Research Article

Total Bacterial Load and Enumeration of Pathogenic *Bacteria vibrio* spp., *Aeromonas* spp. and *Pseudomonas* spp. in the Production Line of Mud Crab (*Scylla olivacea*) Hatchery in Bangladesh

Md. Rashedul Islam[1], Abul Farah Md. Hasanuzzaman[1*], Md. Latiful Islam[2], Ghausiatur Reza Banu[1]

[1]Khulna University, Bangladesh; [2]Bangladesh Fisheries Research Institute (BFRI), Bangladesh.

Abstract | The increasing demand for mud crabs (*Scylla* spp.) has created immense pressure on the wild populations globally. To reduce threats to wild stocks, it has become an urgent issue of increasing mud crab larvae production in captivity. But some chitinolytic bacteria (*Vibrio* spp., *Aeromonas* spp., and *Pseudomonas* spp.) poses major threats in the production line of mud crab larvae. Hence, the present study tried to determine prevalence and load of total and pathogenic bacteria in the different components of the mud crab hatchery. Selective agar media, a series of biochemical tests, and bacteria isolation kits were used for the isolation and identification of bacteria. The genus *Vibrio*, *Aeromonas* and *Pseudomonas* were identified in different components of the larval production line in the hatchery. *V. mimicus*, *V. parahaemolyticus*, *V. harveyi* and *V. alginolyticus* were the major species identified from the *Vibrio* genus. The average concentrations of *Vibrio* spp. in brood, brood's tank water, eggs, larvae, larval tank water, and larval feed were $\log_{10}$ 3.86±0.61 cfu g^{-1}, $\log_{10}$ 3.63±0.57 cfu mL^{-1}, $\log_{10}$ 2.73±0.13 cfu g^{-1}, $\log_{10}$ 2.86±0.21 cfu g^{-1}, $\log_{10}$ 2.93±0.25 cfu mL^{-1} and $\log_{10}$ 3.23±0.37 cfu mL^{-1} respectively. The highest concentration of *Aeromonas* spp. ($\log_{10}$ 3.62±0.24 cfu g^{-1}) and *Pseudomonas* spp. ($\log_{10}$ 2.95±0.22 cfu g^{-1}) was detected in the brood sample of batch 1 and batch 4, respectively. The variations in *Aeromonas* and *Pseudomonas* counts among different batches of different components were statistically non-significant ($p>0.05$). While, most of the components along the larval production line had significantly ($p<0.05$) varied *Vibrio* counts for different batches. Larval feeds were found mostly contaminated with pathogenic bacteria, especially with pathogenic *Vibrio* in the production line of the hatchery; hence, special biosecurity measures are needed in this particular component to substantially reduce horizontal transmission of such pathogens in the larvae; particularly axenic live food culture is to be practiced. The knowledge gained from this study will be useful to the hatchery operators taking appropriate measures to strengthen the biosecurity line for controlling bacterial contamination of the various biological and physical components, and thus for reducing larval mortality within an acceptable range in captivity.

Keywords | *Scylla* spp., pathogenic bacteria, Contamination, Larvae, Biosecurity

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***Correspondence** | Abul Farah Md Hasanuzzaman, Khulna University, Bangladesh; **Email:** afmhzzaman@fmrt.ku.ac.bd
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INTRODUCTION

The global demand for aquaculture products including mud crab causes rapid commercial exploitation of these species in recent years, and is projected to increase in the future. (Hungria et al., 2017). The increasing demand and high price of mud crab in the domestic and international market have motivated farmers to expand mud crab aquaculture utilizing locally available seeds (Azra and Ikhwanuddin, 2016). But, the sustainability of mud crab aquaculture is at risk because the crab farmers still rely mostly on the wild-caught crabs for seed stocks, fattening

and soft-shelled crab production; thus rapid expansion and large-scale aquaculture of mud crabs is still inconvenient due to the small success rate of crablet production in the hatchery systems (Waiho et al., 2018). In Bangladesh, mud crab aquaculture is being rapidly intensified in the climatically vulnerable coastal region as an alternative livelihood for the coastal fisher folks (Hasanuzzaman et al., 2014). Due to high mortality of larvae in the hatchery, farmers depend on wild seeds and harvest mud crabs from the natural sources, which creates immense pressure on the wild stocks.

