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A Novel of Gut Pathogenic Bacteria of Blue Swimming Crab *Portunus pelagicus* (Linneaus, 1758) and Pathogenicity of *Vibrio harveyi* a Transmission Agent in Larval Culture under Hatchery Conditions

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Abstract: The cause of mass mortality of *Portunus pelagicus* larvae reared in a hatchery system was investigated. The gut content of 180 female crabs and egg specimen of 24 female were studied for pathogenic microbes. The gut of female crabs were harboring fish pathogenic bacteria includes *Staphylococcus epidermidis*, *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Micrococcus luteus* and *Pseudoalteromonas piscicida* and eggs were found associated with fish pathogens include *Vibrio harveyi*, *Micrococcus luteus* and *Pseudoalteromonas piscicida*. A causative transmitting pathogen *V. harveyi* through the feces of adult female crab and responsible for heavy mortality during larval rearing was determined by examining samples associated with the gut, hatching tanks, eggs, larvae rearing tanks, live and dead larvae of *P. pelagicus*. All isolates were identified by *16S rRNA* gene sequencing. *Vibrio harveyi* was the major pathogen associated with all sources brought under study. Larvae were found to harbor a higher number of bacteria than larvae rearing tank. Experimental challenges with various doses indicated that the *V. harveyi* isolates were highly pathogenic. Doses 10^5 cfu mL^{-1} produced upto 96.67% mortality and 10^6 cfu mL^{-1} resulted in 100% mortality within 24 h post challenge. The differences among various doses of pathogen were statistically significant ($p<0.05$). The presence of these pathogens in *P. pelagicus* beyond the consequence for larval rearing is of epidemiological and health significance to humans.

Key words: *Portunus pelagicus*, larval rearing, *16S rRNA* gene sequencing, pathogen, epidemiological, blue swimming crab

INTRODUCTION

The blue swimming crab, *Portunus pelagicus* (Linneaus, 1758) (Portunidae) also known as flower crab and sand crab is an important source of animal protein and a favorite recipe in South East Asia and other parts of the world.

This crab form the basis of important commercial (including aquaculture) and recreational fisheries for their distribution in coastal and estuarine waters of the Indo-Pacific (Kailola *et al*., 1993) and Indo-West Pacific region (Xiao and Kumar, 2004). *P. pelagicus* is relatively expensive in comperision to the other sea fishes consumed locally as the flesh of this crab is very tasty and juicy. Currently, most of the *P. pelagicus* crabs are largely sourced from capture fisheries that are unreliable and seasonal (Otto and Jamieson, 2003). The increasing demands have led to a growing interest in the aquaculture of this crab. A better understanding of the basic larval culture conditions is necessary to optimize its production (Romano and Zeng, 2006). *P. pelagicus* has high aquaculture potential because it shows fast growth rate (Josileen and Menon, 2005), high fecundity and relatively short larval duration (Romano and Zeng, 2008). The crab culture gained its importance from the beginning of the last decade owing to the great demand of live crabs and crab products in the export market. The crab culture is presently dependent on wild caught seeds that are not sufficient (Fortes, 1999; Keenan, 1999).

Many countries like Japan. Philippines, India, Indonesia, Thailand, Bangladesh, Vietnam, Australia, Malaysia and USA are actively involved in crab culture and research (Soundarapandian *et al*., 2007). However, in most of the countries until date, hatchery seed production of *P. pelagicus* crab has been experimental and commercial seed production technology is not available anywhere at a commercial scale. In recent years, an attempt to establish a commercial hatchery mass seed

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production experiment of *P. pelagicus* crab was conducted by Soundarapandian *et al.* (2007), Maheswarudu *et al.* (2008) and Ikhwanuddin (in press) but the output is still experimental. An effort was made to breed *P. pelagicus* crab in hatcheries but it resulted in almost 100% mortality or substantial very low survival. Reasons for low survival of larvae are still question mark.

Microbial infections have been a major concern of aquaculture worldwide but to date, the literature does not offers any information on pathogenic microbes with the gut, larval rearing system of *P. pelagicus* and their pathogenic role in the larval culture and survival. The indigenous microflora of fish, particularly the microbial ecology of the digestive tract has been investigated by many researchers (Trust and Sparrow, 1974; Horsely, 1977; Austin and Al-Zahrani, 1998; Munro *et al*., 1994; Ringo *et al*., 1995; Spanggaard *et al*., 2000). This is due to its assumed importance in digestion and disease control (Westerdahl *et al*., 1991; Austin *et al*., 1995; Bly *et al*., 1997). Several *Vibrio* sp. and *Pseudomonas* sp. are known to be fatal pathogens in zoeal stages of mud crabs (Jithendran *et al*., 2009). Owing to non-availability of information on scientific documentation for microbial ecology of the gut in this context the aim of the present study was:

- To evaluate the pathogenic bacteria in the gut and to identify the transmission agent *Vibrio harveyi* and bacterial load in the Hatching Tank (HT), eggs, Larval Rearing Tanks (LRT) and larvae of *P. pelagicus*
- To determine the pathogenicity of isolated *V. harveyi* from various sources on larval survival

MATERIALS AND METHODS

Sample collection and sampling site: The present study was conducted in the marine hatchery and the Laboratory of the Institute of Tropical Aquaculture, Universiti Malaysia Terengganu, Malaysia. Every month 20-30 crab samples including mature male, females and berried females (yellow egg mass) were collected from Tebrau Strait, Johor, Malaysia, (1°22'N and 103°38'E) with different Body Weight (BW) and Carapace Width (CW) (for 12 months from December 2009-November 2010). The collected samples were transferred into 40 L round polythene tanks equipped with aeration. Salinity at site was measured between 31-33‰.

To avoid contamination, the samples were kept in same water until they were dissected for microbes study. The mature male, females and berried females were disinfected, transferred to the brood stock tanks and the Hatching Tanks (HT) filled with treated water and equipped with aeration.

