CHAPTER · 5

SUMMARY

5. SUMMARY

Vibrios are important during hatchery rearing, aquaculture phase and post-harvest quality of black tiger shrimp, *Penaeus monodon*. *Vibrio spp* are of concern to shrimp farmers and hatchery operators because certain species cause Vibriosis. Vibrio species are of concern to humans because certain species cause serious diseases. Vibrios related to post harvest shrimp quality are mainly *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. Rapid Alert System for Food and Feed (RASFF) of the European Commission has issued alert notifications with respect to *P. monodon* and other shrimp exported to Europe from India because of the presence of pathogenic vibrios. Recent rejections vis-à-vis Vibrios in black tiger shrimps were mainly due to the presence of *V. cholerae* and V. *parahaemolyticus*. The export rejections cause serious economic loss to the shrimp industry and might harm the brand image of the shrimp products from the country.

There is a need for an independent study on the incidence of different pathogenic vibrios in shrimp aquaculture and investigate their biochemical characteristics to have a better understanding about the growth and survival of these organisms in the shrimp aquaculture niche. PCR based methods (conventional PCR, duplex PCR, multiplex-PCR and Real Time PCR) for the detection of the pathogenic Vibrios is important for rapid post-harvest quality assessment. Studies on the genetic heterogeneity among the specific pathogenic vibrio species isolated from shrimp aquaculture system provide valuable information on the extent of genetic diversity of the pathogenic vibrios the shrimp aquaculture system. The present study was undertaken with this goal.

Samples of water (n=7) and post-larvae (n=7) were obtained from seven *Penaeus monodon* hatcheries and samples of water (n=5), sediment (n=5) and shrimp (n=5) were obtained from five *Penaeus monodon* aquaculture farms located on the East Coast of India. The microbiological examination of water, sediment, post-larvae and shrimp samples was carried out employing standard methods and by using standard media.

In aquaculture pond samples the mean TPC of pond sediment ($2.9 \times 10^5 \pm 1.4 \times 10^4$ cfu/g) was 2 logs higher than pond water ($3.5 \times 10^3 \pm 790$ cfu/ml). The TPC of pond sediments ranged from 2.8×10^5 to 3.04×10^5 cfu/g while the TPC of pond waters ranged from 2.6×10^3 to 4.4×10^3 cfu/ml in shrimp aquaculture farms. The higher bacterial loads

in pond sediments obtained in this study can be attributed to the accumulation of organic matter at the pond bottom which stimulated bacterial growth. Shrimp head ($4.78 \times 10^5 \pm 3.0 \times 10^4$ cfu/g) had relatively higher bacterial load when compared to shrimp muscle $2.7 \times 10^5 \pm 1.95 \times 10^4$ cfu/g). In shrimp hatchery samples, the post-larvae ($2.2 \times 10^6 \pm 1.9 \times 10^6$ cfu/g) had higher bacterial load than water ($5.6 \times 10^3 \pm 3890$ cfu/ml).

The mean *E.coli* counts were higher in aquaculture pond sediment (204±133 cfu/g) and pond water (124±88 cfu/ml). Relatively lower *E.coli* counts were obtained from shrimp samples (12±11 to 16±16.7 cfu/g). The presence of *E.coli* in aquaculture environment might have been from the source water. *E.coli* was not detected in hatchery waters and post-larvae. Higher mean *E.coli* counts were obtained from pond sediment (204± 132.9) and pond water 123.6 ± 87.8) samples. The *E.coli* counts ranged from 140 to 440 cfu/g in pond sediment and from 48 to 260 cfu/ml in pond water samples. *E.coli* was detected in in three shrimp head and three shrimp muscle samples but the counts were lower. *E.coli* in shrimp head samples ranged between 0 and 40 cfu/g and in shrimp muscle it ranged between 0 and 20 cfu/g. In the present study a negative correlation was observed between total vibrio counts and *E.coli* ($r = -0.54$) in the shrimp culture system which is in accordance with previous reports that state that the counts of vibrios were either negatively correlated or showed no correlation with counts of indicator bacteria (*Escherichia coli*, *Enterococci*, fecal coliforms, and total coliforms). A poor correlation between the level of faecal indicator organisms and the incidence of Vibrios indicate that the Vibrios are a part of the natural microflora of the shrimp culture environment.