Disease is a key problem in aquaculture, regardless of the type of production and/or the species targeted. For instance, major obstacles to larval production in hatcheries include disease caused by opportunistic pathogens, such as vibrios (Sui et al., 2011; Zhang et al., 2014), a lack of knowledge of suitable diets (Basford et al., 2021) and cannibalistic behavior of some crab species (Romano and Zeng, 2017). Crustacean larvae are very much susceptible to disease during their initial developmental stages, where survivability, normal growth and development require specific biotic and abiotic conditions (Quinitio et al., 2001). Mud crab-producing countries across the world are working on various aspects of mud crab larviculture and larval rearing technology, which are being reported year round. Nevertheless, survival of mud carb larvae in commercial hatcheries is till now at unsatisfactory level; in most hatcheries, mass mortality occurs during transformation from zoea to crablet stage due to bacterial and fungal infection (Dan and Hamasaki, 2015). Molt death syndrome during the transition from zoea 5 to megalopa stage is also considered a major obstacle in the commercial production of crab larvae in hatchery systems (Hamasaki et al., 2002; Pates et al., 2017). A number of opportunistic pathogens includes, *Vibrio* spp., *Aeromonas* spp. and *Pseudomonas* spp. infect the zoeal stages of mud crabs and cause mass mortality during the production cycle (Gopal et al., 2005).

The disease problem is particularly severe in hatcheries, and in the past years many shrimp hatchery units were shut down due to invasion by pathogens where mostly by vibrios (Quinitio and Prado-Estepa, 2008). Frequent occurrence of disease has restricted the crab larval production in hatcheries, and this together with lack of suitable feeds are the prime hurdles in the sustainable development of the crab aquaculture sector (Coates and Rowley, 2022). In Bangladesh, two mud crab hatcheries (one government-owned and another private one) have been operated; the both hatcheries had too low survival percentage of larvae to run the hatcheries on a commercial scale. No specific research has been made on bacterial contamination in relation to such larval mortality in these hatcheries. Given with the scarcity of scientific information on the pathogenic bacterial population in crab hatcheries of Bangladesh, the current study is the first to attempt to determine the prevalence of *Vibrio* spp., *Aeromonas* spp., and *Pseudomonas* spp. in the mud crab hatchery production line. The documentation of scientific information on the prevalence of pathogenic bacteria and total bacterial load would be supportive for quality health management of the mud crab hatcheries.

MATERIALS AND METHODS

Sample collection

Desired experimental samples were collected from the crab hatchery of "Bangladesh Fisheries Research Institute (BFRI)", Brackishwater Station, located at Paikgacha of the Khulna district. The samples were collected from four different cycles which were designated as batch 1, batch 2, batch 3 and batch 4 respectively. All the samples were collected from March 2020 to February 2022. From every single cycle berried crab, eggs, larvae, tank water, brood feed, larval feed and crablet samples were taken properly using sterile tools and apparatus. Laboratory analyses have been performed at "Fish and Shellfish Quality Control and Pathology Laboratory" under Fisheries Discipline of Khulna University, Bangladesh.

Berried brood crabs weighing between 200 to 315 g with carapace length (CL) 7.4-8.0 cm and carapace width (CW) 10.2-10.8 cm were taken for analysis. Egg samples from spawned crabs were collected aseptically in small sterile falcon tubes. Tank water was taken direct inserting sterile falcon tubes inside the tank. To collect larval samples, harvested water was filtered through micro mesh sieves and the larvae were transferred in the sterile falcon tubes containing sterile saline water. Rotifer and *Artemia* used as live food for mud crab larvae were cultured inside the hatchery unit; rotifer and *Artemia* culture samples were taken in the falcon tubes. All the samples were immediately stored in an ice box and shipped to the pathology laboratory as earliest possible where the samples were stored at -20°C for further analysis.

Isolation and enumeration of bacteria

The samples were analyzed following the guideline of the Bacteriological Analytical Manual (Elliot et al., 1995) and Technical Specification (ISO, 2007). Brood crabs were dissected aseptically, then pooled samples using gill, gut, hepatopancreas and abdominal muscle samples were used. Brood, larvae, crablet and feed samples were pulverized with pestle and mortar vigilantly and mixed with sterile saline water to prepare the inoculum. A serially tenfold dilution (up to 10^{-6}) of the stock solutions was prepared in alkaline saline peptone water (ASPW) to estimate the most probable number of the pathogenic *Vibrio* spp., *Aeromonas*

spp. and *Pseudomonas* spp. Aliquots (0.1ml) from the stock solution; dilutions were plated on Thiosulfate Citrate Bile Salt Sucrose (TCBS) agar added with 2% sodium chloride for isolation of *Vibrio* spp. and the same aliquots were plated on Glutamate Starch Phenol-red (GSP) agar for the isolation of *Aeromonas* spp. and *Pseudomonas* spp. *Aeromonas* selective agar and *Pseudomonas* selective agar were further used to cross check outcome of *Aeromonas* spp. and *Pseudomonas* spp. respectively. TCBS plates were incubated at 35-37 °C for 24-48 h, while the GSP plates were incubated at 28-30 °C for 24-72 h. In case of total bacterial count, samples were cultured on nutrient agar plate and incubated at 25-30 °C for 18-24 h.

