ISOLATION OF *VIBRIO PARAHAEMOLYTICUS* FROM RAW VEGETABLES IN LOCAL MARKETS AT KUCHING AND SAMARAHAN

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LIST OF ABBREVIATIONS

AFP	American Free Press
AGS	Arginine Glucose Slant
APW	Alkaline Peptone Water
BAM	Bacteriological Analytical Manual
°C	Degree Celcius
CDC	Centers for Disease Control
CFU	Colony Forming Unit
CIFOR	Council to Improve Foodborne Outbreak Response
Cm	Centimetre
CV	CHROMagar *Vibrio*
DNA	Deoxyribose Nucleic Acid
FDA	Food and Drug Administration
g	Gram
H_2S	Hydrogen sulfide
HCl	Hydrogen chloride
ml	Milliliter
mm	Milimeter
NaCl	Sodium Chloride
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
SIM	Sulfide, Indole, Motility
TCBS	Thiosulfate-Citrate-Bile Salt

TDH	Thermostable Direct Hemolysin
TLH	Thermolabile Hemolysin
TRH	TDH-related Hemolysin
TSI	Triple Sugar Ion
T_1N_o	Tryptone Broth with no NaCl
T_1N_1	Tryptone Broth with 1% NaCl
T_1N_3	Tryptone Broth with 3% NaCl
T_1N_6	Tryptone Broth with 6% NaCl
T_1N_8	Tryptone Broth with 8% NaCl
URE	Urease

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Isolation of *Vibrio parahaemolyticus* from Raw Vegetables in Local Markets at Kuching and Samarahan

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ABSTRACT

Currently, *Vibrio parahaemolyticus* are considered as one of the main cause for bacterial gastroenteristis worldwide. Various researches had been conducted and stated that the outbreaks involving raw fruits and vegetables occurred besides those of animal and seafood originated products. This study aims to detect and identify the presence of *Vibrio parahaemolyticus* in raw vegetables found in local wet markets, Kuching, Sarawak. In this study, samplings were conducted from three different stalls found from three different wet markets (Stutong, 7th Mile and Samarindah) respectively. Three types of vegetables were bought and sampled, namely cucumber, tomato and water spinach. Enrichment was done in alkaline peptone water (APW). Thiosulfate-citrate-bile salt (TCBS) agar and CHROMagar *Vibrio* was applied in the methodology in selection of *Vibrio parahaemolyticus.*The bacteria appeared as green colonies on TCBS agar and purple on CHROMagar *Vibrio*. The presumptive colonies were then be streaked on nutrient agar and later stored as stock culture. Biochemical tests were performed in order to identify the isolated colonies. *Vibrio parahaemolyticus* was found in raw vegetables samples obtained from local wet markets but in little quantity (20 occurrences from 270 total samples or 10.7% isolated from TCBS and 9 occurrences from 270 total samples or 3.3% isolated from CHROMagar *Vibrio* due to the poor hygiene level in the markets as well as improper handling of vegetables and cross-contamination with other food sold in the market.

Keywords: *Vibrio parahaemolyticus*; gastroenteritis; raw vegetables; TCBS agar; CHROMagar *Vibrio*