Vibrio loads were higher in *P. monodon* hatchery samples than in aquaculture pond samples. Post-larvae had maximum loads of Vibrios ($2.1 \times 10^5 \pm 1.1 \times 10^5$ cfu/g). Shrimp head portion had relatively higher counts of Vibrios ($3.5 \times 10^4 \pm 2.2 \times 10^4$ cfu/g) than shrimp muscle portion ($1.4 \times 10^4 \pm 1.42 \times 10^4$cfu/g). Hatchery waters had higher Vibrio loads (2400± 2200 cfu/ml) than pond waters (150 ± 42 cfu/ml). In the present study the mean TVC of pond sediment (1.5×10^3 cfu/g) was 10 times higher than the mean TVC of pond water (1.5×10^2 cfu/ml). Sucrose non-fermenting vibrios were higher in shrimp head portion (59%) and hatchery waters (49%) whereas more than 90% of the vibrios in post-larvae, pond water, pond sediment and shrimp muscle portions were sucrose fermenters.

A total of 210 Vibrio cultures isolated and purified from the water (105 Vibrio cultures) and post-larvae samples (105 Vibrio cultures) from hatchery samples were screened for the presence of pathogenic *Vibrio spp* based on their biochemical reactions. *V.alginolyticus* (24.3%) was the most common pathogenic *Vibrio spp* detected in hatchery samples followed by *V. vulnificus* (9.1%), *V. parahaemolyticus* (8.6%) and *V. harveyi* (3.8%). The pathogenic Vibrios were most commonly encountered in the water samples (34.76%) than the post-larvae samples (10.95%). *V.alginolyticus* was the most dominant pathogenic *Vibrio spp* in hatchery water (17%) and post-larvae samples (7.1%). The incidence of *V.parahaemolyticus* (7.1%) was slightly higher than *V.vulnificus* (6.7%) in hatchery water samples whereas the incidence of *V.vulnificus* (2.4%) was slightly higher than *V.parahaemolyticus* (1.4%) in post-larvae. *V.harveyi* was detected in hatchery waters (3.85%) but in post-larvae. The lesser incidence of *V.harveyi* in hatchery samples in the present study can be attributed to hatchery water quality management and the size of the post-larvae samples used for analysis.

A total of 250 Vibrio cultures isolated and purified from farm water (75 Vibrio cultures), farm sediment (75 Vibrio cultures) and farmed shrimp (100 Vibrio cultures) samples were screened for the presence of pathogenic *Vibrio spp* based on their biochemical reactions. *V. alginolyticus* (38.4%) was the most common pathogenic *Vibrio spp* detected in aquaculture samples followed by *V. cholerae* (16.8%). Other pathogenic *Vibrio spp* that were detected in hatchery samples viz., *V.parahaemolyticus, V.vulnificus* and *V.harveyi* were not detected in aquaculture samples. *V.alginolyticus* was the most predominant pathogenic *Vibrio spp* in pond sediment (16.4%), pond water (12.4%) and shrimp samples (9.6%). *V.cholerae* incidence was higher in pond water (7.6%) than in pond sediment (5.2%) and shrimp (4%). Shrimp head portion had relatively higher incidence of *V.alginolyticus* (6%) and *V.cholerae* (3.6%) when compared to shrimp muscle where the incidence levels were 2.4% and 1.6%, respectively.

A total of 96 isolates of *V.alginolyticus* were obtained from aquaculture samples of which 42.7% were isolated from culture pond sediment, 32.3% from pond water, 15.6% from shrimp head portion and 9.4% from shrimp muscle. It indicated that farm sediment had a higher presence of *V.alginolyticus* compared with their presence in farm water. But the incidence of *V.alginolyticus* had been relatively very low in the farmed

shrimp. In the farmed shrimp itself, the head region had a higher percentage of *V.alginolyticus* (15.6%) than the shrimp muscle (9.4%).