Identification of bacteria

External morphology of the colonies that appeared in TCBS agar and GSP agar were recorded for all the samples analyzed. For identification of species, the pure culture of isolated colonies were made on Trypticase Soy Agar (TSA) plates and Luria Bertani (LB) agar plates added with 1% NaCl. Several biochemical tests which included arginine dihydrolase (ADH), lysine decarboxylase (LDC), ornithine decarboxylase (ODC), motility, oxidase, indole production, fermentation of sugars (Klinger Iron Agar test and Triple Sugar-Iron Agar test), salt tolerant test and MR-VP tests were performed. To perform all the reactions, standardized techniques (Alsina and Blanch, 1994; Elliot et al., 1998) have been followed. Additionally, an identification kit (KB007 HiVibrio) was used for the identification of *Vibrio* at the species level.

Statistical analysis

Data were presented as means ± standard deviation. Data were analyzed using the Microsoft Excel and the SPSS version 26. Differences of data collected in different batches were analyzed by performing one-way ANOVA using Tukey's honestly significant difference test. In both cases, the significance level was declared based on P< 0.05.

RESULTS

Prevalence of *Vibrio* spp. *Aeromonas* spp. and *Pseudomonas* spp. in the hatchery units

The samples from different units (brood, water, larvae and feeds) of the mud crab hatchery were found to be infected with chitinolytic bacteria pathogenic to mud crabs. *Vibrio* spp. were the most commonly identified bacteria in broods, larvae, larval tank water and in the feeds of both brood and larvae. *Aeromonas* spp. were isolated from all other units except for crablets, while *Pseudomonas* spp. were only detected in the brood crabs and larval feeds. Broods, brood's tank water, larvae, larval rearing tank water and larval feed samples from all batches were infected with pathogenic *Vibrio* spp. 75% of broods, 65% of larvae and 100% of larval feed samples were contaminated with *Vibrio* spp. In the case of brood feeds, 60% sample from batch 1 and batch 2, and 40% sample from batch 3 were infected with *Vibrio* spp. 40% of the egg samples from batch 2 and batch 3 were found to be infected with *Vibrio* spp. *Aeromonas* contamination was found in half of the broods and brood's tank water samples, 35% of the larvae and 40% of the larval tank water samples, and 60% of the larval feed samples. On the other hand, 60% of the broods, 40% of the brood tank water, and 60% of the larval feed samples were contaminated with *Pseudomonas* species (Figure 1). Both the crablet and its feed were found not to be contaminated with any of the pathogens.

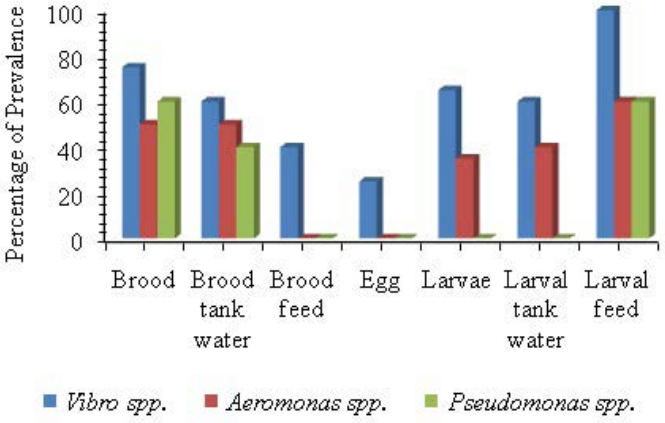

Figure 1: Prevalence rate of pathogenic bacteria in the hatchery units

Determination the load of total bacteria and some chitinolytic bacteria in the hatchery units

