



UNIVERSITI PUTRA MALAYSIA

***ISOLATION, CHARACTERIZATION OF SPECIFIC BACTERIOPHAGES
AND THEIR EFFECTIVENESS AS BIOCONTROLS ON RAW SEAFOODS
CONTAMINATED WITH *Vibrio parahaemolyticus****

TAN CHIA WANQ

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By

TAN CHIA WANQ

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

May 2021

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

ISOLATION, CHARACTERIZATION OF SPECIFIC BACTERIOPHAGES AND THEIR EFFECTIVENESS AS BIOCONTROLS ON RAW SEAFOODS CONTAMINATED WITH *Vibrio parahaemolyticus*

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Vibrio parahaemolyticus is among the leading foodborne pathogens that can cause gastroenteritis, wound infections, and/or septicaemia in humans. Antimicrobial resistance also poses a significant global health issue. Research on alternative approaches such as the application of bacteriophage has become a potential solution to minimize the use of antibiotics. In this study, the prevalence and antibiotic-resistant patterns of *V. parahaemolyticus* were determined, and *V. parahaemolyticus* bacteriophages naturally present in seafood samples were isolated and characterized in detail. The genome of bacteriophages was later sequenced through whole-genome sequencing, followed by genome annotation. Finally, the characterized bacteriophages were used as a biocide to control the growth of *V. parahaemolyticus* in the food samples. According to the results, blood clams were detected at the highest prevalence rate (91.43%), followed by shrimp (88.57%), surf clams (82.86%), and squid (80.00%). Besides, 90.83% of the *V. parahaemolyticus* isolates were detected to be multidrug-resistant (MDR). Six *V. parahaemolyticus* species-specific bacteriophages were isolated from blood clam, shrimp, and surf clam samples. Morphological analysis revealed that bacteriophages ϕ Vp33, ϕ Vp22, ϕ Vp21 and ϕ Vp02 isolated from the shrimp samples belonged to the Podoviridae family. Bacteriophages ϕ Vp08 and ϕ Vp11 isolated from the blood clam and surf clam samples, respectively, were categorised into the Siphoviridae family. The optimal MOI for bacteriophage propagation was determined to be in the range of 0.001 to 1. The latent period of bacteriophages falls in the range of 10 to 20 min, and the burst size was approximately 17 to 51 PFU/cell. All bacteriophages were optimally stable over a wide range of temperature levels (20 to 50°C) and pH levels (5 to 11). After the whole-genome sequencing analysis, the genome of bacteriophages was detected to be between 42,896 and 76,128 bp, with a GC content of 48.93 to 49.33%, and 47 to 101 open reading frames (ORFs). The annotation of each

predicted sequence revealed the presence of functional groups such as DNA regulation, structure, packaging, host lysis, hydrolysis, conjugation, and oxidation-reduction. In terms of the biological control application, bacteriophage suspensions achieved an average log reduction of *V. parahaemolyticus* in 2.85 ± 0.07 to 4.81 ± 0.14 . Bacteriophage cocktails were observed to have an improved in vitro lytic activity compared to a single bacteriophage suspension. Oyster meat samples artificially contaminated with *V. parahaemolyticus* and treated with the bacteriophage cocktail also noticed a significant bacterial load reduction ($P < 0.05$) of 2.77 ± 0.53 to 3.07 ± 0.03 log CFU/g after 48 h of treatment. In conclusion, bacteriophages ϕ Vp33, ϕ Vp22, ϕ Vp21, ϕ Vp02, ϕ Vp08, and ϕ Vp11 isolated from the seafood samples demonstrated strong lytic activity against the growth of *V. parahaemolyticus*. Two bacteriophage cocktails (cocktail A and B) used in the inhibition of biofilm and biocontrol agents in food were found to have statistically significant effects in the reduction of biofilm formation and microbial loads of *V. parahaemolyticus* on the oyster meat samples. Bacteriophages in this study consequently proved to be practicable as biocontrol agents for the control of *V. parahaemolyticus*.

