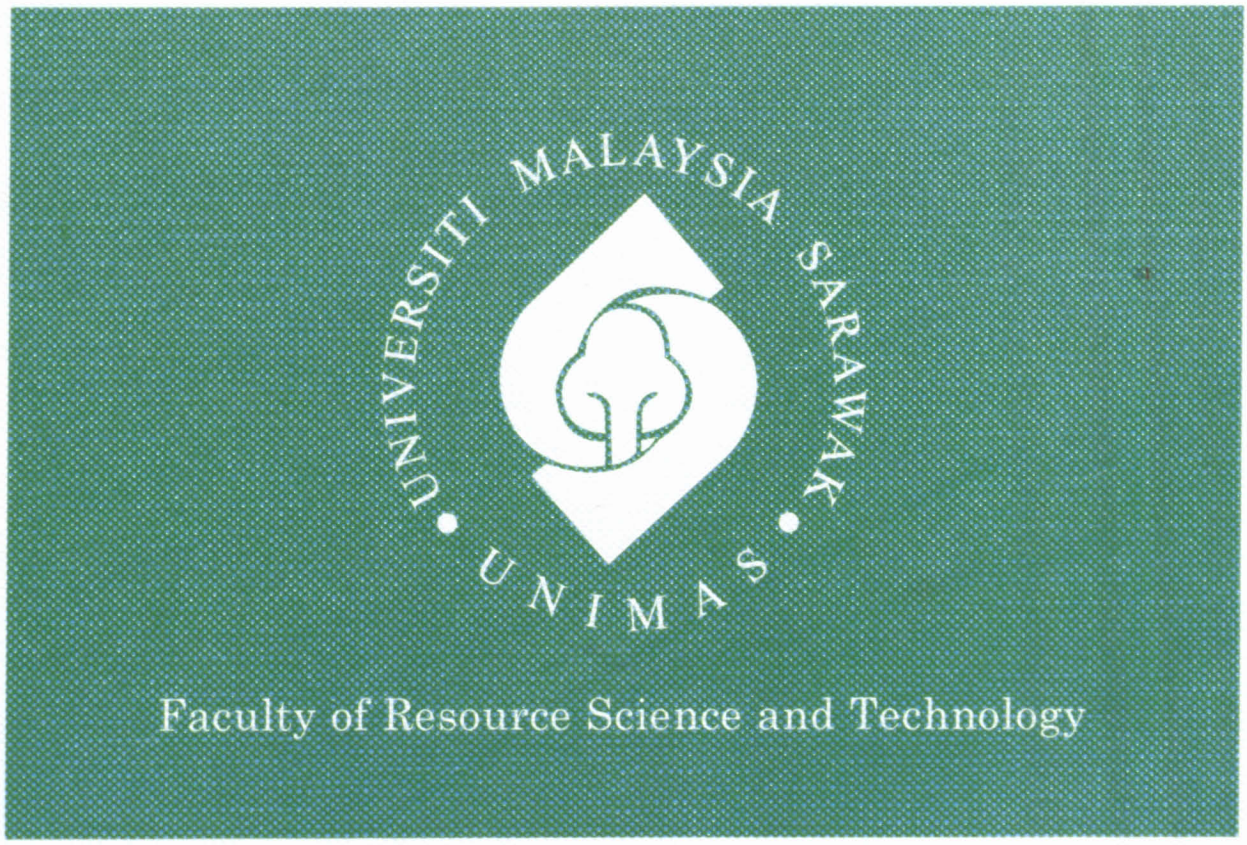

PRESENCE OF *VIBRIO PARAHAEMOLYTICUS* IN AQUACULTURE SHRIMP, FISH AND ENVIRONMENT

Nur Syahirah Binti Shafiin

Bachelor of Science with Honours
(Resource Biotechnology)
2014

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Presence of *Vibrio parahaemolyticus* in aquaculture shrimp, fish and environment

Nur Syahirah binti Shafiin (32247)

A final report submitted in partial fulfilment of the
Final Year Project (STF 3013) Course