ABSTRAK

Vibrio parahaemolyticus merupakan salah satu faktor dalam gastroenteritis bakteria di seluruh dunia kini. Pelbagai aktiviti kajian telah dijalankan dan menyatakan bahawa pencetusan yang melibatkan pemakanan buah-buahan dan sayur-sayuran mentah berlaku selain daripada produk haiwan dan makanan laut. Kajian ini bertujuan untuk mengesan dan mengenal pasti kehadiran Vibrio parahaemolyticus dalam sayur-sayuran mentah yang dijual di pasar tempatan, Kuching, Sarawak. Dalam kajian ini, pensampelan dijalankan untuk tiga gerai yang berlainan yang masing-masingnya berada dalam tiga pasar tempatan (Stutong, Batu 7 dan Samarindah). Tiga jenis sayur-sayuran yang dibeli dan disampel, iaitu timun, tomato dan kangkung. Pengkayaan koloni bakteria dijalankan dalam air pepton beralkali (APW). Agar Thiosulfat-citrat-garam hempedu (TCBS) dan CHROMagar Vibrio digunakan dalam methodologi untuk pemilihan Vibrio parahaemolyticus. Bakteria tersebut berwarna hijau atas agar TCBS dan ungu atas CHROMagar Vibrio. Koloni andaian kemudiaannya dikulturkan atas agar nutrien dan sejurusnya disimpan sebagai kultur simpanan. Ujian biokimia dijalankan untuk mengenal pasti koloni yang dipilih. Vibrio parahaemolyticus dijumpai dalam sampel sayur-sayuran yang dibeli dari pasar tempatan tetapi dalam kuantiti yang sedikit (20 kes daripada 270 jumlah sampel ataupun 10.7% pilihan daripada TCBS dan 9 kes daripada 270 jumlah sampel ataupun 3.3% pilihan daripada CHROMagar Vibrio kerana keadaan yang kurang bersih dalam pasar, pengendalian sayur-sayuran yang tidak betul dan kontaminasi campuran dengan bahan makanan lain dalam pasar tempatan.

Kata kunci: Vibrio parahaemolyticus; gastroenteritis; sayur-sayuran segar; agar TCBS; CHROMagar Vibrio

CHAPTER 1

INTRODUCTION

1.1 Introduction

According to Centers for Disease Control and Prevention (CDC), foodborne disease happens when one or two similar illness occurs after the uptake of a common food. Foodborne illness can happen when the food is contaminated with pathogenic bacteria due to improper preparing and storage of food or poor hygiene practice of the food processors that can be harmful upon consumption (Chang and Chen, 2003). Contaminated or hazardous food has moisture, temperature, pH and nutrients that promote bacterial growth and multiplication. Handling of raw food and serving of meals throughout the day, preparation for different diets and delay in serving can bring problem to food serving venues (Benett *et al.*, 2007). Direct infection or secondary transmission through contact can lead to the transmission of disease.

V.parahaemolyticus had been recognized as one of the cause for bacterial gastroenteritis. *V.parahaemolyticus* is a gram negative cells that require salt to survive (Wong, 2003). This *vibrio* can lead to foodborne illness such as gastroenteritis and traveler's diarrhea in human if raw or undercooked food is consumed or contact with aquatic environment (Khan *et al.*, 2002). Japan was the first country to be infected by *vibrio* through food uptake in the early 1950s (Daniels *et al.*, 2000). Matsumoto *et al.* (2000) and Wong (2003) mentioned that illness caused by *vibrio* had affected North America, India, Southest Asia and Japan. Pandemic strains of *V.parahaemolyticus* have been isolated in most diarrhea cases reported in many Asian countries, for example India, Japan, Bangladesh, Taiwan, Thailand, Vietnam, and

also in United States (Nandi *et al*., 1999; Matsumoto *et al*., 2000; Nishibuchi *et al*., 2000; Wong *et al*., 2000; Bhuiyan *et al*., 2002). Serovar O3:K6 are mostly found in epidemic isolates, but the other serovars can also be a part of pandemic strains (Laohapretthisan *et al*., 2003).

Foodborne illness caused by *V.parahaemolyticus* is usually sporadic type. The illness mainly occurs through consumption of raw or improperly cooked seafood, especially during the warmer seasons (Sakazaki *et al.*, 2006). Clinical symptoms for *V.parahaemolyticus* infections are diarrhea, abdominal cramps, vomiting, headache, nausea, fever and chills with incubation time of 4 to 96 hours (Vuddhakul *et al.*, 2006). Most of the illness happens during warm season (Daniels *et al.*, 2000). During winter, *V.parahaemolyticus* will survive in sediments and hardly be detected (Sakazaki *et al.*, 2006). In unfavorable condition, *V.parahaemolyticus* can turn into viable but non-culturable state (Bates *et al*., 2000).