Experimental design: A two-factor experimental design was used:

- To evaluate the pathogens associated with the gut, targeted pathogen and bacterial load with HT, eggs, LRT, live and dead larvae
- Pathogenicity tests of targeted pathogen on survival of the larvae of *P. pelagicus*

Maintenance of brood stock: Upon producing eggs, one berried female for each experimental attempt and total eight-berried female of *P. pelagicus* with Body Weight (BW) ranges between 134 and 187 g and Carapace Width (CW) of 124-138 mm were maintained with slight modification according to Mireia Andres *et al.* (2010). Berried females quarantined in a bath of seawater with formalin (120 μL^{-1}) for 4 h following 1 h bath in 2 ppm $KMNO_4$. Following the same, the females were transferred 300 L indoor black circular Hatching Tank (HT) provided with sand substrate (filled with treated seawater) for eggs incubation and hatching with temperature 28-30±2°C, salinity 31±2‰, pH 7.4-8.0, dissolved O_2 6.8-7.9 ppm. Every day the broodstock (berried female) was checked for spawning.

Treated water in the Hatching Tank (HT) was aerated continually. The berried females were not fed; feces, discarded eggs and other waste were siphoned out every day from the tanks, accompanied with about a 50% water exchange (treated). UV treated water was stored in 500 L tank and treated with chlorine for 24 h and supplemented EDTA (after 24 h of chlorine treatment) to settle down the heavy metals and then de-chlorinated with sodium thiosulphate for larval and broodstock use.

Larvae rearing: Under the experimental set up of eight attempts (triplicate), 24 experimental aquaria with capacity 15 L each were filled with 12 L treated bacteria free seawater, 31±2‰ salinity were tested with 12 h light and 12 h darkness for larvae rearing.

The newly hatched, energetic swimming larvae (Zoea I, ZI stage) was collected from the hatching tank, counted and randomly distributed into the experimental aquaria with 20 larvae L^{-1}. Throughout the experiment, the larvae were fed daily with a mixture of live prey composed of 30-40 rotifers mL^{-1} (*Brachionus* sp.) with *Nanochloropsis* sp. (8×10^5 cells mL^{-1}) until day 11 and 5 *Artemia* sp. nauplii mL^{-1} from day 9-13. Rotifers were cultured with live microalgae (*Nanochloropsis* sp.) and harvested every

day for the experiment while *Artemia* nauplii were hatched daily from cysts (Great Lake *Artemia*, Salt Lake City, Utah, USA). The larvae were fed rotifers and *Artemia* without enrichment. Every morning during the experiment, rotifers and *Artemia* were harvested from culture tanks. To determine the density of the harvested stocks, 3×1 mL samples were taken and counted to obtain the average using a Sedgewick-Rafter counter under a microscope. Based on this density, the volumes of rotifer and *Artemia* stocks required to achieve designated diet densities which were calculated and added to larvae culture aquaria. Afterwards, live and dead larvae in each replicate were counted and recorded, 20% water (treated) was exchanged daily. A replicate was terminated when all larvae within the replicate culture aquaria had either molted to megalopa or died. Experiments with all replicates were carried out for 14 days. Physiochemical para meters such as salinity, temperature, pH and O_2 were measured daily on site by using YSI 556 MPS (USA) multi parameter equipment.

Bacteriological study

Segregation of the gut: Prior to segregation of the gut, crab specimens were randomly collected from the sample stock and bathed in 10% formalin for 20 min. Subsequently they were again washed with fresh tap water for 5 min and finally washed with sterile de-mineralized water in order to get rid of surface microflora. Sterile dissecting materials were used for this purpose. Aseptically crab specimens were dissected, the whole gut was removed and pulverized with pestle and mortar vigilantly and mixed with sterile seawater to prepare inoculum. Bacteria isolation and total plate count were carried out by serial dilution (up to 3 fold). Randomly selected 15 female crabs per month and a total 180 female crabs were studied for microbes associated with gut for the present study.

Bacteriological sample collection: During the incubation period of berried females (yellow to black egg mass), water samples from Hatching Tanks (HT) for bacteriology study were taken from day 2-6 and samples from Larvae Rearing Tanks (LRT) were collected at day 2, 4, 6, 8, 9 and 13 and were kept in sterile test tubes. Five live crab larvae and five dead larvae from day 2 to the day 9 were collected and kept in sterile microcfuge tubes. Eggs (5 mg) from different places of spongy egg mass clusters in 24 berried females (2 specimens per month from Dec 2009-Nov. 2010) weighing between 138 and 198 g with Carapace Length (CL) 120-147 mm and Width (CW) 59-68 mm (Table 1) were taken with sterlised forceps. Eggs, live and dead larvae samples were washed thrice with sterile seawater to remove off any adhering particles and microbes then

Table 1: Total Bacterial Counts (TBC) and Total Vibrio Count (TVC) cfu mL^{-1} of egg specimens

Berried female	Body Weight (BW) (g)	Carapace (mm) CW	CL	M.A	TCBS
1	144.68	131	63	1.92×10^{4}	7.60×10^{3}
2	198.56	147	68	4.20×10^{3}	3.12×10^{2}
3	166.22	131	61	7.82×10^{3}	5.60×10^{2}
4	183.45	133	59	3.12×10^{5}	1.21×10^{4}
5	192.41	135	63	1.10×10^{4}	8.24×10^{2}
6	148.40	117	58	5.50×10^{3}	6.20×10^{3}
7	160.31	133	60	2.71×10^{3}	1.52×10^{3}
8	155.51	120	59	7.24×10^{4}	3.20×10^{2}
9	186.59	135	68	8.22×10^{3}	2.11×10^{2}
10	138.38	118	57	5.42×10^{3}	0.56×10^{3}
11	152.26	134	61	2.52×10^{4}	5.32×10^{2}
12	172.87	138	65	3.81×10^{3}	1.96×10^{2}
13	151.52	124	59	8.62×10^{4}	2.80×10^{3}
14	166.89	128	67	7.41×10^{5}	2.11×10^{3}
15	168.50	131	60	5.84×10^{4}	2.52×10^{3}
16	198.48	134	66	6.22×10^{4}	3.32×10^{3}
17	154.60	133	64	5.42×10^{3}	1.56×10^{2}
18	172.54	138	62	7.82×10^{4}	2.68×10^{3}
19	155.00	133	59	4.56×10^{4}	2.44×10^{2}
20	175.80	136	67	6.45×10^{3}	1.82×10^{2}
21	156.40	130	60	2.24×10^{4}	1.20×10^{2}
22	192.50	145	68	6.12×10^{4}	2.92×10^{3}
23	188.90	144	66	3.56×10^{5}	2.56×10^{2}
24	143.60	128	58	5.60×10^{4}	2.22×10^{2}
25	181.50	139	67	6.56×10^{4}	3.42×10^{2}

