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Phenotypic and Genotypic Characteristics of *Vibrio Harveyi* Isolated from Black Tiger Shrimp (*Penaeus Monodon*)

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Abstract: In the present study, a total of 30 luminous bacteria were successfully isolated from the hepatopancreas of tiger shrimp (*Penaeus monodon*) in Kedah, Terengganu and Johore. Based on the Baumann and Schubert [12] scheme, all isolates were identified as *V. harveyi*. Thirty biochemical and physiological tests were carried out to reveal the similarity and differentiatial phenotypes among the isolates. Although the isolates were obtained from different locations, they showed similar biochemical and physiological characteristics except for colony morphology, colony color on TCBS and salt tolerance test. Based on luminous activity and the ability to hemolyse against horse red blood cells, all isolates were considered virulent. The percentage of similarity among the isolates from Kedah was ranging from 50 to 100% whilst genetic distance was ranged from 0 to 0.5. The isolates from Terengganu were recorded the lowest ranging of percentage of similarity and genetic distance, from 66.7 to 100% and 0 to 0.333, respectively. Whilst, the isolates from Johore performed the highest ranging percentage of similarity and genetic distance; there were 12.5 to 90% and 0.1 to 0.875 respectively. The percentage of similarity between isolates from Kedah compared to isolates from Terengganu and Johore were ranged from 14.3 to 66.7% and 0 to 88.9% respectively. On the other hand, comparison between isolates from Terengganu and isolates from Johore has shown a quite high percentage of similarity ranging from 18.2 to 93.3%. The susceptibility of antibiotic to the bacterial strains was reported in 173 cases (82.4%). 10 cases (4.7%) were determined as the intermediate sensitive and 27 cases (12.9%) were resistance to certain antibiotics. The isolates were mostly resistant to ampicillin (90%) followed by sulphamethoxazole (40%). However, 96.7% of the isolates in the present study were demonstrated sensitive to chloramphenicol, tetracycline and furazolidone. Furthermore, the isolates were sensitive to nalidixic acid (93.4%), kanamycin (80%), sulphamethoxazole (56.7%) and ampicilin (10%). Intermediate sensitive was observed among of the isolates for kanamycin (20%) and nalidixic acid (6.6%).

Key words: Black tiger shrimp · *Penaeus monodon* · *Vibrio harveyi* · phenotypic · genotypic · antibiogram

INTRODUCTION

Black tiger shrimp (*Penaeus monodon*) is among the economically important species in ASEAN and worldwide. In 1995, the ASEAN Member Countries produced about 558,000 metric tons of *P. monodon*, about 78% of the total world production of shrimp. Penaeid shrimp farmings have become a significant aquaculture activity in many countries in the tropics [1]. Marine shrimp culture in Malaysia started in the 1980's on a small-scale basis by trapping shrimp fry into a storage pond. The culture development of the tiger shrimp grew rapidly towards the end of 1970's and early 1980's with the availability of technical support from the government [1]. The result of continuous culture development and new technological findings has increased production between 2 to 3.5 metric tons/hectare/cycle for semi-intensive culture and 5 to 7 tons for intensive culture [1]. In 1994, it was estimated that 5,790 metric tons of tiger shrimp were produced through brackish-water pond culture activities throughout the country. The production involved 3,284 ponds of 2447.34 hectares and 787 shrimp farmers [1]. However, the exponential growth of shrimp culture is not supported by a sufficient supply of healthy fry [2]. Bacterial diseases have been implicated to be one of the most devastating diseases which can completely destroy

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hatchery productivity for extended periods [2, 3]. Disease outbreaks are recognized as a significant constraint to aquaculture production and trade, affecting both the economic development and socioeconomic revenue of the sector in many countries in the world [1]. At the year of 2000, vibriosis outbreak was reported mainly in shrimp farms at Kedah and Sabah, Malaysia. Economic loss attributed to outbreaks of disease in developing countries in the Asian region was estimated to be at least USD 1400 million in 1990 [1]. The cost of lost production in China alone was approximately USD 1000 million in 1993 [1]. Disease due to the bacteria infection particularly luminous vibriosis has been a major problem in the shrimp industry in the Philippines [4]. In 1991, luminescent vibriosis outbreak was reported in Eastern Java, Indonesia resulting in a decreased of 70% larval production and subsequently doubled the larval price from USD 5.0 to 10.0 per thousand larvae; it is estimated that more than USD 85 millions were lost [5]. In 1980, the production of *P. monodon* in Taiwan was 3,178 tons and increased to 80,271 tons in 1987 but the following year, luminous disease broke out in the shrimp culturing ponds throughout the island and the production declined to 31,171 tons [6]. Based on farm level surveys in 16 Asian countries in 1998, suggested that disease and environment-related problems have caused annual losses of more than USD 3,000 million to aquaculture production [1]. Serious financial losses have also been recorded in other regions of the world. Various factors have been related to the apparent increased incidence of disease such as poor water quality, sometimes resulting from increased self-pollution due to effluent discharge and pathogen transfer via movements of aquatic organisms appear to be and important underlying cause of such epizootics [7]. Abundance of these microorganisms in hatchery samples indicated that they are opportunistic pathogens, which can invade the shrimp tissue, subsequently cause disease when post-larvae are under stressful conditions [7]. As the tiger shrimp industry in Malaysia is gearing towards expansion and intensification, the increase of luminous vibriosis incidences caused by luminous bacteria seem to be parallel to aquaculture industry development. Significant larval mortalities in ASEAN shrimp hatcheries are often associated with luminescent vibriosis which is caused by *Vibrio harveyi* [2, 8]. The main source of luminous vibriosis is midgut contents of broodstock shed into the water together with the eggs during spawning [2, 8]. The finding of [9] showed that gut contents of broodstock and adult shrimp contained the highest number of luminous bacteria. Beside that, the near shore seawater may also be a major source of infection [2, 3]. Supplementation of antibiotics to control the luminous vibriosis has become less effective due to the occurrence of bacterial resistance to a number of antibiotics [10]. Only in this decade, the virulence of *V. harveyi* has been recognized in a small but expanding list of cultured marine animals particularly in penaeids in Asia and Australia [11]. At present, the information on the phenotype and genotype of local *V. harveyi* isolates, both clinical and environmental, are lacking. Therefore, the aim of this study is to investigate the genetic diversity and relationships among *V. harveyi* isolated from shrimp farms in Terengganu, Kedah and Johore. Thus, the objectives of this study are to characterize phenotypic and genotypic *V. harveyi* from tiger black shrimp as well as its antibiogram.

MATERIALS AND METHODS

Isolation of *Vibrio harveyi*: Hemolymph (0.1 ml) was aseptically extracted from live shrimp *Penaeus monodon* using insulin syringe (Becton Dickinson, Singapore). Then the hemolymph was dropped and streaked on the thiosulphate citrate bile salt sucrose agar (TCBS) (Merck, Germany). The loop was burnt and the hemolymph was streak on TCBS plates. The sample was incubated 24 hours room temperature. After 24 hours, only luminous colonies were selected and stored in deep tube tryptic soy agar (TSA) with 2% NaCl as stocks.

Identification of *Vibrio harveyi*: A total of 30 isolates of bacteria were obtained from Kedah, Terengganu and Johore shrimp farms where 10 isolates were obtained from each mentioned states. The isolates were isolated using TCBS agar plate then subcultured on TSA agar plate supplemented with 2% NaCl. They were identified using conventional biochemical tests [2] with five additional tests as follows: casein utilization, lipid utilization, oxidase and fermentation test, blood horse hemolysis test and phenylalanine deaminase test. All isolates were identified as *V. harveyi* based on the scheme of Baumann and Schubert [12].