Bean (1990) and Chang and Chen (2003) stated that fruits and vegetables can act as vehicles to transmit food borne diseases apart from other food products in Taiwan and Western countries. According to Linton *et al.* (2006), many foodborne outbreaks related to fresh vegetables, fruits and fruit juices had been reported in the past ten years. An example of the recent outbreak related to *vibrio* is in Vietnam where there were 130 people infected due to the contaminated food and water. This had drawn the public attention on the poor hygiene condition in wet markers and restaurants (AFP, 2008). Another latest outbreak involving *Vibrio parahaemolyticus* was reported in Singapore in the April 2009. The outbreaks at 'Rojak Geylang Serai' Stall were believed to be cross contamination of rojak and raw seafood ingredients that can be contaminated by *Vibrio parahaemolyticus*. In this outbreak, 154 cases

were reported where 2 deaths were reported and 84 hospitalizations (Channel NewsAsia, 2009).

This worries local people since vegetables are part of their daily diet. Fresh or minimally processed food, such as fruits and vegetables, for example, melon, carrots, alfalfa sprouts and apple ciders have increasing demands among the public. Fresh products have gained their popularity among consumers as they are convenient and ready to be eaten. These products are mostly found in wet markets, supermarkets and convenience stores. However, the degree of hygiene and handling conditions are doubted. The increase of consumption of these fresh products can result in the elevation of food borne disease outbreaks (Labbe and Shetty, 1998). Berrang *et al.* (1989) stated that some of the pathogens can grow in refrigerated food with little or no change in sensory and taste. Therefore, isolation and detection of *Vibrio parahaemolyticus* will aid in preventing the spreading of disease outbreaks. The investigation of foodborne outbreaks can help in determining short term control of the transmission as well as long term prevention strategies in the near future (CDC, 2005).

In this study, *Vibrio parahaemolyticus* was isolated and identified by conventional method. The *vibrio* was plated on thiosulfate-citrate-bile salts (TCBS) and CHROMagar *Vibrio*. The suspected colonies were confirmed with biochemical tests in the laboratories such as oxidase test, arginine glucose slant, arginine dihydrolase test, salt tolerance test, motility test, and urease test (FDA, 2004). *V.parahaemolyticus* form green colonies on TCBS agar since it cannot ferment sucrose (Sakazaki *et al.*, 2006) and they appear as purple colonies on CHROMagar *Vibrio* (Hara-Kudo *et al.*, 2001). CHROMagar *Vibrio* allows better detection of

V.parahaemolyticus since it is able to differentiate the species by color and it is more efficient than classical TCBS agar.

1.2 Objectives

The objectives of this study are:

1. To isolate and detect the presence of *Vibrio parahaemolyticus* in raw vegetables found in local market (Kuching and Samarahan) by using selective agar medium namely TCBS and CHROMagar *Vibrio*.
2. To identify *Vibrio parahaemolyticus* isolates from raw vegetables through a series of conventional methods (biochemical tests).
3. To compare the probability of *Vibrio parahaemolyticus* occurrence between :
    1. Different types of raw vegetables
    2. Different time or season
    3. Different stalls in the wet markets

CHAPTER 2

LITERATURE REVIEW

2.1 Characteristics of *Vibrio parahaemolyticus*

Vibrio parahaemolyticus is one of the 12 *Vibrios* that are known to cause foodborne disease in human (Texas Department of Health, 1999). Most of the *Vibrio* members are gram negative, with straight rod shape or with single curve (Doyle, 1989). Out of the members, *V.cholerae*, *V.parahaemolyticus* and *V.vulnificus* are famous as foodborne pathogens (Hartantyo *et al.*, 2006). *V.prarahaemolyticus* is found mostly in fresh water during summer. Due to this, the occurrence happens every year in seasons, mostly during warmer days of the year (Sakazaki *et al.*, 2006). They mainly live in coastal waters at temperate regions (Baross and Liston, 1973).