M.A: Marine Agar, TCBS: Thiosulphate Citrate Bile Salts agar

Table 2: Mean cfu mL^{-1} of Total Bacterial Counts (TBC) and Total Vibrio Count (TVC) of Hatching Tanks (HT) of 8 attempts

Days	Hatching Tank (HT) M.A	TCBS
2	4.13×10^{3}	1.92×10^{2}
3	5.21×10^{3}	2.41×10^{2}
4	6.55×10^{3}	3.62×10^{3}
5	5.22×10^{4}	2.31×10^{3}
6	5.34×10^{2}	2.16×10^{2}

M.A: Marine Agar, TCBS: Thiosulphate Citrate Bile Salts agar

samples were pulverized in sterlised mortar with pestle to prepare the inoculum using sterile seawater. All samples were serially diluted (3 fold) before plating onto culture media.

Bacterial culture: Different enrichment and selective culture media were used to determine the profusion of bacteria in the gut of the *P. pelagicus*. The mediums employed were Thiosulphate Citrate Bile Salts (TCBS. Difco, USA), MacConkey agar (Merck Germany), nutrient agar (Merck Germany), marine agar (Merck Germany) and GSP (Glutamate Starch Phenol Red) agar (Biolab Hungary). For samples other than the gut, marine agar and TCBS agar were used. All media were prepared in seawater (31±2‰). All plates were incubated at 37°C for 24 h. Pure cultures were obtained by repeated streaking. Total Bacteria Count (TBC) and Total Vibrio Count (TVC) of HT, eggs, LRT, live and dead larvae were performed (Table 2-3). In the present investigation, some biochemical and physiological tests of all isolates were carried out (Table 4-8).

Table 3: Mean cfu mL^{-1} of Total Bacterial Counts (TBC) and Total Vibrio Count (TVC) of LRT, live and dead larvae of eight attempts of larvae rearing

	Larvae Rearing Tank (LRT)		Live larvae		Dead larvae	
Days	M.A	TCBS	M.A	TCBS	M.A	TCBS
2	3.63×10^{3}	2.85×10^{2}	5.58×10^{4}	2.18×10^{3}	4.60×10^{3}	3.78×10^{3}
4	3.58×10^{3}	3.95×10^{2}	4.46×10^{6}	3.63×10^{3}	3.11×10^{5}	1.10×10^{4}
6	5.45×10^{4}	5.22×10^{3}	6.82×10^{6}	1.16×10^{4}	1.13×10^{6}	1.73×10^{4}
8	6.34×10^{4}	3.34×10^{3}	4.95×10^{6}	1.32×10^{4}	7.34×10^{6}	3.46×10^{3}
9	6.70×10^{4}	2.20×10^{3}	7.31×10^{7}	5.88×10^{3}	6.52×10^{7}	6.05×10^{4}
13	7.21×10^{4}	3.22×10^{3}	-	-	-	-

M.A: Marine Agar, TCBS: Thiosulphate Citrate Bile Salts agar

Table 4: Biochemical and physiological characteristics of *S. epidermidis*

Characteristicsn (%)	Results	Characteristics	Results
Growth in NaCl		Maltose	+
0	+	Lactose	+
2	+	D-Trehalose	-
3	+	D-Mannitol	-
7	+	Xylitol	-
10	+	D-Melibiose	-
12	-	Potassium nitrate	+
Growth in sea water	+	β-naphthyl-acid phosphate	+
Growth at °C		Sodium pyruvate	-
4°C	+	Raffinose	-
25°C	+	Xylose	-
35°C	+	Sucrose	+
42°C	+	α-methyl-D-glucose	-
55°C	-	N-acetyl-glucosamine	+
Negative control	-	Arginine	+
D-Glucose	+	Urease	+
D-Fructose	+	Motality	-
D-Mannose	-		

Negative, + positive

Table 5: Biochemical and physiological characteristics of *V. harveyi* (*Vibrio harveyi*)

Characteristics (%)	Results	Characteristics	Results
Gram stain	-	Arginine dihydrolase	-
Growth on TCBS	Green	Amylase production	+
Growth in NaCl		Blood hemolysis	+
0	+	Sensitivity to vibriostat 0/129	+
2	+	Fermentation	+
3	+	Phenylalanine deaminase	-
7	+	Fermentation to acid	
10	+	Glucose	+
12	-	Sucrose	+
Growth in sea water	+	Lactose	-
Growth at°C		Utilization of	
4°C	-	Citrate	-
25°C	+	Glucose	+
35°C	+	Lactose	-
42°C	+	Sucrose	+
55°C	-	Casein	+
Glucose fermentation	+	Lipid	+
Oxidase	+	Glycine	+
Catalase	+	L-Arginine	-
Motility	+	L-Tyrosine	+
Indole	+	L-Serine	+
H_2S formation	-	Acetate	-
Urease	-	Gelatin liquefaction	-
Luminescence	+	-	-

Negative, + positive

Table 6: Biochemical and morphological characteristics of *V. parahaemolyticus* isolated from *P. pelagicus* (*Vibrio parahaemolyticus*)

