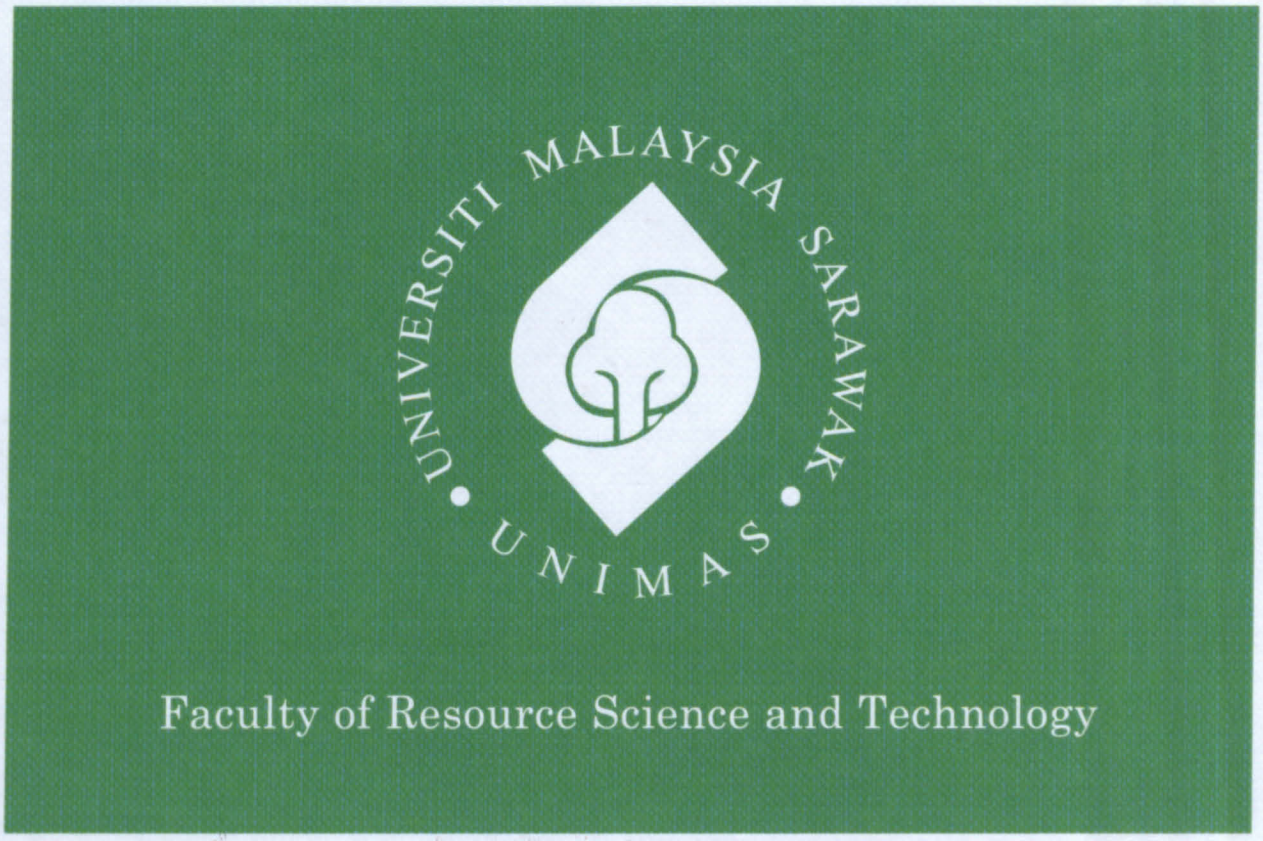

Antibiotic Susceptibility and Detection of *tdh* Gene Among *Vibrio parahaemolyticus* Isolates from An Outbreak Case

Rosdi Anak Kira
(45993)

Bachelor of Science with Honours
(Resource Biotechnology)
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Antibiotic Susceptibility and Detection of *tdh* Gene Among *Vibrio parahaemolyticus* Isolates from An Outbreak Case

Rosdi Anak Kira (45993)

A thesis submitted

in fulfillment for the requirement of Degree of Science

(Resource Biotechnology)

Supervisor: Prof. Dr. Kasing Apun

Co-Supervisor: Dr. Lesley Maurice Bilung

Resource Biotechnology Programme

Department of Molecular Biology

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak

2017

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(Name of the supervisor)