According to Cavallo and Stabili (2002), *Vibrio parahaemolyticus* is usually involved in foodborne related disease that are linked to uptake of raw or undercooked seafood, poor post harvest storage conditions or improper handling of food during preparation. *V.parahaemolyticus* is an important bacterial pathogens for causing gastroenteritis (Mead *et al.*, 1999) and also foodborne diseases in some Asian countries (Joseph *et al.*, 1982). However, the outbreaks reported in Europe are rare (Scientific Committee on Veterinary Measures Relating to Public Health, 2001). The strains of *V.parahaemolyticus* can be recognized by using O:K serotyping scheme (Center for Food Safety and Applied Nutrition, 2005). It is a useful way to identify the species present and to investigate on its epidemiology.

V.parahaemolyticus is moderately halophilic, motile, fermentative bacteria (Kandhasamy *et al.*, 2008). They are non-spore forming rods with 0.5 to 0.8 μM in width and 1.4 to 2.6 μM in length (Drake, 2008). *V.parahaemolyticus* are motile as most of them have single polar flagellum when they are grown in medium while peritrichorus flagellum can be found in young culture on the surface of solid medium (Sakazaki *et al*., 2006). *Vibrio* members are oxidase and catalase positive, but they have the ability to ferment glucose without production of gas (U.S. Food and Drug Administration, 2004). They are able to tolerate with salinity of 1 to 8% sodium chloride but they cannot survive without salt (Sakazaki *et al*., 2006). They survive the best when the media has around 2-3% NaCl (Doyle, 1989).

V.parahaemolyticus can undergo respiratory and fermentative metabolism. This *vibrio* is chemo-organotroph which can grow in medium with D-glucose and NH_4Cl. It has the ability to ferment D-glucose but produce no gas (Doyle, 1989). It retains the ability to increase its population when the temperature lies between 20°C and 42°C (Miwa *et al.*, 2005). *V.parahaemolyticus* survives well at temperature range of 10 to 44°C, but not at 4°C (Sakazaki *et al*., 2006). Miwatani and Takeda (1976) and Oliver and Kaper (2001) stated that *V.parahaemolyticus* can grow very fast as it has the generation time of 9 minutes in the medium and 12 minutes in the seafood.

Environmental stresses such as starvation, cold temperature and suboptimal pH can induce viable but non-culturable state (Gauthier, 2000). The bacteria are still alive as their metabolisms are still on going but they do not form colonies on nutrient media (Gauthier, 2000; Oliver, 2000). According to Chai and Jiang (1996) and Wand and Wong (2004),

V.parahaemolyticus can also enter viable but non-culturable state. They can be revived within 3 days after the temperature are optimal for their growth.

2.2 Antigenic characteristics of *Vibrio parahaemolyticus*

V.parahaemolyticus assembles three antigens, namely thermostable somatic O antigen, thermolabile capsular K antigen and flagellar H antigen. All *V.parahaemolyticus* have the common H antigen (Drake, 2008). K antigen is a type of polysaccharide with many sugar components such as pentoses, hexoses or hexosamines. K antigen is free from the bacterial cell surface when they are exposed to 100°C for 1 to 2 hours (Doyle, 1989). By this, somatic O antigen is released. O antigen is a type of lipopolysaccharide with glucose, galactose, glucosamine, heptose, phosphorus, nitrogen compounds and fatty acid ester (Torii *et al*., 1969). Therefore, serotyping of *V.parahaemolyticus* can be conducted by using antibodies specific to O and K antigens (Drake, 2008). Until today, there are 12 O antigen and over 70 types of K antigens discovered while many other strains still remain ungrouped (FDA BAM, 2001).

It is believed that some virulence factors take part in the pathogenicity of *V.parahaemolyticus.* Some of the examples are hemolysin, adherence factors, enzymes, products of TDH, TRH and URE genes (Drake, 2008). In the past, the pathogenicity of *V.parahaemolyticus* is linked to Kanagawa phenomenon, which is beta-hemolysis on Wagatsuma agar. Later, it was discovered that Kanagawa reaction is influenced by production of TDH protein. It was called TDH proteins because inactivation by heat and its hemolytic activity.

