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Comparative Bacteriological Analysis of Giant Freshwater Prawn (*Macrobrachium rosenbergii*) Cultured in South-Western Coastal Areas of Bangladesh

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Abstract

Background and Objective: With changing pattern of culture practices in the coastal region of Bangladesh, the euryhaline giant freshwater prawn *Macrobrachium rosenbergii* has recently gained popularity among farmers of both semi-intensive and extensive culture systems to improve profitability. Since bacteria are the key player in aquaculture facilities governing health and quality of fish and prawn, comparative bacteriological analyses were undertaken to understand the suitable testing system of bacterial analysis for cultured freshwater prawn.

Methodology: Both on-farm and laboratory conditions in low-resource settings were considered to monitor bacterial population in terms of bacterial count, gram staining and presence of enteric bacteria associated with prawn and its surrounding environment.

Results: On-farm counting was about one log-unit lower total viable count in water, sediment and prawn samples compared to those of laboratory testing. The differences between on-farm and laboratory analysis probably occurred due to the time lapse during sample transportation. Study also showed that gram negative bacteria were dominant and enteric bacteria were present in both the prawn farms with more or less similar frequencies.

Conclusion: It is concluded that the apparent difference in bacteriological condition of the two farms is possibly related to the differences in culture practices, environment and culture conditions and the study has recommended on-farm testing as an ideal bacteriological analysis method.

Keywords: Bacterial flora; Giant freshwater prawn; Physico-chemical properties; Farms

Introduction

Freshwater prawns are important aquaculture species in many tropical and sub-tropical countries and its aquaculture plays a major role in nutrition, employment and foreign exchange earnings. Giant freshwater prawn (*Macrobrachium rosenbergii*) is regarded as the most important farmed prawn species which is now being widely cultured in China, Thailand, Bangladesh, India, Vietnam, Myanmar, Taiwan and even Brazil [1]. Current world production of giant freshwater prawn stands about 229,419 tons which continues to increase due to higher demand and better market price worldwide [2]. There are however, reports that production of some freshwater prawns decreasing in countries like India which is primarily due to disease and poor water quality [3].

Generally, it is believed that the success of aquaculture depends on water quality which is greatly influenced by aquatic microorganisms. Aquatic microorganisms influence the water and sediment quality and are closely associated with the fish physiology, diseases and aquaculture. Bacteria in sediments are major contributors to bio-geochemical processes in benthic ecosystems and bacterial abundance is an important parameter for assessing the roles of bacteria in these environments [4]. It was reported that sediment is the medium to sample than the water column to know the environmental condition because it is less influenced by perturbations caused by rainfall and transient movements of waterborne substances [5]. Also, of interest is the gut flora inside the host which gives a clear picture particularly for water areas of poor quality [6]. Some common bacterial microflora in water such as *Pseudomonas, Aeromonas* and *Vibrio* can cause major fish epizootics under stressful conditions. The bacterial content of the sediments affects the quality of aquaculture products. Despite of this, very little attention has been paid to the bacterial biota associated with cultured giant freshwater prawn [7] and its influence on the initial quality of this species.

In Bangladesh, Khulna, Jessore, Bagerhat and Satkhira regions in the south-west coasts are the major places for prawn culture due to the availability of freshwater or low saline water. Majority of the culture ponds have no perennial outlets and water levels are affected primarily by rainfall, runoff, and sometimes by underground water. Here most of the farmers use organic manures like cow dung compost in addition to chemical fertilizers. It creates the chance of integration of *Salmonella*, *Vibrio*, *Enterobacter*, and other pathogenic bacteria in the ponds [8,9]. In the international export markets, freshwater prawns of Bangladesh have been rejected mainly due to the contamination of *Salmonella*, faecal coliforms and rarely for *Vibrio cholerae*, where *Salmonella* being the major cause [10]. The rejection of prawn consignment in the foreign market involved huge financial loss which ultimately affects the prawn

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farmer. In addition, most diseases in prawn are caused by opportunistic pathogens which are prevalent in the rearing environment [11]. Good Aquaculture Practice (GAP) was, therefore, implemented in prawn culture farms by the government with assistance from European Union (EU) and many donor agencies to ensure production of safe products. As in GAP, farmers are responsible for validating the food safety of their on-farm water and soil but the shrimp and prawn farms in Bangladesh and other developing countries have very low resources. There are, however, urgencies and need to conduct bacteriological tests under low-resource facilities, as time lapse during transportation of raw sample from farm to laboratory is very critical as it adds more time to account for microbiological testing [12]. In the present study, we were interested to consider the quantitative and qualitative aspects of bacterial biota associated with cultured freshwater prawn using two approaches, viz., on-farm and laboratory, and compared the results for better understanding of selecting suitable quantitative method of bacterial analysis.