Dr Kasing Apun
Professor
Department of Molecular Biology
Faculty of Resource Science & Technology
UNIVERSITI MALAYSIA SARAWAK
94300 Kota Samaranan

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Antibiotic Susceptibility and Detection of *tdh* Gene Among *Vibrio parahaemolyticus* Isolates from An Outbreak Case

Rosdi Anak Kira

Resource Biotechnology Programme
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Vibrio parahaemolyticus is a significant cause of foodborne outbreaks associated with consumption of raw or under-cooked food. Pathogenic strains of *V.parahaemolyticus* are attributed by virulent factors such as *tdh* and *trh* genes and associated with multiple antibiotics resistant. This study was carried out to detect the presence of virulence gene and the antibiotic susceptibilities of *V.parahaemolyticus* isolates from a foodborne outbreak case in Limbang, Sarawak. The method used in this study includes morphological and biochemical identification as well as specific PCR targeting *tdh* and *blaSHV* genes. A total of 26 isolates from the outbreak case were used in this study. The morphological identification and biochemical test revealed 50% of the overall isolates were identified as *V.parahaemolyticus*. Specific PCR was used to confirm the present of thermostable direct hemolysin (*tdh*) gene and the result showed that all the isolates do not harbour *tdh* gene. MAR index was measured for six antibiotics and the results revealed a high occurrence of antibiotic resistant towards chloramphenicol (61%), ampicillin (54%) and cephalothin (46%). Specific PCR targeting *blaSHV* gene revealed that none of ampicillin resistant isolates carry β-lactam resistant gene. In summary, the results in this study prove that the *V.parahaemolyticus* isolates from the outbreak case were not pathogenic strains. The occurrence of multi-antibiotic resistance strains among the isolates could be an indication of excessive usage of antibiotics in healthcare and agricultural fields.

Key words: *Vibrio parahaemolyticus*, *tdh* gene, *blaSHV* gene, multiple antibiotics resistant

ABSTRAK

Vibrio parahaemolyticus adalah penyebab wabak penyakit makanan yang sering dikaitkan dengan pengambilan makanan mentah dan separuh masak. Strain patogenik V.parahaemolyticus adalah dikaitkan dengan factor virulen seperti gen tdh dan trh dan ketahanan terhadap pelbagai antibiotik. Kajian ini dijalankan bertujuan untuk mengesan kehadiran gen virulen dan kecenderungan antibiotik di kalangan V.parahaemolyticus diambil dari kes wabak di Limbang, Sarawak. Kaedah yang digunakan dalam kajian ini termasuk pengenalpastian morfologi dan biokimia berserta PCR khusus untuk mengesan gen tdh dan blaSHV. Sejumlah 26 V.parahaemolyticus daripada kes wabak tersebut digunakan dalam kajian ini. Pengenalpastian morfologi dan biokimia menunjukkan bahawa 50% daripada keselurahannya telah dikenalpasti sebagai V.parahaemolyticus. PCR khusus telah digunakan untuk mengesan kehadiran gen thermostable direct hemolysin (tdh) dan keputusan menunjukan bahawa kesemua asingan tidak mempunyai gen tdh. Indeks MAR telah diukur untuk enam antibiotik and keputusannya menunjukkan kemunculan tahap ketahanan antibiotik yang tinggi terhadap chloramphenicol (61%), ampicillin (54%) dan cephalothin (46%). PCR khusus untuk mengesan gen blaSHV menunjukkan bahawa kesemua asingan yang tahan trhadap ampicillin tidak memiliki gen ketahanan β-lactam. Kesimpulannya, keputusan daripada kajian ini membuktikan bahawa V.parahaemolyticus yang berasal dari kes wabak tersebut bukanlah strain yang patogenik. Kemunculan strain yang tahan akan pelbagai antibiotik berkemungkinan menunjukkan penggunaan antibiotik yang berlebihan dalam bidang kesihatan dan pertanian.

