

Monitoring of Total Heterotrophic Bacteria and *Vibrio* Spp. in an Aquaculture Pond

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Abstract: The present study was conducted in order to monitor the total heterotrophic bacteria present at different Days of Culture (DOC). The presence of specific human pathogenic species of *Vibrio* serve as an indicator of public health safety of water and food destined for human consumption. So an assessment of *Vibrio* sp. was conducted in order to evaluate the nature of the pond. THB load in sediments showed its peak of 2.3×10^7 CFU/g at 150th DOC and low of 8.9×10^7 CFU/g at 25th DOC. The *Vibrio cholerae* populations in the sediment high 2.3×10^7 CFU/g at 150th DOC and low in 1.9×10^7 CFU/g at 25th DOC. *Vibrio parahaemolyticus* in sediments was high (5.5×10^7 CFU/gm) at 150th DOC and low (1.3×10^7 CFU/g) at 25th DOC. However, turbidity plays a major role in the distribution of bacterial population in pond water due to heavy organic matter.

Key words: Total heterotrophic bacteria, *Vibrio cholera*, *Vibrio parahaemolyticus* and heavy organic matter

INTRODUCTION

Brackish water aquaculture has become a major frontier of fish and prawn production in the South Asian countries. Aquaculture means the land based farming systems using salt water from the estuaries, back waters and seawater including net, pen enclosures, traditional earthen ponds, concrete block or plastic line, round pond systems and controlled environmental tanks or raceways. Asian region accounts for at least 83% of global aquaculture production (Anonymous, 1990). The brackish water area available in India is esteemed to be about 1.2 million ha (Heran *et al.*, 1992) of which 65000 ha area is now under shrimp farming. Two lakhs ha of brackish water area is available in Tamil Nadu (Santhanakrishnan, 1987) of which only 27000 ha are available readily as potential area for aquaculture. Studies on distribution pattern of heterotrophic bacteria in the seas was started by Zobell (1946) and followed by Kriss (1963) and Sieburth (1971). The distribution and activities of heterotrophic bacteria in polluted waters were introduced by Keller (1960) and his reports suggested that the number of bacteria in the water body is naturally quite variable and depends on a wide variety of factors. Balakrishna (1982) studied the ecology, serology and taxonomy of *Vibrio parahaemolyticus* from neretic environments of Parangipettai.

Microorganisms particularly bacteria plays a vital role in the pond ecosystem. Both beneficial (nutrients recycling, organic matter degrading etc.) and harmful role (as parasites) are being caused by bacteria in the pond ecosystem. Apart from indigenous bacteria in estuarine

water, application of artificial feed and fertilizers, high stocking density, induced breeding and shallow nature of water in intensive and semi intensive farm leads to high bacterial population and thus cause diseases in pond reared animals. Further the dominant roles in the spoilage of marine products are mainly due to bacteria (Palaniappan, 1982). In the environment as well as culture ponds *Vibrios* are responsible for the disease outbreaks.

Among water and food borne pathogens in coastal ecosystems *Vibrios* contribute the major part. The members of the family Vibrionaceae contribute 60% of the total bacterial population (Simidu and Tsukamoto, 1985). Since *Vibrio* species are isolated from water, sediment, invertebrates and fishes they are considered as autochthonous marine and estuarine microflora (Grimens *et al.*, 1986). They are capable of efficiently utilizing a wide spectrum of carbohydrates, proteins and lipids (Kaneko, 1973). The presence of specific human pathogenic species of *Vibrio* can serve as an indicator of public health safety of water and food destined for human consumption (Colwell and Kaper, 1977). *Vibrio cholerae*, a marine *Vibrio* recruiting salt for growth is gram negative bacterium of the family Vibrionaceae. Although there are many serogroups, only 01 and 0139 have exhibited the ability to cause epidemics. *Vibrio cholerae* 01 is divided into 2 serogroups, Inaba and Ogawa and 2 biotypes, classic and E1 tor. Cholera is acquired by ingesting food or water contaminated by fecal material from carriers (shellfish and copepods are natural reservoirs of these carriers). The association of *Vibrio cholerae* with plankton, notably copepods, provides evidence for the new origin of cholera, as well as an explanation for the

sporadic and erratic nature of cholera epidemics (Colwell and Huq, 1999). *Vibrio parahaemolyticus* is a gram negative halophilic bacterium distributed in the temperate and tropical coastal waters and is a leading cause of food borne gastroenteritis (Joseph *et al.*, 1983). People consume seafood that has been improperly cooked/handled are at increased risk of acquiring infections from these pathogens. Over the years, *Vibriosis* have therefore evoked considerable awareness among medical and marine microbiologists. Hence, present study was conducted to monitor the distribution of Total Heterotrophic Bacteria (THB) and pathogens like *Vibrio cholerae* and *Vibrio parahaemolyticus* in abiotic samples like sediments.

