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ARTICLE

Bacterial Microflora on Freshwater Prawn (*Macrobrachium rosenbergii*) and Culture Water Associated with Public Health Concern

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ABSTRACT

Quantitative and qualitative analyses of important bacterial content for public health concern (total bacterial count, total coliforms, faecal coliforms, *Salmonella* and *Vibrio cholerae*) of cultured fresh water prawn and farm water which has significant role in order to manage sustainable aquaculture were carried out. Microbiological parameters of prawn (*Macrobrachium rosenbergii*) and farm water were determined by following the ISO standard methods. Total bacterial Count (standard plate count) found in prawn samples ranged from 5.55 to 5.71 log CFU/g while 4.13 to 4.18 log CFU/mL in water sample. On the other hand, total coliforms found in prawn sample ranged between 1.96 to 2.46 log CFU/g whereas in water sample 2.07 to 2.46 log CFU/mL total coliforms were detected. In case of faecal coliforms, the number ranged between 0.96 to 1.42 log CFU/g in prawn sample and 1.59 to 1.81 in water sample. While *Vibrio cholerae* was absent in both prawns and water sample and *Salmonella* was detected in two tested ponds for both prawn and water sample.

1. Introduction

Fresh water giant prawn, (*Macrobrachium rosenbergii*) is one of the most important sectors in national economy of Bangladesh due to its export potential [1]. A vast area of waterbodies and subtropical climate of Bangladesh make both prawn and shrimp farming more suitable [2]. However, among 10 species of *Macrobrachium*, only *M. rosenbergii* is commercially cultured [3] almost all over the country especially in the south, (mainly Noakhali and Patuakhali district) and south-west, (mainly Khulna, Satkhira, Bagerhat district) part of the country [4]. Due to the increasing demand for prawn in the international market, this sector has expanded every year and also developed in north-central Bangladesh, mainly Mymensingh district [3]. A great number of populations are engaged in prawn farming and it is providing the employment and financial support to change their socio-economic condition [5]. As a whole, the prawn and shrimp industry attain the second largest export sector, earning US$380 million annually of which 20-25% come from prawns [6]. Despite the expansion of this sector, a number of issues

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influence the success of the production including environmental aspects, microbiological parameters and management technology [7]. However, very little attention has been paid to the bacterial flora associated with cultured *M. rosenbergii* and its influence on the initial quality of this prawn species for sustainable production [8, 9].

In 2002, there were about 1, 05,000 prawn farms in Bangladesh covering around 50,000 ha of land [10-12]. There are two prawn farming systems available in Bangladesh; pond and gher and followed almost the same culture practices mainly semi-intensive culture technique with some modifications [11]. The system mainly relies on natural productivity but organic manure like cow dung, poultry manure in addition with chemical fertilizers are used [3]. Most farmers use the farm land as integrated aquaculture-agriculture plot where they cultivate both freshwater prawn with rice and dikes are used for the production of fruits and vegetables [13]. Sometimes, farmers graze their cattle on the dike of the culture ponds. The feces of those ruminants are also mixed with pond water by rain runoff [14]. It creates the chance of growth of *Salmonella, V. cholerae* and other pathogenic bacteria in the ponds [15]. In the foreign markets, a number of consignments from Bangladesh has been rejected mainly due to the contamination of *Salmonella*, total coliforms and fecal coliforms [16]. The rejections are involved with huge economic loss both nationally and internationally. The initial high load of bacteria accelerates the spoilage of fish and shrimp which subsequently cause contamination of pathogenic bacteria like *Salmonella* and *V. cholerae* which have a severely bad effect on human health.

The bacterial flora associated with farmed prawns are considered as useful indicators of quality and safety concern for farm management. Diseases in *M. rosenbergii* mainly caused by opportunistic bacteria which are frequent in the culture environment [17, 18]. In order to develop farm management practices, it is essential to investigate the bacterial populations associated with prawn farms and culture water which can also cause risk to public health. The purpose of this study was to determine the heterotrophic bacterial populations associated with farmed freshwater prawns and their rearing environment. The information obtained should allow a better control of the bacteriological profiles in the farmed prawn and also a better control of possible microbial risks to promote international trade.