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List of abbreviations

APW	Alkaline Peptone Water
ASPW	Alkaline Salt Peptone Water
CDC	Centre for Disease Controls and Preventions
CFU	Colony forms unit
DOF	Department of Fisheries
EU	European Union
H_2S	Hydrogen sulphide
L. vannamei	*Litopenaeus vannamei*
MPN	Most probable number
NH_3	Ammonia
P. hypophthalmus	*Pangasius hypophthalmus*
P. monodon	*Penaeus monodon*
P. schwanenfeldii	*Puntius schwanenfeldii*
PCR	Polymerase Chain Reaction
rpm	rotation per minute
TCBS	Thiosulphate Citrate Bile Sucrose
tdh	Thermostable Direct Hemolysin
trh	Tdh-related hemolysin
US	United States
UV	Ultraviolet
V. alginolyticus	*Vibrio alginolyticus*
V. cholerae	*Vibrio cholerae*
V. parahaemolyticus	*Vibrio parahaemolyticus*
V. vulnificus	*Vibrio vulnificus*
VHB	Viable heterotrophic bacterium

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Presence of *Vibrio parahaemolyticus* in Aquaculture Shrimp, Fish and Environment

By Nur Syahirah binti Shafiin

Resource Biotechnology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

Abstract

Vibrio parahaemolyticus is considered as an important foodborne pathogen causing significant economic problems within the aquaculture industry worldwide. Fish samples and shrimp samples including environmental samples were collected from Bau and Bako, Kuching, Sarawak aquaculture farm respectively. A total of 158 samples were collected from both aquaculture farms between December, 2013 and April, 2014. The simultaneous enumeration and detection of *V. parahaemolyticus* were carried out by using Most Probable Number (MPN) method followed by species specific PCR assay targeting the *toxR* gene (regulatory gene). Thirty two out of 162 (19.8%) isolates of shrimp samples, twenty six out of 324 (8.0%) isolates from water samples and twenty two out of 324 (6.8%) isolates of sediment samples were confirmed positive for *toxR* gene. However, none of the isolates from fish samples were positive for the targeted gene. This study concluded that *V. parahaemolyticus* were present in low concentration of the shrimp and environmental samples examined. Thus, the data obtained in this study can be applied for future studies on the risk assessment of *V. parahaemolyticus* in Malaysian food and aquaculture industry.

Keywords: MPN, PCR, *toxR* gene, *Vibrio parahaemolyticus*

Abstrak

Vibrio parahaemolyticus *dianggap sebagai patogen bawaan makanan penting yang boleh menjejaskan industri akuakultur di seluruh dunia. Sampel ikan telah dikumul dari Bau manakala sample udang telah dikumpulkan dari Bako. Sebanyak 158 sampel telah dikumpulkan daripada kedua-dua ladang akuakultur pada Disember, 2013 hingga April, 2014. Penghitungan serentak dan mengesan* V. parahaemolyticus *telah dijalankan dengan menggunakan kaedah MPN diikuti oleh spesies tertentu PCR mensasarkan gen toxR (gen pengaturan). Tiga puluh dua daripada 162 (19.8%) sampel udang, dua puluh enam daripada 324 (8.0%) sampel air dan dua puluh dua daripada 324 (6.8%) sampel sedimen disahkan positif toxR gen. Walau bagaimanapun, sampel ikan menunjukkan negatif terhadap toxR gen. Kesimpulannya,* V. parahaemolyticus *dikesan di dalam kebanyakan sampel udang dan sampel persekitaran dalam kepekatan yang rendah. Data yang diperoleh dalam kajian ini boleh digunakan untuk kajian masa depan bagi menilai risiko* V. parahaemolyticus *dalam industri makanan dan akuakultur di Malaysia.*

Kata kunci: Gen *toxR,* MPN, PCR, *Vibrio parahaemolyticus*

1.0 Introduction

1.1 Introduction

According to CAC, (2002), over the past 20 years, shrimp accounts for about 20% of the value of exported fishery products. Imports into developed countries accounted for about 40% of intra-developed countries trade, while about 60% comes from developing countries; out of the exports from developing countries 80% goes to developed countries and only 20% stays in the group (Josupeit, 2005). Apart from that, shrimps are one of the major aquaculture products of export importance from the tropics. Eighty percent of the world's aquaculture shrimps are contributed through aquaculture in Asia (Bhaskar *et al.*, 1998).

