



**UNIVERSITI PUTRA MALAYSIA**

***PREVALENCE AND CHARACTERIZATION OF *Vibrio* spp. IN  
SEAWATER AND WET-MARKET COCKLES FROM KUALA SELANGOR,  
MALAYSIA, AND RISK OF ACQUIRING *V. vulnificus* INFECTIONS  
FROM COCKLE CONSUMPTION***

**CHANG WEI SAN**

**FSTM 2019 13**



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By

**CHANG WEI SAN**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra  
Malaysia, in Fulfilment of the Requirement for the Degree of Doctor  
of Philosophy**

**October 2018**

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*Dedicated to my family and relatives for their unconditional love and endless support*  
*Dedicated to my friends for the wonderful friendship, love and joy*  
*Dedicated to everyone whom have invested their lives in my life*



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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

**PREVALENCE AND CHARACTERIZATION OF *Vibrio* spp. IN SEAWATER AND WET-MARKET COCKLES FROM KUALA SELANGOR, MALAYSIA, AND ESTIMATION OF RISK OF ACQUIRING *V. vulnificus* INFECTIONS FROM COCKLE CONSUMPTION**

By

**CHANG WEI SAN**

**October 2018**

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

It has been estimated that global sea surface temperature is approximately 1°C higher than 140 years ago and is one of the primary physical impacts of climate change. The aim of this study was to determine the prevalence of *Vibrio cholerae*, *V. parahaemolyticus* and *V. vulnificus* in cockle and seawater samples. The samples were collected monthly from coastal area of Kuala Selangor over a one year period. To quantify the prevalence of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* in cockle and seawater samples, the most probable number (MPN) method was used in combination with the polymerase chain reaction (PCR) which was used to classify the isolates by detecting their specific genes. Following with the antibiotic resistance study by using E test to characterize *V. parahaemolyticus* strains isolated from the samples. The characteristics of *V. parahaemolyticus* were also assessed by the investigation of the levels of multiple antibiotics resistant *V. parahaemolyticus* in cockles under the effect of different storage conditions. Furthermore, an examination of the survivability of *V. parahaemolyticus* during steaming of cockles was conducted. Finally, a preliminary step-wise risk assessment was carried out to estimate the risk (probability of infection leading to illness) posed by *V. vulnificus* from the consumption of cockles in Malaysia.

The prevalence results showed that *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* were detected in both cockle and seawater samples. Out of the 247 total samples tested, 243(98.4%) samples were positive for *V. parahaemolyticus*. Following with 11 (4.5%) and 23(9.3%) of *V. cholerae* and *V. vulnificus* in total samples, respectively. Pathogenic *V. parahaemolyticus* and *V. cholerae* strains were only detected in cockle samples, with the prevalence of 0.4% and 5.3%,

respectively. Whereas pathogenic *V. vulnificus* strain was detected in both cockle and seawater samples with the prevalence of 8.5%. The prevalence of *V. parahaemolyticus* and *V. vulnificus* in total samples were found positively correlated to the seawater temperature, but not with *V. cholerae*. The study revealed that seawater temperature influences the prevalence of these pathogens. Overall, among those *V. parahaemolyticus* isolates obtained from cockles (n = 22) and seawater (n = 6), the resistance proportions were highest for tetracycline (8; 28.6%). Following with 5 (17.9%) were resistant to trimethoprim-sulfamathoxazole, 3 (10.7%) were resistant to doxycycline. Also, 2 (7.1%) were resistant to cefotaxime and ceftriaxone. All *V. parahaemolyticus* isolates regardless the sources were susceptible to ceftazidime, gentamicin, ciprofloxacin and levofloxacin. MAR index of *V. parahemolyticus* isolates ranged from 0.11-0.44. Seventy percent of the isolates showed MAR index > 0.2. The majority of multiple antibiotic resistance combinations included doxycycline and tetracycline.