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

**PENGASINGAN, PENCIRIAN BAKTERIOFAG TERTENTU DAN
KEBERKESANANNYA SEBAGAI AGEN BIOKONTROL PADA MAKANAN
LAUT MENTAH YANG TERCEMAR DENGAN *Vibrio parahaemolyticus***

Oleh

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Vibrio parahaemolyticus adalah salah satu patogen bawaan makanan terkemuka yang boleh menyebabkan gastroenteritis, jangkitan luka, dan/atau septikemia pada manusia. Selain itu, rintangan antimikroba adalah masalah penting yang menimbulkan masalah ancaman global terhadap kesihatan awam. Penyelidikan mengenai pendekatan alternatif seperti penerapan bakteriofag telah menjadi solusi berpotensi untuk meminimumkan penggunaan antibiotik. Dalam kajian ini, prevalens dan pola tahan antibiotik *V. parahaemolyticus* akan diperiksa. *V. parahaemolyticus* bakteriofag yang terdapat secara semula jadi dalam sampel makanan akan diasingkan dan dicirikan secara terperinci. Genom bakteriofag akan dijelaskan kemudian melalui penjujukan genom keseluruhan, diikuti dengan penjelasan genom. Terakhir, bakteriofag yang dicirikan akan digunakan sebagai biosida untuk meningkatkan tahap keselamatan makanan dengan mengawal pertumbuhan *V. parahaemolyticus* dalam sampel makanan. Dari hasilnya, kerang darah dikesan pada kadar prevalensi tertinggi (91,43%) diikuti oleh udang (88,57%), lala (82,86%), dan sotong (80,00%). Selain itu, 90.83% *V. parahaemolyticus* bakteria mempunyai ketahanan rintangan pelbagai ubat (MDR). Satu *V. parahaemolyticus* bakteria dari kerang darah menunjukkan nilai indeks MAR tertinggi 0.71 dan menunjukkan ketahanan terhadap 17 antibiotik. Enam spesies-spesifik *V. parahaemolyticus* bakteriofag diasingkan dari sampel kerang darah, udang, dan lala. Analisis morfologi menunjukkan bahawa bakteriofag ϕ Vp33, ϕ Vp22, ϕ Vp21 dan ϕ Vp02 yang diasingkan dari sampel udang adalah milik keluarga *Podoviridae*. Bakteriofag ϕ Vp08 dan ϕ Vp11 diasingkan dari kerang darah dan lala dikategorikan dalam keluarga *Siphoviridae*. MOI optimum untuk pembiakan bakteriofag ditentukan 0,001 hingga 1. Tempoh laten bakteriofag berkisar antara 10 hingga 20 minit, dan ukuran pecah kira-kira 17 hingga 51 PFU/sel. Semua bakteriofag stabil secara optimum dalam pelbagai suhu dari 20°C hingga 50°C dan tahap pH dari 5

hingga 11. Analisis penjujukan genom keseluruhan menunjukkan genom bakteriofag dikesan antara 42,896 hingga 76,128 bp, kandungan GC 48,93 hingga 49,33%, dan dikodkan 47 hingga 101 bingkai bacaan terbuka (ORF). Anotasi setiap urutan yang diramalkan menunjukkan adanya kumpulan fungsional seperti peraturan DNA, struktur, pembungkusan, lisis inang, hidrolisis, konjugasi, dan pengurangan oksidasi. Untuk aplikasi kawalan biologi, penggantungan bakteriofag mencapai pengurangan log purata *V. parahaemolyticus* pada 2.85 ± 0.07 hingga 4.81 ± 0.14 . Koktel bakteriofag dilihat mempunyai aktiviti lisis *in vitro* yang lebih baik berbanding dengan suspensi bakteriofag tunggal. Sampel daging tiram dicemari secara buatan dengan *V. parahaemolyticus* dan dirawat dengan koktel bakteriofag juga diperhatikan penurunan beban bakteria yang signifikan ($P < 0.05$) pada 2.77 ± 0.53 hingga 3.07 ± 0.03 log CFU/g setelah 48 jam rawatan. Kesimpulannya, bakteriofag ϕ Vp33, ϕ Vp22, ϕ Vp21, ϕ Vp02, ϕ Vp08, dan ϕ Vp11 yang diasingkan dari sampel makanan laut menunjukkan aktiviti litik yang kuat terhadap pertumbuhan *V. parahaemolyticus*. Dua koktel bakteriofag (koktel A dan B) yang digunakan dalam penghambatan agen biofilm dan biokontrol dalam makanan didapati mempunyai kesan yang signifikan secara statistik dalam pengurangan pembentukan biofilm dan muatan mikroba *V. parahaemolyticus* pada sampel daging tiram. Bakteriofag dalam kajian ini terbukti dapat dilaksanakan sebagai agen biokontrol untuk kawalan *V. parahaemolyticus*.

