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Determination of survival and haemocyte response of mud crab *Scylla olivacea in vivo* challenged with *Vibrio*

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Abstract. *Vibrio* spp. are pathogenic bacteria, and cause shell diseases of Mud crabs. This study was conducted to determine pathogenic load of *Vibrio* sp. to Mud crab *Scylla olivacea* by assessing survival and haemocyte cell variation of Mud crabs *in vivo* challenged with *Vibrio* sp. Mud crabs collected from local market were firstly acclimatized, and then kept in plastic tanks provided feeding with chopped Tilapia for 9 days. In case of the challenge test, 1×10^6, 1×10^7 and 1×10^9 CFU ml^{-1} *Vibrio* sp. were inoculated in the treatment groups T_1, T_2, T_3, respectively; the control group had no *Vibrio* inoculums. This study reported that there was a significant difference of mortality rate ($p<0.05$) between the control and the treatment groups. The control group had no mortality but the treatment groups had 50% cumulative mortality rate (CMR) on 6 day-post challenge (dpc). The first 100% CMR was observed in the T_2 group, and accordingly, the *Vibrio* load $>$ 10^6 CFU ml^{-1} was found to cause vibriosis in *S. olivacea* in a short period of time. In this study, haemocyte profile were characterized: the mean total haemocyte count (THC) was $4.71\pm0.22\times10^7$, $3.57\pm0.61\times10^7$, $4.43\pm0.59\times10^7$ and $5.03\pm0.69\times10^7$ cells ml^{-1} in the C, T_1, T_2 and T_3 groups, respectively. There was significant difference ($p<0.05$) between the control and the treatment groups for the THC, and after 6 dpc, the THC was found decreased in the treatment groups. There was also variation in the percentage of differential haemocyte count (DHC); larger granular cells were found significantly varied between the control and the treatment groups, pointing out the key role of granular cells in the interaction between *S. olivacea* and *Vibrio* spp. The present study provides useful information for understanding the immune response of *S. olivacea* to *Vibrio* infection.

Keywords: Mud crab, *Scylla olivacea*, Haemocyte, *Vibrio*

Introduction

Mud crabs (*Scylla* spp.), the key artisanal coastal fisheries resource in many tropical and subtropical Asian countries, become one of the important export commodities with its high demand in sea food market. Four species of Mud crabs *Scylla serrata, S. olivacea*, *S. paramamosain* and *S. tranquibarica*, are of great commercial importance. *S. olivacea* is the most commonly found Mud crab species in Bangladesh (Rouf *et al.* 2016). Mud crab fishery and aquaculture supports the livelihood of the fisher-folks in Bangladesh (Azad *et al.* 2009, Hasanuzzaman *et al.* 2014). Mud crabs are less susceptible to disease and more resistant to adverse environment conditions, poor water quality and climate change; thus become potential aquaculture species. The present culture system of Mud crab mostly involves fattening of immature (having underdeveloped gonads) and/or underweight (<80 g for female and <100 g for male) Mud crab to produce market-size hard-shell crabs, which are exported live. Over the last 6-7 years, soft-shell Mud crab production venture has been practiced, predominantly in the

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Kolbari and Munshigonj of the Satkhira district. Accordingly, there has been intensification of Mud crab farming along with high stocking density, and such intensification may result in occurrence of diseases; there have been increasing reports on disease incidence in Mud crabs across the world (Lavilla-Pitogo *et al.* 2001, He *et al.* 2003, Lavilla-Pitogo and de la Peña 2004, Poornima *et al.* 2008, Jithendran *et al.* 2010). Now-a-days crab fatteners in Bangladesh have also pointed out the disease-associated problems in their farms (*pers. commun.*). Bacterial diseases are the most ominous concern linked to production loss; among various bacterial diseases, shell disease is most frequently observed. Shell disease, characterized with dark brown or black pigmentation and perforation on shell, is mainly caused by chitinolytic bacteria including *Vibrio, Aeromonas.*