MATERIALS AND METHODS

A pond of area of 0.82 ha was selected for the study is a CAA approved farm situated on the northern bank of River Vellar, Agaram, Parangipettai, Tamil Nadu. The River Vellar originates from Servarayan Hills of Salem district in Tamil Nadu (South India) and travels a distance of 480 kms before draining into the Bay of Bengal at Parangipettai (Lat11°29' N, Lon 79°46' E).

Culture Details: The study was conducted between April-August 2009. The culture pond was Pre-prepared with lime and fertilizers like urea and super phosphate. The chemicals like Zoothamnicide were used to manage the Zoothamnium and Sokaranya as a fungicide. *Penaeus monodon* seeds of PL 14 size were tested by PCR technique for WSSV infection and stocked in culture ponds at a density of 12/m². Probiotics and other supplements applied with feed were Mutagen, Super biotic and Zymetin. The water quality parameters were upheld at DO 5.1-5.9 ppm, pH 7.8-8.6 and salinity 14-25 ppt. The average body weights of the animals were 24 g at the time of harvesting. The total amount of the animals harvested was 1.8 tonnes.

Collection of sediment samples: Sediment samples were collected employing an alcohol rinsed and air dried small PVC pipe. The central portion of the collected sample was aseptically retransferred into new polythene bags for bacteriological analysis. Collections of sediment samples were made at different DOC i.e. at 25, 50, 75, 100, 125 and 150th DOC at the sampling site to elucidate the incidence and distribution of total heterotrophic bacteria, *Vibrio cholerae* and *Vibrio parahaemolyticus*. All samples were transported to the laboratory and bacteriological analysis was performed within 2 hrs immediately after the collection.

Enumeration of THB: THB population was enumerated by adopting the spread plate method. Serial dilutions of sediment samples were prepared using sterile 50% sea water. 0.1 ml of the diluted sample was spread on a petriplate containing Zobell Marine Agar Medium (Hi-Media, Mumbai). The sample was spread using an 'L' shaped spreader. The plates after inoculation were

incubated in an inverted position at 28±2 °C for 24-48 hrs. The colonies were counted and the population density was expressed as Colony Forming Units (CFU) per gm of sample.

Enumeration of *Vibrio* sp.: *Vibrio* population was enumerated by adopting spread plate method. Serial dilutions of sediment samples were prepared using sterile 50% seawater. 0.1 ml of diluted sample was spread on a petriplate containing Thiosulphate Bile Salt Sucrose agar (Hi-Media, Mumbai). TCBS medium weighing 8.9 g was suspended in 100 ml of 50% seawater and heated up to a boiling point to dissolve the medium completely. Then it was cooled to 50°C, poured into the pre-sterilized petriplates and allowed to solidify. The samples were spread using an 'L' shaped spreader. The plates after inoculation were incubated in an inverted position at 37 °C for 20-24 hrs. After incubation, suspected colonies appeared for *Vibrio cholerae* (small or medium sized, yellow or greenish yellow, 2-3 mm in diameter) and *Vibrio parahaemolyticus* (punctuate, <2 mm in diameter, green or bluish green). These colonies were selected, isolated and streaked onto Brain Heart Infusion (BHI) agar for further characterization.



