau (West Africa). *EcoHealth*, *13*(2), 339–349.

Makino, S. I., Kii, T., Asakura, H., Shirahata, T., Ikeda, T., Takeshi, K., & Itoh, K. (2000). Does enterohemorrhagic *Escherichia coli* O157:H7 enter the viable but nonculturable state in salted salmon roe? *Applied and Environmental Microbiology*, *66*(12), 5536–5539.

Mancusi, R., & Trevisani, M. (2014). Enumeration of verocytotoxigenic *Escherichia coli* (VTEC) O157 and O26 in milk by quantitative PCR. *International Journal of Food Microbiology*, *184*(8), 121–127.

Mandal, P., Biswas, A., Choi, K. and Pal, U. (2011). Methods for Rapid Detection of Foodborne Pathogens: An Overview. *American Journal of Food Technology*, *6*(2), 87-102.

Marian, M. N., Sharifah Aminah, S. M., Zuraini, M. I., Son, R., Maimunah, M., Lee, H. Y., … Elexson, N. (2012). MPN-PCR detection and antimicrobial resistance of *Listeria monocytogenes* isolated from raw and ready-to-eat foods in Malaysia. *Food Control*, *28*(2), 309–314.

Martín, B., Jofré, A., Garriga, M., Hugas, M., & Aymerich, T. (2004). Quantification of *Listeria monocytogenes* in fermented sausages by MPN-PCR method. *Letters in Applied Microbiology*, *39*(3), 290–295.

Martinez-Urtaza, J., Powell, A., Jansa, J., Rey, J. L. C., Montero, O. P., Campello, M. G., … Baker-Austin, C. (2016). Epidemiological investigation of a foodborne outbreak in Spain associated with U.S. West Coast genotypes of *Vibrio parahaemolyticus*. *SpringerPlus*, *5*(1), 1–8.

Martinez, R. M., Megli, C. J., & Taylor, R. K. (2010). Growth and laboratory maintenance of *Vibrio cholerae*. *Current protocols in microbiology*, *17*(1), 6A-1.

Maruyama, F., Kenzaka, T., Yamaguchi, N., Tani, K., & Nasu, M. (2003). Detection of bacteria carrying the stx2 gene by in situ loop-mediated isothermal amplification. *Applied and Environmental Microbiology*, *69*(8), 5023–5028.

Mercanoglu Taban, B., & Halkman, A. K. (2011). Do leafy green vegetables and their ready-to-eat [RTE] salads carry a risk of foodborne pathogens? *Anaerobe*, *17*(6), 286–287.

Miettinen, H., & Wirtanen, G. (2005). Prevalence and location of *Listeria monocytogenes* in farmed rainbow trout. *International Journal of Food Microbiology*, *104*(2), 135–143.

Miguéis, S., Santos, C., Saraiva, C., & Esteves, A. (2015). Evaluation of ready to eat sashimi in northern Portugal restaurants. *Food Control*. 47, 32-36.

Miwa, Norinaga, Tomohiro Nishio, Yono Arita, Fumihiko Kawamori, Takashi Masuda, M. A. (2003). Evaluation of MPN method combined with PCR procedure for detection and enumeration of Vibrio parahaemolyticus in seafood. *Journal of the Food Hygienic Society of Japan*, *44*(6), 289–293.

Miyoshi, S. (2006). *Vibrio vulnificus* infection and metalloprotease. *The Journal of Dermatology*, *33*(9), 589-595.

Modarressi, S., & Thong, K. L. (2010). Isolation and molecular sub typing of *Salmonella enterica* from chicken, beef and street foods in Malaysia. *Scientific Research and Essays*, *5*(18), 2713–2720.

MoH. (2016). Health Facts 2016. *Ministry of Health Malaysia, Planning Division Health Informatics Centre*, 1–19. Retrieved from http://www.moh.gov.my/images/gallery/publications/KKM HEALTH FACTS 2016.pdf

Mus, T. E., Cetinkaya, F., & Celik, U. (2014). Occurrence of *Vibrio*, *Salmonella* and *Staphylococcus aureus* in retail fresh fish, mussel and shrimp. *Acta Veterinaria Brno*, *83*(2), 75–78.