Like other invertebrates, Mud crabs have innate immunity which is the first line of inducible host defense against bacterial, fungal, and viral pathogens (Hoebe *et al.* 2004). This innate immune system is composed of cellular and humoral responses, and the cell-mediated defence is principally carried out by haemocytes circulating free amongst all tissues. Crustacean haemocytes have significant role in early nonself recognition, phagocytosis, encapsulation, and elimination of pathogens from the host body (Söderhäll and Smith 1983, Johansson *et al.* 2000). There are several studies of Mud crabs, including farming practice (Kamal *et al.* 2003, Salam *et al.* 2012, Hasanuzzaman *et al.* 2014, Islam 2015); fattening (Ferdoushi and Xiang-guo 2010); socio-economic status of Mud crab collectors and fatteners in Bangladesh (Molla *et al.* 2009); but there is no scientific work of *Vibrio* pathogenicity in *S. olivacea*. Taking into account of such dearth, the present study was developed to assess the immune response of Mud crab (*S. olivacea*) *in vivo* challenged with pathogenic bacteria *Vibrio* sp. To the best of our knowledge, this is the first investigation determining the *Vibrio* load causing mortality of *S. olivacea* as well as revealing haemocyte-response variation due to *in vivo Vibrio* challenge.

Materials and Methods

***Mud crab collection and rearing*:** For conducting this study, 40 healthy, fresh and active Mud crabs of 75-110 g were collected from the depots of Khulna city, and the experiment was conducted in the laboratories of Fisheries and Marine resource Technology Discipline, Khulna University. In order to make crabs prepared for the experiment, crabs were acclimatized in the experimental tanks for 3 days. Then the challenge experiment was conducted in the tanks containing 12 litters water of 10 ppt salinity.

***Isolation and perpetuation of Vibrio* sp.:** *Vibrio* sp. was isolated from apparently diseased Mud crabs collected from wild sources. The shell, gill, gut and intestinal tract were dissected from the diseased crabs using sterile dissecting instruments, and thereafter pool samples were prepared by mixing these dissected organs from each crab. The pooled sample was homogenized with 1 ml distilled water and kept in a 2 ml Eppendorf tube and stored at -4 °C temperature for further use. To obtain isolates, 25 g of each homogenate was placed in 225 ml of alkaline peptone water (APW; pH 8.6), and incubated at 37 °C for 6-8 hrs; a loopful of culture (Enriched APW broth) was then streaked on Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) agar plate, and incubated at 37 °C for 24 hrs. The plates were checked for the characteristic colony of *Vibrio* spp., and large (2-3 mm), green with blue centered colony was selected. A pure

culture of isolated *Vibrio* sp. was done on TCBS agar plate, and pure culture isolate was kept in Eppendorf tube containing autoclaved distilled water (80%) and glycerol (20%) and subsequently stored at -80 °C. During challenge test, fresh *Vibrio* was prepared by adding one loopful of stock culture (V/V) in autoclaved LB_{10} medium and incubated for 24 hr at 30 °C on a shaker under constant agitation.

Pathogen challenge test: The challenge test was conducted with selected *Vibrio* species for 9 days; there were one control group and three treatment groups; each group had replicates. Two crabs were kept in each tank of 12 litters; the control group had no *Vibrio* inoculums, and the challenge test group had T_1, T_2 and T_3 treatments wherein $1x10^6$, $1x10^7$ and $1x10^9$ CFU ml^{-1} *Vibrio* were inoculated, respectively. During the challenge period, crabs were fed (at 5% of body weight) two times daily with chopped Tilapia fish. Crabs were checked two times daily for observing disease and/or infection symptoms as well as mortality. For Haemolymp collection, the Mud crabs were sampled before challenge test and from the control and treatment groups on days post challenge (dpc) when mortality occurred.

Total haemocyte count: Haemolymph (100 µl) was collected from the swimming legs using a 1 ml sterile syringe (25-gauge, 0.45 mm) containing 0.9 ml of an anticoagulant solution (0.114 M trisodium citrate and 0.1 M sodium chloride, at pH 7.45 and with an osmolality of 490 mOsm/kg). The total haemocyte count (THC) was done using a Neubauer haemocytometer chamber (Model: ART1280, China) with a modification of the method described by Bain *et al.* (2001). A drop of the haemolymph suspension was placed on a haemocytometer to estimate the THC using a trinocular research microscope (Labomed, Model: iVu1500, Lx400, USA).