Plate 1: The THB on Zobell marine agar

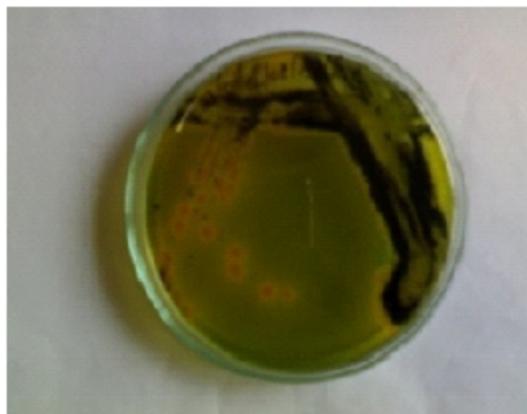


Plate 2: The *Vibrio cholerae* on TCBS agar

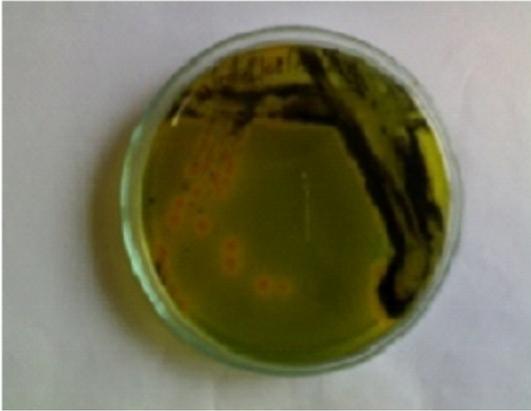


Plate 3: The *Vibrio parahaemolyticus* on TCBS Agar

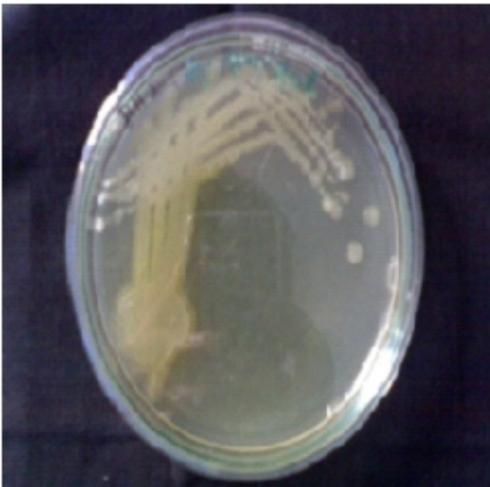


Plate 4: The *Vibrio parahaemolyticus* on BHI Agar

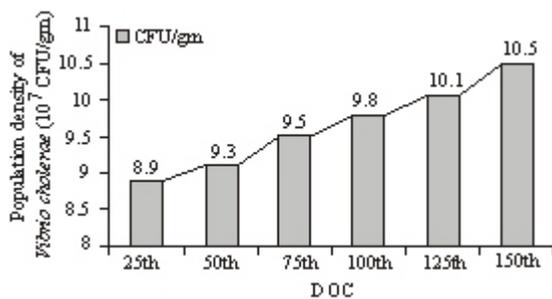


Fig. 1: The density of THB Population

RESULTS

After the incubation period the grown colonies were identified using various colony morphology and biochemical tests according to Bergey's manual.

THB (Total Heterotrophic Bacteria): Plate 1 shows the THB on Zobell Marine Agar. THB load in sediments showed its peak of 10.5×10^7 CFU/g at 150th DOC and

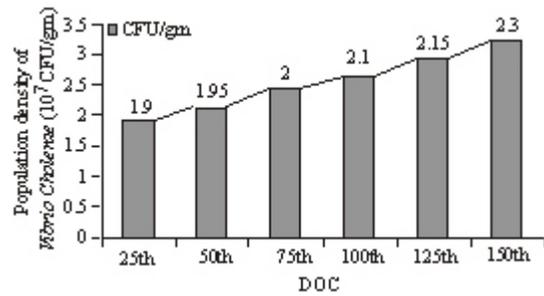


Fig. 2: Density of *Vibrio cholerae* Population

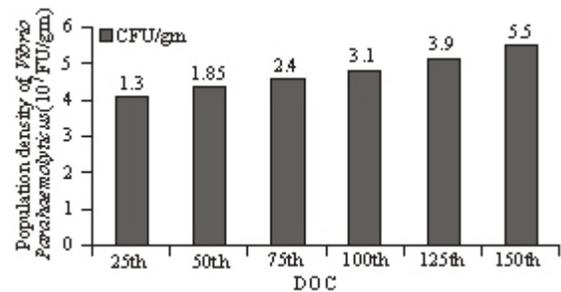


Fig. 3: Density of *Vibrio parahaemolyticus* Population

low of 8.9×10^7 CFU/g at 25th DOC. Generic composition of the isolates of THB showed the predominance of *Vibrio* spp., *Pseudomonas*, *Bacillus* and *Micrococcus*. Density of total heterotrophic bacterial population was shown in Fig. 1.

Vibrio cholerae: *Vibrio cholerae* is a gram negative, motile, catalase positive, oxidase positive and indole producing bacteria. The load is elevated in higher DOC and minimum in initial DOC. The *Vibrio cholera* populations in the sediment is maximum of 2.3×10^7 CFU/g at 150th DOC and low in 1.9×10^7 CFU/g at 25th DOC. Density of *Vibrio cholerae* population (10^7 CFU/g) was illustrated in Fig. 2. Plate 2 shows *Vibrio cholerae* on Thiosulfate Citrate Bile Salts Sucrose agar (TCBS) Agar.

Vibrio parahaemolyticus: *Vibrio parahaemolyticus* is a gram negative, motile, sugar fermenting, catalase positive, oxidase positive, Indole positive, methyl red positive and Voges Proskauer negative bacteria. Population of *Vibrio parahaemolyticus* in sediments was high at 150th DOC (5.5×10^7 CFU/g) and low at 25th DOC (1.3×10^7 CFU/g). Density of *Vibrio parahaemolyticus* population (10^7 CFU/g) was illustrated in Fig. 3. Plate 3 and 4 shows *Vibrio parahaemolyticus* on TCBS agar and BHI Agar.