Extraction of genomic DNA: DNA extraction was conducted by using bacterial genomic kit (Genispin, Malaysia). 3 ml overnight culture of *V. harveyi* in LB (Luria bertani) medium (Merck, Germany) was added to a 1.5 ml microcentrifuge tube. The cells were pelleted down at 4000 *g* for 10 min at room temperature using Minispin (Eppendorf, Germany). Then, the supernatant was

discarded and the cell pellet was resuspended in 100 µl TE buffer. The cell suspension was digested with 10µl of lysozyme (10 mg/ml) at 30°C for 10 min and centrifuged for 5 min at 5000 *g* at room temperature. Then, the cells were resuspended in 200 µl Buffer BTL and treated with 25 µl of a proteinase K (15 mg/ml). The mixture was vortexed and incubated at 55°C for 1 hour in a shaking water bath (BW 05G, Lab Companion, Korea). Then, 20 µl RNase A (20 mg/ml) was added and incubated at room temperature for 2 min. The sample was added with 220 µl Buffer BDL and vortexed before incubated at 70°C for 10 min. Then, 220 µl of absolute ethanol was added and mixed thoroughly by vortexing. The mixture was transferred to assemble in *i*-Spin column in a 2 ml collection tube. Then the sample was centrifuged at 8000 *g* for 1 min to bind DNA. The collection tube was discarded. Then, the column was placed into a second 2 ml tube and washed by pipetting 650 µl of Wash Buffer and centrifuge at 8000 *g* for 1 min. The flow-through was discarded and the collection tube was reused. By using the same empty 2 ml collection tube, the *i*-Spin column was centrifuged at 14000 *g* for 2 min to dry the column. Then the column was placed into a sterile followed by adding 100 µl of TE buffer, pH 8. The tube was allowed to sit for 1 min at room temperature. Lastly, DNA was eluted from the column by centrifuge at 8000 *g* for 1 min.

Quantification of DNA samples: The quantity and quality of extracted DNA was determined by using UV spectrophotometer (Lambda EZ 201, Perkin Elmer, USA) were at absorbance of 260 nm and 280 nm. The purity of DNA was estimated by the ratio of absorbance reading between 260 nm and 280 nm. The absorbance reading at 260nm represents the quantity of DNA [13]. The ratio of A 260: A 280 ranged from 1.6 to 2.0 indicates the DNA purity for PCR reaction [14].

$$\text{DNA purity} = ABS_{260}/ABS_{280}$$

$$\text{DNA} = \text{quantity}(\mu g/ml) = \frac{ABS_{260} \times 50\mu g/ml \times \text{total volume}(\mu l)}{\text{Volume of sample}(\mu l)}$$

RAPD-PCR assay: The PCR master mix consisted of (10 mM tris Hcl, 50 mM KCl, 0.1% Triton ® X 100, 2.5 mM $MgCl_2$, 0.5µM universal primer, 0.5 µl of 0.2mM nucleotide mix, 0.25 µl of 1.25 U of *Taq* DNA polymerase) (Genensis Biotech, Malaysia) and 2µl of DNA isolate in a total reaction volume of 25 µl. A $(GTG)_5$ primer: 5'GTGGTGGTGGTGGTG3' designation was applied in the present study. Amplifications were performed on a thermal cycler (Eppendorf, Germany), which was programmed for an initial denaturation cycle at 95°C for 1 min, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 40°C for 1 min and primer extension at 65°C for 8 min. The programme also included a final primer extension step at 65°C for 16 min. Ten microlitres of RAPD products were analyzed on 2% agarose gel containing ethidium bromide (5 µg/µl) in 1 X TBE buffer at 110V in parallel with 1000-bp and 100-bp DNA ladders (Fermentas, USA) and visualized under UV transilluminator.

Gel electrophoresis and RAPD fingerprint: The gels were prepared by weighing and dissolving 2% agarose powder (NuSieve GTG Agarose, USA) in a boiling 1 X Tris-borate-EDTA (TBE) buffer solution plus 2 µl of ethidium bromide and then cooled to approximately 50°C before pouring into a mold where it solidifies. A comb was placed in the end notches of the gel bed. The gel was submerged in a TBE buffer-filled with ethidium bromide at the concentration 5 µl/ml (Mupid Ex, Japan). DNA samples were mixed with loading dye (G 190 A Blue/Orange Promega Madison, WI USA) before loaded and transferred by using a 10 µl micropipette (Eppendorf, Germany) into wells that were created in the gel by a comb during casting. Electrophoresis was run at 110 V at for an hour. RAPD fingerprint was visualized by using VDS-CL Image Master (Bio Amersham, Israel) and the scored band image was captured.

RAPD analysis and genetic relationship: Bands were visually read from fingerprints generated by a pair of universal primer and a data matrix was generated by giving scores of 0 and 1 for the absence or presence of bands, respectively, at each band position for all isolates. A dendrogram was constructed using the data matrix of all 30 isolates of bacteria based on unweighted pair-group method with arithmetic means (UPGMA) [15] using the Numerical Taxanomy and Multivariate Analysis System (NTSYSpc) version 2.1 [16]. The genetic and degree of similarity of strains were determined based on [17] formulation. Calculation of the percentage of similarity was done manually based on the band scoring that was used for cluster analysis. Calculation was based on the following formula:

$$\text{Percentage of similarity, } F = \frac{2N_{XY} \times 100\%}{N_X + N_Y}$$

Where,

N_{XY} = Number of shared bands
N_X = Ttotal number of bands in lane X, N_Y = total number of bands in lane Y

Antimicrobial tests: Bacterial isolate were inoculated into tryptic soy broth (TSB) (Merck, Germany) tube heavily. The sample of bacteria cell was taken using sterile cotton swabs from the *V. harveyi* cultures before placing them in a tube of sterile saline solution and mix until turbidity equivalent to a 1.0 McFarland standard. The sample from saline solution was taken using a sterile swab and pressed on the inside of the tube above the liquid level to remove excess fluid. The swab was smeared across the middle of the plate and then the smear was continued in a zig-zag pattern across the plate. The procedure was repeated until every square millimeter of the agar was ensured covered with thin, even layer of bacteria. The antimicrobial disks were placed evenly over the surface of the plate with a forceps. When completed, the plate was labeled and incubated for 24 hours at room temperature [18]. A total of seven antimicrobial agents were applied in this study. There were chloramphenicol (30 µg/disk), ampicillin (10 µg/disk), kanamycin (30 µg/disk), tetracycline (30 µg/disk), nalidixic acid (30 µg/disk), furazolidone (15 µg/disk) and sulphamethoxazole (25 µg/disk) (Oxoid, England). The antibiotic susceptibility was determined according to the National Committee for Clinical Laboratory Standards (NCCLS) provided by manufacturer.