Characteristics (%)	Results	Characteristics	Results
Colour on TCBS	Green	Indole	-
Gram stain	-	Gelatin liquefaction	+
Cytochrome oxidase	+	Acid form test	
Growth in %NaCl		Glucose	+
0	+	Mannitol	+
2	+	Inositol	-
4	+	Sucrose	-
6	+	Lactose	-
8	+	Cellobiose	+
10	+	Carbon source test	
Growth in sea water	+	DL-Maltos	-
Growth at°C		L-Rhamnos	+
4°C	-	D-Mannos	+
20°C	+	Sucrose	+
35°C	+	D-Galactose	+
40°C	+	DL-Glycerate	+
44°C	+	D-Glucuronate	-
55°C	-	L- Alanine	+
Lysine decarboxylase	+	Casein	+
Citrate	-	Starch	+
Production of H_2S	-	DNas	+
Urease	+	Collagen	+
Tryptophan deaminase	-		

Negative, + positive

Table 7: Biochemical and physiological characteristics of *M. luteus* (*Micrococcus luteus*)

Characteristics	Results	Characteristics	Results
Gram	+	Gelatinase	-
Nitrate	-	Urease	+
Glucose	-	Catalase	+
Lactose	-	Indole	-
Motility	-	Oxidase	-
Starch hydrolysis	-	Manitol	-
Growth in NaCl		Growth at °C	
0	+	4°C	-
2	+	25°C	+
4	+	35°C	+
6	+	42°C	+
8	+	55°C	-
10	+		
Growth in sea water	+		

Negative, + positive

Table 8: Biochemical and physiological characteristics of *P. piscicida* (*Pseudoalteromonas piscicida*)

Characteristics	Results	Characteristics	Results
Pigmentation	Yellow	Maltose	v
Growth at °C		Mannitol	+
4°C	-	Mannose	v
35°C	+	Fructose	+
40°C	+	Citrate	+
42°C	+	Succinate	+
48°C	+	Acetate	+
55°C	-	Sorbitol	-
Growth in NaCl		Glycerol	-
0	+	Production of	
1	+	Amylase	-
3	+	Lipase	-
5	+	Alginase	-
7	+	Caseinase	+
9	+	Oxidase	+
10	+	Indole	-
Growth in seawater	+	Urease	+
Utilization of		Luminescent	-
Sucrose	+	Growth in sea water media	+

Negative, + positive, v: variable

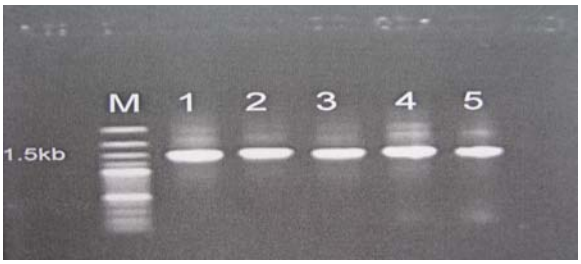

Fig. 1: Gut bacteria; the result of *16S rRNA* gene sequence in PCR amplification product obtained from bacteria DNA. M, GeneRuler indicates 100bp DNA ladder Plus; Lanes 1-5 at 1.5 kb, *16S rRNA* gene PCR products from samples 1-5 show the species-specific profiles (Samples identified, 1-*Staphylococcus epidermidis* 2- *Vibrio harveyi*, 3-*Vibrio parahaemolyticus*, 4- *Micrococcus luteus*, 5-*Pseudoalteromonas piscicida*)

Identification and characterization micro flora

Total genomic dna extraction: Total genomic DNA from overnight culture of all samples were extracted using Wizard® Genomic DNA Purification Kit (Promega, USA) following the manufacturer's instruction. The DNA pellet was rehydrated in 100 μL of Rehydration Solution for 1 h at a temperature of 65°C and stored overnight at 4°C.

Polymerase Chain Reaction (PCR) amplification of 16S RDNA: The 16S ribosomal DNA was amplified by PCR using bacterial universal primers 27F (5'-AGAGTTTGAT CCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTT GTTACGACTT-3'). The PCR reaction was performed in a Bio Thermal cycler (Bio-Rad, USA) with an initial denaturing step at 95°C for 5 min followed by 30 cycles of 95°C for 30 sec, 50°C for 30 sec and 72°C for 1 min and ended with a final extension step of 72°C for 15 min. The PCR product were electrophoresed in 1% agarose gel stained with 1 μg mL^{-1} of ethidium bromide and was visualized using Alpha Imager gel documentation system (Alpha Innotech, UK) (Fig. 1). Sequences obtained were analysed and compared with sequences from GenBank using BLASTn NCBI citation (http://blast.ncbi.nlm.nih.gov).

Pathogenic property challenge test of *V. harveyi*: One day hatch energetic larvae (Zoea-1 Z1) of *P. pelagicus* were used to evaluate the pathogenicity of *V. harveyi* isolated from the gut, HT, eggs., LRT, live larvae and dead larvae with slight modification according to Villamil *et al.* (2003). Larvae were fed as described earlier and were reared in 1 L glass beaker containing sterlised seawater equipped with aeration. In the experiments, *V. harveyi* was added at a final concentration of 10^2, 10^3, 10^4, 10^5 and 10^6 cfu mL^{-1}. In all five experiments, no-pathogen controls were employed. In all experimental challenges, an initial number of 20 larvae L^{-1} were placed in each 1 L of glass beaker and mortality was determined by direct counting of larvae (Total number of larvae died/Initial number of larvae stocked×100) after 24 h of post challenge (Tabe 9). Concentration of doses was adjusted to OD_{630}. Water temperature, dissolved oxygen, pH and salinity were monitored accordingly using YSI 556 MPS (USA) multi parameter equipment.

