Characterization of *Vibrio* isolated from Mangrove crab, *Scylla serrata* larval cultures

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ABSTRACT

This study was conducted to isolate and identify the putative pathogenic *Vibrio* species dominantly present during the first 10 days of mangrove crab, *Scylla serrata* larval culture and test the isolates' susceptibility to antibiotics. Dominant colonies of *Vibrio* were periodically isolated based on their colonial morphology on thiosulfate citrate bile sucrose (TCBS) agar and nutrient agar supplemented with 2% NaCl (NA^+) plates. Conventional biochemical and physiological tests revealed the existence of 4 dominant groups belonging to 4 species including *V. campbellii*, *V. proteolyticus*, *V. alginolyticus* and *V. tubiashii*. Furthermore, through 16S rRNA gene sequencing, the taxonomic position of each representative strain randomly selected from the 4 dominant groups of *Vibrio* spp. Was ascertained. Moreover, the in vitro susceptibility of the 4 representative *Vibrio* spp. to commercially available antibiotic discs including amoxicillin, oxolinic acid, tetracycline, kanamycin, ampicillin, trimethoprim and cefprozil were examined. Results showed that all four species were susceptible to tetracycline, oxolinic acid and trimethoprim, however, the susceptibility of *V. campbellii* and *V. proteolyticus* to trimethoprim were just intermediate. The susceptibility of the *Vibrio* isolates to the rest of the commercial antibiotic discs were either intermediate or resistant. In addition, the minimal inhibitory concentration (MIC) assay was conducted using oxytetracycline (OTC) concentrations ranging from 10 to 50 mg L^{-1}. Results showed that *V. tubiashii* was susceptible to at least 10 mg L^{-1} OTC, while *V. campbellii*, *V. alginolyticus* and *V. proteolyticus* were susceptible to at least 30, 40 and 50 mg L^{-1} OTC respectively. Current data clearly indicate that OTC is a suitable candidate for the treatment and prevention of vibriosis in *S. serrata* larval culture with at least 50 mg L^{-1} required to inhibit all the four putative pathogenic *Vibrio* spp. tested.

KEYWORDS:

antibiotic susceptibility, *Vibrio*, crab larvae

INTRODUCTION

Bacterial infection is one of the major causes of high mortality during mangrove crab larval culture (Cholik 1999; Liessmann 2005; Talib et al. 2013, Amar et al. 2017). *Vibrio* species within the culture system, from the natural food and the larvae itself, have been particularly tagged as the major culprits for the mass mortality of crustacean larvae, including crabs and shrimps (Lavilla-Pitogo and de la Peña 2004; Shelley et al. 2008; Jithendran et al. 2009; 2010). Monitoring of total *Vibrio* counts during mangrove crab larval rearing revealed that the surge in *Vibrio* counts happened during first few days of culture (Mann et al.1999), coinciding with larval mass mortality. While it was obvious that *Vibrio* were indeed the main culprits of early mangrove crab larval mortality, Mann et al. (1999) did not go further to identify the species of *Vibrio* and their characteristics. Although several species of *Vibrio* from mangrove crab larval cultures have been identified (Parenrengi et al. 1993; Akinbowale et al. 2006; Sarjito et al. 2016), the reports were not clear which species were isolated during the early part of larval culture which is the most crucial period.

Antibiotics are still commonly used in mangrove crab larval rearing due to a lack of natural alternatives to prevent bacterial infection (Amar et al. 2017; Pates et al. 2017). The application of antibiotics has been reported to improve larval survival of mangrove crabs (Shelley et al. 2008; Azam and Narayan 2013) and other cultured aquatic organisms (Regidor, 2020), however, the regular, prolonged and improper use of antibiotics may alter the microbial communities within or outside the culture system and may lead to the development of antibiotic-resistant bacterial

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strains (Subashinghe et al. 2000; Somga et al. 2012; Mateus et al. 2014). Knowing the identity of dominant *Vibrio* species in mangrove crab larval culture as well as their susceptibility to antibiotics is vital in the development of larval rearing protocol to address problems with diseases and larval mortality. This study therefore identified the *Vibrio* species associated with the culture of early larval stages of the mangrove crab, *S. serrata* (Zoea I to Zoea III) and tested the susceptibility of such *Vibrio* to antibiotics commonly used in aquaculture.

METHODS

Source of Larvae

Mangrove crab, *S. serrata* larvae were produced from spawners (500-700 g) purchased from mangrove crab buying stations and reared at the Hatchery Complex of the Institute of Aquaculture, University of the Philippines Visayas, following the method of Genodepa (2003). Briefly, larval rearing was done in 4 units circular tanks (500 L capacity), with 400 L of chlorinated, UV-sterilized seawater (22‰ salinity; 28°C temperature) and aeration. Actively swimming newly hatched zoea from the incubation/ hatching tank were attracted to a strong light source and collected using a scoop bowl, about 4 to 6 h from time of hatching and were stocked at 100 L^{-1} in the rearing tanks. Rotifers (*Brachionus sp.*) were fed at a density of 40 to 60 individuals mL^{-1} right after the larvae were stocked and OTC (20 mg L^{-1}) was also added to control the pathogens from both the newly hatched larvae and the rotifers. Both the rotifers and OTC were given only once during the entire 10-day culture duration. Microalgae (*Nannochloropsis sp.*) which served as food of rotifers was introduced in the larval tanks at day 0 and maintained at a density of $5x10^4$ cells mL^{-1} by daily addition of the desired quantity/ volume of the microalgae from asceptic indoor cultures. Newly hatched *Artemia* were introduced and maintained in the larval tanks at a density of 0.5 nauplii mL^{-1} beginning on the second day after larvae molted into Zoea II stage. Water was not changed for the entire 10-day duration of culture.