RESULTS

A total of 30 isolates of bacteria were obtained from Kedah, Terengganu and Johore shrimp farms where 10 isolates were obtained from each mentioned states. The isolates were isolated using TCBS agar plate then subcultured on TSA agar plate supplemented with 2% NaCl. They were identified using conventional biochemical tests [2] with five additional tests as follows: casein utilization, lipid utilization, oxidase and fermentation test, blood horse hemolysis test and phenylalanine deaminase test. All isolates were identified as *Vibrio harveyi* based on the scheme of Baumann and Schubert [12]. Colonial morphologies were recorded after incubation for 24 hours at room temperature on TSA agar plate supplemented with 2% NaCl. A total of twenty four isolates; eight isolates from Johore (J1, J3, J4, J5, J6, J7, J8, J9), six isolates from Terengganu (T5, T6, T7, T8, T9, T10) and ten isolates from Kedah (K1, K2, K3, K4, K5, K6, K7, K8, K9, K10) were considered as dominant isolates based on their single colony morphological appearances as entire margin, circular form, convex elevation and yellow color on TSA plate. Another six isolates; four isolates from Terengganu (T1, T2, T3 and T4) and two isolates from Johore (J2 and J10) showed similar single colony morphological appearance as irregular form, undulated margins and raised elevation. However, different colony color was observed on TSA plate. Isolates T1, T3 and T4 were white in color but T2, J2 and J10 were yellow in color. Biochemical and physiological characteristics of the luminous bacterial isolated from Kedah, Terengganu and Johore shrimp farms were given in Table 1-3 respectively. The green colonies were observed on TCBS agar plate except for one isolate from Kedah and one isolate from Terengganu. Those isolates were yellow in color on TCBS were non sucrose fermenter. The Gram stain result was observed under total magnification 10 X 97 by using a light microscope (Leica, USA) showed all *V. harveyi* isolates were Gram negative short rods. All *V. harveyi* isolates were able to ferment glucose and showed positive result to the oxidase, catalase and motility tests but unable to produce hydrogen sulfide. These isolates were also able to degrade trytophan and produce indole as a final product, sensitive to vibriostat 0/129 (150 µg/disk) and were all glycine decarboxylase, L-tyrosine decarboxylase, L-serine decarboxylase, able to utilize starch and lipid and positive for both Oxidative and Fermentative test. They were also able to ferment glucose. All isolates gave strong positive reactions characteristic of *V. harveyi* in fermentation-indicator medium containing glucose. In addition, these isolates failed to utilize casein, citrate, lactose, L-arginine, acetate acid, phenylalanine and gelatinase-negative. Majority of *V. harveyi* isolates (96.7%) was able grow to in salt tolerance test at 0% to 5%. Only one isolate from Terengganu could grow in salt tolerance test from 0 to 10%. These bacterial isolates showed inhibition at temperatures of 4°C and 55°C but were able to grow well at temperatures of 28 and 37°C. All isolates were able to hemolyse horse's blood which resulted in breakdown of horse blood agar plate around the colony of bacteria known as β-hemolysis. Based on the Baumann and Schubert [12] scheme the all isolates in the present study were identified as *V. harveyi*. The universal primer produced multibanded fingerprints with bands ranging in size from 300 to 8000 nucleotide base pairs. Isolate from Johore (J4) showed only one band and another isolate from Johore (J8), demonstrated 15 bands, the highest number of bands obtained in the present study. As for Kedah and Terengganu isolates, the universal primer generated band ranging from 9 to 11 and 5 to 11 bands, respectively. But for the Johore isolates,

Table 1: Biochemical and physiological characteristics of *V. harveyi* Isolates from Kedah

Characteristics	1	2	3	4	5	6	7	8	9	10
Gram stain	-/S	-/S	-/S	-/S	-/S	-/S	-/S	-/S	-/S	-/S
Glucose fermentation	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+
Indole formation	+	+	+	+	+	+	+	+	+	+
H_2S formation	-	-	-	-	-	-	-	-	-	-
Luminescence	+	+	+	+	+	+	+	+	+	+
Gelatin liquefaction	-	-	-	-	-	-	-	-	-	-
Arginine dihydrolase	-	-	-	-	-	-	-	-	-	-
Amylase production	+	+	+	+	+	+	+	+	+	+
Colony color on TCBS	G	G	G	G	G	G	G	G	G	G
β Blood hemolysis	+	+	+	+	+	+	+	+	+	+
Sensitivity to vibriostat 0/129	+	+	+	+	+	+	+	+	+	+
Oxidase and fermentation	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Phenylalanine deaminase	-	-	-	-	-	-	-	-	-	-
Fermentation to acid										
Glucose	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-	-	-	-
Growth at										
4°C	-	-	-	-	-	-	-	-	-	-
28°C	+	+	+	+	+	+	+	+	+	+
37°C	+	+	+	+	+	+	+	+	+	+
55°C	-	-	-	-	-	-	-	-	-	-
Growth in NaCl at										
0%	+	+	+	+	+	+	+	+	+	+
0.5%	+	+	+	+	+	+	+	+	+	+
1%	+	+	+	+	+	+	+	+	+	+
3%	+	+	+	+	+	+	+	+	+	+
5%	+	+	+	+	+	+	+	+	+	+
7%	-	-	-	-	-	-	-	+	+	+
9%	-	-	-	-	-	-	-	-	-	-
10%	-	-	-	-	-	-	-	-	-	-
11%	-	-	-	-	-	-	-	-	-	-
Utilization of										
Citrate	-	-	-	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+	+	+
Casein	+	+	+	+	+	+	+	+	+	+
Lipid	+	+	+	+	+	+	+	+	+	+
Glycine	+	+	+	+	+	+	+	+	+	+
L-Arginine	-	-	-	-	-	-	-	-	-	-
L-Tyrosine	+	+	+	+	+	+	+	+	+	+
L-Serine	+	+	+	+	+	+	+	+	+	+
Acetate	-	-	-	-	-	-	-	-	-	-
Identification	Vh	Vh	Vh	Vh	Vh	Vh	Vh	Vh	Vh	Vh

Vh = *Vibrio harveyi*, + = positive, -= negative, G = Green, Y = Yellow, S = Short rod

Table 2: Biochemical and physiological characteristics of *V. harveyi* Isolates from Terengganu

Characteristics	1	2	3	4	5	6	7	8	9	10
Gram stain	-/S	-/S	-/S	-/S	-/S	-/S	-/S	-/S	-/S	-/S
Glucose fermentation	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+
Indole formation	+	+	+	+	+	+	+	+	+	+
H_2S formation	-	-	-	-	-	-	-	-	-	-
Luminescence	+	+	+	+	+	+	+	+	+	+
Gelatin liquefaction	-	-	-	-	-	-	-	-	-	-
Arginine dihydrolase	-	-	-	-	-	-	-	-	-	-
Amylase production	+	+	+	+	+	+	+	+	+	+
Colony color on TCBS	G	G	G	G	G	G	G	G	G	Y
Β Blood hemolysis	+	+	+	+	+	+	+	+	+	+
Sensitivity to vibriostat 0/129	+	+	+	+	+	+	+	+	+	+
Oxidase and fermentation	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Phenylalanine Deaminase	-	-	-	-	-	-	-	-	-	-
Fermentation to acid										
Glucose	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	-
Lactose	-	-	-	-	-	-	-	-	-	-
Growth at										
4°C	-	-	-	-	-	-	-	-	-	-
28°C	+	+	+	+	+	+	+	+	+	+
37°C	+	+	+	+	+	+	+	+	+	+
55°C	-	-	-	-	-	-	-	-	-	-
Growth in NaCl at										
0%	+	+	+	+	+	+	+	+	+	+
0.5%	+	+	+	+	+	+	+	+	+	+
1%	+	+	+	+	+	+	+	+	+	+
3%	+	+	+	+	+	+	+	+	+	+
5%	+	+	+	+	+	+	+	+	+	+
7%	+	-	+	-	-	-	-	-	-	-
9%	-	-	+	-	-	-	-	-	-	-
10%	-	-	+/w	-	-	-	-	-	-	-
11%	-	-	-	-	-	-	-	-	-	-
Utilization of										
Citrate	-	-	-	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+	+	-
Casein	+	+	+	+	+	+	+	+	+	+
Lipid	+	+	+	+	+	+	+	+	+	+
Glycine	+	+	+	+	+	+	+	+	+	+
L-Arginine	-	-	-	-	-	-	-	-	-	-
L-Tyrosine	+	+	+	+	+	+	+	+	+	+
L-Serine	+	+	+	+	+	+	+	+	+	+
Acetate	-	-	-	-	-	-	-	-	-	-
Identification	Vh	Vh	Vh	Vh	Vh	Vh	Vh	Vh	Vh	Vh

Vh = *Vibrio harveyi*, + = positive, -= negative, G = Green, Y = Yellow, S = Short rod, W = weak

Table 3: Biochemical and physiological characteristics of *V. harveyi* Isolates from Johore