Muscolino, D., Giarratana, F., Beninati, C., Tornambene, A., Panebianco, A., & Ziino, G. (2014). Hygienic-sanitary evaluation of sushi and sashimi sold in Messina and Catania, Italy. *Italian Journal of Food Safety*, *3*(2). https://doi.org/10.4081/ijfs.2014.1701

Nawa, Y., Hatz, C., & Blum, J. (2005). Sushi Delights and Parasites: The risk of fishborne and foodborne parasitic zoonoses in Asia. *Clinical Infectious Diseases*, *41*(9), 1297–1303.

Neogi, S. B., Chowdhury, N., Asakura, M., Hinenoya, A., Haldar, S., Saidi, S. M., … Yamasaki, S. (2010). A highly sensitive and specific multiplex PCR assay for simultaneous detection of *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*. *Letters in Applied Microbiology*, *51*(3), 293–300.

New, C. Y., Ubong, A., Nur Hasria, K., Nur Fatihah, A., & Son, R. (2016). Risk of transmission of *Vibrio parahaemolyticus* in foods. *International Food Research Journal*, *23*(5), 2249-2257.

Noorlis, A., Ghazali, F. M., Cheah, Y. K., Tuan Zainazor, T. C., Ponniah, J., Tunung, R., … Son, R. (2011). Prevalence and quantification of *Vibrio* species and *Vibrio parahaemolyticus* in freshwater fish at hypermarket level. *International Food Research Journal*, *18*(2), 689–695.

Norazah, A., Zainuldin, M. T., Kamel, A. G., Kamaliah, M. N., & Taha, A. M. (2001). Detection of *Vibrio cholerae* 01 from aquatic environment in Sarawak. *Medical Journal of Malaysia*, *56*(1), 4–9.

Notermans, S. (2000). Risk assessment of *Listeria monocytogenes* in fish products:

some general principles, mechanism of infection and the use of performance standards to control human exposure. *International Journal of Food Microbiology*, *62*(3), 223-229.

Novotny, L., Dvorska, L., Lorencova, A., Beran, V., & Pavlik, I. (2004). Fish: A potential source of bacterial pathogens for human beings. *Veterinarni Medicina*, *49*(9), 343–358.

Oliver, J. D., Pruzzo, C., Vezzulli, L., & Kaper, J. B. (2013). Vibrio species. In *Food microbiology* (pp. 401-439). American Society of Microbiology.

Ottaviani, D., Leoni, F., Rocchegiani, E., Canonico, C., Potenziani, S., Santarelli, S., … Carraturo, A. (2010). Prevalence, serotyping and molecular characterization of *Vibrio parahaemolyticus* in mussels from Italian growing areas, Adriatic Sea. *Environmental Microbiology Reports*, *2*(1), 192–197.

Özer, S., Aslan, G., Tezcan, S., Sevim Bulduklu, P., Serin, M. S., & Emekdaş, G. (2008). Genetic heterogeneity and antibiotic susceptibility of *Vibrio alginolyticus* strains isolated from horse-mackerel (Trachurus trachurus L., 1758). *Turkish Journal of Veterinary and Animal Sciences*, *32*(2), 107–112.

Panicker, G., Call, D. R., Krug, M. J., Bej, K., & Bej, A. K. (2004). Detection of pathogenic *Vibrio* spp. in Shellfish by Using Multiplex PCR and DNA Microarrays. *Applied and Environmental Microbiology*, *70*(12), 7436–7444.

Park, J. Y., Jeon, S., Kim, J. Y., Park, M., & Kim, S. (2013). Multiplex Real-time Polymerase Chain Reaction Assays for simultaneous detection of *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*. *Osong Public Health and Research Perspectives*, *4*(3), 133–9.

Paydar, M., Teh, C. S. J., & Thong, K. L. (2013). Prevalence and characterisation of potentially virulent *Vibrio parahaemolyticus* in seafood in Malaysia using conventional methods, PCR and REP-PCR. *Food Control*, *32*(1), 13–18.