According to Centre of Disease Control (2005), strains that are pathogenic are those that produce thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH). Although TDH and TRH genes are related to pathogenicity in *V.parahaemolyticus,* the strains with these genes are rarely found in aquatic environment (DePaola *et al.,* 2003; Kaufman *et al.,* 2003). Strains of *V.parahaemolyticus* from patients with gastroenteritis were hemolytic but mostly non-hemolytic strains were found on a modified agar for those bacteria isolated from sea organisms (Kato *et al*., 1965). Kanagawa reaction can occur when there is hemolytic reaction due to the presence of thermostable extracellular substance called thermostable direct hemolysin (TDH). TDH gene is believed to be involved in pathogenicity of the bacteria. Sakazaki *et al*. (1986) and Kaper and Nishibuchi (1995) reported most of the strains isolated from patients infected were Kanagawa positive but only a little is positive for the strains found in environment. According to Honda *et al*. (1992), TDH is a pore-forming toxin that contributes to hemolysis, cytotoxicity, enterotoxicity, and cardiotoxicity (Jong and Young, 2001). TDH is active against erythrocytes of dogs, mice, rats, and human, weak against erythrocytes of rabbit and sheep and inactive against horse erythrocytes.

Kanagawa negative outbreaks can also happen when TDH-related hemolysin (TRH) produced by those Kanagawa negative strains (Honda *et al*., 1988). Shirai *et al*. (1990) and Kishishita *et al*. (1992) discovered that TRH-positive *vibrio* have the ability to cause gastroenteritis too. Apart from this, some of the characteristics that participate in the pathogenicity of *V.parahaemolyticus* are lipase, gelatinase and hemolysin (enzymatic), adhensiveness, cytotoxicity and enterotoxicity (biological) and enteropathogenic activities of the strains (Baffone *et al*, 2001).

2.3 Epidemiology

After the first cases concerning *V.parahaemolyticus* infection reported by Fujino *et al*. (1953), there are other reports identified in Japan (Aiiso and Matsuno, 1961; Sakai *et al*., 1970; Takikawa, 1958). *V.parahaemolyticus* contributes to 70% of the foodborne illeness cases reported in Japan during the early 1960s (Sakai *et al*., 1970; Sakazaki, 1979). Other countries affected are such as North America, Central America, South America, Africa, Asia, Europe, Australia and New Zealand (Doyle, 1989; Texas Department of Health, 1999; Daniels *et al.*, 2000; Centre for Disease Control, 2005; Chang and Wong, 2005; Drake, 2008). Pandemic strains of *V.parahaemolyticus* have been isolated in most diarrhea cases reported in many Southeast Asian countries, for example India, Japan, Bangladesh, Taiwan, Thailand, Vietnam, and also in United States (Nandi *et al*., 1999; Matsumoto *et al*., 2000; Nishibuchi *et al*., 2000; Wong *et al*., 2000; Bhuiyan *et al*., 2002). Serovar O3:K6 are mostly found in epidemic isolates, but the other serovars can also be a part of pandemic strains (Laohapretthisan *et al*., 2003).

The number of victims can be a single person to a population (Barker, 1974; Okabe, 1974). Barker (1974) mentioned that secondary spread can happen among the family members. Seasonal trend of the outbreaks can be observed, especially between June and October (Sakai *et al*., 1965). Asymptomatic person can also be affected by this *vibrio* during summer (Sakai *et al*., 1965).

V.parahaemolyticus can survive well in aquatic environment due to its ability to endure halophilic condition. Many reports mentioned that *V.parahaemolyticus* can be isolated from seafood such as fish, shellfish and crustacean as well as sea water (Drake, 2008;

Kandhasamy *et al.,* 2008). *V.parahaemolyticus* can attach to chitin (a part of exoskeleton in plankton) through production of chitinase and it can degrade this plankton to recycle the organic contents (Kaneko and Colwell, 1978; Sakazaki *et al.,* 2006). *V.parahaemolyticus* can also be found in digestive tracts of shellfish (Kandhasamy *et al.,* 2008).