A total of 42 isolates of *V.cholerae* were obtained from aquaculture samples. Of these, 45.2% were isolated from pond water, 31% from pond sediment, 14.3% from shrimp head portion and 9.5% from shrimp muscle. It indicated that shrimp farm water had a higher presence of *V.cholerae* compared with their presence in farm sediment. But the incidence of *V.cholerae* had been relatively very low in the farmed shrimp. In the farmed shrimp, the head region had a higher percentage of *V.cholerae* (14.3%) than the shrimp muscle (9.5%).

The 42 *V.cholerae* isolates obtained from shrimp aquaculture system (19 isolates from pond water; 13 isolates from pond sediment, 6 isolates from shrimp head and 4 isolates from shrimp muscle) gave negative agglutination reaction with polyvalent somatic O antiserum and with *V.cholerae* O139 antiserum thereby grouping them as Non O1 and Non O139 *V.cholerae*.

Utilization of sugars and sugar derivatives by Vibrio cultures isolated from hatcheries / farms was studied. *V.cholerae*, *V.cholerae ctx* (cholera toxigenic *V.cholerae)*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* failed to utilize ribose and xylose. Only *V.parahaemolyticus* isolates utilized arabinose. *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* utilized glucose, fructose, mannose and mannitol. Only *V.alginolyticus* isolates were negative for galactose. *V.cholerae*, *V.cholerae ctx*, *V.alginolyticus* and *V.harveyi* utilized sucrose whereas *V.vulnificus* and *V.parahaemolyticus* failed to ferment sucrose. *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* utilized maltose and all these Vibrios failed to utilize lactose. *V.vulnificus* and *V.harveyi* utilized cellobiose. *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* failed to utilize aesculin and salicin. *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* utilized glycogen.

Utilization of amino acids by Vibrio cultures isolated from hatcheries / farms was studied using 4 critical amino acids, namely, arginine, lysine, ornithine and histidine. *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and

V.harveyi showed decarboxylase activity with lysine and ornithine but not with histidine. All these pathogenic *Vibrio spp* were negative for arginine dihydrolase activity.

All the *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* were studied for their potential to produce enzymes like amylase, gelatinase and other proteases, DNAses, lipases, phospholipases and phosphatase. *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, and *V.parahaemolyticus* showed amylolytic activity. *V.harveyi* isolates were found to be negative for amylase activity. The mean amylolytic activity index was higher in *V.parahaemolyticus* and *V.alginolyticus*. *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* were positive for gelatinase activity; they liquefied gelatin and showed gelatinase activity on gelatin agar. The mean gelatinase activity index was higher in *V.vulnificus*, *V.cholerae* and *V.cholerae ctx*. 25% of *V.cholerae*, 25% of *V.cholerae ctx*, 50% of *V.parahaemolyticus*, 100% of *V.alginolyticus* and 100% of *V.vulnificus* isolates showed proteolytic activity on fish powder agar. *V.harveyi* failed to show proteolytic activity on fish powder agar. *V.alginolyticus* and *V.vulnificus* showed higher proteolytic activity index on fish protein. *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* isolated from shrimp culture environment were positive for proteolytic activity on shrimp protein agar. *V.vulnificus*, *V. harveyi* and *V.cholerae ctx* showed higher proteolytic activity index on shrimp protein. In this study it was noticed that all the pathogenic Vibrio species isolated from shrimp hatchery and farms were able to utilize shrimp protein thereby suggesting their capability to invade the shrimp tissue. The proteolytic activity differed with the protein substrate. 75% of *V.cholerae*, 75% of *V.cholerae ctx*, and 100% of *V.harveyi* isolates failed to show proteolytic activity on fish powder agar whereas all these vibrios showed proteolytic activity on gelatin and shrimp protein. All *V.cholerae* and *V.cholerae ctx* isolates were negative for DNAse activity whereas *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* showed DNAse activity. Neutral lipid (tributyrin) was not utilized by *V.alginolyticus* and *V.harveyi*. Phospholipid (lecithin in egg yolk) was utilized by all the pathogenic vibrio species. Maximum lipolytic activity index on phospholipid was given by *V.alginolyticus* followed by *V.vulnificus* and *V.parahaemolyticus*. The lipolytic activity index of *V.cholerae* and *V.cholerae ctx* was

slightly higher on neutral lipid than on phospholipid. *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* were positive for phosphatase activity.