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LIST OF ABBREVIATIONS

APW	Alkaline peptone water
AST	Antibiotic susceptibility test
ATCC	American Type Culture Collection
BAM	Bacteriological Analytical Manual
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
CFU/cm ²	Colony forming unit centimetre square
CFU/g	Colony forming unit per gram
CFU/ml	Colony forming unit per millilitre
CLSI	Clinical and Laboratory Standards Institute
COVIS	Cholera and Other Vibrio Illness Surveillance
CV	CHROMagar™ <i>Vibrio</i>
dNTPs	Deoxynucleotides
EPS	Exopolysaccharides
GC	Guanine-cytosine
INDEL	Insertion and deletion
LAMP	Loop-mediated isothermal amplification
MAR	Multiple antibiotic resistance
MCP	Major capsid protein
MDR	Multidrug-resistant
MHA	Mueller-Hinton agar
MIC	Minimal inhibitory concentration
MOI	Multiplicity of infection
MPa	Megapascal

MSP	Major sheath protein
NGS	Next generation sequencing
OD	Optical density
ORF	Open reading frame
PEG	Polyethylene glycol
PFU	Plaque forming unit
PFU/g	Plaque forming unit per gram
PHACTS	Phage Classification Tool Set
RFLP	Restriction fragment length polymorphism
RTE	Ready to eat
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SM	Salt of magnesium
SNP	Single nucleotide polymorphisms
T3SS	Type III secretion system
T6SS	Type VI secretion system
TCBS	Thiosulfate citrate bile salts sucrose
TDH	Thermostable direct hemolysin
TE	Tris-EDTA
TEM	Transmission electron microscopy
TLH	Thermolabile hemolysin
TRH	Thermostable direct-related hemolysin
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TTP	Tail tube protein
VBNC	Viable but non-culturable

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Vibrio parahaemolyticus was first discovered in 1950 by Tsunesaburo Fujino, after the outbreak of shirasu food poisoning in Japan. The classification of this microorganism was initially grouped under the genus *Pasteurella*, and is referred to as *Pasteurella parahaemolytica*. This species was later assigned to the *Vibrio* genus, because of its basic similarity with halophilic organisms. The new scientific name *Vibrio parahaemolyticus* was later proposed by Riichi Sakazaki, Setsuo Iwanami, and Hideo Fukumi, and was concluded by Tsunesaburo Fujino (Shinoda, 2011).

Vibrio parahaemolyticus is a natural microflora of marine and coastal water bodies that has been widely disseminated throughout the world (Alagappan *et al.*, 2010). The ecological habitat of *V. parahaemolyticus* can be as free-living as bacterioplankton, and associated with seafood surfaces and parasites in the gastrointestinal tract of fish. Many higher organisms such as crustaceans and molluscan shellfish are frequently associated with *V. parahaemolyticus*. Shellfish and other aquatic organisms are thus often served as vehicles for the transmission of this foodborne pathogen. *V. parahaemolyticus* is identified as among the most prevalent foodborne pathogens in China, Japan, Taiwan, and the USA (Su and Liu, 2007; Wang *et al.*, 2017; Ndraha *et al.*, 2020). Infection of *V. parahaemolyticus* can cause gastroenteritis, wound infection or septicaemia. Gastroenteritis resulting from the consumption of *V. parahaemolyticus*-contaminated food may include abdominal cramps, diarrhoea, fever, nausea, tenesmus, and vomiting (Daniels *et al.*, 1998). The syndromes of gastroenteritis are typically mild and self-limited. However, septicaemia can be fatal in some cases. The onset time after ingestion is 4 to 90 hours, with a mean of 17 hours and the median duration of the illness is 2 to 6 days (DePaola and Jones, 2012).