Materials and Methods

Sampling sites

The present study was carried out in low-resource settings (LRS) of two polyculture farms located at Batiaghata and Rupsha upazilas under Khulna district during the months of December 2013 to May, 2014. Both farms were supplied with ground water as a major source and surrounded by paddy fields. Limited vegetation was present in the shallow areas with bottom mud of approximately 15-25 cm. Polyculture of shrimp and prawn along with finfish was practiced and ponds were supplied with artificial feed three times a day.

Measurement of water quality parameters

On each sampling day water temperature, salinity, dissolved oxygen (DO) and pH of water and sediment were measured using previously calibrated handheld devices. All measurements were done in triplicate.

Sample collection

Sampling was done three times at 1-month interval for bacterial investigations of pond water, sediment and whole prawn (~20 g) in each of the two farms (designated as Farm 1 and 2). In each farm, 3 locations were randomly selected and water samples were collected by submerging clean, sterilized bottles (250 ml) 15-20 cm below the water surface at 3 locations and mixed together. Sediment samples, on the other hand, were collected from same 3 locations employing an alcohol rinsed, air dried small PVC pipe where the sample was aseptically retransferred to wide mouth sterile glass bottles (250 ml) and mixed together. Finally 3-4 giant freshwater prawn (15-20g) were collected from the 3 locations. Sampling was conducted in duplicates and a total of 6 samples were taken from each farm where one set of samples was subjected to immediate analysis at farm-site and rest was kept in ice box with sufficient flake ice to be transferred to the laboratory within 12-14 hrs.

Bacteriological analysis on farm-site

Immediately after collection, all samples were taken to a small confined area covered with card-boards inside the office room with assistance of the farm owner. For water samples, dilutions were made (10^{-1} to 10^{-5}) with sterile physiological saline (0.85% w/v NaCl) and aliquots of 0.1 ml of the serial dilutions were pipetted out and transferred aseptically to the sterile agar plates for total aerobic heterotrophic plate count and EMB- and SS-agar plates for enteric bacterial count by raising the upper lids sufficient enough to admit the tip of the pipette. The samples pipetted were spread by L- shaped glass rods throughout the surface of the media until the samples were dried out. Plates were then wrapped by aluminum foil and kept in room temperature before being transferred to the microbiological laboratory of the Department of Fisheries Technology, Bangladesh Agricultural University, Mymensingh within 12-14 hrs. The plates were incubated at 30°C for another 24-48 hrs. Only the plates having 30 to 300 colonies were considered to calculate bacterial population numbers and expressed as colony-forming units per unit of sample. Sediments were similarly analyzed. Prawn samples, on the other hand, were first ground and homogenized and approximately 5 g of each homogenate was then put in a bottle containing 200 ml of sterile saline solution. One milliliter of each homogenate solution was serially diluted (10^{-1} to 10^{-8}) and treated in the same way as the water samples.

Bacteriological analysis in laboratory

The ice stored samples were also subjected to bacteriological analysis where farm water, sediment and the prawn samples were used.

Isolation and testing of bacteria

For both on-site testing and laboratory testing, bacterial isolates were recovered and subjected to Gram-staining. Presence of enteric bacteria was determined by using selective media EMB- agar and SS-agar. From each stock solution of water, sediment and prawn, 0.1 ml samples were transferred into the selective media. Growth of bacterial colony in EMB-agar and SS-agar media indicate the presence of enteric bacteria. For percent composition, the bacterial colonies were divided into different types according to the colony characteristics of shape, size, elevation, structure, surface, edge, color and opacity, and the number of colonies of each recognizable type was counted. Three to five representatives of each colony type were then streaked on agar plates repeatedly until pure cultures were obtained. The streaked agar plates were incubated at 30°C for two days. Discrete colonies from the streaked agar plates were transferred to agar slants.

Maintenance of stock culture

During the experiment it was necessary to preserve the selected isolates for short term periods. For this purpose, cultures on agar slants were kept at 4°C after 2 days of incubation for stock and these were transferred to new slants at every 6 weeks intervals.

Statistical analysis

Data were analyzed by using Microsoft Office Excel and SPSS version 12.0 (Chicago. USA). Significant differences were determined among treatments at the 5% level ($p< 0.05$).