2. Materials and Methods

2.1 Sample Collection

The study was carried out in Beel Dakatia located in the Phultala, Khulna, Bangladesh (Figure 1) in 2016 where there are about a total of 6982 fresh water prawn farms or ghers with almost same pattern of culture system. Among them, five different freshwater prawn farms were selected randomly and 500 g of farmed *M. rosenbergii* were harvested from 3 different spot in the same pond by using cast net. A total of 15 animals were harvested and collected in sterilize polybags on ice to prevent contamination and transported to the laboratory.

Water samples were collected from different locations, surface, middle and bottom (just above the mud) of the each pond from the three different spots. 500 ml of water sample put in screw cap sterilize glass bottles. Collected samples (prawn and water) were kept in separate insulated box instantaneously and the temperature was maintained below 5^0C by using ice and transported to laboratory within one hour. Microbiological test of prawn samples was initiated on the same day after 2-4 hrs of collection, however, water samples were preserved in the refrigerator and test was started on the next day.

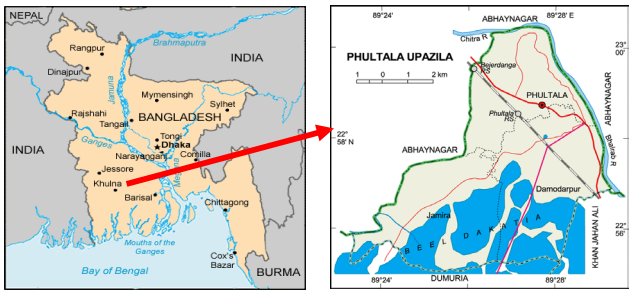

Figure 1. Map showing the study area

2.2 Bacteriological Analysis of Cultured Freshwater Prawn (Macrobrachium rosenbergii) and Farm Water

Total viable aerobic bacterial flora was determined by standard plate count method [19]. From each prawn, 20 g of shell with muscle and digestive tract and 20 ml of farm water collected aseptically into a sterile bags containing 180 ml of 0.1% sterilize peptone solution separately. The prawn sample were blended for two minutes by a stomacher lab blender [20]. Samples were serially diluted up to 10^{-5} and 1 ml of aliquot from each dilution was aseptically poured into duplicate on petri dish and melted (45-50°C) plate count agar (Oxoid, UK) was poured over it and rotated clockwise-anticlockwise, allowed to solidify and incubated at 30^0C for 72 h. Bacterial colonies were counted by using colony counting equipment (Stuart Scientific, UK).

The petri dishes containing 15 to 300 colonies at each dilution were counted. The numbers of bacteria (N) per

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g or per ml of sample was calculated using the following equation and expressed as colony forming units (CFU)/g or CFU/ml [21].

$$N = \frac{\sum C}{\left(n_1 + 0.1n_2\right)d}$$

Where, ∑ C = the sum of colonies counted on all the dishes retained

n_1 = the number of colonies retained in the first dilution

n_2 = the number of colonies retained in the second dilution

d = the dilution factor corresponding to the first dilution

2.3 Enumeration of Total Coliform and Feacal Coliform Bacteria in Freshwater Prawn and Farm Water

Quantitative enumeration of Coliforms were determined by a 3 replicate tube most probable number (MPN) procedure [22] with 1 ml of homogenate sample. Coliform numbers were estimated using Lauryl Sulphate Triptose Broth (LSTB) incubated at 37^{0}C 48 h and sub-culturing all positive tubes in Brilliant-Green Lactose Bile Broth (BGLBB) incubated at 44^{0}C for 24 h. After incubation, the LSTB tubes showing gas production were recorded. Total number of coliforms bacteria was calculated by matching the number of gas positive tubes of the respective decimal dilution to the Most Probable Number (MPN).

Faecal coliform was enumerated from the gas positive inoculums of the LSTB tubes. 1 loop full of inoculums from each of the gas positive LSTB tubes of the specific dilution was transferred to the 10 ml of BGLBB tube containing inverted Durham's tubes and 10 ml Tryptone Water tube separately and incubated at 44^{0}C for 48 h. After incubation, the number of gas formation BGLBB tubes along with the TW tubes of the specific dilution were marked. Then 0.2 to 0.5 ml of Kovac's (indole) reagent was poured to TW tubes and shaken gently and kept for 1 minute. Both gas forming tubes of BGLBB and red ring (indole) forming tubes of Tryptone Water were considered as positive respectively for specific dilutions. Total number of faecal coliforms bacteria was enumerated by comparing the common number of gas positive tubes of BGLB and red rings of the TW tubes with MPN chart [23].