For the investigation of the effects of handling practices in home kitchen level, specifically ambient exposure, refrigeration and icing storage on the levels of high multiple antibiotic resistant indexes *V. parahaemolyticus* in cockles. The results indicated that different storage conditions had a significant effect on the growth of *V. parahaemolyticus*. Given that most *V. parahaemolyticus* are associated with consumption of shellfish and cockle's popularity in Malaysia, examination of the survivability of pathogenic *V. parahaemolyticus* strains in cockles upon steaming process was conducted. Based on the data obtained, Regression analysis of the survival curve showing the linear correlation between log number of pathogenic *V. parahaemolyticus* and the steaming time ( $R^2= 0.88$ ). The results indicate that steaming time of minimum eight minutes is necessary for safe consumption of cockles free of pathogenic *V. parahaemolyticus*.

This simple step-wise risk assessment, concerning acquisition of *V. vulnificus* from consumption of cockles among different major ethnic groups, susceptibility populations and gender in Malaysia, has utilized data from the previous chapters and from diverse sources in an attempt to estimate risk of infection. From the obtained results, Malays were found exposed to the greater risk due to higher consumption pattern of cockles, compared to Chinese. Among susceptible populations, elderly population was found exposed to the greatest risk followed by diabetes, alcoholism and AIDS population. Likewise, male population was found exposed to the greater risk of septicemia infections, compared to female population regardless of their binge drinking habit.

Contamination of cockles and seawater with *V. cholera*, *V. parahaemolyticus* and *V. vulnificus* reflects the contamination of the environment from which the

cockles were harvested. Consumption of contaminated cockles present with these pathogens is likely to cause infections and pose significant risks to human health. Treatment may benefit from the use of ceftazidime, ciprofloxacin, levofloxacin and gentamicin, which were among the antibiotics 100% effective against *V. parahaemolyticus* isolated in this study. Additionally, cockles intended for partial cooked consumption should be eaten immediately following purchase or harvest, and not left at ambient temperature. Apart from this, cockles should be cooked sufficiently before consumption to safeguard the public health.



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia  
sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**PREVALENS DAN PENCIRIAN *Vibrio* spp. DALAM AIR LAUT DAN  
KERANG PASAR DARI KUALA SELANGOR, MALAYSIA DAN RISIKO  
MENDAPAT JANGKITAN *V. vulnificus* DARIPADA PEMAKANAN  
KERANG**

Oleh

**CHANG WEI SAN**

**Oktober 2018**

**Pengerusi : Profesor Son Radu, PhD**  
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Suhu permukaan laut global telah dianggarkan kira-kira 1°C lebih tinggi daripada 140 tahun yang lalu dan merupakan salah satu kesan fizikal utama perubahan iklim. Tujuan kajian ini adalah untuk menentukan prevalens *Vibrio cholerae*, *V. parahaemolyticus* dan *V. vulnificus* dalam sampel kerang dan air laut. Sampel telah diambil pada setiap bulan dari kawasan pantai Kuala Selangor dalam tempoh masa satu tahun. Untuk mengukur prevalens *V. cholerae*, *V. parahaemolyticus* dan *V. vulnificus* dalam sampel kerang dan air laut, kaedah nombor paling mungkin (MPN) telah digunakan pegabungan dengan tindak balas berantai polimerasi (PCR) dimana kaedah ini digunakan untuk mengelaskan pencilan-pencilan dengan mengesan gen-gen khusus mereka. Diikuti dengan kajian kerintangan antibiotik dengan menggunakan E test untuk mencirikan strain-strain *V. parahaemolyticus* yang telah dipencilkan daripada sampel. Ciri-ciri *V. parahaemolyticus* juga telah dinilai dengan penyiasatan terhadap beberapa tahap rintang pelbagai antibiotik *V. parahaemolyticus* dalam kerang di bawah keadaan penyimpanan yang berbeza. Tambahan pula, pemeriksaan tentang kebolehhidupan *V. parahaemolyticus* semasa pengukusan kerang telah dijalankan. Akhir sekali, penilaian saringan risiko step-wise telah dijalankan untuk menganggarkan risiko (kebarangkalian untuk mendapat jangkitan yang membawa kepada penyakit) yang disebabkan oleh *V. vulnificus* daripada pemakanan kerang di Malaysia.