Kata kunci: V.parahaemolyticus, gen tdh, gen blaSHV, ketahanan terhadap pelbagai antibiotik

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LISTS OF ABBREVIATIONS

%	Percentage
et al.	Et alia
°C	Degree Celsius
spp.	Species
&	And
β	Beta
CLSI	Clinical and Laboratory Standard Institute
LB	Luria-Bertani
TCBS	Thiosulphate-citrate-bile salt sucrose
SIM	Sulfur reduction -Indole -Motility test
MR	Methyl Red
VP	Voges-Proskauer
UV	Ultra Violet
DNA	Deoxyribonucleic acid
PCR	Polymerase Chain Reaction
dNTP	Deoxynucleotide triphosphate
ddH2O	Double-distilled water
MgCl2	Magnesium Chloride
MH	Mueller-Hinton
NaCl	Sodium Chloride
Taq	Thermus aquaticus
Bp	Base pair
µl	microlitre
mL	millilitre
µM	micromolar
mM	millimolar
mg	milligram
g	gram
h	hour/hours
min	minute/minutes
s	seconds
V.	*Vibrio*
tdh	Thermostable Direct Hemolysin
trh	Thermostable Direct Hemolysin-Related

CHAPTER 1: INTRODUCTION

1.1 Introduction

The members of the genus *Vibrio* includes Gram-negative, oxidase-positive with rod or curved rod-shaped facultative anaerobes. According to Feldhusen (2000), 25% of the foodborne diseases are caused by *Vibrio parahaemolyticus* as compared to other *Vibrio* spp. *V.parahaemolyticus* is a gram-negative halophilic bacterium and is a natural inhabitant of marine and estuarine environments around the world (Meador *et al.*, 2007). The gastrointestinal illness caused by *V. parahaemolyticus* is typically accompanied by symptoms including vomiting, diarrhea, headache, nausea, low-grade fever, and abdominal cramps (Alipour *et al.*, 2014). The symptoms due to infection of *V. parahemolyticus* can occur within 24 hours of ingestion (Zulkifli *et al.*, 2009).The pathogenesis of *V.parahaemolyticus* is attributed by the presence of virulence factors namely thermostable direct hemoylsin (*tdh*) and tdh-related hemolysin (*trh*) genes (Honda & Iida, 1993). The *tdh* and *trh* genes are responsible for hemolysis and cytotoxicity activity in the host's cell (Letchumanan *et al.*, 2015).

The outbreaks of foodborne illnesses caused by *V. parahaemolyticus* are generally associated with retail food (Tunung *et al.*, 2010). In Taiwan and Japan, reported outbreaks of food poisoning caused by *V.parahaemolyticus* were due to consumption of two popular Japanese food, sashimi and sushi (Novotny *et al.*, 2004). In Malaysia, a study by Letchumanan *et al.* (2015) had demonstrated a high level of *V.parahaemolyticus* contamination (185 out of 320 isolates) in shrimps purchased from local wet market.

Vibrio spp. is usually susceptible to most clinically used antibiotics (Letchumanan *et al.*, 2015). However, over the years, antibiotic resistance strains has emerged in the environment due the excessive use of antibiotics and other chemotherapeutic agents in

human, agriculture, and aquaculture fields (Cabello *et al.*,2013). Antibiotic resistant *V. parahaemolyticus* strains has been isolated and detected from shrimps in Malaysia (Letchumanan *et al.*, 2015). This phenomenon has raised concern due to the increase number of resistance pathogenic *V.parahaemolyticus* strains in the environment toward clinically used antibiotics.

Currently, the standard method for the detection and identification of *V. parahaemolyticus* is by using selective medium such as Thiosulfate-citrate-bile salts sucrose (TCBS) agar (Vincent *et al.*, 2015) as well as biochemical identification. Biochemical identifications such Voges-proskauer test, salt tolerance test, sulfide test, indole test, motility test and oxidase test are indicative of *V.parahaemolyticus* (Khan *et al.*, 2007). Molecular technique such as polymerase chain reaction (PCR) can be used to detect virulent gene such as *tdh* gene using DNA primers that are specific for this genes (Tada *et al.*, 1992). The presence of thermostable direct hemolysin gene (*tdh*) and thermostable direct hemolysin-related gene (*trh*) are responsible for the virulence and pathogenicity of *V. parahaemolyticus* (DePaola *et al.* 2003; Robert-Pillot *et al.* 2004). PCR assays are useful for the detection of *tdh* and *trh* genes among *V. parahaemolyticus* strains. Another molecular technique that can be applied in the identification of *V.parahaemolyticus* is the detection of *toxR* gene. *toxR* gene is present in all *V. parahaemolyticus*, either pathogenic or non-pathogenic (Dileep *et al.*, 2003; Sujeewa *et al.*, 2009) . However, the presence of *toxR* gene does not indicate that *V.parahaemolyticus* is virulent or pathogenic.