Results

Physico-chemical parameters of water from prawn farms

The average values for physico-chemical parameters of water samples taken from the two giant freshwater culture farms are shown in Table 1. According to the sampling time, it was from late summer to early autumn where water temperature was found to be decreasing gradually. It is, therefore, has significant effect on physicochemical parameters of farm water which is a very important aspect of prawn farming. Although some water quality parameters varied between the 2 farms, they were found to be insignificant ($p>0.05$). Water temperature during the sampling period ranged from 25.7 ± 3.3 to 27.3 ± 3.4°C while a slightly higher temperature was recorded for ambient air. Pond

Parameters	Temperature (°C)		pH		Salinity (ppt)	DO (mg/l)
	Water	Air	Water	Sediment		
Farm 1	25.7 ± 3.3	28.6 ± 3.9	7.1 ± 0.4	3.7 ± 0.2	7.6 ± 1.5	4.9 ± 0.2
Farm 2	27.3 ± 3.4	29.8 ± 3.7	6.6 ± 0.5	3.7 ± 0.2	9.3 ± 1.1	4.9 ± 0.2

Table 1: Average physico-chemical parameters of pond water collected during 3 months from freshwater prawn culture farms.

water pH was slightly alkaline (7.3 ± 0.4 to 6.6 ± 0.0) while sediment was found to be of acidic nature (3.7 ± 1.5 to 3.7 ± 0.2). It was found that both farms had saline water with values ranging from 7.6 ± 1.5 to 9.3 ± 1.1 ppt while the DO varied from 4.9 ± 0.2 to 4.9 ± 0.2 mg/l. These common water parameters observed in the prawn farms were reported to be suitable for prawn aquaculture [13-15], and probably suits microbial growth as well.

Quantitative analysis of bacterial flora

After the stipulated incubation period, quantitative analysis of bacterial flora was conducted using standard plate count technique. As described in the rationale, we were interested to compare bacteriological estimation using on-farm and laboratory testing and accordingly we found that during the period of study, on-farm testing showed that bacterial loads in water, sediment and prawn from farm 1 ranged from 2.56×10^4 8.65×10^4 Cfu/ml, 6.01×10^8- 1.51×10^9 Cfu/g and 2.95×10^6 - 2.33×10^7 Cfu/g (Figure 1). The same sample when tested in the laboratory after refrigerated transportation, bacterial load increased with values in farm water, sediment and prawn ranging from 1.54×10^5-8.31×10^5 Cfu/ml, 4.55×10^9 - 9.52×10^9 Cfu/g and 4.30×10^7 - 1.18×10^8 Cfu/g in prawn during the period of study. More or less similar observation was found for farm 2 on-farm testing where bacterial loads were slightly higher than the previous farm where bacterial loads in water, sediment and prawn ranged from 6.52×10^4 - 1.30×10^5 Cfu/ml, 9.63×10^8 - 1.12×10^9 Cfu/g and 2.50×10^6 -9.16×106 Cfu/g, respectively. Bacterial loads also increased in laboratory testing where the values for water, sediment and prawn were 7.30×10^5 - 1.89×10^6 Cfu/ml, 9.29×10^9 - 3.18×10^{10} Cfu/g and 4.25×10^7 - 3.08×10^8 Cfu/g, respectively.

Qualitative analysis of bacterial flora

As a preliminary examination of bacterial population, we employed gram staining method which is a useful method that differentiates bacterial species by the chemical and physical properties of their cell walls by detecting peptidoglycan. It was observed that during three different samplings, the number of bacterial isolates in water, sediment and prawn were 4.33 ± 0.58, 5.67 ± 0.58 and 6.67 ± 0.58 respectively in farm 1. When we calculated these numbers as percentage, Gram +ve was 27.66% and Gram –ve was 72.33% in farm 1. As for the case of farm 2, the number of bacterial isolates in water, sediment and prawn were 5.00 ± 1.00, 7.00 ± 1.00 and 6.67 ± 1.15 respectively, representing 16% Gram +ve and 84% Gram –ve bacteria. Generally, the number of fermentative Gram –ve bacteria becomes dominant in well fed prawn, and they are considered to be able to thrive harsh environmental conditions in saline waters [16,17]. The water salinity values in our sampling farms were ranging between 7 and 9, and it probably has some effect on high gram –ve bacteria. Similar observation was also reported by Uddin and Al-Harbi [18] where they observed more than 80% bacteria in carp, catfish and water were gram –ve bacteria.