Antibiotics are utilized as antibacterial agents to fight off bacterial infections in humans. They are broadly used as growth-promoting agents as well as feed additives in food animals. Hence, the invention of antibiotics has completely revolutionised medical treatment, and saved millions of lives from bacterial infections. However, unmonitored, extensive use and misuse of antibiotics in medicine, agriculture, and aquaculture over the years has led to the development and global spread of antibiotic-resistant bacteria. Antimicrobial residues present in the environment resultant in selection pressure for antimicrobial-resistant bacteria. Moreover, the ever-increasing emergence of multidrug-resistant (MDR) bacteria which is resistant over two classes of antimicrobial agents poses a serious global health threat.

Bacteriophages, or phages, are bacteria viruses that have the ability to attack and destroy bacterial cells. Bacteriophages constitute a majority of organisms in the biosphere, and it is estimated that the global population of bacteriophages on Earth exceeds 10^{30} (Mushegian, 2020). Prior to the discovery of antibiotics, it was found that bacteriophages could be used as potential therapeutic agents to treat infectious diseases. However, the use of phage therapy had gradually declined at that time, primarily due to arguable phage preparation experiments, poor understanding of phage biology and the discovery of antibiotics. More recently, there has been a surge of interest in the study and application of bacteriophages as bio-controlling agents. In addition, gaining higher knowledge in molecular biology, advances in transmission electron microscopy and global antibiotics resistance threats eventually relighted the potential use of bacteriophages.

Bacteriophages will bind to their bacterial host, replicate with the use of host cellular mechanisms and cause lysis of bacterial cells. Most bacteriophages are highly host specific and usually infect only certain bacteria species or specific subspecies. From a clinical standpoint, bacteriophages are innocuous to health, and do not attack normal gut flora (Ghannad and Mohammadi, 2012). Bacteriophages can be used as natural antimicrobials alongside farm-to-fork production throughout the entire food chain (Jones *et al.*, 2020). In the food industry, bacteriophages can be widely applied along the food chain to enhance food safety. The application of bacteriophages in phage therapy can potentially reduce or even prevent the colonization of bacterial pathogens in farm animals, thus reducing the morbidity and mortality rate in livestock. Moreover, they can be used for the protection of food products at the pre- and postharvest stage as preservatives to extend the expiry date of food products, and as bio-sanitizers to decontaminate raw products and equipment surfaces used in production plants (Połaska and Sokołowska, 2019).

1.2 Problem Statement

Vibrio parahaemolyticus is a Gram-negative halophilic bacterium that naturally occurs in marine environments, and is recognised to be among the leading foodborne pathogens that is frequently isolated from a variety of seafood sources (Wang *et al.*, 2015). Gastroenteritis, which is characterised by abdominal pain, diarrhoea, fever and nausea, is an example of a common syndrome caused by the consumption of food contaminated with *V. parahaemolyticus*. Open wounds in contact with *V. parahaemolyticus* may also result in wound infection and life-threatening septicemia.

Based on the number of vibriosis infections reported to the Cholera and Other Vibrio Illness Surveillance (COVIS) system and Centers for Disease Control and Prevention (CDC) from 1996 to 2014, *V. parahaemolyticus* was identified to be the most common foodborne pathogen which caused 39–51% of *Vibrio* infections as compared to other *Vibrio* species such as *V. vulnificus*, *V. cholerae* (non-O1 and non-O139), *V. alginolyticus*, *V. fluvialis*, *V. mimicus*, and *V. hollisae* (Newton *et al.*, 2012; CDC, 2019). The epidemiology of foodborne disease outbreaks caused by *Vibrio parahaemolyticus* has resulted in high medical costs on a global scale.