Broods: The bacteriological analysis showed that total bacteria count (TBC), total *Vibrio* count (TVC), total *Aeromonas* count (TAC) and total *Pseudomonas* count (TPC) in the brood samples ranged between $\log_{10}$ 5.78 and $\log_{10}$ 6.83 cfu g^{-1}, $\log_{10}$ 2.66 and $\log_{10}$ 4.86 cfu g^{-1}, $\log_{10}$ 2.74 and $\log_{10}$ 3.88 cfu g^{-1}, $\log_{10}$ 2.78 and $\log_{10}$ 3.66 cfu g^{-1} respectively. The highest TBC and TVC were recorded in the batch 3, while the highest TAC and TPC were recorded in the batch 1 and batch 4 respectively (Table 1). In the study period, the average concentration of total bacteria, *Vibrio* spp., *Aeromonas* spp. and *Pseudomonas* spp. were $\log_{10}$ 6.21±0.43 cfu g^{-1}, $\log_{10}$ 3.86±0.61 cfu g^{-1}, $\log_{10}$ 3.40±0.31 cfu g^{-1} and $\log_{10}$ 2.71±0.31 cfu g^{-1} respectively. Data analysis showed that, the concentration of *Vibrio* spp. was significantly higher ($p<0.05$) in the batch 1 and batch 3 compared to the batch 2 and batch 4. However, the data showed no significant differences ($p>0.05$) for *Aeromonas* spp. and *Pseudomonas* spp. count among different batches.

Brood's tank water

Tank water from the brood rearing unit was sampled during the collection of the broods. TBC, TVC, TAC and TPC varied from $\log_{10}$ 5.60 to $\log_{10}$ 7.58 cfu mL^{-1}, $\log_{10}$

Table 1: Total bacteria and pathogenic bacterial load in broods from mud crab hatchery

Sample source	Bacterial count (log_{10} cfu g^{-1})			
	Total bacterial count	*Vibrio* spp. count	*Aeromonas* spp. count	*Pseudomonas* spp. count
Batch 1	6.40±0.35	4.18±0.38 [a]	3.62±0.24	2.72±0.15
Batch 2	5.94±0.19	3.40±0.29 [b]	3.26±0.33	2.57±0.34
Batch 3	6.69±0.12	4.44±0.43 [a]	3.36±0.28	2.59±0.34
Batch 4	5.82±0.27	3.41±0.53 [b]	3.35±0.32	2.95±0.22

* Different superscript letters in the same column indicates significant differences ($p<0.05$)

Table 2: Total bacteria and pathogenic bacterial load in brood's tank water of mud crab hatchery

Sample source	Bacterial count (log_{10} cfu mL^{-1})			
	Total bacterial count	*Vibrio* spp. count	*Aeromonas* spp. count	*Pseudomonas* spp. count
Batch 1	7.13±0.48 [a]	4.14±0.50 [a]	3.29±0.37	ND
Batch 2	6.06±0.45 [b]	3.32±0.36 [b]	2.84±0.15	ND
Batch 3	6.94±0.58 [a]	3.74±0.65 [a,b]	3.08±0.33	2.51±0.26
Batch 4	5.95±0.40 [b]	3.33±0.36 [b]	2.88±0.17	2.58±0.38

*ND- not detected

* Different superscript letters in the same column indicates significant differences ($p<0.05$)

2.62 to log_{10} 4.78 cfu mL^{-1}, log_{10} 2.54 to log_{10} 3.81 cfu mL^{-1} and log_{10} 2.15 to log_{10} 3.08 cfu mL^{-1} respectively. The highest TBC and TVC were calculated in the batch 3, while the highest TAC and TPC were calculated in the batch 1 and batch 4 respectively (Table 2). Though *Vibrio* spp. and *Aeromonas* spp. were detected in the samples collected from all batches, *Pseudomonas* spp. was not detected in the tank water samples collected from the batch 1 and batch 2. In the entire study period, the average count of total bacteria, *Vibrio* spp., *Aeromonas* spp. and *Pseudomonas* spp. were log_{10} 6.52±0.70 cfu mL^{-1}, log_{10} 3.63±0.57 cfu mL^{-1}, log_{10} 3.02±0.32 cfu mL^{-1} and log_{10} 2.54±0.32 cfu mL^{-1} respectively. The mean *Vibrio* concentration of the batch 2 and batch 4 were significantly ($p<0.05$) lower compared to the mean *Vibrio* count of the batch 1. However, the data showed no significant difference ($p>0.05$) of *Aeromonas* ssp. and *Pseudomonas* spp. count among the batches.