Aquaculture shrimp is grown in controlled freshwater or marine environment. Since 1970s, marine shrimp farming was commercialised and the production grew steeply and fulfill the market demands of the United States, Japan, and Western Japan. In Asia, particularly in China and Thailand, about 75% of aquaculture shrimp is produced there and Thailand became the largest exporting nation in the world. However, for some EU countries, the exports of block frozen black tiger shrimp (*Penaeus monodon*) from Malaysia had been rejected due to the presence of *Vibrio parahaemolyticus* (Mohammad *et al.*, 2005). In Malaysia, the production from aquaculture in 2009 for food increased to 333 451 tonnes which showed an increment of 37.2% compared to 243 129 tonnes in the year 2008 (Department of Fisheries, DOF, 2009).

Shrimp ponds are stressful environments compared to estuaries or other enclosed water bodies (Direkbusaram *et al.*, 1998). The confined environment of pond ecosystem by having high content of organic matter, feed, fecal wastes, accumulation of phytoplankton, high stocking density, ammonia (NH_3), hydrogen sulphide (H_2S) and human interference

has resulted in increment of bacteria (Sharmila *et al.*, 1996). Under normal conditions though, temperature increments will also resulted in greater diversity of *Vibrio* species (Barbieri *et al.*, 1999).

In Malaysia, the production from aquaculture industry has showed an increment of 37.2% in year 2009 with 333 451 tonnes compared to 243 129 tonnes in the year 2008 (Department of Fisheries, DOF, 2009). Brackish water aquaculture is the main contributor to this sub-sector at 54.2% or 181 820 tonnes while freshwater aquaculture contributed to 45.8% or 152 630 tonnes to the national food fish production (Anon., 2011). This value showed that the consumers in Malaysia began to accept aquaculture fish as alternative food resources since the scarcity of the sea fish products. Ibrahim *et al.* (2011) has stated red tilapia fish (*Oreochromis sp.*), patin fish (*Pangasius sp.*) and keli fish (*Clarius sp.*) are the main fresh water aquaculture fish products in Malaysia.

Vibrio spp. is commonly found in coastal, estuarine waters, brackish waters and freshwater (Li *et al.*, 1999; Imziln and Hassani, 1994; Majusha *et al.*, 2005; Zulkifli *et al.*, 2009; Ibrahim *et al.*, 2010). *Vibrio* spp. have been recognized as the major cause of foodborne outbreaks in many countries including Japan, India, Korea, China, Taiwan and Malaysia (Noorlis *et al.*, 2011). *V. parahaemolyticus* and *V. vulnificus* are the two major pathogenic *vibrios* which contribute to the food borne illness and death (Mead *et al.*, 1999; Oliver, 2006).

V. parahaemolyticus belongs to the *Vibrionaceae* family, and was first identified as foodborne pathogen after large outbreaks occurred in Japan in 1950 (Fujino *et al.*, 1953). The outbreak reported worldwide was usually associated with the consumption of raw or undercooked seafood. *V. parahaemolyticus* has been reported to become the leading pathogen that caused foodborne illness in Malaysia (Cann *et al.*, 1981).

Generally, *Vibrio* spp. was found to be the dominant forms in the aquaculture ponds (Zhomei *et al.,* 1993). Among vibrios, *V. parahaemolyticus* play an important role in aquaculture farming as its presence may affect the water quality and aquaculture product's quality. The presence of *V. parahaemolyticus* may cause vibriosis in shrimp and consequently contribute to the economic loss. Hence, the presence of *Vibrio* spp. in aquaculture environment is used as environmental indicator (Lopez-Torres, 2001).

1.2 Objectives of the study

This study was undertaken with the following objective:

i. To determine the concentration and presence of *V. parahaemolyticus* in shrimp, fish, water and sediment samples collected from aquaculture farms using Most Probable Number (MPN) method and species specific PCR, respectively.