Characteristics	1	2	3	4	5	6	7	8	9	10
Gram stain	-/S	-/S	-/S	-/S	-/S	-/S	-/S	-/S	-/S	-/S
Glucose fermentation	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+
Indole formation	+	+	+	+	+	+	+	+	+	+
H_2S formation	-	-	-	-	-	-	-	-	-	-
Luminescence	+	+	+	+	+	+	+	+	+	+
Gelatin liquefaction	-	-	-	-	-	-	-	-	-	-
Arginine dihydrolase	-	-	-	-	-	-	-	-	-	-
Amylase production	+	+	+	+	+	+	+	+	+	+
Colony color on TCBS	G	G	G	G	Y	G	G	Y	G	G
β Blood hemolysis	+	+	+	+	+	+	+	+	+	+
Sensitivity to vibriostat 0/129	+	+	+	+	+	+	+	+	+	+
Oxidase and fermentation	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Phenylalanine Deaminase	-	-	-	-	-	-	-	-	-	-
Fermentation to acid										
Glucose	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	-	+	+	-	+	+
Lactose	-	-	-	-	-	-	-	-	-	-
Growth at										
4°C	-	-	-	-	-	-	-	-	-	-
28°C	+	+	+	+	+	+	+	+	+	+
37°C	+	+	+	+	+	+	+	+	+	+
55°C	-	-	-	-	-	-	-	-	-	-
Growth in NaCl at										
0%	+	+	+	+	+	+	+	+	+	+
0.5%	+	+	+	+	+	+	+	+	+	+
1%	+	+	+	+	+	+	+	+	+	+
3%	+	+	+	+	+	+	+	+	+	+
5%	+	+	+	+	+	+	+	+	+	+
7%	-	-	-	-	-	-	-	-	-	-
9%	-	-	-	-	-	-	-	-	-	-
10%	-	-	-	-	-	-	-	-	-	-
11%	-	-	-	-	-	-	-	-	-	-
Utilization of										
Citrate	-	-	-	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	-	+	+	-	+	+
Casein	+	+	+	+	+	+	+	+	+	+
Lipid	+	+	+	+	+	+	+	+	+	+
Glycine	+	+	+	+	+	+	+	+	+	+
L-Arginine	-	-	-	-	-	-	-	-	-	-
L-Tyrosine	+	+	+	+	+	+	+	+	+	+
L-Serine	+	+	+	+	+	+	+	+	+	+
Acetate	-	-	-	-	-	-	-	-	-	-
Identification	Vh	Vh	Vh	Vh	Vh	Vh	Vh	Vh	Vh	Vh

Vh = *Vibrio harveyi*, + = positive, -= negative, G = Green, Y = Yellow, S = Short rod

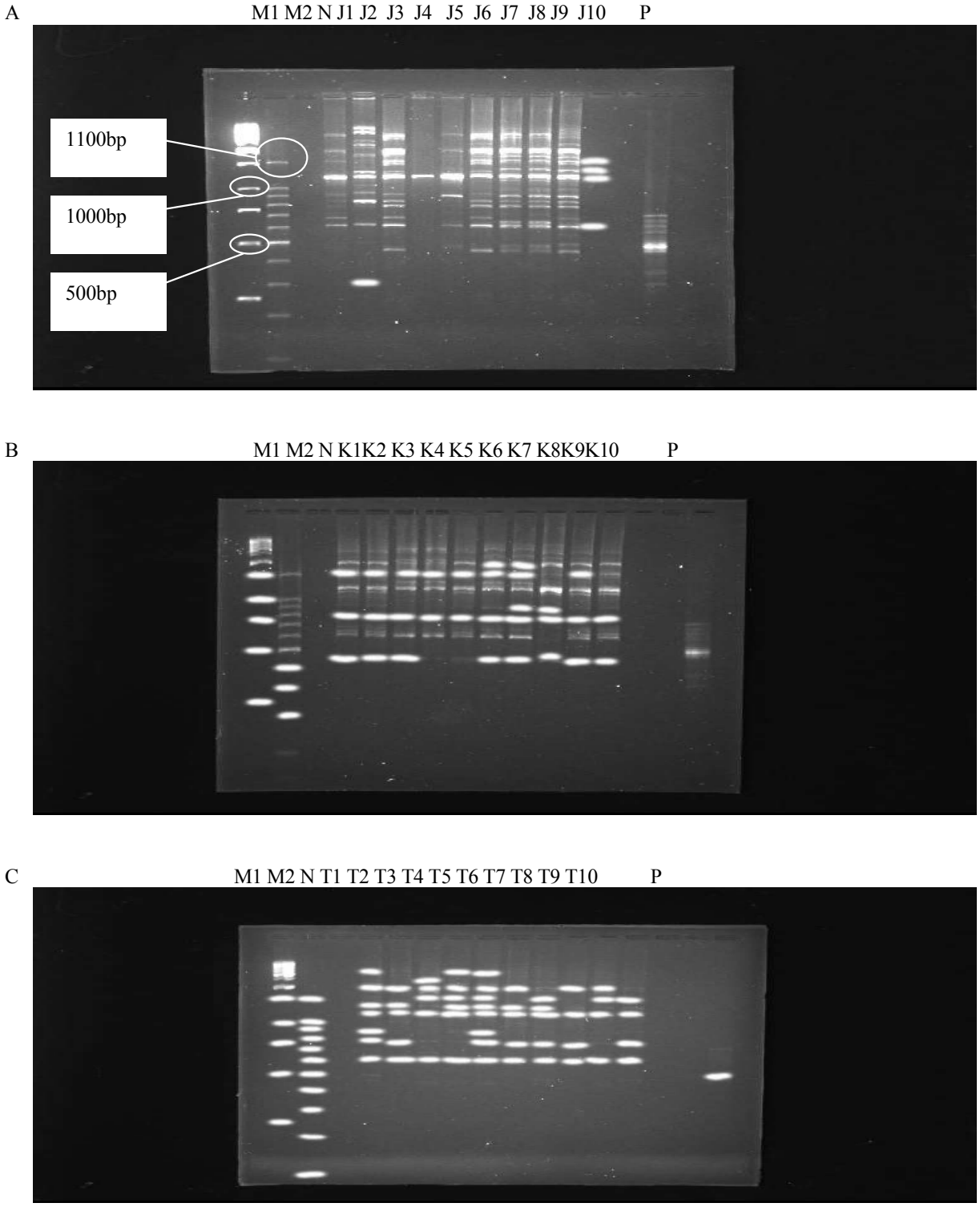

Fig. 1: (A-C) RAPD-PCR profiles of 30 bacterial strains generated after amplification with universal primer. M1=1 kb marker, M2=100 bp marker, N=Negative control, P=Positive control, A=Johor isolates, B=Kedah isolates, C=Terengganu isolates