Many of the pathogenic enteric bacteria are of great concern for tropical fish and shrimps including *E. coli* and *Salmonella* spp. and estimating their presence and abundance becomes important. We therefore, used both on-farm and laboratory testing approach

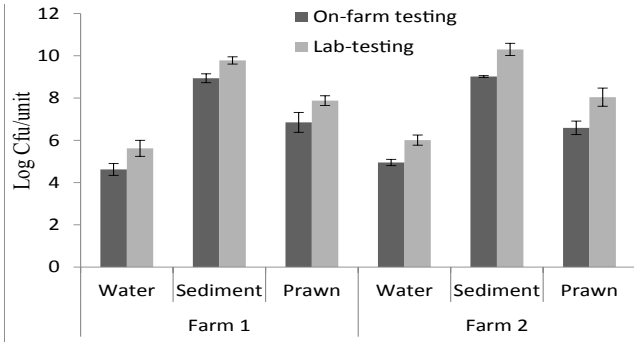

Figure 1: Comparative analysis of bacterial load between on-farm testing and laboratory testing in two giant freshwater prawn farms. Error bars indicate standard deviation.

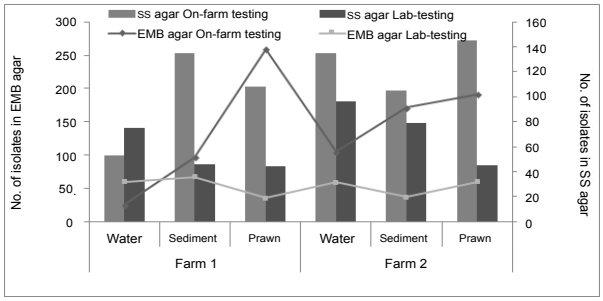

Figure 2: Enteric bacteria recovered from water, sediment and prawn samples collected from two giant freshwater prawn farms.

to estimate enteric bacteria by selective media (EMB and SS agar). On-farm testing revealed that presence of enteric bacteria in water, sediment and prawn from farm 1 in two different media types counted as in EMB media 24.6 ± 26.3 Cfu/ml, 97.3 ± 61.7 Cfu/g and 259.6 ± 195.3 Cfu/g respectively and in SS media 53.0 ± 8.0 Cfu/ml, 135.3 ± 91.5 Cfu/g and 108.3 ± 88.1 Cfu/g respectively (Figure 2). These results suggest that the same samples when tested in the laboratory after refrigerated transportation, number of enteric bacteria changed with values in farm water, sediment and prawn as in EMB media 60.00 ± 23.43 Cfu/ml, 67.33 ± 16.50 Cfu/g and 35.00 ± 9.90 Cfu/g respectively and in SS media 75.33 ± 41.49 Cfu/ml, 46.00 ± 29.51 Cfu/g and 44.67 ± 28.29 Cfu/g respectively. More or less similar results were obtained for farm 2 where on-farm testing showed presence of enteric bacteria in water, sediment and prawn in two different media types counted as in EMB media 105.3 ± 104.1 Cfu/ml, 171.6 ± 155.1 Cfu/g and 191.3 ± 153.1 Cfu/g respectively and in SS media 135.0 ± 71.7 Cfu/ml, 105.6 ± 87.7 Cfu/g and 145.6 ± 199.1 Cfu/g respectively. The same samples when tested in the laboratory after refrigerated transportation, number of enteric bacteria changed with values in farm water, sediment and prawn as in EMB media 59.0 ± 35.5 Cfu/ml, 37.0 ± 19.5 Cfu/g and 60.6 ± 38.0 Cfu/g respectively and in SS media 96.0 ± 53.3 Cfu/ml, 79.6 ± 18.7 Cfu/g and 45.3 ± 30.2 Cfu/g respectively. These changes of enteric bacteria in selective media are probably related to time lapse during transportation of samples from farm to laboratory. The method used, however, have some limitations as these selective media are primary screening media for the isolation and differentiation of *Salmonella*, *Shigella* and *E. coli* spp., and requires subsequent species confirmation test. As we were interested to conduct these analyses in a low-resource

setting with as minimal materials as possible, we used these primary screening media alone for the present study.

Discussion

There has been an increasing need to establish on-farm rapid and reliable bacteriological test methods for detecting foodborne pathogens in fish, shrimp and prawn, and in GAP, it is almost impossible for the farmers to manage the culture system. In the present study, we used basic bacteriological facilities and compared on-farm and laboratory bacteriological testing in low-resource settings associated with giant freshwater prawn cultured in south-western coastal areas of Bangladesh.