Recently, a foodborne outbreak had occurred in Limbang District of northern Sarawak. The symptoms reported includes diarrheal, vomiting and abdominal pain which is similar to the symptom of infection caused by *Vibrio* spp. (Broberg *et al.*, 2011). The outbreak was reported to originate from contaminated food purchased from local market. The presence of pathogenic *Vibrio parahemolyticus* strains in food is a major concern

because *V.parahaemolyticus* can contaminate raw and improperly cooked food. Thus, this study aims to identify *V.parahaemolyticus* in the food samples from the outbreak case and determine the antibiotic susceptibility of this bacterium.

1.2 Objectives

The objectives of this study are:

1) To identify *V.parahaemolyticus* isolates using morphological and biochemical tests
2) To confirm the pathogenicity of the isolates by using specific PCR targeting *tdh* gene
3) To determine the antibiotic susceptibility and detect the antibiotic resistant gene of the *V.parahaemolyticus* isolates

1.3 Hypothesis

The hypothesis of this study is the presence of pathogenic and multiple antibiotic resistant *V.parahaemolyticus* strains exist in food samples from the outbreak case.

CHAPTER 2: LITERATURE REVIEW

2.1 *Vibrio parahaemolyticus*

Vibrio parahaemolyticus belongs to the genus *Vibrio*, one of the five genera in the *Vibriocanaceae* family. *V.parahaemolyticus* is a Gram-negative bacteria and it is generally oxidase and catalase positive. It is nonsporulating, motile and has curved rod shape. It grows in a medium that contains glucose with no gas production. *V.parahaemolyticus* is able to grow under optimum temperature of 30-37°C and able to tolerate 3-5% NaCl. The optimum pH for growth is 7.8 to 8.6 within a range of 4.8 to 11 and grows optimally under aerobic conditions (Oliver *et al.*, 2005).

V.parahaemolyticus is an important human pathogen that is widely distributed in estuarine and marine environments and often associated with gastroenteritis following on the consumption of contaminated raw or insufficiently cooked seafood (Paydar *et al.*, 2013). In Southeast Asian countries such as Malaysia, there is a high probability of *V. parahaemolyticus* outbreaks, due to the ambient optimum temperature and appropriate climate for the growth of this species (Paydar *et al.*, 2013). Virulent *V. parahaemolyticus* isolates in retail food from local market have been reported in Malaysia (Letchumanan *et al.*, 2015). Thus, the prevalence of pathogenic *V. parahaemolyticus* in retail food is of public health concern.

2.2 *Vibrio parahaemolyticus* reported in Foodborne Outbreaks

Many *Vibrio* species are pathogenic to humans and have been associated with foodborne diseases .According to Janda *et al.* (1988), the number of *Vibrio* spp. classified as pathogenic strains is at least 11, which includes *Vibrio cholerae* as the main cause of diarrhea and *V. parahaemolyticus* as the cause of foodborne gastroenteritis (Özer *et al.*, 2008). According to Feldhusen (2000), 25% of the foodborne diseases are caused by *Vibrio*

parahaemolyticus as compared to other *Vibrio* spp. and it is considered as a major cause for these illnesses (Johnson *et al.* 2009). Over the last few decades, there have been numerous reports of *V. parahaemolyticus* outbreaks associated with consumption of retail food.

In the United States, an outbreak during July-September 1998 which involves residents of Connecticut, New Jersey and New York were reported due to the consumption of oysters and clams contaminated with *V.parahaemolyticus* (Velazquez-Roman *et al.*, 2013). In Taiwan and Japan, reported outbreaks of food poisoning caused by *V.parahaemolyticus* were due to consumption of sashimi and sushi, two popular Japanese food (Novotny *et al.*, 2004). *V.parahaemoluticus* is naturally distributed in the marine coastal region of Malaysia due to the optimum temperature (30-37° C) of the seawater. This natural inhabitant contributes to the transmission of *V.parahaemolyticus* in seafood and causes gastroenteritis following on the consumption of contaminated seafood products. A study by Letchumanan *et al.* (2015) had demonstrated a high level of *V.parahaemolyticus* contamination (185 out of 320 isolates) in shrimps collected from local wet market. Another study in Kuching, Sarawak showed a total of 15 fish samples of 3 different fish types (*Kembung, Bawal and Sangeh*) were positive of *tl* gene, which indicates that they were contaminated by *V.parahaemolyticus* (Vincent *et al.*, 2015).