Bacteriological Analysis

Duplicate samples of rearing water (100 mL) and crab larvae (20 individuals) were asceptically collected from each of the 4 larval rearing tanks between 0700H to 0800H daily from day 0 to day 10. Triplicate samples of the microalgae (100 mL), *Artemia* (50 individuals) and rotifers (50 mL) were likewise aseptically collected prior to introduction of these natural feeds into the larval rearing tanks. The crab larvae, rotifer and *Artemia* samples were washed thrice in sterile normal saline solution (NSS) to remove surface microflora. All samples were homogenized and spread onto nutrient agar supplemented with 2% NaCl (NA^+) and thiosulfate citrate bile sucrose (TCBS) agar in 3 replicates and incubated overnight at room temperature (28°C). Total plate count (TPC) and presumptive *Vibrio* count (PVC) were determined after 24 h and were expressed in colony forming units per milliliter (CFU mL^{-1}).

Isolation of Vibrio Species

Isolation of bacteria were conducted at the Microbiology Laboratory of the Institute of Aquaculture, University of the Philippines Visayas. Isolates of dominant bacterial colonies with unique colony morphology such as color, margin, elevation on TCBS agar plates and luminescence in NA^+ plates were individually picked and repeatedly streaked onto fresh NA^+ to obtain pure isolates. Purified bacterial isolates were inoculated in an agar stab culture and stored at 4°C in a laboratory refrigerator until further analysis.

Characterization of the Isolates

Bacterial isolates were first screened through Gram-staining following the method of Moyes et al. (2009). The colony appearance, luminescence in NA^+ plates and color in chromogenic *Vibrio* agar were examined and then colonies having the same characteristics were categorized under the same group. Isolates were characterized using biochemical tests following the method of Alsina and Blanch (1994) which included tests for oxidase, oxidative-fermentative, decarboxylase (arginine, lysine, ornithine), gelatin hydrolysis, catalase, methyl red, carbohydrate fermentation/gas production, triple sugar iron agar, indole, nitrate reduction, citrate utilization, Voges-Proskauer and resistance to ampicillin. Physiological tests such as growth in different salinities (0, 3, 6, 8, and 10% NaCl) and temperatures (4°C and 28°C) were also performed.

Molecular identification of isolates

Pure isolates of the 4 *Vibrio* species were submitted to Philippine Genome Center, University of the Philippines Diliman for 16S rRNA sequencing. The resulting sequences were compared to the sequences of organisms in the GenBank using the National Center for Biotechnology Information

(NCBI) Basic Local Alignment Search Tool (BLAST) (https://www.ncbi.nlm. nih.gov/). Representative strains which were more than 97% identical to the isolates were selected to construct a phylogenetic tree through the neighbor-joining method in the NCBI website. The species closest to the clade of the reference sequence was then chosen as the identity of the bacterial isolate.

Antibiotic sensitivity test

The inhibitory effects of the different antibiotic discs (amoxicillin, 30 µg; oxolinic acid, 2 µg; tetracycline, 30 µg; kanamycin 30 µg; ampicillin 10 µg; trimethoprim, 5 µg; and cefprozil, 30 µg) were tested on the identified *Vibrio* species using disc-diffusion assay following the method of Bauer et al. (1966). Bacterial isolates were sub-cultured on NA^+ and incubated at room temperature (28°C) for 18 to 24 h, and then 4 to 5 colonies of each bacterial isolate were inoculated in sterile normal saline solution (NSS). The density of each bacterial suspension was adjusted to approximately $1.5x10^8$ CFU mL^{-1} by adding sterile NSS until turbidity was comparable to No. 0.5 McFarland standard solution. A sterile cotton swab was dipped into each bacterial suspension, then streaked onto 3 replicate Mueller-Hinton agar (MHA) plates and incubated for 15 minutes (Tendencia 2004). Antibiotic discs were then strategically placed on the MHA plates using sterile forceps and then the plates were incubated at 28°C. After 24 h of incubation, the inhibition zones were determined by measuring the clear zones around the antibiotic disc using a transparent ruler. The clear zone diameters were then compared to the zone of inhibition interpretative standards (CLSI, 2015) and reported either as intermediate (I), susceptible (S), or resistant (R).

Susceptibility at Different OTC Concentrations

The minimum concentration of OTC to inhibit the bacterial isolates were determined based on the method of Epoke et al. (2003). OTC powder (10, 20, 30, 40 and 50 mg) were diluted in 100 mL of sterile distilled water to obtain the desired concentrations of 10, 20, 30, 40, and 50 mg L^{-1} respectively. Each antibiotic concentration was then impregnated onto sterile paper discs (Whattman No.1, 6 mm diameter) using 10 µL of the prepared concentrations and air dried for 15 min. The discs were then tested for their inhibitory effects following the method mentioned earlier.