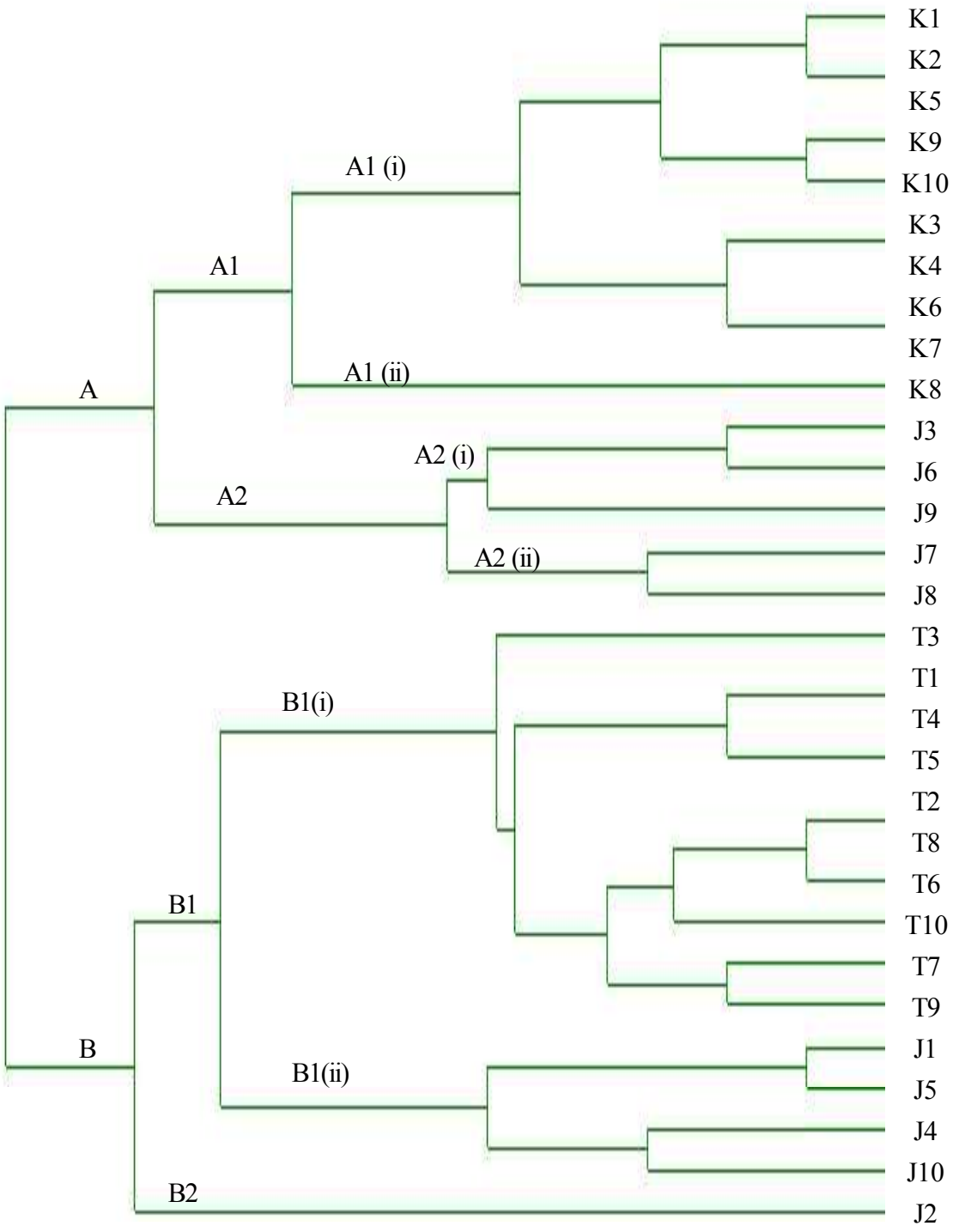

Fig. 2: Dendrogram of Kedah's isolates (K1-K10), Terengganu's isolates (T1-T10) and Johore's isolates (J1-J10) of *V. harveyi*

Table 4: The genetic distance between isolates

	Kedah	Terengganu	Johore
Kedah	0.000-0.500	0.333-0.857	0.111-1.000
Terengganu	0.333-0.857	0.000-0.333	0.067-0.818
Johore	0.111-1.000	0.067-0.818	0.100-0.875

Table 5: The percentage of similarity, (%F) between isolates

	Kedah	Terengganu	Johore
Kedah	50.0-100%	14.3-66.7%	0.0-88.9%
Terengganu	14.3-66.7%	66.7-100%	18.2-93.3%
Johore	0.0-88.9%	18.2-93.3%	12.5-90.0%

Table 6: The susceptibility of antibiotics to the bacterial isolates

Isolate	AM	C	K	NA	RL	TE	FR
K1	R	S	S	S	R	S	R
K2	R	S	S	S	R	S	S
K3	R	I	I	S	I	S	S
K4	R	S	S	I	R	S	S
K5	R	S	I	S	R	S	S
K6	R	S	S	S	S	S	S
K7	R	S	I	S	R	S	S
K8	R	S	I	S	R	S	S
K9	R	S	S	S	S	S	S
K10	R	S	S	S	S	S	S
T1	R	S	S	S	R	S	S
T2	R	S	I	S	S	S	S
T3	R	S	S	S	R	S	S
T4	R	S	S	S	R	S	S
T5	R	S	S	S	S	S	S
T6	R	S	I	S	R	S	S
T7	R	S	S	S	R	S	S
T8	R	S	S	S	R	S	S
T9	R	S	S	S	S	S	S
T10	R	S	S	S	S	S	S
J1	R	S	S	S	S	S	S
J2	S	S	S	S	S	S	S
J3	R	S	S	I	S	R	S
J4	R	S	S	S	S	S	S
J5	S	S	S	S	S	S	S
J6	R	S	S	S	S	S	S
J7	R	S	S	S	S	S	S
J8	R	S	S	S	S	S	S
J9	R	S	S	S	S	S	S
J10	S	S	S	S	S	S	S

AM = Ampicillin 10 µg/disk, K = Kanamycin 30 µg/disk, TE = Tetracycline 30 µg/disk, NA = Nalidixic Acid 30 µg/disk, FR = Furazolidone 15 µg/disk, RL = Sulphamethoxazole 25 µg/disk, C = Chloramphenicol 30 µg/disk, J = Johore; J1, J2, J3, J4, J5, J6, J7, J8, J9, J10, K = Kedah; K1, K2, K3, K4, K5, K6, K7, K8, K9, K10, T = Terengganu; T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, R = Resistant, I = Intermediate, S = Sensitive

the universal primer generated a wide range of bands from 1 until 15 bands. NTSYSpc programme analysis separated the 30 *Vibrio harveyi* isolates from three states into two distinct clusters (A and B) (Fig. 3). The A cluster was divided into two subclusters (A1 and A2). A1 was divided into 2 groups; A1 (i) and A1 (ii). The A1 (i) was subdivided into 3 small subgroups. The first subgroup comprised isolates K1, K2 and K5. K2 and K5 showed similar fingerprint pattern. The second subgroup included only K9 and K10 isolates. The third subgroup consisted of K3, K4, K6 and K7. K3 and K4 were identified as same strain since both isolates showed similar fingerprint pattern. K6 and K7 also demonstrated similar fingerprint pattern. The A1 (ii) group was only consisted of K8 alone in its own cluster. The A2 subcluster was also consisted of 2 groups; A2 (i) and A2 (ii). The A2 (i) was subdivided into 2 small subgroups. The first subgroup consisted of J3 and J6. The second subgroup was J9 alone in its own cluster. And the A2 (ii) group was only consisted of J7 and J8 isolates. As forhe B cluster, it was divided into two subclusters (B1 and B2); B1 was consisted of 2 groups; B1 (i) and B1 (ii), while, B2 was only consisted of one isolate from Johore (J2) alone in its own cluster. The B1 (i) was subdivided into five subgroups. The first subgroup was only consisted of T3. The second subgroup included T1, T4 and T5. The members of the third subgroup were consisted of T2, T8 and T6. T10 was alone in the fourth subgroup and the fifth subgroup was comprised of T7 and T9. The Table 4 and Table 5 showed Nei and Li's [19] genetic distance and percentage of similarity between the thirty isolates in the present study. Both genetic distance and percentage of similarity were invertly correlated. The percentage of similarity among Kedah isolates ranged from 50 to 100% whilst genetic distance was ranged from 0 to 0.5. The isolates from Terengganu showed the lowest range of percentage of similarity and genetic distance recorded from 66.7 to 100% and 0 to 0.333, respectively, in the present study. Meanwhile, isolates from Johore showed the highest range of percentage of similarity and genetic distance from 12.5 to 90% and 0.1 to 0.875, respectively. The percentage of similarity between isolates from Kedah compared to isolates from Terengganu and Johore were ranged from 14.3 to 66.7% and 0 to 88.9%, respectively. On the other hand, comparison between isolates from Terengganu and isolates from Johore showing rather high percentage of genetic similarity ranging from 18.2 to 93.3%.