2.3 Isolation & Identification of *Vibrio parahaemolyticus*

Food-processing often cause injury to *V. parahaemolyticus* cells and injured cells may not be recovered by plating in selective media. Therefore, enrichment should be done prior to plating on selective media. Enrichment of *V.parahemolyticus* involves special media to ensure the optimum recovery of both injured and healthy cells (Wong, 2003). Alkaline Peptone Water (APW) or LB broth supplemented with 3% NaCl is usually used as enrichment medium for the isolation of *Vibrio parahaemolyticus* as this bacterium is a halophilic bacterium.

Thiosulphate Citrate Bile Salt Sucrose agar (TCBS) and CHROMagar Vibrio are two most commonly used medium in the isolation and detection of *V.parahaemolyticus* (Kaysner & Depaola, 2004). Most *Vibrio* species have a considerable growth on TCBS while the growth of most non-*vibrios* spp. is inhibited on this medium. When a sample containing *V.parahaemolyticus* is cultured on TCBS agar, green or blue-green colonies will appear on the agar (Zulkifli *et al.*, 2009). According to Hara-Kudo *et al.* (2001), *V. parahaemolyticus* colonies on TCBS are very difficult to distinguish visually from the colonies of other bacteria, because they might be covered by a yellow color produced by sucrose-fermenting bacteria.

Another medium that can be used in the isolation and detection of *V.parahaemolyticus* is CHROMagar™ Vibrio. CHROMagar™ Vibrio is more accurate and specific than TCBS agar because it uses chromogenic technology that resulted in colonies that can be distinguished on the basis of color development (Di Pinto *et al.*, 2011). The colony colors that appear on TCBS agar and CHROMagar™ Vibrio for *V.parahaemolyticus* are presented in Table 2.3 and Figure 2.3.

Table 2.3: The colony colors of *V.parahaemolyticus* on (TCBS) and CHROMagar.

Organism	Colony color on TCBS agar	Colony color on CHROMagar™ Vibrio
Vibrio parahaemolyticus	Green	Mauve