Statistical Analysis

Statistical analyses of the resulting data on the bacteriological analysis of the samples during the 10-day culture period and the data on the susceptibility of the four *Vibrio* isolates to different concentrations of OTC were performed using SPSS for Windows Version 20. Paired t-Test was used to determine the differences in bacterial density (TPC) and *Vibrio* counts (PVC) between adjacent days of culture in both rearing water and crab larvae. The resulting bacterial density (TPC) and PVC from the natural food samples (green algae, rotifer, and *Artemia* nauplii) and the susceptibility test (based on the diameter of the zones of inhibition) of the four *Vibrio* isolates to different OTC concentrations were analyzed for homogeneity of variance using Levene's test, then subjected to one-way analysis of variance (ANOVA). Further, either Tukey's test for equal variances, or Games–Howell test for unequal variances were performed to determine significant differences among treatment means.

RESULTS

Bacteriological Analysis

The trend of TPC in both the rearing water and the crab larvae were similar (Figure 1). Bacterial concentration (TPC) increased sharply from day 1 to day 2, then decreased and maintained low levels from day 3 to day 7 before slightly increasing again towards the end of the 10-day culture period (day 8 and day 9 in rearing water and crab larvae respectively). PVC in rearing water (Figure 2-A) also fluctuated and the trend was like TPC results, but this time the peak densities were at day 1 and day 6. In the larvae, PVC (Figure 2-B) remained low from day 0 to day 5, then began increasing from day 6 up to day 9 but slightly decreased at day 10. The TPC and PVC in the natural feeds are presented in Table 1. Among the natural feeds *Artemia* had the highest bacterial load and *Vibrio* count, followed by rotifers and green algae.

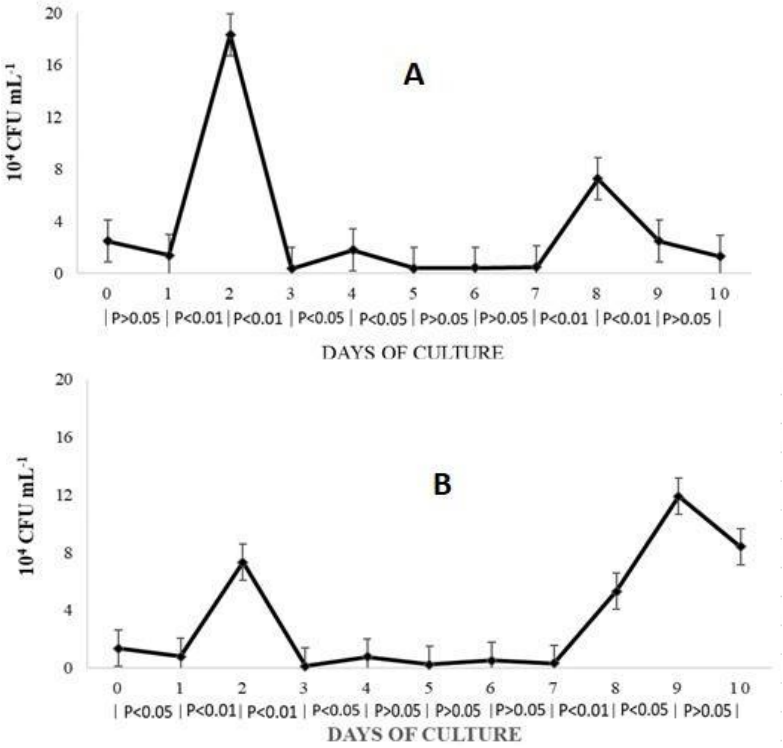

Figure 1. Mean (±SE, n=4) bacterial load (TPC) in (A) rearing water and (B) crab larvae during the 10-day culture of *S. serrata* larvae in static tanks. Significant differences between days of culture are shown (p<0.01=highly significant; p<0.05=significant; p>0.0 =not significant)

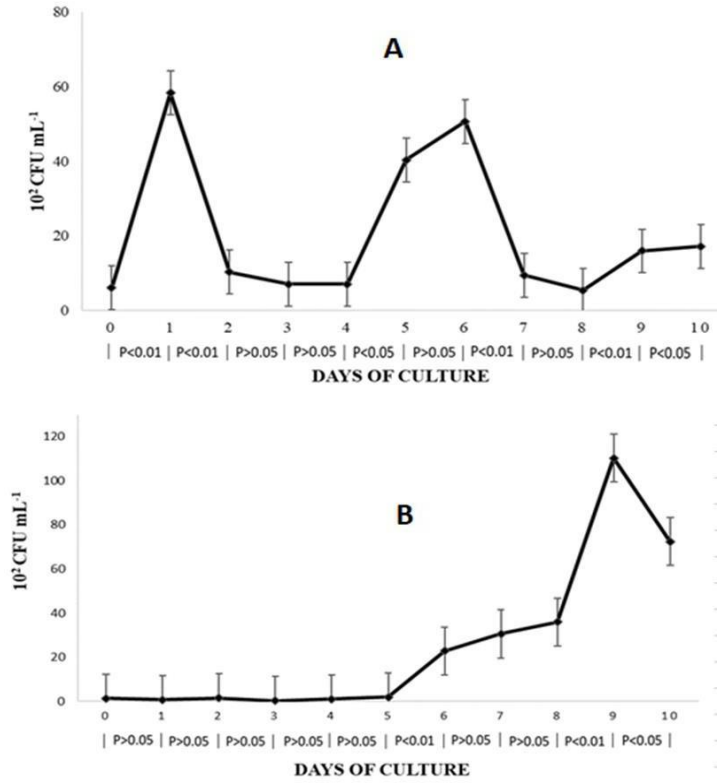

Figure 2. Mean (±SE, n=4) presumptive *Vibrio* counts (PVC) in (A) rearing water and (B) crab larvae during the 10-day culture of *S. serrata* larvae in static tanks. Significant differences between days are shown (p<0.01=highly significant; p<0.05=significant; p>0.05=not significant).