2.0 Literature Review

2.1 Family *Vibrionaceae*

Family *Vibrionaceae* was proposed earlier in 1965 by Véron as a convenient grouping for fermentative bacteria that posses polar flagella and positive oxidase reaction (Véron, 1965). According to seventh edition of Bergey's Manual of Determinative Bacteriology, *vibrios* and related fermentative bacteria were classified into a number of families which consists of five genera (Holt, 1994). The genera classified are *Vibrio, Aeromonas, Enhydrobacter, Photobacterium*, and *Plesiomonas.* However, genus *Aeromonas* have been transferred into family *Aeromonadaceae* while *Plesiomonas* into family *Enterobacteriaceae* which *Plesiomonas* had been suggested transferred to genus *Proteus* in the family *Enterobacteriaceae.* The transferring of genus to new family was due to its 5S rRNA which closely related to that of *Proteus mirabilis* (Holt, 1994).

2.1.1 *V. parahaemolyticus*

V. parahaemolyticus belongs to the *Vibrionaceae* family, gram negative enteric bacterium, curved-rod shaped bacterium, halophilic, and facultative aerobic which does not form spores. It is an oxidase-positive facultative anaerobe that can ferment glucose without gas production (Butt *et al.,* 2004). It has a polar flagellum which enables its high motility in liquid media, and its lateral flagella allow it to migrate across semi-solid surfaces by swarming (Yeung *et al.,* 2004).

Vibrio parahaemolyticus was first identified as a cause of food borne illness in 1950 as a large outbreak reported in Japan due to consumption of sardines (Fujino *et al.,* 1953). *Vibrio* spp. is one of the most commonly occurring bacteria in shrimp aquaculture and naturally occurs in aquatic environment (Vanderberghe *et al.,* 2003). Extensively, *V. parahaemolyticus* has been known to be ubiquitously present in brackish and marine

waters, and hence, infection to human is frequently associated with the consumption of contaminated seafood or raw or undercooked shellfish (Guoxiang *et al.,* 2009).

Non pathogenic vibrios also can be present in food and environmental samples thus, as a results, the total *Vibrio* counts are not indicative enough for the presence of pathogenic vibrios. The presence of virulence genes is considered as current markers of pathogenicity in *V. parahaemolyticus* (Chiou *et al.,* 2000). Moreover, according to webpage of Public Health of Canada, it is proven that the virulent strains isolated from patients, showed a characteristics that is not observed in other non-pathogenic *V. parahaemolyticus* strains which produce thermo stable direct hemolysin (TDH), and, or TDH-related hemolysin (TRH). According to Goarant *et al.* (1999), some of the pathogenic *V. parahaemolyticus* have been reported as the causal agents of shrimp infections. Contrary, the other species of same family, *V. alginolyticus* have been reported as probiotics for shrimp aquaculture (Vandenberghe *et al.,* 2003; Direkbusaram *et al.,* 1998).

2.2 Outbreaks of *Vibrio parahaemolyticus*

V. parahaemolyticus and *V. vulnificus* are the two major pathogenic *vibrios* which contribute to the food borne illness and death in United States (Mead *et al.,* 1999; Oliver, 2006). The first confirmed outbreak was reported in 1971 and was associated with consumption of crabs (Dadisman *et al.,* 1973), while in Asia, which includes Taiwan, Japan, India, China, Korea and Malaysia approximately half of the food poisoning was due to *V. parahaemolyticus* infection (Chiou *et al.,* 1991; Noorlis *et al.,* 2011). Mead *et al.* (1999) has reported 5000 food borne illness occurred annually in United States was due to the *Vibrios* infection and *V. parahaemolyticus* became the leading cause of the infection (Hlady *et al.,* 1993; Hlady and Klontz, 1996).

Daniels *et al.* (1998) has reported 40 outbreaks of *V. parahaemolyticus* infection between 1973 and 1998 in US and it occurred mostly during the warmer months. Later, in July-September 1998, another outbreak of *V. parahaemolyticus* infections were reported among residents of Connecticut, New York and New Jersey and they were related to the consumption of oysters and clams harvested from Long Island Sound (CDC, 1999). This is the first reported outbreak of *V. parahaemolyticus* associated to the consumption of shellfish harvested from New York waters.