Paydar, M., & Thong, K. L. (2013). Prevalence and Genetic Characterization of *Vibrio vulnificus* in Raw Seafood and Seawater in Malaysia. *Journal of Food Protection*, *76*(10), 1797–800.

Ponniah, J., Robin, T., Paie, M. S., Radu, S., Ghazali, F. M., Kqueen, C. Y., … Malakar, P. K. (2010). *Listeria monocytogenes* in raw salad vegetables sold at retail level in Malaysia. *Food Control*, *21*(5), 774–778.

Preeprem, S., Mittraparp-arthorn, P., Bhoopong, P., & Vuddhakul, V. (2014). Isolation and Characterization of *Vibrio cholerae* Isolates from Seafood in Hat Yai City, Songkhla, Thailand. *Foodborne Pathogens and Disease*, *11*(11), 881–886.

Puah, S., Chua, K., & Tan, J. (2017). Prevalence of *Staphylococcus aureus* and *Salmonella enterica* in ready-to-eat sushi and sashimi. *Tropical Biomedicine*, *34*(1), 45-51

Ralph, A. and Currie, B. (2007). *Vibrio vulnificus* and *V. parahaemolyticus* necrotising fasciitis in fishermen visiting an estuarine tropical northern Australian location. *Journal of Infection*, *54*(3), 111-114.

Ramos, R. J., Miotto, L. A., Miotto, M., Silveira Junior, N., Cirolini, A., da Silva, H. S., … Vieira, C. R. W. (2014). Occurrence of potentially pathogenic *Vibrio* in oysters (Crassostrea gigas) and waters from bivalve mollusk cultivations in the South Bay of Santa Catarina. *Revista Da Sociedade Brasileira de Medicina Tropical*, *47*(3), 327–333.

Randa, M. a, Polz, M. F., & Lim, E. (2004). Effects of temperature and salinity on *Vibrio vulnificus* population dynamics as assessed by quantitative PCR. *Applied and Environmental Microbiology*, *70*(9), 5469–5476.

Rasooly, A., & Herold, K. E. (2008). Food microbial pathogen detection and analysis using DNA microarray technologies. *Foodborne Pathogens and Disease*, *5*(4), 531–550.

Robert-Pillot, A., Copin, S., Himber, C., Gay, M., & Quilici, M.-L. (2014). Occurrence of the three major Vibrio species pathogenic for human in seafood products consumed in France using real-time PCR. *International Journal of Food Microbiology*, *189*, 75–81.

Sandra, A., Afsah-Hejri, L., Tunung, R., Tuan Zainazor, T. T. C., Tang, J. Y. H., Ghazali, F. M., … Son, R. (2012). *Bacillus cereus* and *Bacillus thuringiensis* in ready-to-eat cooked rice in Malaysia. *International Food Research Journal*, *19*(3), 829–836.

Saravanan, V., Sanath Kumar, H., Karunasagar, I., & Karunasagar, I. (2007). Putative virulence genes of *Vibrio cholerae* from seafoods and the coastal environment of Southwest India. *International Journal of Food Microbiology*, *119*(3), 329–333.

Saux, M. F. Le, Hervio-Heath, D., Loaec, S., Colwell, R. R., & Pommepuy, M. (2002). Detection of cytotoxin-hemolysin mRNA in nonculturable populations of environmental and clinical *Vibrio vulnificus* strains in artificial seawater. *Applied and Environmental Microbiology*, *68*(11), 5641–5646.

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., … Griffin, P. M. (2011). Foodborne illness acquired in the United States-Major pathogens. *Emerging Infectious Diseases*, *17*(1), 7–15.

Senderovich, Y., Izhaki, I., & Halpern, M. (2010). Fish as reservoirs and vectors of Vibrio cholerae. *PloS one*, *5*(1), 8607.

Shen, X., Cai, Y., Liu, C., Liu, W., Hui, Y., & Su, Y. C. (2009). Effect of temperature on uptake and survival of *Vibrio parahaemolyticus* in oysters (Crassostrea plicatula). *International Journal of Food Microbiology*, *136*(1), 129–132.