Table 9: Mortality (%) in *P. pelagicus* larvae after 24 h exposed to different doses(cfu mL^{-1}) of *V. harveyi* (Mean±SD)

	Mortality at indicated dose (%)				
Isolate	10^2	10^3	10^4	10^5	10^6
Control	16.67±2.89	13.33±2.89	15.00±5.00	15.00±5.00	15.0±5.0
Gut	41.67±5.77	48.33±2.89	66.67±2.89	91.67±2.89	100.0±0.0
HT	46.67±7.64	56.67±7.64	73.33±5.77	96.67±2.89	100.0±0.0
Eggs	33.33±2.89	46.67±2.89	68.33±5.77	88.33±5.77	100.0±0.0
LRT	41.67±5.77	51.67±5.77	71.66±2.89	91.67±2.89	100.0±0.0
Live larvae	43.33±2.89	56.67±2.89	73.33±7.64	90.00±5.00	100.0±0.0
Dead larvae	38.33±5.77	51.67±5.77	71.67±5.77	91.67±2.89	100.0±0.0

Statistical analysis of data: Means of different concentrations and morality were compared by ANOVA analysis of variance using statistical software (SPSS 16.0 for windows). Post hoc test was carried out using Duncan multiple range tests if they were significant.

RESULTS AND DISCUSSION

Experimental attempts (triplicates) of larvae rearing showed substantially very low survival 1.0-2.0% and in some attempts survival was recorded 0%. The cause of mass mortality of *Portunus pelagicus* larvae rearing in a hatchery system was examined and the reason was figured out. The causative pathogen associated with the gut, Hatching Tanks (HT), eggs, Larvae Rearing Tanks (LRT), live and dead larvae of *P. pelagicus* was investigated in detail.

All isolates were identified using *16S rRNA* gene sequences analyses. PCR amplification of *16S rRNA* gene sequence result divulged the presence of diversity of pathogenic floral consortium in the gut; it includes *Staphylococcus epidermidis*, *Vibrio harveyi*, *Vibrio parahaemolyticus* *Micrococcus luteus* and *Pseudoalteromonas piscicida* Fig. 1 and the eggs were harbouring *Vibrio. harveyi*, *Micrococcus luteus* and *Pseudoalteromonas piscicid* while *V. harveyi* was identified as the target pathogen with HT, LRT, live and dead larvae of *P. pelagicus* (Table 10). The gut pathogens *S. epidermidis*, *V. harveyi*, *V. parahaemolyticus M. luteus*

and *P. piscicida* all together were found only in 62 (34.44%) out of 180 female gut specimens (Fig. 2). Among the isolates *V. harveyi* and *V. parahaemolyticus* were found 100% in the gut of all samples while rest of the microbes were found in variations from specimen to specimen, *S. epidermidis* 88 females (48.9%), *M. luteus* 95 females (52.7%) and *P. piscicida* 119 females (66.11%) in 180 female guts respectively Table 11 and 12. *V. harveyi* was found 100%in all the specimen sources in the present study. Egg pathogens that include *V. harveyi. M. luteus* and *P. piscicida* altogether were observed in 10 berried female egg specimens out of the 24 specimens (41.67%). *P. piscicida* was found in 15 egg specimens (62.50%) while *M. luteus* was detected in 11 egg specimens (45.83%) (Table 13 and 14).

Table 10: Isolates from various source of *P. pelagicus* (*16S rRNA* gene sequencing result)

Isolate source	Species	Max. identity (%)
Gut	*S. epidermidis*	99
	V. harveyi	99
	V. parahaemolyticus	99
	M. luteus	99
	P. piscicida	99
Hatching Tank (HT)	*V. harveyi*	99
Eggs	*V. harveyi*	99
	M. luteus	99
	P. piscicida	99
Larvae Rearing Tank (LRT)	*V. harveyi*	100
Live larvae	*V. harveyi*	99
Dead larvae	*V. harveyi*	99

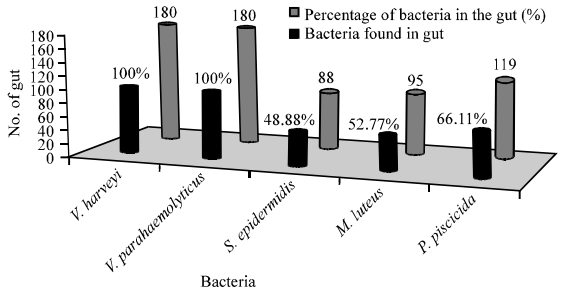

Fig. 2: Species wise existence of microflora in the gut of 180 female crabs

Biochemical characteristics of the egg isolates were similar to isolates from the gut (Table 5, 7 and 8). For HT, LRT and larvae were similar to *V. harveyi* isolated from the gut (Table 5). Total Bacteria Count (TBC) and Total Vibrio Count (TVC) HT, LRT, live and dead larvae of eight experimental attempts (Mean count) shown in Table 2-3 and TBC and TVC of eggs of 24 females are elucidated in Table 1.

Plates with 30 and above colonies were taken to determine the bacteria counts from all samples. Bacteriological study showed that larvae were found to harbor a higher number of bacteria than Larvae Rearing Tank (LRT). Vibrio counts were observed increasing in Larval Rearing Tanks (LRT) as compared to Hatching Tanks (HT).

Increase in bacterial load cfu mL^{-1} in LRT and larvae were determined as time dependant, more the larval rearing days high the bacterial load. Burgey's manual of determinative bacteriology, morphological, physiological and biochemical characteristics of bacteria, BD BBL Crystal Identification systems (Becton, Dickinson and company, USA), API Staph. Kit were also used for identification of bacteria during the present study and final results were confirmed by using *16S rRNA* gene sequences analyses.Sequences obtained were analysed and compared with sequences from data base Gen Bank using BLASTn (megablasn) National Center for Biotechnology Information (NCBI) USA online data bank citation (http://blast.ncbi).