DISCUSSION

Extensive work, both on THB and human pathogens have been carried out along south east coast of India. Alavandi (1989) observed heterotrophic bacteria in coastal waters of Cochin, were the presence of *Vibrio* sp. was abundant. The results of the study clearly indicate an

increasing trend of bacterial populations towards the end of culture operation. It reconciles with the earlier findings by Sharmila *et al.* (1996) in semi intensive ponds around Tuticorin.

In any aquatic system, environmental parameters such as temperature, salinity, pH and dissolved oxygen play a foremost part in the distribution of bacteria (Palaniappan, 1982). But, pond being a confined environment the optimum environmental parameters (temperature 27-31°C; salinity 14 -25 ppt, pH 7.8-8.6 and DO 5.1 -5.9 ppm) could be maintained throughout the culture period by proper pond management involving water exchange and lime application. The bacterial loads might have changed due to the organic matter deposited at the pond bottom irrespective of environmental factors and water exchange, which was supported by Sharmila *et al.* (1996). They suggested that environmental parameters did not influence the distribution of bacterial load in the pond ecosystem because there was no dramatic change in the environmental parameters. Due to the influence of turbidity, heavy organic matter plays a major role in the distribution of bacterial population in pond water (Sharmila *et al.*, 1996). High organic stuff is possible in shrimp culture ecosystem due to organic manure, fertilizers, high stocking density, feed waste, fecal matter, algal bloom and human interference (Moriarty, 1997; Lloberra *et al.*, 1991).

In shrimp culture ecosystem, most of the bacteria play a negative role as they compete with shrimps for food and oxygen, causing stress and disease (Moriarty, 1997). Generally gram-negative bacteria were found to be the dominant forms in the shrimp culture ponds (Sung *et al.*, 2003) as noticed in the present investigation. Among the THB, *Vibrio cholerae*, *Vibrio parahaemolyticus* play a vital role in shrimp culture ecosystem (Ruangpan and Kitao, 1991) as they damage water quality causing diseases and mortality to the shrimp as primary and secondary pathogens. *Vibrio* sp. is the autochthonous flora of the coastal pond ecosystem (Aiyamperumal, 1992; Ruangpan and Tubakaew, 1994). This is in comparison with present finding, previous showed *Vibrio parahaemolyticus* incidence and its population showed increasing trend towards DOC as observed in the case of THB. The later stage of the culture operation faced serious viral disease outbreak and mortality when more populations of THB and *Vibrio parahaemolyticus* were observed. Bacterial populations increase may be due to microbial contaminated water discharge from other farms located in the same coastal belt. The opportunistic pathogens cause diseases only under favorable conditions (Lightner, 1984). Sindermann (1979) has pointed out that *Vibrios* are the major disease causing bacteria normally found in the environment (Yasuda and Kitao, 1980; Sharmila *et al.*, 1996) as in the present study. A sudden increase of bacterial load could develop bacterial infection directly and making shrimps susceptible to infection indirectly as found by Ping and Meimei (1994).

Vibrio parahaemolyticus was found high during later stages at the time of disease outbreak. The population of *Vibrio parahaemolyticus* reached the peak at the time of mortality which is just a week after viral disease outbreak. It appears that *Vibrio parahaemolyticus* might have acted as a secondary pathogen in the viral disease outbreak. This agrees with the previous work of Nash *et al.* (1992) and Karunasagar *et al.* (1996) who suggested with the aim of the secondary infection of *Vibrios* in *Penaeus monodon* occurs due to stress, high stocking density, unstable environment and Virion particles. The present work suggests that *Vibrio parahaemolyticus* can also act as primary pathogen to White Spot Disease as population of the bacterial species increases with the onset of the viral disease. Also, the bacterial species might have facilitated WSSV to enter the chitinous body of shrimp due to its chitinolytic activity (Jose *et al.*, 2009). Since then it has come to be recognized as one of the leading causes of food borne disease in different parts of the world. Aoki (1967) isolated *Vibrio parahaemolyticus* from seawater, plankton and fish collected in the Pacific Ocean and in the open seas of Southeast Asia. In aquaculture system the pathogenic *Vibrios* load should be under control (below 1000 CFU/ml). A proper pond bottom and microbial management will certainly arrest disease. It is important to maintain the culture successfully through proper pond preparation, seeding quality larvae with moderate stocking density, maintaining stable phytoplankton bloom, good water quality, less feed waste and routine monitoring. It will reduce the bacterial load and ultimately reduce the chance of disease outbreak. The present investigation resulted in outbreak of viral infections at the time of increase in bacterial load especially with that of *Vibrio* species, suggesting the susceptibility of the culture species by the presence of increased bacterial load. The present study concludes that proper water quality management and continues monitoring of benthic microbes are the key factors to avoid viral outbreaks in culture ponds.

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