Table 1. Mean (±SE, n=3) total plate count (TPC) of bacteria and presumptive *Vibrio* count (PVC) of natural food used in larval rearing of *S. serrata*.

Natural Food	TPC (CFU mL^{-1})	PVC (CFU mL^{-1})
Green algae (*Nannochloropsis* sp.)	$5.1x10^4 \pm 1.7x10^2$ **a**	$4.5x10^1 \pm 0.3x10^1$ **a**
Rotifer (*Brachionus* sp.)	$2.5x10^5 \pm 8.8x10^3$ **b**	$2.2x10^4 \pm 1.5x10^3$ **b**
Brine shrimp (*Artemia* sp.) nauplii	$2.5x10^7 \pm 3.2x10^6$ **c**	$2.1x10^6 \pm 1.5x10^5$ **c**

Note: Columns with different superscript letter are significantly different (P<0.05).

Isolation and Identification of Vibrio Species

A total of 20 bacterial colonies were isolated from NA^+ and TCBS during the 10-day culture of *S.serrata* larvae (Table 2). Three unique bacterial colonies from the TCBS agar and one luminous colony from the NA^+ were noted: labelled as NC1 (spread on NA^+, green colony on TCBS, entire margin, convex elevation); labelled as HPa (spread on NA^+, yellow colony on TCBS, entire margin, convex elevation); labelled as LD1 (yellow colony on TCBS, entire margin, flat elevation); and labelled as LUM (luminous in NA^+, green colony on TCBS agar, entire margin, convex elevation). All four isolates were Gram-negative, comma-shaped and oxidase-positive bacteria. Based on their morphological and biochemical characteristics, isolates NC1, HPa, LD1 and LUM were presumptively identified as *Vibrio proteolyticus*, *V. alginolyticus*, *V. tubiashii* and *V. campbellii* respectively (Table 3).

Comparison of the results of the partial 16S rRNA sequence of the isolates with sequences of organisms obtained in the gene bank using the NCBI BLAST showed highest similarity of NC1, HPa, LD1 and LUM to *Vibrio proteolyticus* NR 113610.1 (98.98%), *V. alginolyticus* NR122060.1 (99.69%), *V. tubiashii* NR 113791.1 (99.26%) and *V. campbellii* NR 11382.1 (99.05%) respectively, confirming the presumptive identification. The constructed phylogenetic trees of the identified *Vibrio* species further confirmed the result of the BLAST homology searches since they were the closest species related to the bacterial isolates (Figure 3).

Table 2. Bacterial colonies isolated during the 10-day larval culture of *S. serrata*

Isolate	Colony in TCBS			Colony in NA^+		Color in Vibrio Chromogenic Agar	Gram Stain	Code
	Color	Margin	Elevation	Luminescence	Swarming			
1	Green	Entire	Convex	Positive	Negative	Brown	Negative	LUM
2	Green	Entire	Convex	Positive	Positive	White	Negative	NC1
3	Yellow	Entire	Convex	Negative	Positive	White	Negative	HPa
4	Yellow	Entire	Flat	Negative	Negative	White	Negative	LD1
5	Yellow	Entire	Flat	Negative	Negative	White	Negative	LD1
6	Green	Entire	Convex	Negative	Positive	White	Negative	NC1
7	Yellow	Entire	Convex	Negative	Positive	White	Negative	HPa
8	Green	Entire	Convex	Positive	Negative	Brown	Negative	LUM
9	Green	Entire	Convex	Negative	Positive	White	Negative	NC1
10	Green	Entire	Convex	Negative	Positive	White	Negative	NC1
11	Yellow	Entire	Flat	Negative	Negative	White	Negative	LD1
12	Green	Entire	Convex	Negative	Positive	White	Negative	NC1
13	Yellow	Entire	Convex	Negative	Positive	White	Negative	HPa
14	Yellow	Entire	Convex	Negative	Positive	White	Negative	HPa
15	Green	Entire	Convex	Negative	Positive	White	Negative	NC1

Table 2 continued

16	Green	Entire	Convex	Positive	Negative	Brown	Negative	LUM
17	Yellow	Entire	Convex	Negative	Positive	White	Negative	HPa
18	Yellow	Entire	Flat	Negative	Negative	White	Negative	LD1
19	Yellow	Entire	Convex	Negative	Positive	White	Negative	HPa
20	Green	Entire	Convex	Negative	Positive	White	Negative	NC1

Table 3. Phenotypic and biochemical tests result of the different *Vibrio* species isolated from *S. serrata* larval culture during the 10-day rearing.