Srisuk, C., Chaivisuthangkura, P., Rukpratanporn, S., Longyant, S., Sridulyakul, P., &

Sithigorngul, P. (2010). Rapid and sensitive detection of *Vibrio cholerae* by loop-mediated isothermal amplification targeted to the gene of outer membrane protein ompW. *Letters in Applied Microbiology*, *50*(1), 36–42.

Su, Y. C., & Liu, C. (2007). *Vibrio parahaemolyticus*: A concern of seafood safety. *Food Microbiology*, *24*(6), 549–558.

Sujeewa, A. K. W., Norrakiah, A. S., & Laina, M. (2009). Prevalence of toxic genes of Vibrio parahaemolyticus in shrimps (Penaeus monodon) and culture environment. *International Food Research Journal*, *16*(1).

Sun, J. R., Yan, J. C., Yen, C. Y., Lee, S. Y., & Lu, J. J. (2007). Invasive infection with *Streptococcus iniae* in Taiwan. *Journal of Medical Microbiology*, *56*(9), 1246–1249.

Suo, B., He, Y., Tu, S.-I., & Shi, X. (2010). A multiplex real-time polymerase chain reaction for simultaneous detection of Salmonella spp., Escherichia coli O157, and *Listeria monocytogenes* in meat products. *Foodborne Pathogens and Disease*, *7*(6), 619–28.

Suppin, D., Rippel-Rachle, B., Schopf, E., & Smulders, F. J. M. (2004). A survey of the microbiological condition of sushi from Viennese retail operations. *Food Safety Assurance and Veterinary Public Health. Volume 2: Safety Assurance during Food Processing*, 377–379.

Suzita, R., Abdulamir, A. ., & Abu Bakar, F. (2009). A Mini Review : Cholera Outbreak via Shellfish. *American Journal of Infectious Disease*, *5*(1), 40–47.

Suzita, R., Abu Bakar, F., Son, R., & Abdulamir, A. S. (2010). Detection of *Vibrio cholerae* in raw cockles (Anadara granosa) by polymerase chain reaction. *International Food Research Journal*, *17*(3), 675–680.

Tan, Y. F., Haresh, K. K., Chai, L. C., Ghazali, F. M., & Son, R. (2008). Prevalence of *Campylobacter* spp. in retailed ready-to-eat sushi. *International Food Research Journal*, *15*(3), 331–336.

Tantillo, G. M., Fontanarosa, M., Di Pinto, A., & Musti, M. (2004). Updated perspectives on emerging vibrios associated with human infections. *Letters in applied microbiology*, *39*(2), 117-126.

Tang, J. Y. H., Mohd Noor, N. H., Mazlan, N., Yeo, C. C., Abu Bakar, C. A., & Radu, S. (2014). Survival of *Vibrio cholerae* O1 and *Vibrio parahaemolyticus* in fried and boiled Malaysian fish sausage. *Food Control*, *41*(1), 102–105.

Tang, Y., Cao, Y., Yu, Y., Yan, S., Wang, Y., Pan, Y., & Zhang, W. (2017). Real-Time Recombinase Polymerase Amplification Assay for the Detection of *Vibrio cholerae* in Seafood. *Food Analytical Methods*, *10*(8), 2657–2666.

Teh, C. S. J., Chua, K. H., Puthucheary, S. D., & Thong, K. L. (2008). Further

evaluation of a multiplex PCR for differentiation of *Salmonella paratyphi* A from other Salmonellae. *Japanese Journal of Infectious Diseases*, *61*(4), 313–314.

Teh, C. S. J., Chua, K. H., & Thong, K. L. (2010). Simultaneous differential detection of human pathogenic and nonpathogenic *Vibrio* species using a multiplex PCR based on *gyr*B and *pnt*A genes. *Journal of Applied Microbiology*, *108*(6), 1940–1945.

Thompson, C. K., Wang, Q., Bag, S. K., Franklin, N., Shadbolt, C. T., Howard, P., … Hope, K. G. (2017). Epidemiology and whole genome sequencing of an ongoing point-source *Salmonella Agona* outbreak associated with sushi consumption in western Sydney, Australia 2015. *Epidemiology and Infection*, *145*(10), 2062–2071.