Hasil kajian menunjukkan bahawa *V. cholerae*, *V. parahaemolyticus* dan *V. vulnificus* telah dikesan dalam kedua-dua sampel kerang dan air laut. Daripada 247 jumlah keseluruhan sampel yang telah diuji, 243 (98.4%) sampel adalah positif untuk *V. parahaemolyticus*. Diikuti dengan 11 (4.5%) dan 23 (9.3%)



masing-masing daripada *V. cholerae* dan *V. vulnificus* daripada jumlah keseluruhan sampel. Strain patogenik *V. parahemolyticus* dan *V. cholerae* hanya dikesan dalam sampel kerang, dengan prevalens sebanyak 0.4% dan 5.3%. Manakala, strain patogenik *V. vulnificus* dikesan dalam kedua-dua sampel kerang dan air laut dengan prevalens sebanyak 8.5%. Prevalens *V. parahaemolyticus* dan *V. vulnificus* daripada jumlah keseluruhan sampel dijumpai berkadar positif dengan suhu air laut tetapi tidak dengan *V. cholerae*. Kajian ini menunjukkan bahawa suhu air laut mempengaruhi prevalens patogen ini.

Secara keseluruhan, di antara pencilan *V. parahaemolyticus* yang diambil dari kerang (n = 22) dan air laut (n = 6), pecahan kadar rintangan adalah tertinggi bagi tetracycline 8 (28.6%). Diikuti dengan 5 (17.9%) adalah rintang terhadap trimethoprim-sulfamathoxazole, 3 (10.7%) adalah rintang terhadap doxycycline. Juga, 2 (7.1%) adalah rintang terhadap cefotaxime dan ceftriaxone. Semua pencilan *V. parahaemolyticus* tanpa mengira sumber adalah tidak rintang kepada ceftazidime, gentamicin, ciprofloxacin dan levofloxacin. MAR indeks bagi pencilan *V. parahemolyticus* adalah antara 0,11-0,44. Tujuh puluh peratus daripada pencilan menunjukkan MAR indeks > 0.2. Majoriti kombinasi rintangan pelbagai antibiotik termasuk doxycycline dan tetracycline.

Bagi pengajian kesan amalan pengendalian dalam peringkat dapur rumah, terutamanya pendedahan ambien, penyejukan dan penyimpanan secara ais pada *V. parahaemolyticus* yang mempunyai beberapa tahap indeks kerintangan antibiotik pelbagai yang tinggi dalam kerang. Keputusan menunjukkan bahawa tempoh masa penyimpanan dan suhu mempunyai kesan yang ketara ke atas pertumbuhan *V. parahaemolyticus*. Memandangkan kebanyakan *V. parahaemolyticus* dikaitkan dengan pengambilan kerang dan populariti kerang di Malaysia, pemeriksaan kebolehhidupan strain patogenik *V. parahaemolyticus* dalam kerang selepas proses pengukusan dikajikan. Berdasarkan data yang diperolehi, patogenik *V. parahaemolyticus* tidak dikesan dalam sampel yang dikukus daripada tempoh pengukusan enam minit ke atas. Analisis regresi lengkung kebolehhidupan, menunjukkan korelasi linear antara nombor log patogenik *V. parahaemolyticus* dan tempoh mengukus ( $R^2 = 0.88$ ). Keputusan menunjukkan bahawa masa pengukusan minimum selama lapan minit diperlukan untuk pemakanan kerang yang selamat dan bebas daripada patogenik *V. parahaemolyticus*.