Differential haemocyte count: Differential haemocyte counts (DHC) were performed following the methods of Celi *et al.* (2014). Briefly, a drop of haemocytes cell suspension was smeared on a slide to prepare a monolayer and allowed to air dry, and then, the monolayer was fixed with few drops of absolute methanol and inundated for 6 minutes. The haemocyte monolayer was stained with Giemsa solution (1:10 dilution) for 10 minutes and dehydrated with 70% ethanol for1 minute and immersed in xylene for 6 minutes (Celi *et al.* 2014). Then, the cells were observed under the microscope, and haemocytes were differentiated based on morphology and metachromatic staining of cytoplasm, cytoplasmic granules and nuclear chromatin (Hrubec and Smith 2000). Haemocyte differential cells were presented as non-granular haemocyte (NGH), large granular haemocyte (LGH), small granular haemocyte (SmGH), semi granular haemocyte (SGH). At least 200 cells on each slide were counted in random areas, and the DHCs were calculated by using the following equation:

DHC (%)={(Number of different haemocyte cell types)/ (Total haemocyte cell counted)} × 100

Determination of survival: During the challenge test period, the survival of crabs for each experimental group was recorded by the following formula:

$$\text{Survival rate } (\%) = Ni - Nf / (Ni) \times 100$$

Ni = Initial number of animals

Nf = Final number of animals.

Data analysis: All data analysis was carried out using Microsoft office Excel (ver. 2007) and SPSS (version 20). Generalized linear model was used to determine the significant variation of the survival rate, total and differential haemocytes counts among the experimental groups.

Results

At the beginning of the challenge test period, all Mud crabs were very healthy fresh and active but after 2-3 days, some abnormal symptoms were observed in the treatment groups compared to the control group, and gradually the abnormal symptoms were prominently evident (Table I; Fig.1).

Table I. Physical appearance of Mud crabs in the experiment

Characteristics	Control group	Treatment groups
Activity	Normal	Slow and sluggard on day 1
Feeding rate	Normal	low feeding on day 2
Body color	Normal	Yellowish color on day 6
Shell condition	Normal	Soft shell
Carapace	Normal	Small black spot on the carapace on day 8
Appendages	Normal	Soft and loosen appendages on day 7
Mortality	No occurrence of mortality	Highest mortality on day 8

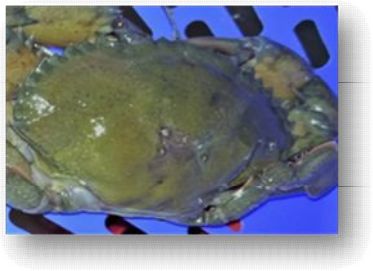

Fig. 1(a). *Vibrio* infected Mud crab

Fig. 1(b). Healthy Mud crab

Mortality of Mud crab: There was no mortality in the control group during the 9 day-challenge test period. The first mortality occurred in the treatment groups T_1 and T_2 on 2 dpc but no disease symptoms were found (Table II.). The mortality rate in the treatment groups on 6 dpc and 7 dpc was as same as on 2 dpc. The highest mortality was observed on 8 dpc. All crabs died in the T_2 and T_3 groups and the first 50% cumulative mortality rate (CMR) was found on 6 dpc (Fig.2).

Table II. Mortality (%) of the experimental Mud crabs

Day after challenge	Mortality (%)				Disease symptoms
	Control[a]	Treatment$_1$[b]	Treatment$_2$[b]	Treatment$_3$[b]	
Day 0[a]	0	0	0	0	
Day 1[a]	0	0	0	0	
Day 2[b]	0	25	25	0	No
Day 3[a]	0	0	0	0	
Day 4[a]	0	0	0	0	
Day 5[a]	0	0	0	0	
Day 6[b]	0	25	25	0	White patch on shell
Day 7[b]	0	0	25	25	Soft abdomen shell
Day 8[c]	0	25	25	25	Small black spot on the carapace
Day 9[c]	0	0	-	50	Soft abdomen shell

Different superscripts in the same column and in the same raw indicate significant difference ($p<0.05$).