The result of the study on the growth of pathogenic vibrios at different temperature showed that all the pathogenic vibrios showed good growth between 22°C and 42°C. At 45°C, only *V.alginolyticus* showed growth. *V.parahaemolyticus* and *V.alginolyticus* showed growth at lower temperature (4°C to 8°C). *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* were negative for growth at 0°C and 55°C temperatures. Study on the growth of pathogenic vibrios at different pH showed that these organisms grew at pH between 7 and 9. None of the pathogenic vibrios isolated from shrimp culture system showed growth either at pH 4 or pH 12.

Effect of salt on the growth of pathogenic Vibrio cultures isolated from shrimp culture system was studied. Maximum growth of *V.cholerae* was observed between 0% and 2% salt concentration (log 8 cfu/ml to 8.3cfu/ml) followed by 3% (log 7cfu/ml) and 6% (log 5.6 cfu/ml) salt concentrations. No growth was observed above 6% salt concentration. In the present study the *V.cholerae* isolate showed growth at 6% salt concentration. This *V.cholerae* isolate belonged to the *V.cholerae* Non O1 and Non O139 group. The Non O1 and O139 *V.cholerae* are a diverse group with high genetic variation between them. The growth at higher salt concentration (6%) might be attributed to the genetic potential of the isolate. In the case of choleratoxigenic *V.cholerae* (*V.cholerae* ctx) no growth was observed at 6% and above salt concentrations. Maximum growth of *V.vulnificus* was observed at 3% followed by 2% and 1% salt concentrations. No growth was observed at 0% and at above 8% salt concentration. Maximum growth of *V.parahaemolyticus* was observed at 3% (log 9.77 cfu/ml) followed by 2% (log 9.47 cfu/ml), 1% (log 9.3 cfu/ml) and 6% (log 9.3 cfu/ml) salt concentrations. No growth was observed at 0%, 12% and 15% salt concentration. The ability of this *V. parahaemolyticus* isolate obtained from shrimp hatchery to grow at a very low salinity of 0.3% is reported in this study. The growth of *V. parahaemolyticus* at lower salinities was reported in few cases. Although salinity is a critical parameter, it does not completely explain the environmental distribution of all Vibrios because halophilic species such as

V.parahaemolyticus can survive in suboptimal Na^+ concentrations. The result of the study on the influence of salt on the growth of *V.alginolyticus* showed that maximum growth was observed at 2% salt concentration (log 9.6 cfu/ml) followed by 3% (log 9.47 cfu/ml) and 0.5% salt concentrations (log 8.78 cfu/ml). No growth was observed at 0% and 15% salt concentrations.

Effect of salt on the enzymatic activity pathogenic Vibrio cultures isolated from shrimp culture system was studied. All isolates showed amylolytic activity at 0.8% salt concentration. At 0% salt concentration, only *V.cholerae* and *V.cholerae ctx* showed amylolytic activity. Amylolytic activity was maximum at 0.8% salt concentration for *V.cholerae*, *V.cholerae ctx*, *V.parahaemolyticus*; at 0.5% concentration for *V.alginolyticus* and at 1% concentration for *V.vulnificus* and *V. harveyi*. At higher salt concentration (7%) only *V.parahaemolyticus* and *V.alginolyticus* produced amylase. 0.3% salt concentration was sufficient for *V.cholerae*, *V.cholerae ctx*, *V.alginolyticus*, *V.parahaemolyticus* to produce gelatinase. For *V.vulnificus* 0.5% salt concentration was found necessary. The maximum proteolytic activity index was observed with *V.vulnificus* at 0.5% followed by *V.cholerae* at 0.3% and *V.cholerae ctx* at 0.3%. At higher salt concentration (7%) only *V.parahaemolyticus* was able to produce gelatinase. *V.alginolyticus* showed an increasing trend of DNAse activity from 0.5%, to 7% salt concentration. The DNAse activity of *V.parahaemolyticus* increased from 0.5% to 3% and thereafter decreased.