Therefore, it has been suggested that these recent outbreaks may have been closely related with warmer water temperatures, which had cause increment in *V. parahaemolyticus* levels and thus increased the probability of infection. According to Williams and LaRock (1985), as this bacterium is a thermophilic and halophilic, *V. parahaemolyticus* will predominate in a condition of high temperature and high salinity.

Apart from that, according to Kaneko and Colwell (1975), 70% of gastroenteritis in Japan occurred due to *V. parahaemolyticus* infection and associated with consumption of raw seafood. Although food poisoning caused by *V. parahaemolyticus* mostly occurred in Japan and Southeast Asia (Nishibuchi, 2004), there were also cases reported in other parts of the world. For instance, *V. parahaemolyticus* accounts 35% to 50% more than the other bacterial food borne illness that occurred annually in Taiwan (Chiou *et al.*, 1991; Wang *et al.*, 1996).

2.3 Isolation and detection of *V. parahaemolyticus*

The isolation of *V. parahaemolyticus* can be performed by the use of selective enrichment with Alkaline Peptone Water (APW) and plating of the enrichment culture onto Thiosulphate Citrate Bile Sucrose (TCBS) agar. However, similar to other vibrios, *V. parahaemolyticus* will show ambiguous results and may be overlooked if plated on

nonselective medium, thus, it should be plated on this agar for selective isolation of *V. parahaemolyticus*. On TCBS, *V. parahaemolyticus* isolates appear as distinct green colonies.

The newer method which includes the technique of PCR helps in amplification and detection of virulence strains in samples collected and has been proven to be the most reliable and suitable method for the identification of *V. parahaemolyticus* (Karunasagar *et al.*, 1997; Kim *et al.*, 1999). PCR based on *toxR* gene and on a chromosomal locus of unknown function reported to be specific for *V. parahaemolyticus* (Lee *et al.*, 1995) has been found to be useful for confirmation of this species (Karunasagar *et al.*, 1997).

25 of environmental isolates collected from selected aquatic environment in Bachok, Kelantan, Malaysia showed positive results for primer targeting *toxR* gene at locus 368 bp using PCR method (Vimala *et al.*, 2009). The environmental isolates included shrimp, water and sediment samples. Besides that, Sujeewa *et al.* (2009) also reported incidence of *V. parahaemolyticus* in shrimp and water isolates collected from culture ponds. One hundred and twenty eight out of 251 (51.0%) isolates showed positive result for presence of regulatory gene of *V. parahaemolyticus, toxR* gene. Twenty five out of 35 (68.6%) of prawn isolates, thirty three out of 35 (94.3%) of shrimp isolates, and twenty seven out of 35 (77.1%) of crab isolates showed positive result for confirmation of *toxR* gene in samples tested using PCR method (Subhashini *et al.*, 2011).

Alam *et al.* (2003) has stated that the PCR has been proven to be a very useful technique for its ability to amplify the specific DNA segment by a factor of 106 or more within hours and hence allowing the detection of very limited amount of cells. It has been demonstrated that the PCR technique can detect a low number of specific bacteria against a large background of other prokaryotic and eukaryotic cells and organic materials which may present in the samples (Tsai and Olson, 1992; Thiem *et al.*, 1994; Laser *et al.*, 1995).

3.0 Materials and Method

3.1 Materials

The materials that were used in this study as the following:

i. Alkaline Peptone Water (with addition of 3% NaCl)

ii. Ice box containing ice

iii. Lampan *(Puntius schwanenfeldii)*

iv. Patin *(Pangasius hypophthalmus)*

v. Pond sediment

vi. Pond water

vii. Red Tilapia *(Oreochromis sp.)*

viii. Salt Polymyxin Broth

ix. Sterile distilled water

x. Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar plate

xi. Whiteleg shrimp *(Litopenaeus vannamei)*

3.2 Methods

3.2.1 Sample collection

Three types of fish, Lampan *(P. schwanenfeldii)*, Patin *(P. hypophthalmus)* and Red Tilapia *(Oreochromis sp.)* were collected from aquaculture farm in Bau, Kuching while whiteleg shrimp (*L. vannamei*) were collected from aquaculture farm in Bako, Kuching. Shrimp samples were taken from three ponds identified as Pond 1, Pond 2 and Pond 4. Ten grams of shrimps and fish samples were collected using a cast net, and packed individually into sterile stomacher bag. Ten militer of pond water and 10 g of sediment samples were collected in 50 ml falcon tube from each designated pond. All samples were stored in 4 °C inside the ice box and transported to UNIMAS Microbiology Laboratory for further processing.