Thong, K. L., Tech, C. S. J., & Chua, K. H. (2014). Development and evaluation of a multiplex polymerase chain reaction for the detection of *Salmonella* species. *Tropical Biomedicine*, *31*(4), 689–687.

Traoré, O., Martikainen, O., Siitonen, A., Traoré, A. S., Barro, N., & Haukka, K. (2014). Occurrence of Vibrio cholerae in fish and water from a reservoir and a neighboring channel in Ouagadougou, Burkina Faso. *The Journal of Infection in Developing Countries*, *8*(10), 1334-1338.

Tunung, R., Ghazali, F. M., Noranizan, M. A., Haresh, K. K., Lesley, M. B., Nakaguchi, Y., … Son, R. (2011). Rapid detection and enumeration of pathogenic *Vibrio parahaemolyticus* in raw vegetables from retail outlets. *International Food Research Journal*, *18*(1), 67–78.

Tunung, R., Margaret, S. P., Jeyaletchumi, P., Chai, L. C., Zainazor, T. C. T., Ghazali, F. M., … Son, R. (2010). Prevalence and quantification of *Vibrio parahaemolyticus* in raw salad vegetables at retail level. *Journal of Microbiology and Biotechnology*, *20*(2), 391–396.

Ucko, M., & Colorni, A. (2005). Mycobacterium marinum infections in fish and humans in Israel. *Journal of Clinical Microbiology*, *43*(2), 892–895.

US Food & Drug Administrator. (2011). *Fish and Fishery Products Hazards and Controls Guidance*. US Department of Health and Human Services Food and Drug Administration Center for Food Safety and Applied Nutrition.

US Food and Drug Administration. (2010). BAM Appendix 2: Most probable number from serial dilutions. *Bacteriological Analytical Manual*, 52-57.

Vengadesh, L., Son, R., & Yoke Kqueen, C. (2012). Molecular quantitation and characterization of Vibrio cholerae from different seafood obtained from wetmarket and supermarket. *International Food Research Journal*, *19*(1), 45–50.

Wang, L., Shi, L., Su, J., Ye, Y., & Zhong, Q. (2013). Detection of *Vibrio parahaemolyticus* in food samples using in situ loop-mediated isothermal amplification method. *Gene*, *515*(2), 421–425.

Wang, R., Huang, J., Zhang, W., Lin, G., Lian, J., Jiang, L., … Wang, S. (2011). Detection and identification of *Vibrio parahaemolyticu*s by multiplex PCR and DNA-DNA hybridization on a microarray. *Journal of Genetics and Genomics*, *38*(3), 129–135.

Wang, R., Zhong, Y., Gu, X., Yuan, J., Saeed, A. F., & Wang, S. (2015). The pathogenesis, detection, and prevention of *Vibrio parahaemolyticus*. *Frontiers in Microbiology*. https://doi.org/10.3389/fmicb.2015.00144

Wei, S., Zhao, H., Xian, Y., Hussain, M. A., & Wu, X. (2014). Multiplex PCR assays for the detection of *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and *Vibrio cholerae* with an internal amplification control. *Diagnostic Microbiology and Infectious Disease*, *79*(2), 115–118.

Wong, H. C., You, W. Y., & Chen, S. Y. (2012). Detection of toxigenic *Vibrio cholerae*, *V. parahaemolyticus* and *V. vulnificus* in oyster by multiplex-PCR with internal amplification control. *Journal of Food and Drug Analysis*, *20*(1).

Wu, Y., Wen, J., Ma, Y., Ma, X., & Chen, Y. (2014). Epidemiology of foodborne disease outbreaks caused by *Vibrio parahaemolyticus*, China, 2003-2008. *Food Control*, *46*, 197–202.

Xu, Y. G., Sun, L. M., Wang, Y. S., Chen, P. P., Liu, Z. M., Li, Y. J., & Tang, L. J. (2017). Simultaneous detection of *Vibrio cholerae*, *Vibrio alginolyticus*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* in seafood using dual priming oligonucleotide (DPO) system-based multiplex PCR assay. *Food Control*, *71*, 64–70.

Yamazaki, W. (2011). Sensitive and rapid detection of cholera toxin-producing *Vibrio cholerae* using loop-mediated isothermal amplification. In *Microbial Toxins* (pp. 13-22). Humana Press, Totowa, NJ.