Tests	LUM	NC1	HPa	LD1
Color on TCBS	G	G	Y	Y
Luminescence	+	-	-	-
Swarming on NA+	-	+	+	-
Color on Chrome Agar	B	W	W	W
Gram Stain	-	-	-	-
Cell form	cs	cs	cs	cs
Growth on Temp.				
4ºC	-	-	-	-
28ºC	+	+	+	+
Growth on NaCl				
0 ppt	-	-	-	-
3 ppt	+	+	+	+
6 ppt	+	+	+	+
8 ppt	+	+	+	-
10 ppt	+	+	+	-
Catalase	+	+	+	-
Oxidase	+	+	+	+
Decarboxylase Tests				
Arginine	-	-	-	-
Lysine	+	+	+	+
Ornithine	-	-	-	-
Carbon Utilization				
Arabinose	+	-	+	-
Glucose	+	+	+	+
Glucose (Gas)	-	-	-	-
Sucrose	-	-	+	+
Inositol	+	+	+	-
Mannitol	+	-	+	-
Citrate Reduction	+	+	+	+
Nitrate Reduction	+	+	+	+
Gelatin Hydrolysis	+	+	+	-

Table 3 continued

OF Test	F	F	F	F
Open Tube	Y	Y	Y	Y
Close Tube	Y	Y	Y	Y
Methyl Red	+	+	-	+
Voge's Proskaeur	-	-	-	-
Indole	+	+	+	+
Resistance to Ampicilin	R	I	R	I
Identification	*V. campbellii*	*V. proteolyticus*	*V. alginolyticus*	*V. tubiashii*

Notes: G-green colony, Y-yellow colony, cs-comma-shaped, F-fermentative, Y-yellow color, R-resistant, I-intermediate, B-brown, W-white, OF-Oxidative/Fermentative

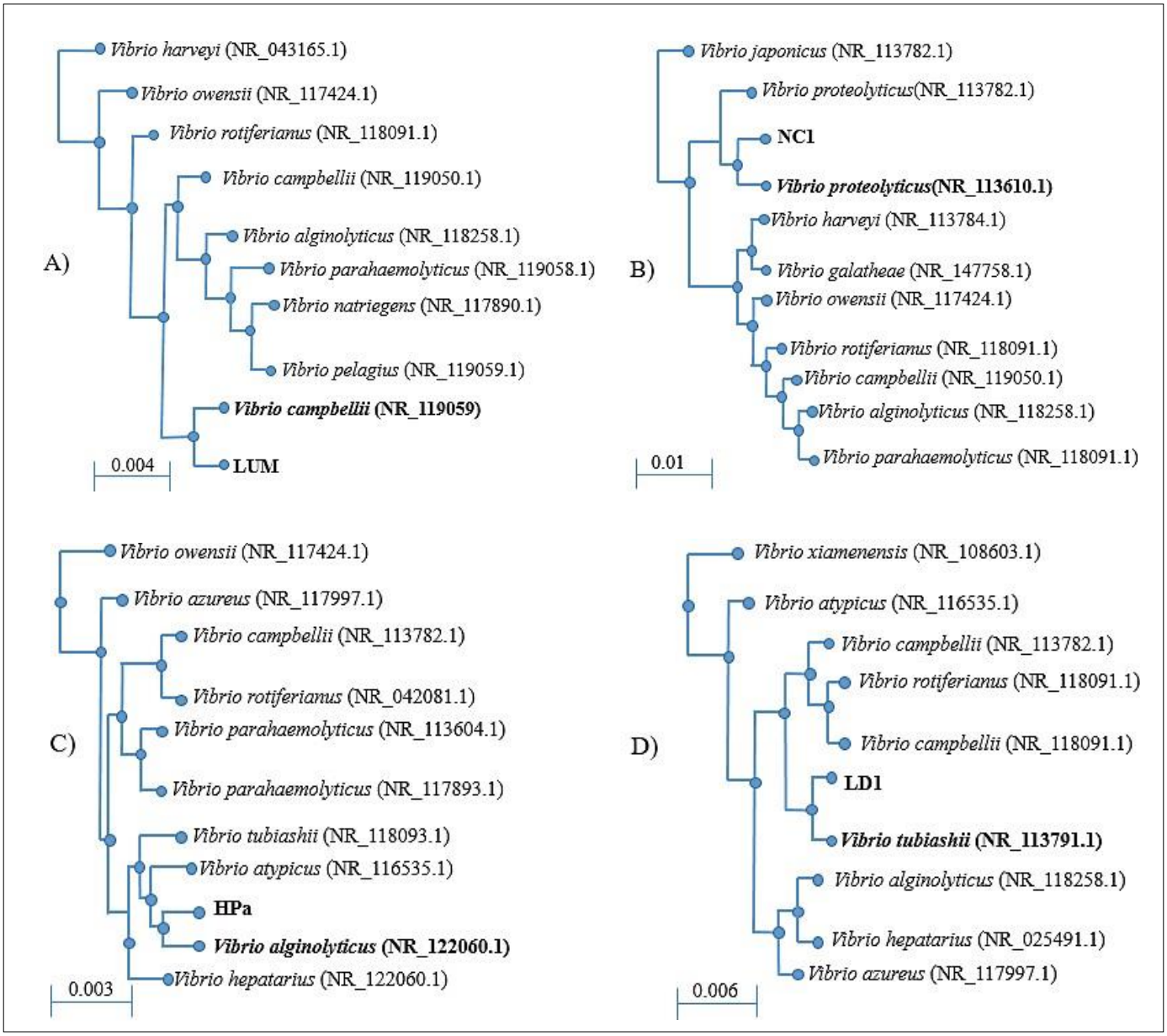

Figure 3. Phylogenetic tree of bacterial isolates (A) LUM, (B) NC1, (C) HPa and (D) LD1 from *S. serrata* larval culture, constructed using neighbor-joining method.