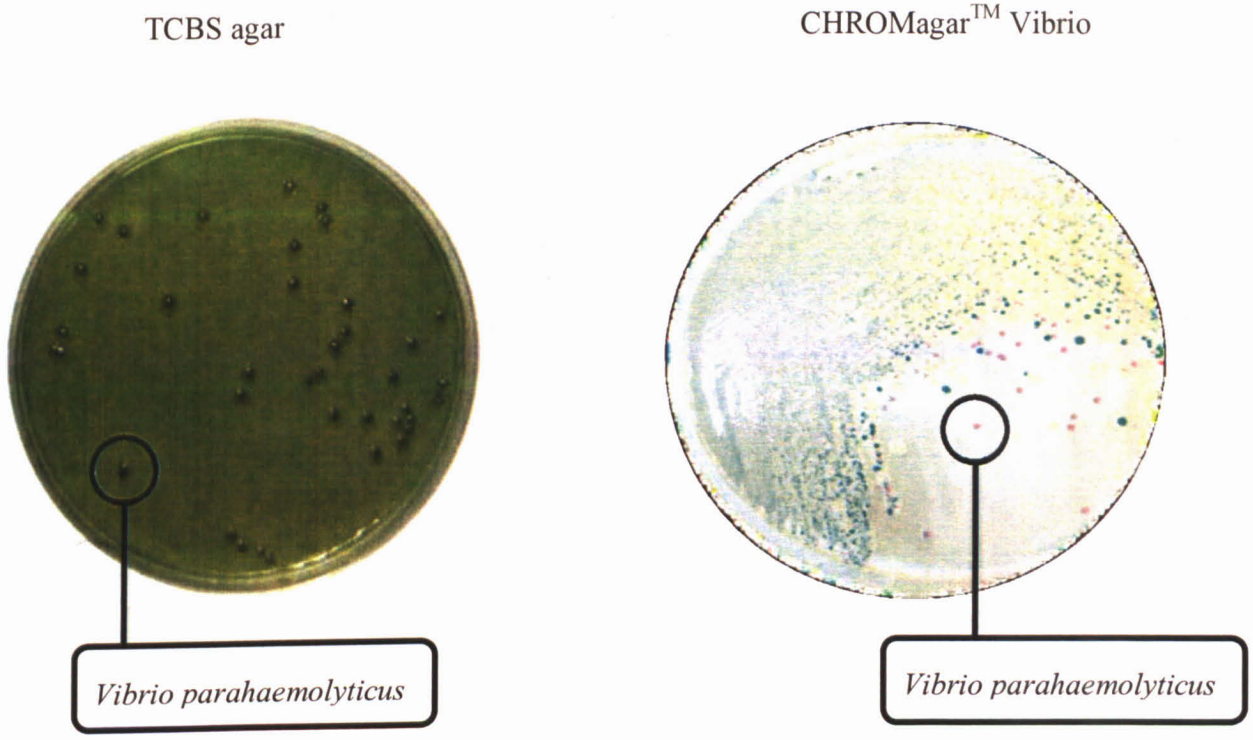

Figure 2.3: The colony colors of *V.parahaemolyticus* on (TCBS) and CHROMagar (Source: E&O Laboratories Ltd , www.eolabs.com)

2.4 Morphological Characteristics of *Vibrio parahaemolyticus*

V. parahaemolyticus is a Gram-negative, halophilic, small curve-rod shaped bacterium and can exist as either a swimmer cell with single polar flagella or swarmer cell with lateral flagella (McCarter, 1999; Su & Liu, 2007). The increase in viscosity of the growth environment will induces the conversion from swimmer to swarmer cell type (Broberg *et al.*, 2011). *V.parahaemolyticus* is found to inhabit brackish and estuarine waters, and requires salt for survival and growth.

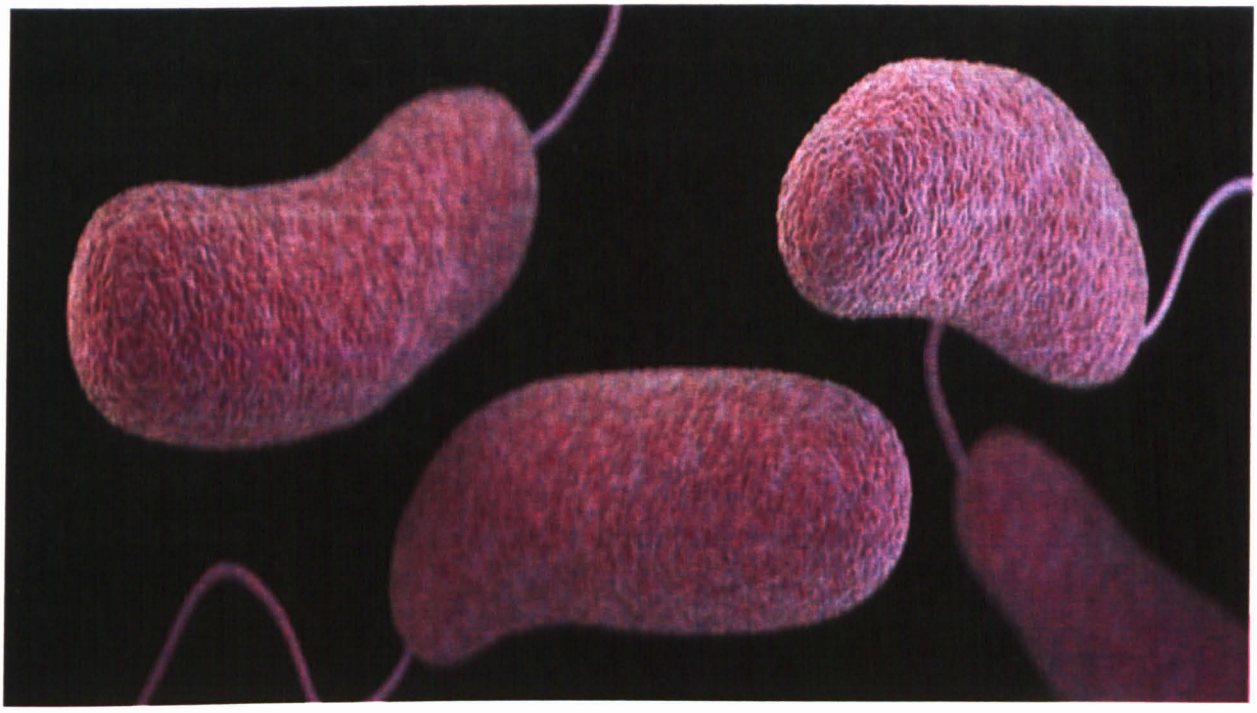

Figure 2.4: The morphology of *Vibrio parahaemolyticus* showing curved-rod with flagella (Source: www.cdc.gov/vibrio)