2.4 Detection of *Salmonella* in Fresh Water Cultured *Macrobrachium rosenbergii*

Salmonella were determined by following the procedure as described by Lalitha and Surendran [24]. 25 g of sample (shell and digestive tract) was taken aseptically in to a sterile poly bag with 225 ml of sterilized buffered peptone water and was blended by the stomacher for 120 sec. Blended homogenates were transferred aseptically to sterile 500 ml screw cap bottles and incubated at 37 ^{0}C for 18 h for pre-enrichment. For selective enrichment, after incubation 0.1 ml of each pre-enrichment broth was transferred to 10 ml RVS (Rappaport-vassiliadis soya peptone) medium and 1 ml to 10 ml of MKTTn (Muller Kauffmann Tetrathionate novobiocin) medium and was incubated at 41.5^{0}C and 37^{0}C respectively for about 24 h. The inoculums from selective enrichment broth were inoculated by streaking out in large-size petri dish (140 mm diameter) of sterile XLD (Xylose lysine Deoxycholate) and HEA (Hektoen Enteric) agar media to obtain the well-isolated colonies and petri dishes were incubated in inverted position at 37^{0}C for 24 h.

After incubation of the selective plating media, the colonies of the XLD and HEA were examined and sample codes namely P3S1, P3S3, P5S1 and P5S2 for prawn sample and P3B and P5B for water sample were selected for confirmation test on the basis of the following colony character [25]:

(1) Typical colonies of *Salmonella* grown on XLD agar have a black centered and a lightly transparent zone of pink to reddish color due to the color change of the indicator. *Salmonella*, H_2S negative variants (e.g. S. *paratyphi*) grown on XLD agar are yellow with or without blackening.

(2) Typical colonies of *Salmonella* grown on HEA agar are bluish green with or without a black center.

For the confirmation test, one prominently characterized typical colony was selected from each of the XLD and HEA agar plates. The selected colonies were streaked on the sterile pre-dried nutrient agar plates and incubated at 37^{0}C for about 24 h. These pure cultures were used for biochemical test.

2.5 Biochemical Confirmation Test

Biochemical confirmation test was determined following the taxonomic guides of Bergey's Manual of Determinative Bacteriology [25] for both prawn and farm water sample.

2.5.1 TSI (Triple Sugar Iron) Agar and LIA (Lysine decarboxylate Iron agar) Test

The pure cultures from the nutrient agar were streaked on the slant surface and stabbed to the butt in the both TSI agar and LIA media test tubes. The agar media with test tubes were incubated at 37^{0}C for 24 h.

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Typical *Salmonella* cultures show alkaline (red) slants and acid (yellow) butts with gas formation (bubbles) and formation of hydrogen sulfide (blackening of the agar) When a lactose positive Salmanella was isolated, the TSI slant showed yellow color (Table 1 and Table 2).

Table 1. Biochemical test for *M. rosenbergii*

Sample code	Agar medium	Reaction in the tubes		Comment	Decision
		Slant	Butt		
P3S1	TSIA	Red slant (alkaline)	Yellow with acid	Positive reaction for *Salmonella*	*Salmonella* may be present
	LIA	No change of media colour	Deep purple	Positive	*Salmonella* may be present
P3S3	TSIA	Red slant (alkaline) and black with H_2S gas	Black with H_2S gas	Positive	*Salmonella* may be present
	LIA	purple colour	black colour	Positive	*Salmonella* may be present
P5S1	TSIA	Red slant (alkaline)	Yellow with acid	Positive	*Salmonella* may be present
	LIA	No change of media colour	black colour	Positive	*Salmonella* may be present
P5S2	TSIA	Red slant (alkaline)	black with H_2S gas	Positive	*Salmonella* may be present
	LIA	No change of media colour	Deep purple colour	Do	Do

Note: P3= Pond 3, P5= Pond 5, S1= Sample 1, S2= Sample 2, S3= Sample 3

Table 2. Biochemical test for farm water

Sample code	Agar media	Reaction in the tube		Comment	Decision
		Slant	Butt		
P^3B	TSIA	Red slant (alkaline)	Yellow with acid	Positive reaction for *Salmonella*	*Salmonella* may be present
	LIA	Purple colour	Deep purple	Positive	*Salmonella* may be present
P^5B	TSIA	Slant black with H_2S gas	Yellow with acid	Positive	*Salmonella* may be present
	LIA	No change of media colour	Black colour	Positive	*Salmonella* may be present