A total of 30 *V. harveyi* isolates were tested with seven types of antimicrobials (Table 6). The susceptibility of antibiotics to the bacterial strains was reported in 173 cases (82.4%), 10 cases (4.8%) were determined as the

Table 7: The total susceptibility rates of antibiotics

Antibiotic	S	%	I	%	R	%
AM	3	10.0	-	-	27	90.0
C	29	96.7	1	3.3	-	-
K	24	80.0	6	20.0	-	-
NA	28	93.4	2	6.6	-	-
RL	17	56.7	1	3.3	12	40.0
TE	29	96.7	-	-	1	3.3
FZ	29	96.7	-	-	1	3.3

AM = Ampicillin 10 µg/disk, K = Kanamycin 30 µg/disk, TE = Tetracycline 30 µg/disk, NA = Nalidixic Acid 30 µg/disk, FZ = Furazolidone 15 µg/disk, RL = Sulphamethoxazole 25 µg/disk, C = Chloramphenicol 30 µg/disk, R = Resistant, I = Intermediate, S = Sensitive

intermediate sensitive and 27 cases (12.8%) showed that the bacterial species were resistant to the certain antibiotics. Table 7 showed the total of susceptibility rates of antibiotic. The isolates were mostly resistant to ampicilin and sulphamethoxazole; 90% and 40%, respectively. *V. harveyi* (96.7%) were demonstrated to be sensitive to chloramphenicol, tetracycline and furazolidone. Sensitive results were also available for nalidixid acid on 28 isolates (93.4%), kanamycin on 24 isolates (80%), sulphamethoxazole on 17 isolates (56.7%) and ampicilin on 3 isolates (10%). Kanamycin and nalidixic acid intermediate sensitive rates were 6 isolates (20%) and 2 isolates (6.6%), respectively.

DISCUSSION

The purpose of this study was to describe morphological, biochemical and physiological characteristics of *V. harveyi* colonizing hepatopancreas of tiger shrimp from commercial tiger shrimp farms in Kedah, Terengganu and Johore, Malaysia. Based on the morphological appearances, some similarities were observed among the isolates from Kedah, Terengganu and Johore. A total of twenty four isolates, they were eight isolates from Johor, six isolates from Terengganu and all isolates from Kedah have dominant characteristics based on their single colony morphological appearances as entire margin, circular form, convex elevation and yellow color on TSA plate. Another six isolates, they are four isolates from Terengganu (T1, T2, T3 and T4) and two isolates from Johor (J2 and J10) showed similar single colony morphological appearance as irregular form, undulated margins and raised elevation. However, they have different color on TSA plate. Isolates T1, T3 and T4 appeared white color but T2, J2 and J10 appeared yellow color on TSA plate. All the isolates from three locations were luminous, Gram negative and possess short rod cell morphology; these morphological appearances were similar to isolated *V. harveyi* from *P. monodon* juveniles in Philippines and Southern of Thailand described by Leano *et al.* [20] and Ruangsri *et al*. [21], respectively. This finding has shown although isolates from different locations but they have similar colony morphological appearances. According to Hernandez and Olmos [22], phenotypes of bacterium are related to its genotypes properties. This is because the genomic of a bacterium is responsible to phenotypic a bacterium. However, various environment factors are also contribute to characterize phenotypes of a bacterium. According to Leano *et al*. [20], *V. harveyi* isolates from *P. monodon* juveniles in Philippines were positive for both oxidative and fermentative tests. The finding by Leano *et al.* [22] was also similar to the result of this study. However, based on the study by Tendencia [23] showed that *V. harveyi* isolates from seabass, *Lates calcarifer*, in Philippines positive only for fermentative test but not for oxidative test. On the other hand, Baumann and Schubert [12] showed that most of the *V. harveyi* strains were positive to both oxidase and fermentative tests. Thus, based on the oxidase and fermentative tests result; it is clearly showed that *V. harveyi* isolates of the present study and Leano *et al.* [20] possessed similar characteristics. Majority of *V. harveyi* isolates from three states showed similar biochemical and physiological characteristics except in colony color differentiation on TCBS, ability to ferment sucrose and tolerance level to sodium chloride concentration. Majority of the isolates (90%) were green colony on TCBS indicating the ability to ferment sucrose. In the present study, isolates that were yellow color on TCBS was observed unable to ferment sucrose. These result was in contrast to the finding of Ruangsri *et al.* [21] where *V. harveyi* isolated from shrimp were observed to be both green and yellow colony color on TCBS agar but they were able to ferment sucrose. Another finding by Suwanto *et al.* [2] showed that all isolates from shrimp that were green color on TCBS were unable to ferment sucrose. However, the findings by Tendencia [23] and Alcaide *et al*. [24] showed that *V. harveyi* isolated from cage-cultured seabass *Lates calcarifer* Bloch in Philippines and from seahorse *Hippocampus* sp. in Spain, respectively had yellow colony color on TCBS agar and those isolates were unable to ferment sucrose, similar to the finding of the present study. Jahreis *et al*. [25] stated that bacteria were able to utilize sucrose possessed gene *csc B* in their genomic DNA. Thus, in the present study, isolates that yellow in color on TCBS may be absent the gene that enable isolates to utilize sucrose, on the other

hand, isolates that green in color on TCBS may possess gene *csc B* in their genomic DNA. Majority of isolates in this study was tolerant to sodium chloride concentration ranging from 0 to 7% but only one isolate from Terengganu could grow weakly up to 10% of sodium chloride concentration. Based on the research conducted by Ruangsri *et al*. [21], ten isolates of *Vibrio harveyi* were isolated from black tiger shrimp in Southern Thailand; only one isolate could grow weakly up to 10% of sodium chloride concentration and the rest of the isolates were found to be tolerant to sodium chloride concentration ranging from 2% to 8%. Suwanto *et al*. [2] and Ruangsri *et al.* [21] reported that the growth of isolates from Java Island, Indonesia and Southern Thailand, respectively, was inhibited on media without sodium chloride supplementation; however, all isolates from three states in Malaysia could grow well on media without supplementation with sodium chloride. Beside that, another finding of Tendencia [23] showed that *V. harveyi* isolates from cage-cultured seabass in Philippines showed growth inhibition on the media without sodium chloride supplementation. Thus, the present study showed that local isolates possessed a distinct growth characteristic towards salt tolerance compared to isolates from Java Island, Indonesia, Southern Thailand and Philippines. According to Baumann and Schubert [12], 90% or more strains of *V. harveyi* possessed lipase enzyme in order to hydrolase lipid for obtaining energy to grow. The isolates from this study were found to be positive for lipid and casein tests. According to Tendencia [23], *V. harveyi* isolates from seabass were able to hydrolase lipid. But another important finding was that 17 out of 19 isolates of virulent *V. harveyi* were able to utilize lipid and 16 out of 19 isolates were able to hydrolase casein [26]. In this study, all isolates from three states in Malaysia were able to utilize lipid and casein. This may indicate that those virulent *V. harveyi* were able to utilize lipid and casein to obtain energy as well as enable to utilize other substrates such as glucose and sucrose like other non-virulent isolates. In this study, the phenotypes of isolates showed almost 50% similarity to the isolates from Java Island, Indonesia and Southern Thailand based on the finding of Suwanto *et al*. [2] and Ruangsri *et al*. [21], respectively. All *Vibrio harveyi* isolates in this study as well as reported results from Java Island and Southern Thailand were positive for oxidase, catalase and motility tests. They were also able to utilize glucose, starch, tryptophan and sensitive to vibriostat 0/129 150 µg/disk. All isolates were sensitive to vibriostat indicating confirmed grouping to the Vibrionaceae. Another study conducted by Abraham and Palaniappan [9] showed that *V. harveyi* isolates from semi intensive penaeid shrimp hatcheries in India were also positive for motility, utilization of glucose, starch and tryptophan. The study clearly showed that those isolates were able to obtain energy sources from glucose, starch and tryptophan. However, the isolates from two countries (Java Island, Indonesia and Southern Thailand) as well as the isolates in present study were unable to obtain energy through arginine hydrolysis based on the negative result for the arginine hydrolysis test. Different characteristics between the isolates obtained from the present study as compared to the isolates from Java Island and Southern Thailand were that isolates from the present study could not utilize citrate and unable to produce hydrogen sulfide. On the other hand, all isolates from Java Island [2] and Southern Thailand [21] were positive for both citrate utilization and hydrogen sulfide production tests. Isolates from tiger shrimp hatchery in India [9] were also able to utilize citrate and produce hydrogen sulfide. Beside that, isolates from those three countries (Java Island, Indonesia, Southern Thailand and India) were positive for gelatin liquefaction test. Thus, the phenotypes of the isolates in this study showed staring differentiation in terms of citrate utilization and H_2S production as compared to the isolates from Java Island, Southern Thailand and India. In the present study, the isolates were observed to grow well at the temperatures of 28°C and 37°C but the growth was inhibited at the temperatures of 4°C and 55°C. The finding of Suwanto *et al*. [2] has shown similar result. However, according to research conducted by Ruangsri *et al.* [21], one out of ten isolates from Southern Thailand could grow at a temperature of 4°C. By referring to Baumann and Schubert [12], growth of *V. harveyi* was observed to be inhibited at the temperatures of 4°C, 30°C and 35°C. Thus, all isolates in this study fell into a normal range of temperatures for *V. harveyi* to grow. Suwanto *et al.* [2] reported that a total of fifty five isolates of *V. harveyi* from Java Island were positive for L-Arginine decarboxylase, L-Tyrosine decarboxylase, L-Serine decarboxylase, Glycine decarboxylase and Acetate decarboxylase. However, the isolates from the present study were only able to decarboxylase three out of five mentioned amino acids; they were L-Serine, L-Tyrosine and Glycine. Another important finding in this study was that isolates were unable to utilize phenylalanine. Similar characteristics were also observed between these isolates and findings of Tendencia [23] where the isolated *V. harveyi* from cage-cultured seabass in Philippines were negative for L-Arginine decarboxylase test. According to Baunmann and Schubert [12], strains of *V. harveyi* were able to utilize L-Arginine, L-Serine, L-Tyrosine, acetate and glycine but unable to utilize