3.2.2 Enumeration of samples

All of the samples collected were homogenized in preparation for enrichment with Salt Polymyxin Broth. Ninety millilitre of Salt Polymyxin broth were added to 10 ml of samples in stomacher bag and incubated at 37 °C for 24 hours. Salt Polymyxin Broth is a superior selective enrichment broth for *Vibrio* species compared to Alkaline Peptone Water (APW) (Subhashini *et al.*, 2011).

3.2.2.1 Viable plate counts

Enriched cultures of *Vibrio* species were diluted to form ten-fold dilutions. Dilution of 10^{-1}, 10^{-2} and 10^{-3} from each samples were prepared with 9 ml of sterile distilled water. About 0.1 ml of dilutions from 10^{-1}, 10^{-2} and 10^{-3} were transferred onto thiosulphate citrate bile salt sucrose (TCBS) agar plate for viable plate counts. Spread plating technique was carried out. The TCBS agar plates were incubated at 37 °C for 18 hours. Then, single

colonies of *V. parahaemolyticus* on the agar were counted and recorded. Two to three colonies of *V. parahaemolyticus* were selected and kept as stock culture.

3.2.2.2 Most Probable Number (MPN)

Three-tube MPN method was used in enumeration of bacteria samples. Dilution prepared from 10^{-1}, 10^{-2} and 10^{-3} of each samples were introduced into MPN tubes containing 9 ml of APW and incubated at 37 °C for 18 hours. All turbid tubes were subjected for further analysis in detection of *V. parahaemolyticus* regulatory gene, *toxR* using species specific PCR.

3.2.3 Molecular Analysis

3.2.3.1 DNA extraction

Turbid MPN tubes were subjected for DNA extraction using boiled cell method (Vengadesh *et al.,* 2012). Briefly, 1 ml portion of overnight cultures were centrifuged at 10,000 rpm for 2 minutes. The cell was harvested three times to increase the cell concentration. Then, the supernatant were removed and the pellet was suspended with 500 µl of sterile distilled water. The centrifuge tubes were vortexed vigorously. Later, the cell suspension were boiled at 100 °C for 10 minutes and cooled immediately at -20 °C for 10 minutes. The mixtures were centrifuged for 13,000 rpm for 3 minutes. The supernatant were transferred into new 1.5 mL centrifuge tube and were used for further analysis using species specific PCR.

3.2.3.2 Species specific PCR

Species specific PCR were carried out to detect *toxR* gene with the oligonucleotide primer as shown in Table 3. The PCR mixture was prepared in a total volume of 25 µl containing 10X PCR buffer (100 mM Tris-HCl, pH 8.3 at 25°C; 500 mM KCl; 15 mM $MgCl_2$; 0.01% gelatin), 25mM $MgCl_2$, 10 Mm dNTPs, *toxR* gene primer, 5 unit of Taq

polymerase and 2 µl of template DNA. The PCR mixtures were prepared according to the volume shown in Table 1 and performed based on conditions as shown in Table 2.