Antibiotic sensitivity test

The zones of inhibition of the different antibiotics tested on the four dominant *Vibrio* species and their corresponding interpretation of results are presented in Table 4. All four species (*V. campbellii*, *V. proteolyticus*, *V. alginolyticus* and *V. tubiashii*) were susceptible to tetracycline and oxolinic acid. Results further showed that *V. campbellii* was resistant to most of the tested antibiotics while *V. alginolyticus* and *V. tubiashii* were susceptible to most.

The diameter of inhibition of the different concentrations of OTC used against four *Vibrio* species are presented in Table 5. The zones of inhibition of the *Vibrio* species generally increased with increasing OTC concentration, however in some species, the diameters of inhibition did not vary ($p>0.05$) between the resistant and the intermediate as well as between the intermediate and the susceptible. Among the four species, *V. proteolyticus* was the most resistant as it was susceptible only at 50 mg L^{-1}.The second most resistant was *V. alginolyticus*, as it was susceptible to at least 40 mg L^{-1}. The third in the rank based on OTC resistance was *V. campbellii*, which was susceptible only to at least 30 mg L^{-1}. The least resistant to OTC was *V. tubiashii* as it was already susceptible to 10 mg L^{-1}.

Table 4. Mean (±SE, n=3) zones of inhibition (mm) of the different antibiotics used against *Vibrio* species isolated from *S. serrata* larval culture during the 10-day rearing and interpretation of results.

Antibiotics (µg)	*V. campbellii*		*V. proteolyticus*		*V. alginolyticus*		*V. tubiashii*		Interpretation of Zone of Inhibition (mm)*		
	Diameter (mm)	Interpre-tation	Diameter (mm)	Interpre-tation	Diameter (mm)	Interpre-tation	Diameter (mm)	Interpre-tation	S	I	R
Tetracycline (30)	21.3 ±.3	S	21 ±.3	S	24.5 ±.3	S	24.2 ±.2	S	≥19	15-18	≤14
Amoxicillin (30)	0 ±0	R	12.3 ±.3	R	22 ±0	S	13 ±0	R	≥18	14-17	≤13
Oxolinic Acid (2)	23.2 ±.2	S	24.8 ±.2	S	16 ±0	S	23.2 ±.2	S	≥11	-	≤11
Trimethoprim (5)	13.3 ±.3	I	11.2 ±.2	I	18.2 ±.2	S	20.2 ±.2	S	≥16	11-15	≤10
Cefprozil (30)	11 ±0	R	0 ±0	R	17 ±0	I	15 ±0	I	≥18	15-17	≤14
Ampicillin (10)	0 ±0	R	15 ±0	S	0 ±0	R	15 ±0	S	≥15	12-14	≤11
Kanamycin (30)	10.2 ±2	R	16 ±.3	R	14.5 ±.3	I	14.2 ±.2	I	≥17	14-16	≤13

*Note: S – Susceptible; I – Intermediate; R – Resistant (CLSI 2015)

Table 5. Mean (±SE, n=3) diameter of inhibition (mm) of the different concentrations of OTC used against four *Vibrio* species isolated from *S. serrata* larval culture during the 10-day rearing and interpretation of results.

Oxytetracycline (mg L^{-1})	*V. campbellii*		*V. proteolyticus*		*V. alginolyticus*		*V. tubiashii*	
	Diameter (mm)	Interpretation	Diameter (mm)	Interpretation	Diameter (mm)	Interpretation	Diameter (mm)	Interpretation
10	13.7 ±.3^{a}	Resistant	13.3 ±.3^{a}	Resistant	15.2 ±.4^{a}	Intermediate	19.0 ±0^{a}	Susceptible
20	17.0 ±.6^{ab}	Intermediate	15.7 ±1.2^{ab}	Intermediate	16.0 ±.6^{ab}	Intermediate	20.7 ±.3^{a}	Susceptible
30	19.3 ±.9^{b}	Susceptible	16.3 ±1.2^{ab}	Intermediate	17.2 ±1.0^{ab}	Intermediate	25.0 ±.6^{b}	Susceptible
40	19.3 ±.3^{b}	Susceptible	16.7 ±2.0^{ab}	Intermediate	19.0 ±.6^{b}	Susceptible	25.0 ±.6^{b}	Susceptible
50	21.3 ±1.3^{b}	Susceptible	20.3 ±.9^{b}	Susceptible	23.0 ±1.2^{c}	Susceptible	28.3 ±0^{c}	Susceptible

*Note: Columns with the same superscript are not significantly different ($P>0.05$); Interpretation of OTC inhibition diameters (CLSI 2015): Resistant ≤14mm; Intermediate = 15-18mm; Susceptible = ≥19mm

DISCUSSION

It is almost impossible to exclude bacteria from mangrove crab larval cultures. Once hatching has occurred, bacterial numbers begin to rise despite the high water exchange rates because of the hatching scum that serve as bacterial substrate and nutrient source (Mann et al. 1999). In addition, the natural food for mangrove crab rearing (i.e., rotifers, algae and *Artemia*) are also vectors for the introduction of pathogenic organisms into larval tanks (Genodepa et al. 2004). Although hazard control procedures to prevent or reduce bacterial loads during mangrove crab larval rearing (Blackshaw et al. 1999) are now part of the current rearing protocols, the chances that mangrove crab larvae will survive longer than four days remain slim unless antibiotics are used.