phenylalanine. Another important finding of this study was that all the 30 isolates (100%) showed β haemolytic activity against horse erythrocytes. According to Pollack *et al.* [18], β haemolytic activity refers to the ability to lyse the whole cell of erythrocytes. The study of Zhang and Austin [26] described those bacterial hemolysins especially in Vibrios could be one of important pathogenic factors due to the fact that they could cause hemorrhagic septicemia and diarrhea in the host (fish and human). Beside that, the study of Zhang and Austin [26] showed that isolated *V. harveyi* obtained from a diversity of hosts and geographical locations were also able to lyse erythrocytes from donkey, rabbit and sheep. The study of Harding [27] showed that pathogenicity and luminescence of *V. harveyi* may be interlinked and controlled by quorum sensing. Thus, all the *V. harveyi* isolates in the present study was considered virulent based on their ability to lyse red blood cells and luminous characteristic. The hepatopancreas of shrimps reportedly to be the main target organ of most bacterial pathogens [20]. According to Soto-Rodriguez *et al.* [28], Vibrios such as *V. harveyi* that implicated vibriosis were usually found in hepatopancreas and haemolymph of shrimp. Morphological, biochemical and physiological profiles of *V. harveyi* isolates in the present study indicated that *V. harveyi* have various phenotypic characteristics. This finding was in agreement with a statement of Nealson *et al*. [29] who stated that the luminous bacteria is complex and could exhibit a variety of lifestyles.

RAPD can be applied as a tool to generate genetic fingerprint and genetic relationship database for bacteria. It is important for epidemiological investigation of during disease outbreak and tracing the source of infection. In addition, RAPD analysis can also be used to assist treatment of bacterial diseases, whereby the similar treatment can be applied for bacteria showing clonal similarity. The total number of DNA fragment amplified depended on the length of the primer used; shorter primer has a higher chance of annealing at more than one complementary site within the genome [30]. As the matter of facts, the size of primer applied in the present study was 15-mer 5'GTGGTGGTGGTGGTG3'. The primer produced 1 to 15 DNA fragments ranging from 400 to 10000 bp. Somarny *et al.* [31] amplified 1 to 10 DNA fragments of 250 to 6000 bp by using 10-mer primer (12 OPAE) (Operon Technologies, USA). Furthermore, Haim *et al*. [32] suggested that $(GTG)_5$-PCR was useful for identification of *Vibrio* species bacteria. This study generated a large number of polymorphic bands among the isolates from Kedah, Terengganu and Johore. However, among isolates from each state showed monomorphic bands. For instance, all Kedah's isolates possessed two monomorphic bands 700 bp and the band located between 1100 bp to 900bp, respectively. Terengganu's isolates also possessed two monomorphic bands 500 bp and the band located between 1100 bp to 900 bp, respectively. However, all Johore's isolates possessed only a monomorphic band which located between 1100 bp to 900 bp. According to Rus-Kortekaas *et al*. [33], some bacteria share the bands of same size, known as monomorphic band. The bands are considered as polymorphic when they are present in some sample but absent in others. In the present study, only one monomorphic band with the size between 1100 bp to 900 bp was observed among the 30 isolates. In the present study, DNA polymorphism was observed in 22 out of 30 isolates, revealing genetic heterogenecities of *V. harveyi*. Another significant finding of this study was that the isolates from Johore showed no correlation between genetic and geographical distance. For instance, 50% isolates from Johore were clustered together with isolates from Kedah while the rest of them were clustered with isolates from Terengganu. In addition, isolates from Johore showed high percentage of similarity ranging from 12.5% to 90%. Therefore, the isolates from Johore in the present study exhibited high degree of genetic diversity since they possessed the highest genetic distance among each other in spite of the fact they were isolated from same shrimp farm. This finding was in contrast to the study by Somarny *et al*. [31] showed that although five isolates of *V. harveyi* were isolated from different sources but those isolates possessed lower genetic distance to each other as compared to the isolates from Johore in the present study. However, dendrogram in the study of Calcagno *et al*. [34] showed *Paracoccidioides brasiliensis* isolated from Venezuela and Brazil was grouped together with isolates from Colombia. Thus, it is assumed that bacteria can possess a highly genetic variation in the same niche. In this study, the genetic distance among 3 locations (Kedah, Terengganu and Johore) was more than 0.5 although they were located nearly 400 km far away than one another. Therefore there was a very little likelihood of bacterial mobility from Kedah and Terengganu to Johore or vice versa. Furthermore, Kedah and Terengganu were separated by Titiwangsa mountain range that divided Peninsular Malaysia into two parts, i.e. East coast and West coast. The study conducted by Somarny *et al.* [31] showed that *V. harveyi* isolates from Banting and Pulau Carey in Selangor, were close in genetic distance as both places located about 50 km to each other. On the other hand, Kerpan and Serkam in Kedah were far in genetic distance

as both were far in geographical location. Another study by Goarant *et al*. [35] showed that *Vibrio penaeicida* isolates performed heterogeneity according to their geographical origin, New Caledonia and Japan. In spite of the presence and absence of some DNA fragments between the isolates from both places, although, seven different types of 18 mers primers (KF, KN, RSP, KZ, KG, SP and KpnR) (Genset, Paris) were used, similar results were observed discriminated the isolates originating from Japan and those from New Caledonia. According to Versalovic *et al*. [36], it is recommended that at least a minimum of 8 to 15 bands per sample to be used for a rigorous comparative analysis. However, many researches on RAPD-PCR in *Vibrio* sp. showed low number of bands per sample. A study of Sudheesh *et al*. [37] revealed that the number of bands produced by seven OPD 10-mers primers (Operon Technologies, USA) amplifying the genomic DNA of 25 isolates of *V. alginolyticus* and *V. parahaemolyticus* was ranging from 0 to 11. Another study conducted by Somarny *et al.* [31] demonstrated the number of DNA fragments amplified from a given sampled ranged from 1 to 10 by using 12 OPAE 10-mers (Operon Technologies, USA) primers on genomic characterization of five *V. harveyi* isolates. Many studies related to RAPD-PCR analysis applied only one primer. According to Gillespie *et al*. [38], a 10-mer primer OPE-04 (Operon Technologies, USA) (5'-GTGACATGCC-3') was able to identify *Streptococcus* and *Enterococcus* according to their species. Another study of Ertas *et al.* [39] used a random 11-mer primer OPA-11 (Fermentas, USA) to reveal genetic diversity of *Campylobacter jejuni* and *E. coli*. A study of Krawczyk *et al.* [40] used a primer RAPD-4 (5'-AAGAGCCCGT-3') (RAPD Analysis Primer Set, Pharmacia Biotech) as a tool to access genetic property of *Serratia marcescens* isolates from three outbreaks ongoing in the Public Hospital in Gdansk, Poland. Furthermore, the study of Sesena *et al.* [41] was only using a 9-mer primer OPL-05 (5'-ACGCAGGCA-3') (Sabadell, Spain) to assess genetic diversity of 323 strains of *Lactobacilli* isolated from an Almargo eggplant manufacturing plant. A 10-mer primer OPM-01 (5'-GTTGGTGGCT-3') (Operon Technologies, USA) was used to generate RAPD PCR profiles for 91 strains of *Listeria monocytogenes* from raw milk, food and veterinary, medical and food-environmental sources [42]. Thus it is no doubt to use only one primer as a tool to assess genetic of bacteria in a study. Furthermore, the $(GTG)_5$ primer that applied in the present study was commonly used to study genetic relationship among *Vibrio* species [32].