Brood's feeds: Tilapia fish collected from local sources were degutted, chopped into small pieces, then was thoroughly to remove dirt and pathogens to use as feed for the broods. Feed collected from different batches were found to be infected with *Vibrio* spp. but free of *Aeromonas* spp. and *Pseudomonas* spp. The TVC in feed sample ranged from log_{10} 2.75 cfu g^{-1} to log_{10} 3.08 cfu g^{-1}; the highest count was recorded in the batch 3 and the lowest was in the batch 2, while the average concentration was recorded as log_{10} 2.88±0.25 cfu g^{-1}. The differences of *Vibrio* count among different batches were non-significant ($p>0.05$). On the other hand, the TBC of brood's feed ranged from log_{10} 5.68 to log_{10} 6.65 cfu g^{-1}. The highest load was recorded in the batch 1 and the lowest was recorded in the batch 2, and the average count was log_{10} 6.25±0.32 cfu g^{-1}. The data showed no significant difference ($p>0.05$) in total count among the batches.

Eggs from spawned crab: The egg samples collected from different batches were analyzed. The TBC ranged from log_{10} 4.64 to log_{10} 5.56 cfu g^{-1}. The highest concentration was detected in the batch 3 and the lowest count was found in the batch 4. The average concentration of total bacteria was log_{10} 4.93±0.39 cfu g^{-1}. All the samples from four different batches were free of *Aeromonas* spp. and *Pseudomonas* spp., and no pathogenic *Vibrio* spp. were detected in the samples from the batch 1 and batch 3. However, one sample (20%) of the batch 2 and two samples (40%) from the batch 3 were found to be infected with *Vibrio* spp. The TVC in egg samples varied from log_{10} 2.45 to log_{10} 2.92 cfu g^{-1}. The highest concentration of *Vibrio* spp. was recorded in the batch 3 and it was lowest in the batch 2. The average concentration of *Vibrio* spp. was log_{10} 2.73±0.13 cfu g^{-1}. The data showed no significant difference ($p>0.05$) in TBC and TVC among different batches.

Larvae: The analysis of larval samples showed that all the zoea stages of all batches were free of *Pseudomonas* contamination and the zoea stages of the batch 1 were free of *Aeromonas* contamination. The TBC, TVC and TAC of the larval samples raged between log_{10} 4.52 and log_{10} 5.68 cfu g^{-1}, log_{10} 2.28 and log_{10} 3.64 cfu g^{-1}, log_{10} 2.56 and log_{10} 3.28 cfu g^{-1}. The maximum average of TBC and TVC was detected in zoea 3 of the batch 1, and for TAC, the maximum average was noticed in zoea 4 of the batch 4 (Table 3). The data showed no significant ($p>0.05$) difference for TBC, TVC and TAC among the different batches. However, *Vibrio* spp. count of zoea stage 3 and 4 was found significantly higher ($p<0.05$) than other zoea stages of the batch 1 and batch 3.

Table 3: Total bacteria and pathogenic bacterial load at the zoea stages of mud crab larvae

Sample Source	Bacterial Types	Bacterial load (log_{10} cfu g^{-1}) at larval stages				
		Zoea 1	Zoea 2	Zoea 3	Zoea 4	Zoea 5
Batch 1	TBC	4.68±0.15	4.85±0.24	5.56±0.21	5.18±0.12	4.84±0.15
	TVC		2.93±0.16[b]	3.49±0.18[a]	3.04±0.15[a]	2.88±0.12[b]
	TAC					
	TPC					
Batch 2	TBC	4.64±0.15	4.84±0.24	5.08±0.31	4.86±0.35	4.66±0.28
	TVC		2.56±0.16	2.64±0.10	2.52±0.15	2.38±0.10
	TAC				2.88±0.25	2.76±0.15
	TPC					
Batch 3	TBC	4.72±0.28	4.94±0.32	5.52±0.18	5.11±0.22	4.78±0.16
	TVC	2.66±0.11[b]	2.72±0.12[b]	3.21±0.21[a]	2.92±0.15[a]	2.68±0.16[b]
	TAC				2.75±0.14	2.88±0.10
	TPC					
Batch 4	TBC	4.66±0.35	4.72±0.36	4.95±0.26	4.76±0.28	4.65±0.24
	TVC		2.72±0.15	2.93±0.22	2.88±0.16	2.52±0.12
	TAC			2.64±0.12	3.08±0.16	2.91±0.15
	TPC					

*Empty fields indicates- 'not detected'

* Different superscript letters in the same row indicates significant differences (p<0.05)

Table 4: Total bacteria and pathogenic bacterial load in the water samples from larval rearing tank

Study periods	Bacterial count (log_{10} cfu mL^{-1})			
	Total bacterial count	*Vibrio* spp. count	*Aeromonas* spp. count	*Pseudomonas* spp. count
Batch 1	5.34±0.30	3.53±0.10[a]	2.79±0.12	ND
Batch 2	5.16±0.29	3.02±0.19[b]	2.65±0.06	ND
Batch 3	4.91±0.13	3.21±0.0.23[a,b]	2.88±0.07	ND
Batch 4	4.98±0.15	2.90±0.14[b]	2.75±0.06	2.67±0.10