Table 1: Materials for PCR used in the detection of *toxR* gene

Materials	Volume (µl)
Sterile distilled water	12.5
10 X PCR buffer	4.0
25 mM $MgCl_2$	3.0
10 mM deoxyribonucleotide phosphates	1.0
Primer-F	1.0
Primer-R	1.0
5 units Taq DNA	0.5
Template DNA	2.0
Total	25.0

Table 2: Conditions used in species specific PCR

Step	Temperature (°C)	Duration (minute)	
Pre-denaturation	96	5	
Denaturation	94	1	35 cycles
Annealing	63	1.5	
Extension	72	1.5	
Final extension	72	7	

Table 3: Oligonucleotide primer used in the study

Primer	Target gene	Primer sequence	Fragment size	Reference
toxR-F	*toxR*	GTC TTC TGA CGC AAT CGT TG	368	Kim *et al.*, 1999
toxR-R	*toxR*	ATA CGA GTG GTT GCT GTC ATG	368	Kim *et al.*, 1999

3.2.3.3 Agarose Gel Electrophoresis (AGE)

Five microlitres of the PCR products from each samples was mixed with 1 µl of 5 X loading dye (Vivantis Technologies, Selangor, Malaysia). 1.0 % (w/v) agarose gel loaded with mixtures of PCR product and loading dye were conducted at 90 V with 300 mA for 1 hour in TBE buffer. A DNA molecular ladder (100bp ladder) (Vivantis Technologies, Selangor, Malaysia) was included in each gel as molecular weight markers. The gel was stained with 0.5 µg/ml of ethidium bromide solution for 10 minutes and visualized under UV transilluminator.

4.0 Results

4.1 Enumeration of bacteria

Inoculum from serial dilution transferred onto TCBS agar plate produced green and yellow colonies. *V. parahaemolyticus* was distinguished from other types of *Vibrio* sp. by producing green colonies as shown in Figure 1. The inoculum transferred onto TCBS agar mostly produced yellow colonies as shown in Figure 2. The results for viable plate counts obtained from both aquaculture farms were tabulated as shown in Appendix I and II. The estimated microbial load of *V. parahaemolyticus* was ranged from 0.01 to 131 CFU/ml.

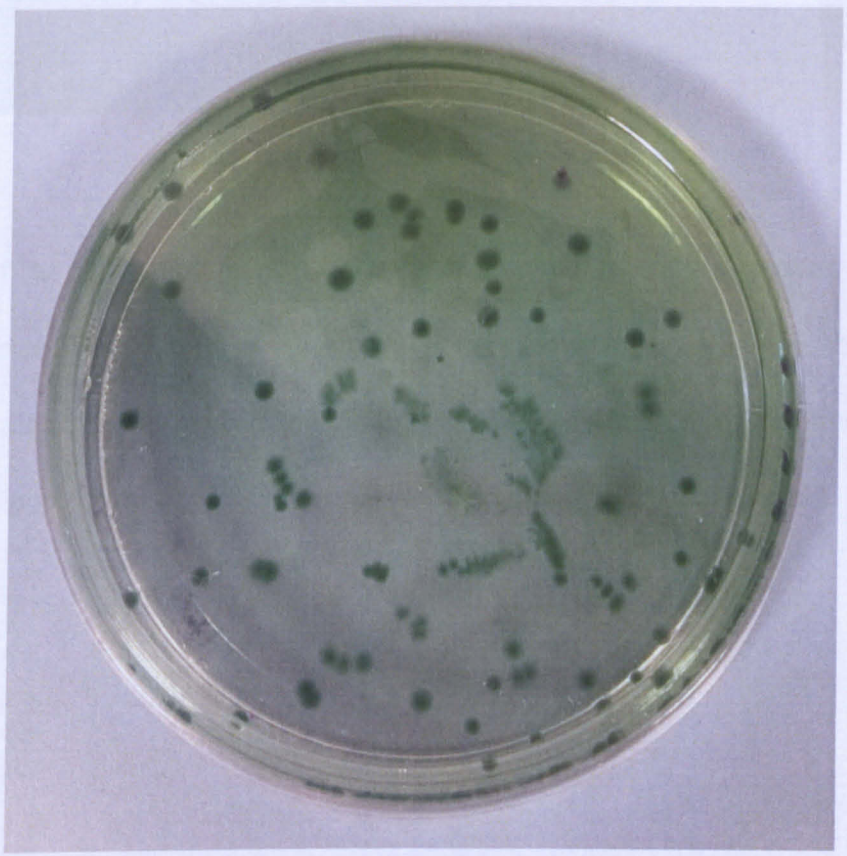

Figure 1: *V. parahaemolyticus* on TCBS agar