To ensure that the larvae in this study will survive until the end of the 10-day culture period, OTC was added to the larval tanks together with a high density of rotifers (40 to 60 mL^{-1}) during larval stocking. The OTC was expected to reduce the bacterial load in the whole culture system, particularly in the newly hatched larvae and the rotifers which were known to carry pathogens. The use of high rotifer density was intended to avoid the need to add rotifers daily and consequently reduce the chances of introducing pathogenic organisms (Genodepa 2003). Despite such precautionary measures, bacteria were still ample in the rearing water and larvae during the 10-day culture period. The application of OTC might have reduced the bacterial load during the first few days of culture, but the effectivity was likely limited by the concentration used and the degradation of the antibiotic (Samuelsen 1989).

The increase in total bacteria (TPC) in both rearing water and larvae at the start of the rearing period may have been due to the proliferation of OTC resistant bacterial strains. Nevertheless, TPC levels dropped in the succeeding days, suggesting that the effect of OTC may not be immediate, and prolonged exposure to OTC is necessary to inhibit bacterial growth. This could be one of the reasons why the short-term (1-3 h) treatment of rotifer and *Artemia* with OTC prior to feeding them to mangrove crab larvae was observed in this laboratory to be ineffective. The increase of PVC in the rearing water on day 1 can be attributed to the rotifers and green algae that were added into the tanks at day 0. The results presented in Table 1 confirms the reports that natural food can carry high concentrations of *Vibrio* into rearing systems (Munro et al. 1994; Genodepa 2003). On the contrary, PVC level in the crab larvae was comparatively low during the first five days of larval culture. It is possible that the larvae were not immediately affected by the increase of *Vibrio* count in the water, as it took some time for *Vibrio* to build up in the larval gut because of the inhibitory effect of OTC. The increased density of *Vibrio* after day 5 may be linked to the introduction of *Artemia* nauplii which were reported to be carriers of *Vibrio* (Chen 1993; Torres and Partida 2001; Savas et al. 2005) and found in this study to have significantly higher TPC and PVC compared to green algae and rotifers (Table 1). It is possible that OTC has started to degrade and have limited inhibitory effect on *Vibrio* when *Artemia* was added into the larval tanks.

Finding dominant *Vibrio* species (*V. campbellii*, *V. proteolyticus*, *V. alginolyticus*, and *V. tubiashii*) in this study was not surprising because the presence of different *Vibrio* species in mangrove crab larval cultures were also encountered in other countries. In India, Parenrengi et al. (1993) reported the presence of *V. harveyi*, *V. alginolyticus*, and *V. parahaemolyticus* from mangrove crab zoea, while Sarjito et al. (2016) mentioned the presence of other species such as *V. vulnificus*, *V. nereis*, *V. fischeri* and *V. fluvialis*. In addition, Jithendran et al. (2010) reported that *V. harveyi* and *V. campbellii* were predominant in mangrove crabs infected with White Spot Syndrome Virus. In Vietnam, *V. harveyi* was also identified as major pathogen of mangrove crab larvae and juveniles in hatcheries (Sang 2016). In Australia, *V. harveyi*, *V. alginolyticus*, *V. proteolyticus*, *V. tubiashii* plus another one described only as *Vibrio sp.* were isolated from mangrove crab larval tanks/ rearing water (Akinbowale et al. 2006).

Various authors have reported the isolation of *V. campbellii* from farmed shrimps (Hameed 1995; George et al. 2006; Haldar et al. 2010; 2011; Wang et al. 2015) but some authors have misidentified *V. campbellii* as *V. harveyi* due to the high similarity among *Vibrionaceae* (Gomez-Gill et al. 2004; Haldar et al. 2010). Several authors have considered *V. campbellii* responsible for the disease known as Zoea II syndrome (Soto-Rodriguez et al. 2006b; Burge et al. 2009; Cuellar-Anjel et al. 2014; Kumar et al. 2017) as well as septic hepatopancreatic necrosis disease (Stern and Sonnenholzner 2014; Morales-Covarrubias et al. 2018). While *V. campbellii* has been reported as non-pathogenic and non-luminous (de la Peña et al. 2001; Lin et al. 2010), there were also studies revealing that *V. campbellii* could cause

luminescent disease in shrimps (Gomez-Gil et al. 2004; Phuoc et al. 2008; Wang et al. 2015). Results of this study support the findings that *V. campbellii* could be luminescent; the luminous *Vibrio* colony isolated in this study was ascertained to be *V. campbellii* based on both the biochemical analysis and 16s rRNA sequencing.

There are many reports stating that *V. alginolyticus* could cause various diseases in crustaceans such as shrimps and lobsters (Handlinger et al. 1999; Jayaprakash 2006; Soto-Rodríguez et al. 2006a; Soto-Rodríguez et al. 2006b; Bourne et al. 2007; Goulden et al. 2012; Cuellar-Anjel et al. 2014; Stern and Sonnenholzner 2014; Kumar et al. 2017; Morales-Covarrubias et al. 2018; Radhakrishnan and Kizhakudan 2019). Mortality of mangrove crab larvae has also been associated with high incidence of *V. alginolyticus* (Shelley et al. 2008), however, some studies have revealed that *V. alginolyticus* is beneficial since it has a probiotic effect. The report of Vandenberghe et al. (1998) indicated that *V. alginolyticus* has an antagonistic effect on *V. harveyi* in the culture of Penaeus chinensis. Similarly, *V. alginolyticus* has been proven as probiotic against *V. harveyi* in mangrove crab larval culture by Liessmann (2005).