*ND- not detected

* Different superscript letters in the same column indicates significant differences (p<0.05)

Tank (larval rearing) water: The larval tank water had a TBC from log_{10} 4.74 to log_{10} 5.65 cfu mL^{-1}. Though *Vibrio* spp. and *Aeromonas* spp. was identified in the samples collected from all batches, *Pseudomonas* spp. was only detected in the batch 4. The highest average TBC and TVC was found in the batch 1, while the highest mean TAC was found in the batch 3 (Table 4). The data of TBC and TAC showed no significant (p= 0.431 and p= 0.122 respectively) differences among the batches, but the data of TVC, showed a significant difference (p= 0.03) among the batches.

Larval feed: *Artemia* and rotifers are widely used as live feed for larvae of many crustacean species in the hatchery units. In the larval feed, TBC ranged from log_{10} 4.86 to log_{10} 6.60 cfu mL^{-1} in *Artemia* culture and log_{10} 4.92 to log_{10} 6.18 cfu mL^{-1} in rotifer culture. In both cases of *Artemia* and rotifer cultures, TBC was recorded higher in the batch 1 and batch 3 compared to the batch 2 and batch 4 (Figure 2). In case of pathogenic bacterial infection, *Vibrio* spp. were frequently detected in all batches of *Artemia* and rotifer cultures. The TVC in *Artemia* and rotifer culture ranged from log_{10} 2.74 to log_{10} 3.56 cfu mL^{-1} and log_{10} 2.83 to log_{10} 3.62 cfu mL^{-1} respectively. The *Vibrio* count in the batch 2 for both *Artemia* and rotifer was significantly lower (p<0.05) than in other batches. In *Artemia* culture, *Aeromonas* spp. was identified in the batch 1, 2 and 3, while *Pseudomonas* spp. was identified in the batch 3 and 4. On the other hand in rotifer culture, both *Aeromonas* spp. and *Pseudomonas* spp. were identified in the batch 2, 3 and 4, but not detected in the batch 1. The TAC in *Artemia* culture ranged from log_{10} 2.81 to log_{10} 3.04 cfu mL^{-1} and the TAC was found from log_{10} 2.93 to log_{10} 3.18 cfu mL^{-1}in rotifer culture. The TPC ranged from log_{10} 2.68 to log_{10} 2.98 cfu mL^{-1} in *Artemia* culture and from log_{10} 2.93 to log_{10} 3.08 cfu mL^{-1} in rotifer culture. The data analysis

showed that both TAC and TPC did not significantly differ (p>0.05) among different batches of the *Artemia* and rotifer cultures.

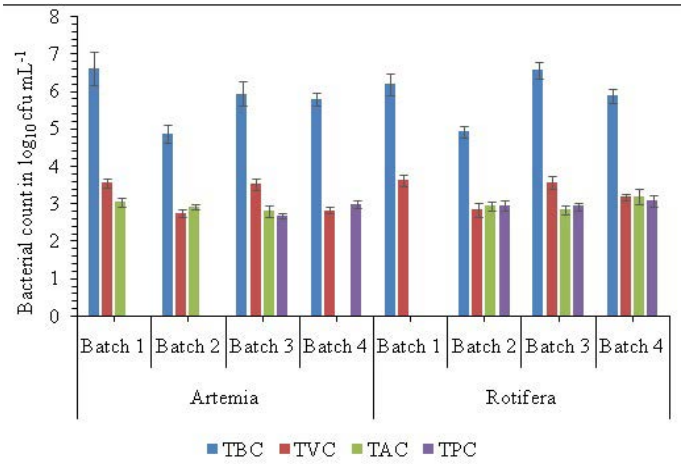

Figure 2: Total and pathogenic bacterial count in *Artemia* and rotifer cultures

Crablet rearing unit: Just after molting, megalopa stage of mud crab larvae turned into crablet which were immediately transferred into crablet rearing unit from larval rearing unit. The TBC of crablet samples was between $\log_{10}$ 4.93 and $\log_{10}$ 2.91 cfu g^{-1}. The crablet feed and tank water had the TBC from $\log_{10}$ 4.20 to $\log_{10}$ 4.34 cfu g^{-1} and $\log_{10}$ 4.65 to $\log_{10}$ 5.68 cfu g^{-1} respectively. There was no *Vibrio* spp., *Aeromonas* spp. and *Pseudomonas* spp. infection in the crablet, crablet feed and crablet rearing tank water samples collected from the hatchery unit.