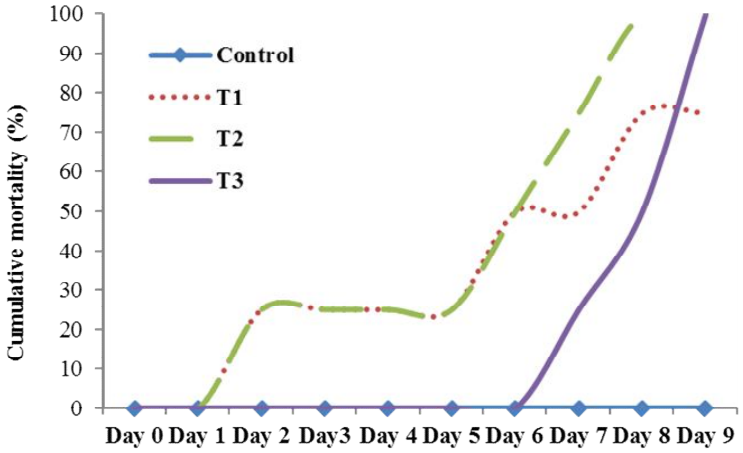

Fig. 2. Cumulative mortality % of Mud crab challenged with *Vibrio* sp.

Effect of Vibrio* sp. *load on Mud crab mortality: The test model effect demonstrated that the *Vibrio* sp. challenge rendered significant effect on the mortality of Mud crab ($p<0.05$) (Table II). The faster CMR was detected in the T_2 group wherein $1x10^7$ CFU ml^{-1} *Vibrio* sp. were inoculated (Fig.2). The exposure of crab to *Vibrio* sp. with a higher concentration and for long duration might lead to their higher mortality.

THC and DHC in Mud crab: The THC of the Mud crabs ranged between $4.5\text{-}6.18x10^7$ cells ml^{-1} before challenge test (Table III). After inoculating *Vibrio* sp., there was significant difference ($p<0.05$) of THC between the control and the treatment groups (Fig.3); the highest THC was found in the T_3 group till 7 dpc.

Table III. THC (cell/ml) and DHC (%) in Mud crabs before challenge test

Tank	THC cells ml^{-1}	DHC %			
		Large granular	Semi granular	Small granular	Non granular
T1	4.5×10^7	46.33±3.64	18.33±3.04	19.66±1.79	18.04±2.30
T2	6.03×10^7	42.33±2.16	20.02±6.89	17.33±3.35	21.33±4.28
T3	6.18×10^7	45.01±3.64	15.33±2.39	21.07±3.76	19.66±3.34
Control	4.94×10^7	57.66±1.79	15.06±3.24	19.03±3.74	10.66±3.64

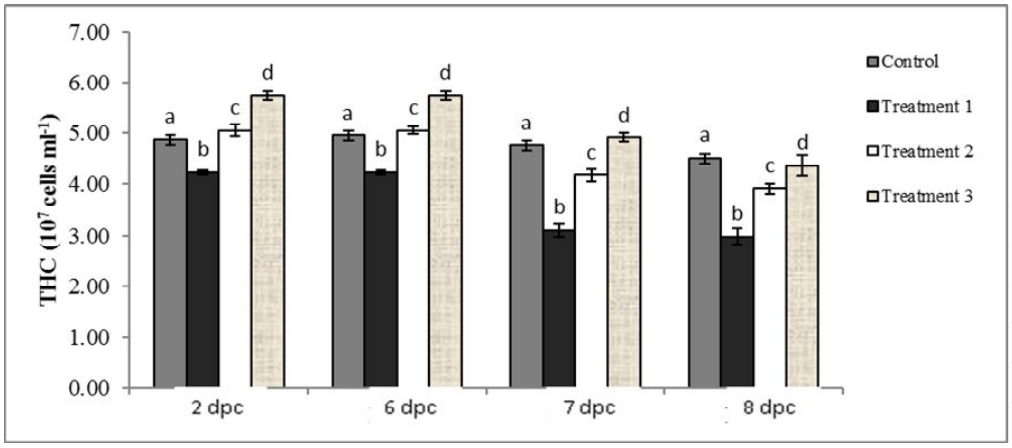

Fig. 3. THC (cells/ml) of Mud crabs after challenge test.