Penilaian risiko langkah-bijak yang mudah ini, telah menganggarkan risiko memperoleh *V. vulnificus* daripada pemakanan kerang di kalangan populasi etnik yang berbeza, populasi mudah terdedah terhadap penyakit dan jantina di Malaysia, telah menggunakan data dari bab-bab sebelum ini dan daripada pelbagai sumber dalam usaha untuk menganggarkan ukuran risiko ini. Daripada keputusan yang diperolehi, bangsa Melayu telah didapati terdedah

kepada risiko yang lebih tinggi kerana pola pemakanan kerang yang lebih tinggi, berbanding bangsa Cina. Di kalangan populasi mudah terdedah terhadap penyakit, populasi warga tua telah didapati terdedah kepada risiko yang tertinggi diikuti oleh populasi diabetes, ketagihan arak dan AIDS. Begitu juga, populasi lelaki telah didapati terdedah kepada risiko yang lebih tinggi daripada untuk mendapat jangkitan septisemia, berbanding populasi wanita tanpa mengira tabiat minum alkohol mereka.

Pencemaran kerang dan air laut dengan *V. cholerae*, *V. parahaemolyticus* dan *V. vulnificus* menunjukkan pencemaran alam sekitar yang dimana kerang didapati. Pemakanan kerang tercemar dengan patogen ini mungkin menyebabkan jangkitan dan menimbulkan risiko kepada kesihatan manusia. Rawatan mungkin mendapat faedah daripada penggunaan ceftazidime, ciprofloxacin, levofloxacin dan gentamicin, adalah di antara antibiotik 100% berkesan terhadap *V. parahaemolyticus* yang ditemui dalam kajian ini. Di samping itu, kerang bertujuan untuk penggunaan separa masak perlu dimakan sebaik sahaja selepas pembelian atau diperolehi, dan tidak ditinggalkan pada suhu bilik. Selain daripada itu, kerang perlu dimasak secukupnya sebelum dimakan untuk melindungi kesihatan awam.

## ACKNOWLEDGEMENTS

I would like to express my heartfelt gratitude and appreciation to Professor Dr. Son Radu, the chairman of my supervisory committee for the invaluable guidance and support throughout my study. I really appreciate everything you have taught me! Your mentorship and words of encouragement mean a lot to me. I'm also very grateful for the opportunities you have given me. A million thanks to my co-supervisors, Associate Professor Dr. Yaya Rukayadi and Dr. Nor Khaizura Mahmud @ Ab Rashid from Faculty of Food Science and Technology for the advice and motivation throughout my study. Thank you very much!

Sincere thanks to all my dearest laboratory colleagues: Najwa, Sin Yee, Malcolm Tan, Vivian New, Ubong, Raymond Kuan, Zect Chin, Thung and Ramzi. Thank you for the help and assistance given to me. I will remember the laughter and sweet memories while working with all of you. My sincere gratitude is extended to all staffs especially En. Zulkefli and Kak Ikin.

Not forgetting to acknowledge all my dearest sisters especially Chang Hui Lee, Low Kui San and Lee Wei Chuan who support and accompany me every step of the way. Thank you for being just as weird as I am and making ordinary moments extraordinary. You all never fail to make me laugh. There is never a dull moment with you all. To Ho Hui See and Chan Leng Lai, thank you for being a true friend - even when we are apart. Friendships forever!

Last but not least, I like to express my thousand heartfelt thanks to my beloved father, mother, cousins and brothers. Thank you very much for everything, the sacrifices and endless love for me. For mom and dad, i feel so honored and blessed to have you as my parents, and want to express my gratitude for your care and support over the years. Thank you for instilling me with a strong passion for learning and for doing everything possible to put me on the path to greatness. I will never forget the important values you have passed down to me—particularly perseverance and honesty. Words cannot describe how important you both are mean to me. There are no better words to use today than "thank you," Mom and Dad. You have given me the greatest gift of all: an education. For my cousins, thank you for the lovely companionship!