It is interesting to note that both *V. proteolyticus* and *V. tubiashii* were isolated in this study, as this is probably the first record of the isolation of these species of *Vibrio* from mangrove crab larvae in the Philippines. Both species were likewise isolated from the mangrove crab larval rearing tank/water in Australia, and so far, only this paper and that of Akinbowale et al. (2006) documented the presence of both *V. proteolyticus* and *V. tubiashii* in mangrove crab larval tanks. It was reported that *V. proteolyticus* is a common pathogen isolated from *Macrobrachium rosenbergii* (Jayaprakash et al. 2005) and *Artemia* spp. (Verschuere et al. 2000; Marques et al. 2006). Through proteomics, it was known that *V. proteolyticus* can cause actin cytoskeleton rearrangement, leading to cell death (Ray et al. 2016). Meanwhile, *V. tubiashii* was first isolated from both larvae and juveniles of bivalve mollusks (Tubiash et al.1965). Since then, several studies have reported the virulence of *V. tubiashii* on bivalves, particularly on oysters, scallops and clams (Hasegawa et al. 2008; Richards et al. 2014; Prado et al. 2015, Rojas et al. 2016).

While antibiotics have been effective prophylactic agents in mangrove crab larval cultures (Shelley 2004; Genodepa et al. 2004; Shelley et al. 2008; Azam and Narayan 2013; Thirunavukkarasu et al. 2014; Pates et al. 2017), concern about potential outbreak of multidrug resistant microbial strains has been growing (Vignesh et al. 2011). Several authors have reported that *Vibrio* isolated from most crustaceans and finfishes, wild mangrove crab, shrimp farms, finfishes and crustaceans, as well as from aquaculture facilities revealed that the *Vibrio* genera were multidrug resistant (Akinbowale et al. 2006; Aftabuddin et al. 2013; Arunagiri et al. 2013; Garcia-Aljaro et al. 2014; Heenatigala and Fernando 2017). Similarly, this study showed that the isolated *V. campbellii*, *V. proteolyticus* and *V. tubiashii* were multidrug resistant while *V. alginolyticus* was only resistant to ampicillin. In addition, Akinbowale et al. (2006) reported that 75% of the 62 *Vibrio* species isolated from aquaculture facilities were resistant to at least one antibiotic. So far, *Vibrio* resistance was highest for amoxicillin (45%) followed by ampicillin (40%), while it was comparably low for tetracycline (5%) and there was no resistance to trimethoprim and oxolinic acid. Similarly, results of this study showed the *Vibrio* isolates' resistance to ampicillin and amoxicillin and susceptibility to oxolinic acid, tetracycline and trimethoprim.

Among the three potential antibiotics to treat *Vibrio* infection in this study, OTC was considered for further investigation as it is widely approved for veterinary use, least expensive and easily available. According to Shelley and Lovatelli (2011) bacterial pathogens in mangrove crab larval rearing can be controlled by using 25-50 mg L^{-1} OTC, however, there were many instances wherein considerably good survival was attained even at lower OTC doses. An example is the report of Azam and Narayan (2013), where the use 10 and 25 mg L^{-1} OTC in the mangrove crab larvae obtained 25% and 53% survival up to megalopa stage respectively, instead of the usual less than 10%. While rearing success is dependent on many factors, Cruz-Lacierda et al. (2000) noted minimal success in treating vibriosis in shrimp hatcheries in the Philippines when lower OTC concentrations are used. Looking at Table 5, it is probably unsafe to consider the use of OTC concentration that resulted in intermediate susceptibility only, because of the lack of significant differences ($p>0.05$) in diameters of inhibition zones between the resistant and intermediate classifications, like in the case of *V. campbellii* and *V. proteolyticus*.

It was not surprising to find out that 50 mg L^{-1} OTC was required to inhibit the in-vitro growth of the *Vibrio* species isolated in this study. Hameed and Rao (1994) reported that even at OTC concentration of 150 mg L^{-1}, the *Vibrio* density in seawater could only be reduced from 10^{12} cfu mL^{-1} to 10^{3} cfu mL^{-1} after 24 h. Baticados et al. (1990) also reported that the minimum bactericidal concentration of OTC for luminous *Vibrio* species isolated from diseased *P. monodon* larvae was greater than 100 mg L^{-1}. While it is almost impossible to prevent antibiotic resistance in *Vibrio*, it is vital to know which antibiotics and dosages will be effective. This study therefore reports the presence of four dominant species of *Vibrio* (*V. campbellii*, *V. proteolyticus*, *V. alginolyticus*, and *V. tubiashii*) isolated during early stages of mangrove crab larval culture, together with their characteristics and responses to antibiotics. Except for *V. alginolyticus*, the isolated *Vibrio* were all multidrug resistant but were susceptible to tetracycline, oxolinic acid and trimethoprim. Susceptibility trials suggest that OTC may potentially prevent or treat possible diseases in mangrove crab larvae caused by the four identified *Vibrio* species, but at least 50 mg L^{-1} is needed to inhibit all of them. The results of this study may help explain the possible reasons for the mortalities encountered in mangrove crab larval rearing and provide new insights for the improvement of rearing protocols, particularly on the use of antibiotics.

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