Since shrimp and prawns are bottom dwellers, they accumulate many types of bacteria from the surrounding soil and water and the food they ingest in the culture system. All microbes grow at wide range of temperatures with 30 to 40°C being the most suitable. In the present study, the water temperature of the prawn farms was slightly lower, but we observed higher bacterial load in prawn samples during on-farm and laboratory testing. The reason of such higher load is not well understood, but may be related to poor water quality. As for other water quality parameters we found that pH values varied from 6.60 to 7.50, which was more or less similar to the findings of Ali et al. [19], Dewan et al. [20], Wahab et al. [21] and Kohinoor et al. [22]. These values were also within the suitable range for prawn culture. All of the above studies agreed with the present study pH 7.0 or nearly neutral which was suitable for bacterial growth. DO concentrations in the farm were more or less similar to the study of Kohinoor et al. [22], Hasan [23] and Paul [24]. DoF [25] reported that DO from 5 to 8 mg/l were suitable for prawn culture but in the sampling farm the range of DO was slightly lower. Increased temperature may be the cause of lower DO. The most striking was the salinity observed in the prawn farms. Our measured salinity of both farms water ranged from 6-11 ppt which might have been caused by vaporization of water (in farm 1 mainly) and intrusion of brackish water (in farm 2).

Bacteriological analysis including aerobic plate count in farm and in laboratory e.g. determination of bacterial load in farm water, sediment, prawn and the presence of pathogenic bacteria by using some selective media were done. The present study results of bacterial load in farm (measured as colony-forming units Cfu/ml for water and Cfu/g for sediment and prawn) ranged from $4.82 \pm 3.33 \times 10^4$-$9.26 \pm 3.36 \times 10^4$ Cfu/ml in water, $9.37 \pm 4.99 \times 10^8$-$1.04 \pm 0.79 \times 10^9$ Cfu/g in sediments and $4.76 \pm 3.81 \times 10^6$-$1.04 \pm 1.12 \times 10^7$ Cfu/g in prawn when analysis was done by using samples in farm and bacterial load ranged from $5.21 \pm 3.42 \times 10^5$-$1.13 \pm 0.66 \times 10^6$ Cfu/ml in water, $6.30 \pm 2.79 \times 10^9$-$2.27 \pm 1.18 \times 10^{10}$ Cfu/g in sediments and $8.27 \pm 3.77 \times 10^7$-$1.49 \pm 1.40 \times 10^8$ Cfu/g in prawn when analysis was done by using samples in laboratory. The enumerated enteric bacteria in the present study was almost similar to those reported for farmed giant freshwater prawn cultured in earthen ponds in Saudi Arabia [26], bacterial micro flora associated with farmed freshwater prawn and the aquaculture environment [27] and enteric bacteria and water quality of freshwater prawn in culture environment from Kerala, India [28]. Total bacterial count and enteric bacteria observed in the present study were also comparable to that reported for farmed freshwater prawn in India [29,30]. Bacterial loads of water, sediment and prawn samples of two farms were different because the management conditions were not similar and the water sources of the farms were different. The organic matter influences the load and composition of microbial population [31]. Sediment bacterial composition and load greatly influence by effluent characteristics. On the other hand bacterial flora in prawn is the reflection of aquatic environments [32]. It affects the storage life and quality of fishery products [33]. The results showed the variation of bacterial flora among the farms. The variation also may influenced by feeding regime used by the farm owners between the two prawn farms.

In the present study, enteric bacterial levels in prawn were high as previously reported for tiger shrimp farms in India and in Philippines [30]. 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. The composition of the bacterial micro flora found in prawn farms is typical of freshwater environments and as generally recognized, is dominated by Gram – ve bacteria [35]. The results found in this study are comparable and similar to the results of the studies that have been conducted in different countries by different researchers that have already been discussed. On the other hand, the highest number of total coliform and faecal coliform were enumerated in traditional or extensive management system. Therefore, use of raw cow dung, poultry faeces and raw meats, might be the possible source of pathogenic bacteria in freshwater prawn farms. The presence of pathogenic bacteria in the farm indicated unhygienic environment and the sources of contamination of prawn farm such as bird faeces, mammals and reptiles.

Conclusion

Until now, very few studies have been conducted on the enumeration and detection of bacterial flora associated with cultured giant freshwater prawn. In the present study, we found that on-farm testing of bacteriological enumeration can be adopted as a practice by the prawn farmers and use as a measure for quality management and safety.

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