Table 11: Species wise percentage of microbes found in the gut

Total female (gut)	Microbe species					Found (No of female gut)	Percentage
180	*V. harveyi*	*V. parahaemolyticus*	-	-	-	180	100.00
Do	*V. harveyi*	*V. parahaemolyticus*	*S.epidemidis*	*M. luteus*	*P. piscicida*	62	34.44
Do	*V. harveyi*	*V. parahaemolyticus*	*S.epidemidis*	*M. luteus*	-	7	3.89
Do	*V. harveyi*	*V. parahaemolyticus*	*S.epidemidis*	*P. piscicida*	-	8	4.44
Do	*V. harveyi*	*V. parahaemolyticus*	*S.epidemidis*	-	-	11	6.11
Do	*V. harveyi*	*V. parahaemolyticus*	*M. luteus*	*P. piscicida*	-	11	6.11
Do	*V. harveyi*	*V. parahaemolyticus*	*M. luteus*	-	-	15	8.33
Do	*V. harveyi*	*V. parahaemolyticus*	*P. piscicida*	-	-	38	21.11

Table 12: Summary (%) of individual microbe found in the female gut (from Table 11)

Total female (gut)	*V. harveyi* and *V. parahaemolyticus* (%)	*S. epidemidis* (%)	Found (No of female gut)	*M. luteus* (%)	Found (No of female gut)	*P. piscicida* (%)	Found (No of female gut)
180	100	34.44	62	34.44	62	34.44	62
Do	-	3.89	7	3.89	7	4.44	8
Do	-	4.44	8	6.11	11	6.11	11
Do	-	6.11	11	8.33	15	21.11	38
Total	-	48.88	88	52.77	95	66.10	119

Table 13: Species wise percentage (%) of microbes found in the eggs specimens

Total egg specimens	Microbe species			Found (No egg specimen)	Percentage
24	*V. harveyi*	*M. luteus*	*P. piscicida*	10	41.67
Do	*V. harveyi*	*M. luteus*	-	1	4.16
Do	*V. harveyi*	*P. piscicida*	-	5	20.83
Do	*V. harveyi*	-	-	24	100.00

Table 14: Summary (%) of individual microbe found in female egg specimens

Total female (eggs)	*V. harveyi* (%)	*M. luteus* (%)	Found (No. of egg specimen)	*P. piscicida* (%)	Found (No. of egg specimen)
24	100	45.83	11	62.50	15
Total	100	45.83	11	62.50	15

nlm.nih.gov). Results obtained are shown in Table 10. The sequence results from Gen Bank showed similarity with various strains of same species among all isolates.

Biochemical and physiological tests of isolates: In the present investigation, biochemical and physiological tests of each isolates were carried out. Results obtained are shown in Table 4-8.

Pathogenicity: Experimental challenges proved the pathogenicity of *V. harveyi* isolates was highly pathogenic, various doses caused severe mortality while 10^5 cfu mL^{-1} was producing 96.67% and 10^6 cfu mL^{-1} resulted in 100% mortality within 24 h post dose (Table 9 and Fig. 3 and 4). HT isolate was determined more pathogenic. In all five experiments, no-pathogen controls were employed Mortality rate (%) was calculated as (Total number of larvae died/Initial number of larvae stocked×100). The differences among various doses of pathogen were statistically significant ($p<0.05$).

Previous researches on pathogenic microorganisms isolated from the crabs were in term of human health principle. Uaboi-Egbenni *et al.* (2010) examined pathogen in the gut of swimming crab, *Callinectes* sp. for the purpose of public health and epidemiological implications not for aquaculture. Najiah *et al.* (2010) conducted a general study focusing on antibiotic resistant bacteria with wild mud crab *Scylla serrata.* The pioneer research of Faghri *et al.* (1984), he found tissues of several types of crabs such as tanner crab, *Chionoecetes opilio*, Dungeness crab, *Cancer magaster*, King crab, *Paralithodes camtschatica* and Rock crab and *Cancer irroratus* which could serve as accumulation sites for human pathogens particularly in crabs collected from contaminated area. In another study by Tison *et al.* (1982) reported that the presence of *Vibrio* species in marine ecosystems have led to a re-evaluation of the taxonomy of this group and the definition of several new species

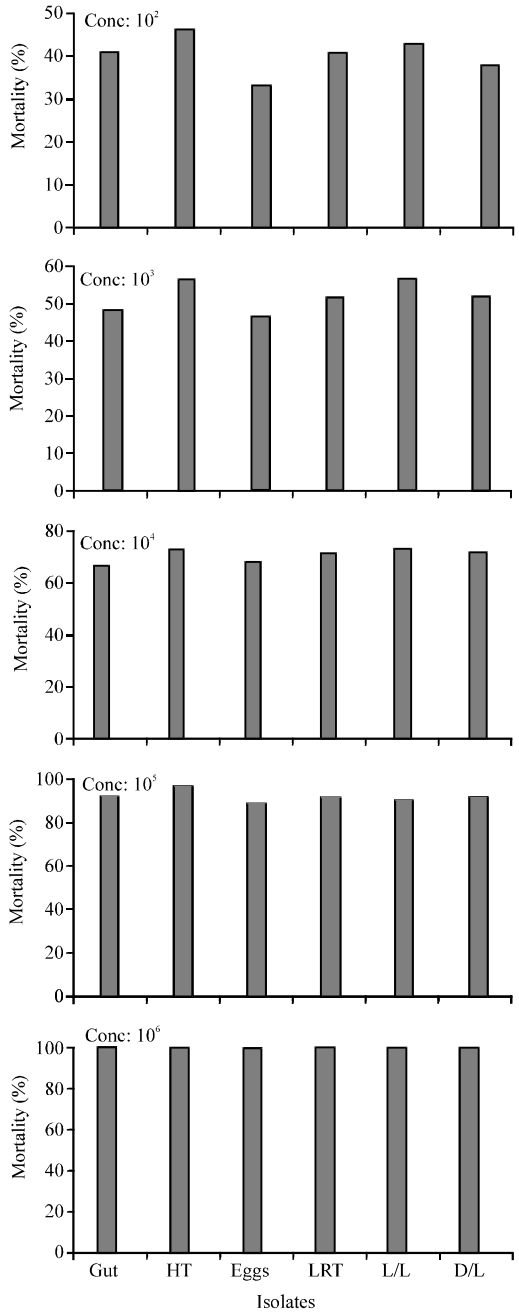

Fig. 3: Larval mortality (%) of *Portunus pelagicus* after 24 h exposure to various concentrations of *V. harveyi* isolates from various sources (L/L = Live Larvae, D/L = Dead Larvae)

that are potential human pathogens. Lavilla-Pitogo *et al.* (2001) did some preliminary research on disease side on mud crab *Scylla* sp. broodstock, he observed significant diseases such as shell disease in broodstock tanks. Another research done by Parenrengi *et al.* (1993), he