Vibrio harveyi has been implicated as the casual agent for "luminous" disease or vibriosis in shrimps [4]. To treat this disease, the shrimp farmers preferred to use β-lactam antibiotics such as ampicillin since these groups of antibiotics did not cause significant side-effects [43]. Unfortunately, many types of β-lactam antibiotics were no longer able to prevent vibriosis [43]. In addition, many *V. harveyi* strains also showed resistance to multiple antibiotics such as tetracycline, chloramphenicol, streptomycin and spectinomycin [8]. According to Shariff *et al.* [8], the common antimicrobial used in aquaculture in Malaysia are sulphamethoxazole, tetracycline, furazolidone, chlorampenicol, oxolinic acid and nalidixic acid. Among the stated antimicrobials, sulphamethoxazole is normally applied in hatcheries against *Vibrio* sp. The present study demonstrated that up to 90% of the isolates were resistant to ampicilin. This result was similar to the finding of Otta *et al*. [44] where 92% of isolated *V. harv*eyi from *P. monodon* hatcheries in India were resistant to ampicilin. Ampicilin is categorized as β broad spectrum beta lactam antibiotic and it functions as inhibitors of bacteria cell wall biosynthesis [45]. Thus, the finding of the present study indicated that most of the isolated *V. harveyi* in Malaysia possessed β lactamases enzyme to overcome the β lactam antibiotics such as ampicilin. This study supports the statement of Molina-Aja *et al*. [46] that β-lactam resistance is now widespread in vibrios isolated from a variety of location and sources. The isolates in the present study were found to be highly sensitive to tetracycline based on the observation of largest average of inhibition zone and the highest percentage of total isolates resistant against this antibiotic. The study of Otta *et al*. [44] showed 97% isolates from shrimp hatcheries in India demonstrate highly sensitive against tetracycline. Tetracycline is known as non-β lactam antibiotic that inhibit protein synthesis thus prevent the growth of bacteria [45]. Nalidixic acid belongs to a group of broad spectrum antibiotics called the quinolones [45]. It works by entering the bacterial cell and inhibiting a chemical called DNA-gyrase which is involved in the production of genetic material (DNA) [45]. As a matter of fact, nalidixic acid prevents the bacteria from reproducing and their growth is stopped. Based on Otta *et al.* [44] studies, 80% of a total 87 isolates of *Vibrio* spp. including *V. harveyi* isolated from tiger shrimp hatcheries in India were sensitive to nalidixic acid and none of the isolates showed resistant to this type of antibiotic. The finding is similar to the present study, where up to 96% of isolates demonstrated sensitive and only 6% performed intermediate sensitive to this type of antibiotic.

Kanamycin is an aminoglycoside antibiotic and responsible in inhibition of protein synthesis [45]. Eighty six percent of a total 87 isolates from India were sensitive to kanamycin and 13% showed resistant to this antibiotic [44]. On the other hand, the present study demonstrated that none of the isolates were resistant to this type of antibiotic; 80% of the isolates were sensitive and only 20% were intermediate sensitive. Sulphamethoxazole is a non-β lactam antibiotic but is an anti-metabolites antibiotic [45]. It functions by interfering with enzyme in the metabolites system, thus, metabolites could not occur in the bacteria cell; therefore, the bacterial growth would be inhibited. Ottaviani *et al*. [47] showed that 66.7% *V. harveyi* isolates were resistant to sulphamethoxazole and the rest 33.3% isolates were intermediate sensitive. On the other hand, the isolates in the present study demonstrated 36.7% resistant but majority performed sensitive to this antibiotic. Chloramphenicol is known as polypeptides antibiotic [45]. It plays inhibition role of protein synthesis in bacteria. Another important finding of Otta *et al.* [44] showed that 87 isolates from India's tiger shrimp hatcheries demonstrated the highest sensitive to chloramphenicol with the same concentration that applied in the present study. In the present study, 96.7% isolates demonstrated sensitive to furazolidone with the concentration 15 µg/disk. Thus, it's clearly showed furazolidone can be applied in local shrimp farming against vibriosis due to *V. harveyi*. Furazolidone inhibits a variety of bacterial enzymes, an activity that minimizes the development of resistant organisms. Furazolidone, a synthetic nitrofuran, is active against a broad spectrum of bacteria [45]. A study of Tendencia and De La Pena [10] showed that supplementation of antibiotics to control the luminous vibriosis has become less effective due to the occurrence of bacterial resistance to a number of antibiotics. Thus, alternative method to combat against luminous bacteria that associated with disease must be carried out. For example, a marine bacterial strain, *Pseudomonas* I-2, was found to produce inhibitory compounds against shrimp pathogenic vibrios including *Vibrio harveyi, V. fluvialis, V. parahaemolyticus, V. damsela* and *V. vulnificus* [48]. This bacterial strain can be seeped into aquaculture as a probiotic to control the luminous vibriosis. Another important finding of Selvin and Lipton [49] found that the secondary metabolites of brown seaweed, *Dendrilla nigra*, form an excellent source for developing potent antibacterial agents to combat bacterial diseases of shrimp and replace the conventional antibiotics. Furthermore, the crude fucoidan from brown seaweed in Thailand also demonstrated the ability to inhibit the growth of *V. harveyi* [50]. Therefore, it is crucial to understand the basis of antibiotic resistance in this microorganism that is associated with shrimp larvae and shrimps. The results of this study should be able to provide basic research for shrimp farmers that are publishable and in addition, strategies such as incorporated shrimp feed with antibiotic and determination of Minimum Inhibition Concentration (MIC) of antibiotic against vibriosis due to *V. harveyi* can be developed to prevent the occurrence of vibriosis.

CONCLUSION

Conventional biochemical and physiological tests were successfully identified 30 luminous bacteria isolated from tiger shrimp's hepatopancreas as *Vibrio harveyi*. Thus, this method can be applied in the shrimp farm or elsewhere for diagnosis work. Furthermore, this method is inexpensive and need less equipment compared to other method. It can be concluded that *V. harveyi* isolated from three states of Peninsular of Malaysia (Kedah, Terengganu and Johore) exhibited high degree of genetic diversity and strain variation as revealed by the present study. RAPD-PCR was indeed a very useful tool to reveal the level of genetic variation among the same strain of bacteria. Luminous bacteria from shrimp cultures in Terengganu, Kedah and Johore were resistant to ampicilin. Tetracycline, nalidixic acid, furazolidone, chloramphenicol, kanamycin sulphamethoxazole were found can be used to against *V. harveyi*. However, five antibiotics namely chloramphenicol, oxolinic acid, tetracycline, oxytetracycline and nitrofuroin have been banned for use in Malaysia's aquaculture.

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