2.5 Biochemical Characteristics of *Vibrio parahaemolyticus*

2.5.1 Voges-Proskauer (VP) tests

The purpose of Voges-Proskauer (VP) test is to identify bacteria that are capable of 2, 3-butanediol fermentation. The medium used in this test is MRVP medium which contains glucose, peptone and phosphate buffer. Acid end products produced by some organisms are not stable and can be further metabolize into a more neutral end product such as 2,3-butanediol. During the test, culture was observed after 15 minutes for the formation of pink complex that indicates positive result for 2, 3-butanediol fermentation. According to Thakur *et al.* (2003), *V. parahaemolyticus* gave negative result for this test.

2.5.2 Sulfur reduction – Indole – Motility (SIM) test

2.5.2.1 Sulfur Reduction

The sulfur reduction test is conducted to differentiate enteric organisms. SIM medium is used as the medium for SIM test. If an organism is capable of reducing sulfur into hydrogen sulfide, the hydrogen sulfide produced will combine with the iron in the medium and will form a black precipitate (Difco, 1984). The black precipitate formed is ferric sulfide (FE_2S_3). The formation of black precipitate indicates a positive result for sulfur reduction test. *V.parahaemolyticus* gave negative result for sulfur reduction test.

2.5.2.2 Production of Indole

Indole production test is conducted using SIM medium. The capability of a microorganism to break down the amino-acid tryptophan will leads to the production of indole (MacFaddin, 1980). This distinctive characteristic can be used for the identification and classification of bacteria. The presence of indole is further detected by the addition of an indole reagent to the SIM medium. Upon the addition of indole reagent, a pink coloration will be produced in the reagent if the microorganism is able to break down tryptophan and produce indole (MacFaddin, 1980). *V.parahaemolyticus* gave positive result to indole test.

2.5.2.3 Motility test

Motility test is conducted by creating a stab line using straight wire on SIM medium to determine whether the organism is motile or non-motile. Motile organisms use their flagella to migrate away from the stab line and leads to the production of turbidity throughout the medium. On the other hand, non-motile organisms will grow along the stab line and they are not able to migrate away from the stab line. This will leave the surrounding medium clear (Difco, 1984). The observation of the spreading growth is very helpful to

detect the motility of the tested organisms. *V.parahaemolyticus* gave positive result to motility test.

2.5.3 Oxidase test

Oxidase test is another differential test that can be performed on all gram-negative bacteria that are to be identified. The test strip contains N, N, N`, N`-tetra-methyl-p-phenylenediamine dihydrochloride which acts as an artificial electron acceptor for the enzyme oxidase. The enzyme cytochrome oxidase participate electron transport chain. Organisms which produced cytochrome oxidase can transfer electrons from a donor molecule to oxygen (Isenberg, 2004). *V.parahaemolyticus* gave positive result to oxidase test.

2.5.4 Salt Tolerance Test

V.parahaemolyticus is a halophilic bacterium and has a physiological requirement for salt (NaCl). They inhabit marine, brackish, and estuarine waters, where fluctuations in salinity pose a constant challenge to their adaptive response (Whitaker *et al.*, 2010). *V. parahaemolyticus* is able to grow in enrichment media such as LB broth supplemented with 3, 6 and 8% salt content. Palasuntheram (1981) reported that *V. parahaemolyticus* requires a minimum of 0.086 M (0.5%) NaCl for growth.