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

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## LIST OF ABBREVIATIONS

µl	Microliter
µm	Micrometer
ATCC	American Type Culture Collection
BAM	Bacteriological Analytical Manual
bp	base pair
CDC	Centre for Disease Control
CLSI	Clinical Laboratory and Standards Institute
DNA	Deoxynucleic acid
dNTP	Deoxyribonucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and Agricultural Organization
FDA	Food and Drug Administration
HIV	Human immunodeficiency virus
ICMSF	International Commission on Microbiological Specifications for Food
MAR	Multiple Antibiotic Resistance
MgCl <sub>2</sub>	Magnesium Chloride
mM	MilliMolar
MOH	Ministry of Health
MPN	Most Probable Number
NaCl <sub>2</sub>	Sodium Chloride
OD	Optical Density
PCR	Polymerase Chain Reaction
<i>Taq</i>	<i>Thermus aquaticus</i>
TBE	Tris-Boric acid-EDTA
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
U. S	United States
UV	Ultra violet
V	Volt
WHO	World Health Organization
WTO	World Trade Organization
x g	Unit gravity
β	Beta

# CHAPTER 1

## INTRODUCTION

### 1.1 Background

The vibrios are aquatic bacteria, 72 species have been identified at the present time, 12 of which associated to human infections. The most significant pathogenic species account for the majority of *Vibrio* illnesses in humans are *Vibrio cholerae*, *V. parahaemolyticus*, and *V. vulnificus* (Thompson et al., 2004; Bonnin-Jusserand et al., 2017). *Vibrio cholerae* caused the disease cholera and remains a persistent causative agent of mortality and morbidity in Africa and Asia. *Vibrio parahaemolyticus* is a halophilic bacterium that thrives in coastal, estuarine and marine environments. This species typically caused acute gastroenteritis in human. In rare cases, wound infections can occur in individuals with pre-existing open sore. Infection is rarely fatal, and the majority of cases attributed to the ingestion of raw or undercooked shellfish, or drinking contaminated water. Infections may lead to septicemia and death if untreated promptly, though reported case is rare (Drake et al., 2007). Likewise, *Vibrio vulnificus* is usually found in warm, coastal seawater. This bacterium has emerged as a significant threat in Korea and Taiwan, associated with the ingestion of raw seafood, especially oysters. Infections can occur through wounds exposure to seawater and lead to deadly septicemia in people with pre-existing medications (Matsumoto et al., 2010).

Numerous studies of *Vibrio* species showed that increasing water temperature has resulted in evident multiplication of these bacteria in the environment (Kaboré et al., 2017; Froelich et al., 2017; Di et al., 2017). The species can be accumulated by filter feeding nature of molluscan bivalves that sieve through surrounding food particles. This may result in the entrapment of pathogenic strains to humans in the tissues of edible bivalves. This is concerning since many food poisoning outbreaks associated with the consumption of raw or partially cooked bivalves have been reported throughout the world (Potasman et al., 2002; Bonnin-Jusserand et al., 2017; Camkerten et al., 2017). In addition to the ecology, studies have shown that temperature has an effect on *Vibrio* pathogenicity. It is reported to play a role in serogroup conversion of *V. cholerae* non-O1 to O1 (Montilla et al., 1996).

Bivalve mollusks such as clams, oysters, mussels and cockles are filter feeders in nature and they tend to entrap microorganisms from the surrounding environment. They are usually grown and harvested just below the surface of the muds or sandy silts in the intertidal zones of estuarine waters. Therefore,

they are likely to harbour high concentrations of microbial pathogens including vibrios (Beaz-Hidalgo et al., 2010). The blood cockle species *Anadara granosa* is a bivalve in the phylum mollusca and locally known as “kerang” and “sea hum” in Malaysia. They are frequently consumed by Malaysian as an essential ingredient in popular local dishes in curry laksa and char kuey teow or cooked in sambal belacan and served with nasi lemak. Additionally, *A. granosa* is of considerable economic importance in Malaysia. In 2013, Malaysia had produced 40,186.5 metric tonnes of cockle for seafood industry (Department of Fisheries Malaysia, 2013).