Differential haemocyte count (DHC) during challenge test: The DHC analysis reveals that the highest LG count (16.46%) was found in T_3 on 6 dpc and the lowest LG count (7.23%) was found in T_2 on 8 dpc (Table IV). There was no significant difference ($p>0.05$) found within the control group for LG cells during the experiment period but the LG counts were significantly different ($p<0.05$) among the experimental groups. In case of SG cells, the highest count (39.06%) was found in the T_2 on 2 dpc and the lowest count (21.96%) in the T_1 on 8 dpc. There was significant difference ($p<0.05$) between the control group and the treatment groups, but no significant difference between T_2 and T_3. The highest SmG count was in the T_1 (47.62%), and the lowest SmG cells in the T_2 group (29.89%) on 8 dpc, and significant difference ($p<0.05$) between the control and the treatment groups was observed. In case of NG cells, no significant difference was found between the control group and the treatment groups. The lowest NGH count (9.84%) was found in the control group on 7 dpc, and the highest (26.31%) on 6 dpc in the T_1 group.

Table IV. DHC % (Mean ± SD) of Mud crabs during the challenge period

Groups	Differential haemocyte cell	Day post challenge			
		2 dpc	6 dpc	7 dpc	8 dpc
Control	LG	11.61±4.77	12.57±4.21	13.02±2.65	12.01±2.08
	SG	31.67±4.55	32.89±2.82	37.49±1.71	38.18±2.94
	SmG	40.63±4.01	42.21±4.89	40.01±1.71	35.92±5.88
	NG	16.09±3.86	12.36±2.98	9.84±6.24	13.72±4.19
Treatment 1	LG	10.94±2.22	9.10±2.65	9.66±2.50	8.56±3.10
	SG	28.08±4.57	24.53±4.40	22.61±3.11	21.96±.96
	SmG	42.16±3.39	39.62±3.10	46.98±2.82	47.62±5.12
	NG	18.79±3.39	26.31±3.56	19.74±4.51	21.52±4.27
Treatment 2	LG	12.53±2.99	14.34±3.51	11.94±2.58	7.23±1.83
	SG	39.06±3.30	34.63±4.27	33.89±4.35	29.24±1.71
	SmG	34.37±5.06	32.24±2.70	29.89±2.08	25.01±4.35
	NG	16.01±4.20	18.78±3.30	24.69±1.50	25.06±2.52
Treatment 3	LG	15.32±4.03	16.46±4.64	12.03±5.08	10.94±3.30
	SG	33.23±4.79	31.63±4.79	30.69±2.22	29.13±4.04
	SmG	37.51±4.79	40.12±3.30	37.31±2.49	39.81±3.30
	NG	13.97±5.45	11.54±4.11	20.01±2.22	20.13±4.19

Discussion

Vibrio spp. is one of the main pathogens to cause high levels of mortality in the culture industry of Mud crabs. Lavilla-Pitogo *et al.* (2001) reported shell disease in the Mud crabs after being held in tanks for three months, and speculated *Vibrio* spp. as the causative agent. The present study was attempted to evaluate the immune competence of *S. olivacea* against pathogenic bacteria *Vibrio.* During 9 day-long challenge test period, all crabs of the control group were very healthy and alive; no disease symptoms or mortality was found. But in the Mud crabs of the treatment groups, some abnormal symptoms (brown or light red patch on shell, soft abdomen shell and small black spot on carapace) were observed from 2 dpc and gradually the abnormal symptoms were prominently evident and lastly mortality occurred, which pointing out the pathogenicity of *Vibrio* sp. Such symptoms are characteristic features of shell diseased crabs; there are discolored patches on the carapace initially appeared on the carapace, soft exoskeleton and calcified tissue underneath exoskeleton of Mud crabs challenged with *Vibrio* (Lavilla-Pitogo *et al.* 2001).