Species identified and their proportion

Selective agar media, several biochemical tests and identification kits were used for identifying the genus *Vibrio*, *Aeromonas* and *Pseudomonas* at the species level. The *Vibrio* species identified from the samples were *V. alginolyticus* (8%), *V. parahaemolyticus* (12%), *V. mimicus* (16%), *V. harveyi* (12%), *V. fluvialis* (6%), *V. vulnificus* (8%), *V. cholerae* (6 %) and *V. spendidus* (6%), while about 26 percent of the *Vibrio* colonies grown in TCBS plates were unspecified. *A. hydrophila* was accounted for 80% of the isolates of *Aeromonas* spp., and the remainder identified as *A. caviae* and *A. veronii*. On the other hand, all the *Pseudomonas* species were identified as *P. aeruginosa*.

Water quality parameters of larval rearing tank

In captive condition, maintaining water quality parameter within optimum range are very much crucial for the survival of mud crab larvae. All the water quality parameters of the larval rearing tanks were found within the acceptable limit. The water temperature ranged from 29.1 to 31 °C, water salinity ranged from 27 to 30 ppt, pH ranged from 7.7 to 8.8, dissolve oxygen ranged from 5.5 to 6.7 mg L^{-1} and NH_4 ranged from 0.00 to 0.22 mg L^{-1}. The differences of the mean value of the water quality parameters among different batches were statistically non-significant (p>0.05).

DISCUSSION

The present study aimed to identify the prevalence rate and load of pathogenic *Vibrio* spp. *Aeromonas* spp. and *Pseudomonas* spp. in the mud crab hatchery units, as the presence and excess load of such pathogenic bacteria can be fatal for the larval production. In mud crab hatchery, vibriosis is a major concern causing high mortality and inconsistent survival of mud crab larvae (Mann et al., 1998). Among *Vibrio* spp., *V. hraveyi, V. parahaemolyticus,* and *V. alginolyticus* are highly pathogenic to mud crab zoea (Boer et al., 1993). Opportunistic marine bacteria may infect any stage of the production cycle but the possibility of vertical transmission could not be ignored. Transmission of pathogenic *Vibrio harveyi* through feces of adult female was responsible for heavy mortality during larval rearing of blue swimming crab *P. pelagicus* (Talpur et al., 2011).

Among the *Vibrio* spp. isolated in this study, *V. alginolyticus, V. fluvialis, V. harveyi, V. parahaemolyticus* and *V. vulnificus* were predominant, and these *Vibrio* spp. are considered as pathogenic to adult and larval crustacean (Hisbi et al., 2000; Gopal et al., 2005, Liu et al., 2016). Gunasekaran, et al. (2019) reported *V. alginolyticus* involvement in mortality of *S. serrata* in hatchery unit and in culture farms. *V. harveyi* is often an opportunistic bacteria and pathogenic at 10^2-10^3 cfu mL^{-1} to zoeal stages of mud crab (Jithendran et al., 2010). The present study also identified *Aeromonas* spp. and *Pseudomonas* spp. infection in the hatchery units. *Aeromonas* spp. are common micro flora of wild and reared crustacean and are often considered as opportunistic pathogen (Lightner, 1993). *Aeromonas* spp. has been associated with the soft shell syndrome in *P. monodon* (Baticados et al., 1986; Uddin et al., 2008). The genus *Pseudomonas* was found to cause red spot syndrome in *Litopenaeus vannamei,* which lead to mass mortality of larvae and post larvae in the hatchery (Soltani et al., 2010).

Vibrio spp. *Aeromonas* spp. and *Pseudomonas* spp. were dominant in the broods and brood rearing tanks; The TBC, TVC, TAC and TPC in brood crabs calculated in the present study were found similar as described by Lavilla-Pitogo et al. (2001). Warmer temperature might be the reason for higher concentration of *Vibrio* spp. in the batch 1 and 3 compared to other two batches. There are very few studies available on pathogenic bacterial association with mud crab eggs and larvae. The *Vibrio* load in zoea in the present study was found almost similar to the *Vibrio* load in shrimp larvae mentioned by several authors (Soto-Rod-