2.6 Virulence properties of *Vibrio parahaemolyticus*

The pathogenicity of *V. parahaemolyticus* is associated with the presence of virulence factors which includes; *tdh* gene or the *trh* gene (DePaola *et al.*, 2003; Robert-Pillot *et al.*, 2004; Roque *et al.*, 2009). *tdh* gene code for thermostable direct hemolysin while *trh* codes for *tdh*-related hemolysin (Honda & Iida *et al.*, 1993). *V. parahaemolyticus* strains that produce virulence factors are considered to be pathogenic and can cause acute

gastroenteritis (Robert-Pilot *et al.*, 2004). According to Lee & Pan (1993), PCR assays have been developed using oligonucleotide primers designed to target and amplify the genes for the detection of *tdh* and *trh* genes in order to distinguish the pathogenicity of *V.parahaemolyticus* strains. Paydar *et al.* (2013) reported a strong correlation between the presence of haemolysin gene and pathogenicity of *V.parahaemolyticus*. In the study, the presence of *trh* gene in 12% (6/50) and *tdh* gene in 4% (2/50) of seafood isolates were observed. This indicates a high risk of contamination in the seafood isolates. However, environmental *V.parahaemolyticus* strains are reported to be non-pathogenic due to the absence of *tdh* gene; however a small percentage of environmental strains could harbor this virulence factor (Velazquez-Roman *et al.*, 2012; Haley *et al.*, 2014).

2.7 Antibiotic Resistance in *Vibrio* spp.

Antibiotic resistance has become a major medical and public health problem as it is associated with disease management (Okoh & Igbinosa, 2010). According to Letchumanan *et al.* (2015), *Vibrio* spp. is usually highly susceptible to most clinically used antibiotics. Some clinically used antibiotics for the treatment of *Vibrio* spp. infections includes: cephalothin, cefuroxime, cefotaxime, ceftazidime, tetracycline, doxycycline and fluoroquinolone (Al-Othrubi *et al.*, 2014). However, antibiotic resistance strains has emerged in the environment due the excessive use of antibiotics and other chemotherapeutic agents in human, agriculture, and aquaculture fields (Cabello *et al.*,2013). Kitaoka *et al.* (2011) reported that antibiotic resistance in *Vibrio* spp. can develop through efflux pumps, spontaneous chromosomal mutation, conjugative plasmids, trimethoprim sulfamethoxazole (SXT) elements and integrons, and integrating conjugative elements (ICEs).

2.7.1 Ampicillin Resistance Pattern in *Vibrio parahaemolyticus*

Ampicillin is an antibiotic under the class beta-lactams antibioties together with penicillin and amoxicillin. According to Shaikh *et al.* (2015), beta-lactam antibioties are the most common drugs in the treatment of bacterial infections. Beta-lactam antibiotics have been widely used since 1980 for the treatment of infections caused by gram-negative bacteria. Bradford (2001) reported that resistance against these antibiotic groups had occurred worldwide. In United States, Joseph *et al.* (1978) reported that over 90% of 160 *V. parahaemolyticus* isolates were resistant to ampicillin and exhibited β-lactamase activity. Another study by Han *et al.* (2007), have identified 95 ampicillin-resistant *V.parahaemolyticus* isolated from Louisiana Gulf and retail raw oysters. Han *et al.* (2007), suggests that ampicillin has a potentially low efficiency in empirical treatment of *V. parahaemolyticus* infections. Letchumanan *et al.* (2015) have also reported 82% of the *V.parahaemolyticus* isolates from retailed shrimps were resistant to ampicillin. According to Li *et al.* (2015), the intrinsic ampicillin resistance in *V.parahaemolyticus* is contributed by β-lactamase. The gene encoding for β-lactamase is an intrinsic gene in *V. parahaemolyticus* and is more conserved in this species compared to other gene markers (Li *et al.*, 2015).

CHAPTER 3: MATERIALS & METHODS

3.1 Materials

The lists of materials used in this study are listed in the Appendix A and the flow chart for the outline in this study are listed in Appendix B.

3.2 *Vibrio parahaemolyticus* isolates

A total of 26 isolates from an outbreak case in Limbang, Sarawak were used in this project. The isolates were isolated by previous researcher on TCBS agar. The origin of the isolates is listed in the Table 3.1.

Table 3.1: Types of food samples from outbreak case

No	Isolates	Food samples
1	VC01A	Stall No. 26
2	VC01B	Chicken meat
3	VC01C	Squid
4	VC02A	Stall No. 21
5	VC02B	Squid
6	VC02C	Clam
7	VC03A	Stall No. 19
8	VC03B	Hoven's carp gills
9	VC04A	Stall No. 21
10	VC04B	Mackerel gills
11	VC04C	Clam
12	VC05A	Stall No. 15
13	VC05B	Chicken Wings