2.4 Prevalence of *Vibrio parahaemolyticus* in Raw Vegetables

Fruits and vegetables constitute an important part in our daily diets. According to Canada Paediatric Society (2008), there are more people eating fresh fruits and vegetables as these food products are recommended for healthier diet. These food products can be imported since the demands for fresh produce increases as a result of increased consumption of fruits and vegetables. If the food products are imported from countries that do not practice proper and hygienic methods of production, there is a chance for the occurrences of microbial contaminations.

Zhang (2005) stated that as there are more people consuming vegetables, foodborne illness related to fresh products consumption increases as well. Some of the factors contributing to this scenario are the change in eating habit, the centralization of produce distribution, rising popularity of ready fruits and vegetables and the rise in produce imports. CIFOR (2008) stated that some of the reasons for changes in diet are industrial consolidation and globalization, health concerns, dietary suggestions and culinary trends. The consumption of food will contribute to foodborne outbreaks if the food is not cooked or handled properly.

According to Chang and Chen (2003), food borne illness in Taiwan occurs most frequently at homes and followed by schools, restaurants and working places respectively. This is because the food products can be obtained easily. Todd (1992) stated that in Canada, restaurants are the most frequent location for outbreaks, followed by food stands and homes. From above, it is clearly seen that there is difference among occurrence locations for Asia and developed Western countries due to different eating habit.

CDC (2005) reported that foodborne disease linked to uptake of fresh fruits and vegetables make up 12% of the reported cases in United States. Bean (1990) and Chang and Chen (2003) stated that fruits and vegetables can act as vehicles to transmit food borne diseases apart from other food products in Taiwan and Western countries. Wong *et al*. (1999) and Chang and Chen (2003) revealed that *Vibrio parahaemoyticus* can be detected from seafood, meat and meat products, cereal products, egg products, fruits and vegetables, boxed meals and others.

Fresh or minimally processed food, such as fruits and vegetables, for example, melon, carrots, alfalfa sprouts and apple ciders have increasing demands among the public. Thus, this can lead to elevation of food borne disease outbreaks (Labbe and Shetty, 1998). Fresh products have gained their popularity among consumers as they are convenient and ready to be eaten. These products are mostly found in supermarket and convenience stores. Temperature is an important issue in preventing food borne disease outbreaks. Berrang *et al*. (1989) stated that some pathogens can grow in refrigerated food with little or no change in sensory and taste.

Sushi had been famous among the public for its taste and healthfulness. The ingredients in sushi are vinegar rice, seafood, vegetables, and raw fish. Some of sushi is prepared with cooked seafood while some are served raw (such as sashimi). Food-borne illness involves sushi are *Vibrio parahaemolyticus*, *Staphylococcus aureus*, *Salmonella* species and *Listeria monocytogenes* (Safe Food News, 2006). Cross contamination can happen during sushi preparation which uses raw and cooked fish.

2.5 Route of Transmission

The minimum dose for infection by *V.parahaemolyticus* is around 10^5 to 10^7 Kanagawa positive cells after the uptake of contaminated food products. Virulent cell population can be expanded through genetic transformation of Kanagawa negative cells or host selection and colonization of Kanagawa positive cells in vivo (Doyle, 1989).

Seafood is always linked to foodborne illness caused by *V.parahaemolyticus* (Sakazaki *et al*., 2006). In Japan, raw fish meat and shellfish are the main source for the infection as the residents like to eat raw fish and products in their custom. Raw vegetables can also be a vector to the illness through contamination of kitchen utensils. In European countries, *V.parahaemolyticus* seldom occurs because eating raw fish is not famous among the residents (Sakazaki *et al.*, 2006).

Cases involving foodborne outbreak caused by *V.parahaemolyticus* are reported mostly in East European, United States, United Kingdom and also Africa (Sakazaki *et al.*, 2006). Seafood consumed is cooked for a short period before they are eaten. Crab and shrimp