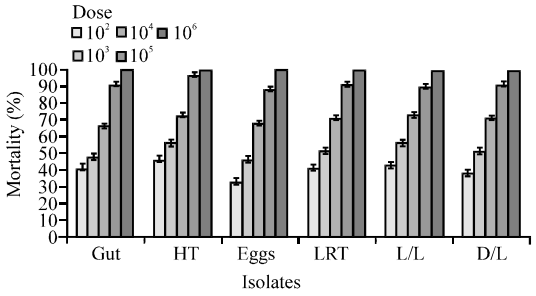

Fig. 4: Larval mortality (%) with standard error bars of *Portunus pelagicus* after 24 h exposure to various concentrations of *V. harveyi* isolates from various sources (L/L = Live Larvae, D/L = Dead Larvae)

reported that *V. harveyi* was more pathogenic in comparison to the other luminous bacteria causing mass deaths in crab larvae.

Present study was aimed to document the scientific knowledge and to evaluate the assemblage of pathogenic flora in the gut of female crab and bacterial load with HT, eggs, LRT and larvae of *P. pelagicus*. Further to determine the transmission of responsible pathogenic agent (*V. harveyi*) through the feces of adult female crab in larval culture system for mass mortality in hatchery rearing larvae. Experimental attemptsof the larvae rearing in hatchery generated very low survival upto 2.0% while in some of the replicates no survival yield was observed. Lower survival and mass mortality among larvae of *P. pelagicus* crab under hatchery conditions was observed owing to *V. harveyi* infections. The disinfected berried females were not fed until the hatching. However, the water samples of HT for bacteriological study revealed that the existence of bacterial load was transmitted through the feces of adult berried female crab. According to Lavilla-Pitogo *et al*., (1992) primary source of *V. harveyi* in hatcheries appears to be the midgut contents of female broodstock, shed during spawning.

No direct proportion in relation to weight, length and width of carapace of berried female was observed in term of (TBC) and (TVC) cfu mL^{-1} of egg specimens. Egg pathogens *V. harveyi*, *M. luteus* and *P. piscicida* were observed simultaneously in 10 berried female egg specimens out of the 24 egg specimens (41.67%).

V. harveyi was found in 24 (100%), *P. piscicida* in 15 (62.50%) and *M. luteus* in 11 (45.83%) egg specimens were observed. However, female broodstock was disinfected properly and was kept in HT with treated water but the occurrence of pathogens with eggs and *V. harveyi* with HT, LRT and larvae was believed to be transmitted from the gut of adult female crab. LRT were holding maximum cfu mL^{-1} 7.21×10^4 on M.A at day 13 and 5.22×10^3 cfu mL^{-1} on TCBS agar at the day 6 of the larvae rearing. Increase in cfu mL^{-1} in LRT was due to contamination of live food or multiplication of bacteria owing to availability of nutrients of the live food. Increase in *Vibrio* cfu mL^{-1} paved the way for death penalty among larvae rearing. The cfu mL^{-1} increase with live larvae was time dependant and highest 7.3×10^7 cfu mL^{-1} on M.A was observed on day nine and 1.32×10^4 cfu mL^{-1} on TCBS at the day 8 of the larvae rearing. The increase in bacteria cfu mL^{-1} with live larvae may be grazing of rotifers, *Nanochloropsis* algae and *Artemia* as food prey. Increase in cfu mL^{-1} in (TBC) and (TVC) in dead larvae was time dependant and highest 6.52×10^7 cfu mL^{-1} on M.A and 6.05×10^4 cfu mL^{-1} on TCBS respectively at the day 9 of larval rearing.

The gut studies of female crabs showed that *V. harveyi* and *V. parahaemolyticus* were the dominant pathogens with 100% existence in the gut of 180 female specimens brought under present study while rest shared with the gut of female specimens include *S. epidermidis* 88 females (48.9%), *M. luteus* 95 females (52.8%) and *P. piscicida* 119 females (66.11%). *P. piscicida* was noticed as third most dominant pathogen, *M. luteus* fourth and *S. epidermidis* fifth in the gut study of female specimens. *S. epidermidis* was first reported as a fish pathogen by Kusuda and Sugiyama (1981) in farmed yellow tail (*Seriola quinquiradiata*) and red sea bream (*Chrysophrus major*) in Japan. *S. epidermidis* caused mass mortality of cultured Tilapia in Taiwan (Huang *et al*., 1999). *S. epdermidis* caused infection in gillhead sea bream (*Sparus aurata*) juvenile (3-5 g) in a net cage and out break resulted fish loses upto 12% in one day (Kubilay and Ulukoy, 2004). *V. harveyi* found globally in marine environments is a serious pathogen for a wide range of marine animals (Austin and Zhang, 2006). The Vibriosis bacteria have shown high mortality in cultured shrimp worldwide (Lightner and Lewis, 1975; Lightner *et al*., 1992; Lavilla-Pitogo *et al*., 1996).