Note: P3= Pond 3, P5= Pond 5, B= Bottom

2.5.2 Urea Agar Test

Pure cultures of sample code P_3S_1, P_3S_3, P_5S_1, P_5S_2, P_3B and P_5B from the nutrient agar were streaked to the urea agar slants surface of the test tubes and were incubated at 37^0C for 2 to 4 h. The colour of the samples were not changed that indicates the presence of *Salmonella*.

2.5.3 Test for Indole Reaction

The bacterial colonies from the nutrient agar of the suspected samples were inoculated in tubes containing 5 ml of the tryptone medium and incubated at 37^0C for 24 h. After incubation, 1 ml of the Kovac's reagent was added to it. A positive reaction (formation of red ring) was found with all the tubes and indicates the presence of *Salmonella*.

However, all the selective test and all the biochemical test were positive, so it decided that the detected bacteria was *Salmonella*.

2.6 Statistical Analysis

Total bacterial counts were expressed as CFU/g for prawn sample and CFU/ml for water sample after log10 transformation of the values. Student t-test was used to compare significant difference ($P<0.05$) of means of microbial counts in prawn and water samples.

3. Results and Discussion

There have been few studies about the enumeration and detection of bacteria associated with giant freshwater cultured prawn (*Macrobrachium rosenbergii*) and farm water. However, in case of prawn, three samples were collected from three different locations of the each pond and it was observed that the result of total bacterial load (SPC) of the samples were very close to each other of the same pond. It may be due to that the ponds were in a cluster and adjacent to each other and the water source of the farm was the same. Among three category, SPC was much higher than other two. The SPC (Standard Plate Count) was found within the range of 5.55 to 5.71 log CFU/g and highest number of bacterial load (SPC) found in pond 3 (5.71±0.07 log CFU/g) and the lowest (5.55±0.05 log CFU/g) were collected from the pond 4 (Figure 2).

Similarly the results of total coliforms and faecal coliforms were found within little difference to each other from the same pond. The composition and level of the microorganisms associated with the intestinal tract is related to the environment as well as to the food consumed by fish [26-28]. Total coliforms ranged from 1.96 to 2.46 and faecal coliforms 0.96 to 1.42. The highest number of total coliforms and faecal coliforms were collected from pond 5 counted as mean log count (per g) 2.46±0.18 and 1.42±0.23 respectively while the lowest number found in pond 2 (Figure 2 and Table 3) *Salmonella* were detected only from the pond 3 and pond 5 (Table 3).

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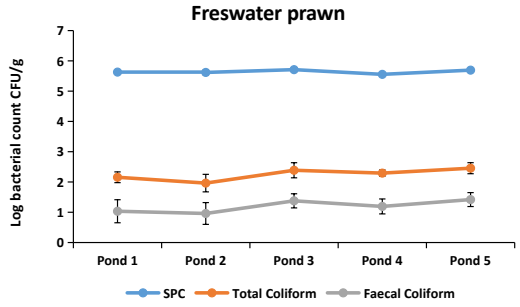

Figure 2. Total bacterial count, total coliform and faecal coliform in *M. rosenbergii* from five (5) ponds. The error bars indicate the standard deviation of three replicates.

In the farm water samples, bacterial load was counted as log CFU/mL. The SPC (Standard Plate Count) ranged from 4.13 to 4.18, total coliforms 2.07 to 2.46 and faecal coliforms 1.59 to 1.81 CFU/ml. The highest number of bacterial load (SPC), total coliform and faecal coliform were found from pond 3 and 5 respectively whereas subsequently the lowest number collected from pond 4 and 1 (Figure 3 and Table 3).

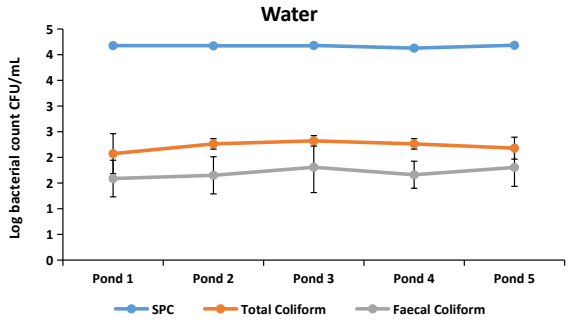

Figure 3. Total bacterial count, total coliform and faecal coliform in farm from five (5) ponds. The error bars indicate the standard deviation of three replicates.