riguez et al., 2006; Hassan et al., 2017). Spawned crab may be treated with suitable antibiotics or medical plant extract to reduce the bacterial load in eggs and lessen the chance of vertical transmission. The infection rate and load of *Vibrio* were found higher at the middle stage of larviculture, such as at zoea 3. As time progress during larviculture bacterial load increased and this might be due to intervention of live feeds which often carry pathogenic bacteria. On the other hand decreasing *Vibrio* load at later stages (zoea 4, zoea 5) might be the effect of probiotics which ensure adequate growth of beneficial bacteria that inhibit the proliferation of pathogenic *Vibrio* and other chitinolytic bacteria. Studies demonstrated that both rotifer and *Artemia* are vector for introducing potentially harmful bacteria such as *Vibrio* spp. In spite of special efforts are being made to keep the cultures as clean as possible, high-density cultures create high organic load hence rapidly colonized by bacteria which results in poor growth and survival of fish and crustacean larvae (Dhert et al., 2001). Furthermore, the lack of a strong biosecurity in the *Artemia* and rotifer culture units may increase the risk of contamination by various pathogens, as such culture units provide a suitable environment with adequate nutrients from the feeds (type of algae) given for *Artemia* and rotifer enrichment for the growth of various pathogenic bacteria. Any breach of the biosecurity line could result in tank water contamination, which could explain variations in both the total count and the pathogenic bacterial load in larval rearing tank water from different batches. The larval feeds from all batches were infected with *Vibrio* spp., mostly by *V. alginolyticus*. Though concentration was low, prevalence of both *Aeromonas* spp. and *Pseudomonas* spp. were common in larval feeds. The TVC, TAC and TPC were found almost similar to the findings of Interminense et al. (2014). Overall, in the present study, the load of pathogenic *Vibrio*, *Aeromonas*, and *Pseudomonas* identified in the different components of the hatchery was similar to the findings of many authors (Nakamura et al., 1995; Roza and Hatai, 1999; Lavilla-pitogo and de la Pena, 2004; Quinitio and Parado-Estepa, 2008; Dan and Hamasaki, 2015) who conducted such experiments in the production line, particularly with *S. serrata* and *S. paramamosain.*

The total load of *Vibrio* and *Aeromonas* was much higher in the hatchery systems particularly in the warmer months while maximum load of *Pseudomonas* was observed in the wet season and in the winter months. Similar result was reported (Gopal et al., 2005; Liu et al., 2016) in farms and wild condition but inside the controlled environment of hatchery unit such result indicates improper handling either of physical or biological components or breakdown of bio-security line by any means. Water quality parameters, particularly salinity, temperature and pH are considered to be the most important ones for larval rearing and culture of fish and crustacean (Ojwala et al., 2018). Suitable water quality variables ensure proper utilization of nutrients by plankton and better growth and survival of larvae and juveniles. Having all of the water quality variables in the larval rearing tanks within their optimum range indicated that these variables had no adverse effect on larval survivability. The notable finding from the hatchery unit was a pathogen-free crablet.

CONCLUSION

Successful production and survival of mud crab larvae in hatcheries is the prime need to ensure the sustainability of this sector as wild stock is the only source of crabs being harvested and stocked in the mud crab farms of Bangladesh. Through this study, scientific knowledge regarding the association of total bacteria and some pathogenic bacteria in the mud crab hatchery production units has been documented. The prevalence rate and load of pathogenic bacteria detected in broods, eggs and larval feeds might be matters of great concern because vertical and horizontal transmissions of such pathogens in larval production units are quite possible. Therefore, more experimental efforts are needed to diminish the chances of pathogen transmission from brood to offspring, and contamination from external sources during hatchery operation. The scientific use of prebiotics, probiotics and antibiotics to produce viable percentages of crablets, which are indeed inevitable to flourish the aquaculture of mud crab (*Scylla olivacea*) in Bangladesh.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

NOVELTY STATEMENT

This study is the first one that has been carried out to determine the bacterial load and identification of pathogenic bacteria in the larval production line of mud crab (Scylla olivacea) hatchery in Bangladesh. Therefore, all the findings of this research are novel.

AUTHORS CONTRIBUTION

Md. Rashedul Islam: Collection and analysis of samples; data mining; interpretation and analysis of data; write up

of the manuscript draft.

Abul Farah Md. Hasanuzzaman: Conceptualization and design of the research work; data analysis; comprehensive review of the manuscript.

Md. Latiful Islam: Concept of sampling design and collection of water quality data. Ghausiatur Reza Banu: Conceptualization of the research work; supervision of the experiments; revision of the manuscript draft.

ETHICAL APPROVAL

The Research Ethics Committee of Khulna University Research Cell, Bangladesh, had given ethical approval (Ref No: KU AEC-2021/08/15; Date: August 17, 2021). All procedures carried out involving crab during the present study followed the institutional guideline, following the international and national guidelines.

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