Yamazaki, W., Ishibashi, M., Kawahara, R., & Inoue, K. (2008). Development of a loop-mediated Isothermal amplification assay for sensitive and rapid detection of Vibrio parahaemolyticus. *BMC Microbiology*, *8*(1), 163.

Yano, Y., Yokoyama, M., Satomi, M., Oikawa, H., & Chen, S. S. (2004). Occurrence of *Vibrio vulnificus* in fish and shellfish available from markets in China. *Journal of Food Protection*, *67*(8), 1617–1623.

Yeung, P. S. M., & Boor, K. J. (2004). Epidemiology, pathogenesis, and prevention of foodborne *Vibrio parahaemolyticus* infections. *Foodborne Pathogens and Disease*, *1*(2), 74–88.

Yoke Kqueen, C., Learn Han, L., Noorzaleha, A. S., Son, R., Sabrina, S., Jiun-Horng, S., & Chai-Hoon, K. (2008). Characterization of multiple-antimicrobial-resistant *Salmonella enterica* Subsp. enterica isolated from indigenous vegetables and poultry in Malaysia. *Letters in Applied Microbiology*, *46*(3), 318–324.

Zappelini, L., Martone-Rocha, S., Dropa, M., Matté, M. H., Tiba, M. R., Breternitz, B. S., & Razzolini, M. T. P. (2017). Effective characterization of *Salmonella Enteritidis* by most probable number (MPN) followed by multiplex polymerase chain reaction (PCR) methods. *Environmental Science and Pollution Research*, *24*(5), 4828–4834.

Zhang, Z., Lou, Y., Du, S., Xiao, L. L., Niu, B., Pan, Y., & Zhao, Y. (2017). Prevalence of *Vibrio parahaemolyticus* in seafood products from hypermarkets in Shanghai. *Journal of the Science of Food and Agriculture*, *97*(2), 705–710.

Zhao, X., Lin, C.-W., Wang, J., & Oh, D. H. (2014). Advances in Rapid Detection Methods for Foodborne Pathogens. *J. Microbiol. Biotechnol*, *24*(243), 297–312.

Zhao, X., Zhong, J., Wei, C., Lin, C. W., & Ding, T. (2017). Current perspectives on viable but non-culturable state in foodborne pathogens. *Frontiers in Microbiology*. https://doi.org/10.3389/fmicb.2017.00580

Zijlstra, C., & Van Hoof, R. A. (2006). A Multiplex Real-Time Polymerase Chain Reaction (TaqMan) Assay for the Simultaneous Detection of Meloidogyne chitwoodi and M. fallax. *Phytopathology*, *96*(11), 1255–1262.

Zulkifli, Y., Alitheen, N. B., Son, R., Yeap, S. K., Lesley, M. B., & Raha, A. R. (2009). Identification of *Vibrio parahaemolyticus* isolates by PCR targeted to the *tox*R gene and detection of virulence genes. *International Food Research Journal*, *16*(3), 289–296.

LIST OF PUBLICATIONS

Bonny, S. Q., Hossain M. M., KL Thong, Ali, M. E. Kader, M. A., & Rahman, M. A. (Under Review). Multiplex MPN-PCR for the Enumeration of Three Major Vibrios in Raw Fishes. Food Control (Accepted) **[Q1, ISI Journal, Impact Factor: 3.496]**

CONFERENCE PROCEEDINGS

1. **Bonny, S. Q**., Thong, K. L., Ali, ME, (2017). Comparison of PCR, MPN-PCR and Multiplex MPN-PCR Assay for Discriminatory Identification of Three Zoonotic Vibrios in Fish; UI Scholar Summit – Universitas Indonesia, Universitas Indonesia, Jakarta, Indonesia; 10-11 Oct 2017.
2. **Bonny, S. Q**., Thong, K. L., Ali, ME, (2017). Occurrence and Quantification of Staphylococcus aureus in ready-to-eat Canned Fish; Biological Sciences Graduate Congress 2017, National University of Singapore, Singapore; 19-20 Dec 2017.