The value observed in the present study were comparable to the results for farmed fresh water prawn and brackish water shrimp in India [29-31]. Total coliforms, faecal coliform values and detection of *Salmonella* observed in the present study are similar to those of reported for farmed freshwater prawn (*M. rosenbergii*) culture in earthen ponds in Saudi Arabia [32].

In the present study, faecal coliforms levels in *M. rosenbergii* were high as previously reported for tiger prawn farms in India [29] and in Philippines [33]. This microbial group is important in foods as indicator of hygienic quality of foods and also as spoilage flora [34]. If the influent water does not contain high numbers of these organisms, incidence of such high numbers of these organisms in prawn may be attributed to the feed or animal manure commonly used to fertilize ponds.

SPC counts (log CFU/g) were significantly higher in prawn samples than in water ($P<0.05$) in the freshwater prawn grow-out cultures. Total coliform and faecal coliform counts for water, and prawn did not differ significantly ($P>0.05$) (Table 3).

Salmonella were detected only in pond 3 *V. cholerae* was not found in any sample for both prawn and water sample. However, a few researchers reported that they have detected *V. cholerae* in fresh water shrimp farms and some of the researchers has reported only the genus of the Vibrio, not the species, someone found many other Vibrios other than *V. cholerae* [35]. Since cow dung was used for pond preparation and cow dung and poultry faeces was used as manure in pond 3 and 5, the total bacterial load, total coliforms and faecal coliform were higher in those ponds and may be the source of *Salmonella* [15].

4. Conclusion

This is a baseline study of normal flora associated with prawn farms. In this study, a significant number of SPC,

Table 3. Mean microbial counts (log_{10} $CFUg^{-1}$) of prawn and water sample from freshwater farm

	log_{10} $CFUmL^{-1}$ ± SD•									
Counts	**Pond 1**		**Pond 2**		**Pond 3**		**Pond 4**		**Pond 5**	
	†Prawn	**Water**	**†Prawn**	**Water**	**†Prawn**	**Water**	**†Prawn**	**Water**	**†Prawn**	**Water**
*SPC	5.63 ±0.04	4.18 ±0.03	5.62 ±0.08	4.17 ±0.02	5.71 ±0.07	4.18 ±0.05	5.55 ±0.05	4.13 ±0.02	5.69 ±0.04	4.18 ±0.02
Total coliform	2.16 ±0.18	2.07 ±0.39	1.96 ±0.29	2.26 ±0.10	2.39 ±0.25	2.32 ±0.10	2.29 ±0.11	2.26 ±0.10	2.46 ±0.18	2.18 ±0.21
Faecal coliform	1.03 ±0.38	1.59 ±0.36	0.96 ±0.36	1.65 ±0.36	1.38 ±0.23	1.81 ±0.49	1.19 ±0.25	1.66 ±0.26	1.42 ±0.23	1.80 ±0.37
Salmonella	Absent	Absent	Absent	Absent	Present	Present	Absent	Absent	Present	Present
V. cholera	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent

Note:

†For prawn CFU/g

•SD= Standard deviation

*Significantly different from farm water and prawn sample ($P<0.05$)

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total coliform and faecal coliforms were found in farmed freshwater prawn (*M. rosenbergii*). Identification of *Salmonella* is a risk factor as well. The presence of this species in freshwater prawns can cause harmful effects on human health. The rearing practices such as feeding and pond fertilization could have influenced on the microflora in prawn. Thus it is important to regulate the bacterial load in the freshwater prawn aquaculture system by adopting good farm management practices like regular water exchange and feed regulation in order to safeguard against infectious agents. However, further research is also required to investigate the impacts of feed, rearing condition and host microbe interaction. Farmed *M. rosenbergii* should be washed with clean iced water, maintained at a temperature below 5°C during sorting and grading and transported with minimum delay to the processing plant to eliminate quality losses during primary handling. These appropriate measurements will help to earn foreign currency and improve the national health status.

Conflict of interest

The authors declare that they have no conflicts of interest.

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