In the USA, almost 35,000 individuals domestically acquire foodborne *V. parahaemolyticus* illnesses annually (Scallan *et al.*, 2011). In Japan, it is estimated that 500 to 800 *V. parahaemolyticus* outbreaks affect approximately 10,000 people annually, where sashimi and sushi are responsible for 26% and 23% of outbreaks, respectively (FAO/WHO, 2008). In Malaysia, *V. parahaemolyticus* outbreaks were likely unreported, but several studies reported that the high prevalence rate of *V. parahaemolyticus* was frequently detected in seafood (Letchumanan *et al.*, 2015; Malcolm *et al.*, 2015; Tan *et al.*, 2017).

The incidence of antibiotic-resistant bacteria associated with food products is another serious healthcare issue. The development of foodborne pathogens that are resistant to several antibiotics classes has been reported in numerous studies. For example, Oh *et al.* (2011) reported that 65.1% of 218 *V. parahaemolyticus* strains isolated from farmed fish in Korea showed antimicrobial resistance to multiple antimicrobial agents. Yang *et al.* (2017) also reported that 68.38% of 98 *V. parahaemolyticus* isolates from seafood in China developed resistance to over three antibiotics. The higher prevalence of multidrug-resistant *V. parahaemolyticus* is most likely, due to the widespread use of antibiotics in the past, which allows foodborne pathogens to acquire antimicrobial resistance genes and express drug resistance. Hence, research on other alternative approaches such as the use of bacteriophages is a promising solution that could replace or potentially reduce the usage of antibiotics in food production and processing in the near future.

The application of *V. parahaemolyticus* bacteriophages as biocontrol agents in food products was limited in extent. To our knowledge, no *Vibrio* spp. specific bacteriophage-based products have been developed commercially for usage in aquaculture, biotherapy and food products. Only a few reports were found to demonstrate the usage of *V. parahaemolyticus* bacteriophages in reducing the concentration of *V. parahaemolyticus* in oysters (Jun *et al.*, 2014a; Rong *et al.*, 2014). For example, Jun *et al.* (2014a) reported the use of *V. parahaemolyticus* pVp-1 bacteriophages on live oysters through surface application and bath immersion. Rong *et al.* (2014) reported that the application of VPP1 bacteriophages during the depuration of oysters. The results were obtained with a significant reduction of *V. parahaemolyticus* following bacteriophage treatment. Hence, there is no doubt that *V. parahaemolyticus* bacteriophages can be used as biocontrol agents in the food and aquaculture industry to inhibit the growth of the *V. parahaemolyticus* foodborne pathogen. In addition, the use of bacteriophages could help to reduce the microbial contamination of food, improve food safety, supply safe food, and ensure a high level of public health protection (Principi *et al.*, 2019; Vikram *et al.*, 2021). In the near future, it is believed that a list of novel bacteriophage-based products which target *V. parahaemolyticus* as well as many other different foodborne pathogens and food spoilage bacteria will be available in the market, and will be widely applied in industry.

1.3 Objectives

Based on the hypotheses in the problem statement, the objectives of the present study are as follows:

- a) To determine the prevalence and antibiotic-resistant patterns of *V. parahaemolyticus*
- b) To screen and isolate for host specificity of *V. parahaemolyticus* bacteriophages
- c) To characterize *V. parahaemolyticus* bacteriophage isolates that cause cell lysis in *V. parahaemolyticus*
- d) To perform whole-genome sequencing and bioinformatics analysis of *V. parahaemolyticus* bacteriophages genome
- e) To determine the in-vitro lytic ability of *V. parahaemolyticus* bacteriophages and examine the effectiveness of *V. parahaemolyticus* bacteriophages on biofilm and oyster meat samples

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