*Vibrio* associated diseases originating from physical exposure of seawater or through consumption of contaminated seafood can threaten public health especially if the implicated strains are strongly resistant to more than one clinically important antibiotic (Dengo-Baloi et al., 2017). The main concerns for the clinical treatment are the development of resistance and the rapid adaptation to new generated antibiotics by the pathogenic strains. Many antibiotic resistance profile demonstrated by different *Vibrio* species have been reported (Kang et al., 2017; Dengo-Baloi et al., 2017; Xie et al., 2017). For instance, the bacterial resistance to penicillin and ampicillin was most frequently detected among the *Vibrio* species in coastal waters (Elmahdi et al., 2016).

Microbiological risk assessment (MRA) is a science based approach to explore the complexity of food systems and to assess the potential presence of hazards in food from production, processing, and preparation environments into statements of likelihood and magnitude of a acquiring certain risk which give impacts on public health in adverse term (Lammerding, 2013; Masood et al., 2014). The collaborative study by the Food and Agricultural Organization (FAO) and the World Health Organization (WHO) has provided valuable insights and considerable resources for conducting risk assessments. The FAO/WHO Joint Experts on Microbial Risk Assessment (JEMRA) have published several series of extensive risk assessments on priority pathogen/food combinations which including *Vibrio*, *Salmonella*, *Listeria* and *Campylobacter* species (Rocourt et al., 2003; WHO, 2005).

Many microbiological risk assessment studies have been carried out on seafood (FDA, 2005; Yamamoto et al., 2008; Sobrinho et al., 2014). From this approach, proper measures and actions can be taken to mitigate the situation through risk management and risk communication. Besides that, the current risk assessment can also be updated and improved by new research and information when they become available (Robertson et al., 2005; Chai, 2008).



## 1.2 Problem statement

Influences of temperature on the metabolism and growth rates of *Vibrio* species have long been established. Given the significant health consequences caused by *Vibrio* species, the prevalence of these pathogens in local seafood and seawater should not be overlooked. Also, the development of resistance and the rapid adaptation to new generated antibiotics by the pathogenic *Vibrio* strains are important food safety concerns. Investigation of the evolution of antibiotic-resistant ability and characteristics of the *Vibrio* species are particularly important to give an overall in-depth understanding of these pathogens over time. Quantitative risk assessment (QRA) modeling is increasingly used as a tool for the evaluation of health risks and supporting the management of safe food production. While in Malaysia, Tan et al. (2016) and Norrakiah et al. (2013) conducted risk assessment of *V. parahaemolyticus* in bloody clams and tiger shrimps, respectively. However, the focus was on *V. parahaemolyticus*.

In the present study, the prevalence of three major pathogenic *Vibrio* species: *V. cholera*, *V. parahaemolyticus* and *V. vulnificus* in cockles and seawater was determined, but also the antibiotic sensitivity and characteristics of the isolates from the samples. Estimation of the risk of acquiring *V. vulnificus* infections from the consumption of cockles was also carried out by using step-wise risk assessment.

## 1.3 Objectives

The objectives of this study are:

1. To determine the prevalence of *Vibrio* spp. isolated from seawater samples and wet market samples of cockles collected from Kuala Selangor, Malaysia, and the antimicrobial susceptibility profiles of the bacterial isolates.
2. To investigate the effects of ambient and low temperature storage conditions and steaming on the high multiple antibiotic resistant index and survivability of *V. parahaemolyticus* inoculated on cockles.
3. To estimate the risk of acquiring *V. vulnificus* infections from the consumption of cockles using step-wise risk assessment.



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