V. harveyi was more pathogenic in comparison to the other luminous bacteria causing mass deaths in crab larvae (Parenrengi *et al*., 1993). In the present study *V. haveryi* isolated from various sources were tested for pathogenicity against Zoeal (Z1) of *P. pelagicus*. Mortality (Table 9) was high after 24 h exposure to isolate from HT at all concentrations in comparison with other isolates. Isolate from live larvae at 10^2 cfu mL^{-1} showed 43.33% mortality followed by 41.67, 33.33, 41 and 38.33% mortality due to isolates from gut, eggs, LRT and dead larvae, respectively. *V. harveyi* has caused 100% mortality in *P. monodon* larvae at challenge doses of 10^2 cfu mL^{-1} (Lavilla-Pitogo *et al*., 1990). At 10^3 cfu mL^{-1} isolates from HT and live larvae showed more virulence resulted in

56.67% mortality by each isolate while less mortality 48.33% was due to isolate from the gut and 46.67% due to egg isolate. Isolate from HT and live larvae at 10^4 cfu mL^{-1} were more pathogenic resulted in 73.33% mortality by each isolate and the isolate from gut produced less deaths 66.67% followed by 68.33% mortality of eggs isolate.

The infection at 10^5 cfu mL^{-1} with isolate from HT resulted in 96.67% mortality followed by 91.67% deaths due to the isolates from the gut, LRT and dead larvae respectively while eggs isolate produced less mortality 88.33%. Exposure to 10^6 cfu mL^{-1} of all isolated for 24 h resulted in 100% mortality. The pathogenicity results demonstrate that a higher dose of all isolates produced high infection resulted in 100% mortality. Infections due to *V. harveyi* at different concentrations cfu mL^{-1} resulted severe mortality have serious implications in larvae rearing of *P. pelagicus* under hatchery condition.

V. parahaemolyticus was observed as the causative agent of infection in Iberian toothcarp *Aphanius iberus* and have reproduced the same gross signs observed in the outbreak after bacterial challenge (Alcaide *et al*., 1999). *V. parahaemolyticus* has emerged as an important virulent pathogen of the shrimp (Sudhesh and Xu, 2001). *V. parahaemolyticusis* a representative pathogenic bacterium to abalone post-larvae resulting mortalities (Cai *et al*., 2007). *V. parahaemolyticus* has emerged worldwide as one of the major causes of gastroenteritis outbreaks associated with the shellfish (Fuenzalida *et al*., 2006). Various bacteria in the marine and estuarine environment such as *vibrio* sp. are prospective human pathogen (Broza *et al*., 2007; Senderovich *et al*., 2010). As crabs are in close contact with milieu that is rich in pathogenic bacteria, chances of infection by bacteria can be high (Hudson and Lester, 1994). *M. luteus* has been reported to be another pathogen in aquaculture life. *M. luteus* caused Rainbow Trout Fry Syndrome (RTFS) (Austin and Stobie, 1992).

The yellow-pigmented species *P. piscicida* produces a neuromuscular toxin that kills a variety of fish and crab species (Bein, 1954; Hansen *et al*., 1965). This organism was associated with whitening of eggs followed by mortalities within 24 h among eggs of the damsel fish, *Amphiprion clarkii* and *Amblygly phidodon curacau* (Nelson and Ghiorse, 1999). This microbe was found associated with eggs of 15-berried females (62.5%) out of the 24 female for the present study. Presence of this pathogen with eggs or its transmition through adult female feces may result in killing of larvae as described by Bein (1954) and Hansen *et al*. (1965). Among the guts of 180 female crabs brought under present study *P. piscicida* had shared 66.11% (119 female gut) and with egg specimens it shared 15 specimens (62.50%) as third and second dominant pathogen, respectively. The pathogenicity of *S. epidermidis M. luteus* and *P. piscicida* has not been worked out under the present study therefore no assumption can be made on them. The literature information confirms that the *S. epidermidis*, *M. luteus* and *P. piscicida* are fish and shellfish pathogen and have implication for organisms.

CONCLUSION

The scientific knowledge regarding association of pathogenic flora with the gut of *P. pelagicus*, bacterial load with HT, eggs, LRT and larvae of *P. pelagicus* was documented. Despite of disinfection of female broodstock, the bacteriological samples under the present study has proved that *V. harveyi* was transmitting from the gut to HT, eggs, LRT and larvae through feces of adult female crab and was determined as a representative fatal pathogen responsible for severe mortality in crab larvae reared under hatchery condition.

The present study concludes that the *P. pelagicus* dwelling in Tebrau strait of Johor (Malaysia) waters and brood stock collected from there are intensely harbouring a consortium of gut pathogenic bacteria that includes *S. epidermidis*, *V. harveyi*, *V. parahaemolyticus*, *M. luteus* and *P. piscicida*. The *V. harveyi* and *V. parahaemolyticus* was found 100% in the gut of all 180 female crab specimens and *V. harveyi*, *M. luteus* and *P. piscicida* were found associated with eggs. The pathogenicity challenge test of *V. harveyi* isolated from all sources had a fatal effect and the isolate from HT was more pathogenic. The findings proved that the gut of *P. pelagicus* harboured with shellfish pathogen and human threatening (*S. epidermidis*, *V. parahaemolyticus* and *M. luteus*) beyond the consequence for larval rearing is of epidemiological and health significance to humans.

RECOMMENDATIONS

Future studies will to determine the stability of the microflora of individual crab's gut depending on its feeding in hatchery system and the season. Such studies will determine whether the microflora is established in the crab's gut or representing the flora of the surrounding water or environment.

Virulence and antibiotics tests may be carried out to observe the pathogenicity and sensitivity of all isolates. The epidemiological part may be considered for human health. The emphasis may be laid to isolate the indigenous probiotic from the *P. pelagicus* to control the infection of *V. harveyi* in the larval culture. To avoid any infection from the gut borne bacteria, the blue swimming

crab should be cooked properly before consumption and as crabs are in close contact with milieu that are rich in pathogenic bacteria, the chance of infection by bacteria is high. Whether the crab is touched, the hands should be washed with any antiseptic or antibacterial solution.

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