The present study determined the pathogenic load of green *Vibrio* sp.; the treatment group T_1 with $1x10^6$ CFU ml^{-1} *Vibrio* inoculum had 75% mortality but no severe disease symptoms were observed. On the other hand, $1x10^7$ and $1x10^9$ CFU ml^{-1} *Vibrio* inoculation caused 100% mortality of Mud crabs with diseased symptoms. Such pathologic variation was likely associated with *Vibrio* load and exposure duration. According to Srimeetian *et al.* (2017), 100% cumulative mortality was found on 1 and 2 day after challenging the healthy Mud crabs with 10^8-10^{10} and 10^7 CFU ml^{-1} *V. parahaemolyticus*. During 30 day-challenge period, the cumulative mortality was 22.22 % with 10^6 CFU ml^{-1} but no cumulative mortality was detected with 10^5-10^1 CFU ml^{-1} of *V. parahaemolyticus* inoculum. Lavilla-Pitogo *et al.* (2001) has also reported 10^4-

10^7 CFU g^{-1} *Vibrio* count in severely diseased exoskeleton of Mud crab broodstock held in the tank for 3 months. Accordingly, the present study pointed out that the green *Vibrio* load greater than 10^5 CFU ml^{-1} might cause mortality of *S. olivacea* when exposed for long period of time.

In host-pathogen interaction studies, the profile of haemocytes is widely used to evaluate the health status of the target species. Haemocytes play very crucial role in the defence system of crustaceans against microbial pathogens: phagocytosis, haemocyte clotting, hardening of exoskeleton, healing of cuticle damage, encapsulation (Johansson and Soderhall 1989, Noga *et al.* 2000). The present study estimated THC in the range of 2.98-5.75x10^7 cells ml^{-1}, which is almost similar to the mean THC (2.86x10^7 cells ml^{-1}) of *S. serrata* (Kumar *et al.* 2013). The haemocytic response of *S. olivacea* to *Vibrio* infection was characterized; the THCs of the healthy crabs were not significantly varied in the healthy crabs but there was varied trend of the THCs in the *Vibrio* challenged crabs along the challenge period. The higher THC of the Mud crabs in the treatment groups might be associated with initial stage of vibrosis, when the haemocytes are likely increased in the circulating system and differentiated variedly to provide defence against invading pathogens. On the other hand, the reduction of the THC on 7 and 8 dpc was an indicative of immunological suppression in response to critical stage of vibriosis characterized with higher mortality of Mud crabs in the present investigation. It is of note that compared to the control group, the THC in the T_1 group was found lower to which *Vibrio* load was likely linked since the haemocyte count can be varied with infection type and environmental stress (Persson *et al.* 1987, Smith and Jhonston 1992); however, this fact has to be elucidated through further prudent studies.

The present study also revealed that the *Vibrio* infection caused variation in the haemocyte sub-type count of *S. olivacea*, as reported in crustaceans exposed to pathogens by Sequeira *et al.* (1996) and Zhou *et al.* (2018). In comparison with the control group, the LG, SG and SmG morphotypes of the haemocytes varied significantly. There was no distinct trend in granulocytes of Mud crabs in the control group while the LG and SG counts of the treatment groups follow a pattern; like the THC in the T_2 and T_3 groups, there was increasing number of LG cells till 6 dpc, and thereafter the counts were reduced. Regarding semi-granular cells, there is decreasing number of these cells along the challenge period. In the interaction between *S. olivacea* and *Vibrio* sp., such observations pointed out the role of granular cells which are actively involved in phagocytosis (Noga *et al.* 2000, Zhang *et al.* 2013) and in hydrolytic, oxidative, phenoloxidase enzyme activities as well as superoxide anion production (Matozzo and Marin 2016). However, the immune function of haemocyte sub-population of *S. olivacea* in response to vibriosis is to be explored explicitly through further enzymatic and gene expression studies. In conclusion, this study demonstrated that the *Vibrio* infection caused mortality of *S. olivacea,* and the load greater than 10^6 CFU ml^{-1} was found to cause disease symptoms in *S. olivacea* under experimental condition. There was change in THC and DHC between the control and the treatment groups, and such variation in THC and DHC suggests the role of *S. olivacea* haemocytes against vibriosis.

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