The effect of NaCl concentration on the swarming behaviour of *V.alginolyticus* was studied. The swarming zone of *V.alginolyticus* increased from 1% salt concentration to 3% salt concentration and thereafter showed a decreasing trend. No swarming was observed at 7% to 10% salt concentrations and no growth was observed at salt concentration of above 12%. The results from this study suggests that agar with 7% salt can be used to study *V.alginolyticus* properties on agar media; with minimal swarming problem of this species.

Effect of salt and temperature on the utilization of sugars (sucrose and mannitol) and amino acids (arginine, lysine and ornithine) by pathogenic Vibrio cultures isolated from shrimp culture system was studied.

Effect of preservatives/ chemicals on the growth of pathogenic Vibrio cultures isolated from shrimp culture system was studied. Potassium chloride (KCl) at 5% level inhibited the growth of *V.cholerae*, *V.cholerae ctx*, *V.vulnificus* and *V.harveyi*. Inhibition by KCl was lower in the case of *V.parahaemolyticus* and *V.alginolyticus* isolates and they showed a 2 to 3 log decrease in counts even at a level as high as 5%. From this study it was found that sodium citrate had negligible effect on the growth of pathogenic Vibrios isolated from shrimp culture system. A maximum reduction of 1 log value was observed at 7% level. It was observed that 3% level of sodium tri polyphosphate (STPP) inhibited *V.vulnificus* and *V.harveyi*. Others species showed 1- 3 log reduction in counts. At 7% level all the pathogenic vibrios were inhibited. Potassium sorbate at 1% level inhibited the growth of *V.cholerae ctx*, *V.vulnificus*, *V.parahaemolyticus* and *V.harveyi*. The counts decreased from 5-8 log to nil. *V.cholerae* and *V.alginolyticus* showed a reduction of 2 log in counts at 1% level but were completely inhibited at 3% level. From this study it is known that all pathogenic Vibrio cultures isolated from shrimp culture system can be inhibited at 3% potassium sorbate level. This result finds application in the seafood processing plants wherein the use of potassium sorbate for the control of pathogenic vibrios can be explored.

Cholera toxin production encoded by the *ctxAB* genes is the major factor in the pathogenesis of cholera. A PCR method that selectively amplifies a specific DNA fragment within the *ctxAB* operon of *V.cholerae* was used. All the 42 isolates were tested using *ctxAB* primers. Three *V.cholerae* isolates were positive in this PCR and all of them yielded a single specific amplicon of 777bp size. 93% of the *V.cholerae* isolates were negative for the presence of cholera toxin genes. The detection of *ctx* positive isolates (7%) among Non O1 and Non O139 *V.cholerae* isolates from *P.monodon* shrimp aquaculture system is being reported for the first time in this study.

PCR method employing species-specific primers that target the house keeping gene *sodB* of *V.cholerae* was used. All the 42 isolates of *V.cholerae* obtained from aquaculture pond water, sediment, shrimp head and muscle yielded the specific amplicon (248bp) thereby confirming their identity as *V.cholerae*.

The draw back of the PCR targeting *ctxAB* genes is that it detects only enterotoxigenic strains of *V.cholerae*. It does not detect non-toxigenic (*ctxAB* negative)

strains. The lacunae of using the PCR targeting species-specific *sodB* primers is that it identifies *V.cholerae* but does not state its toxigenic status with respect to *ctxAB* genes. An experiment was planned as *V.cholerae*-duplex PCR for detection and confirmation of toxigenic *V. cholerae*. For this, the method of Tarr et al (2007) and USFDA-BAM method (2001) were integrated. The amplification cycle conditions for the *V.cholerae*-duplex PCR were standardized and a simple template preparation procedure was used for the *V.cholerae*-duplex PCR. The *V.cholerae*-duplex PCR was initially standardized using *V.cholerae* (MTCC 3906), *V.vulnificus* (MTCC 1145), *V.alginolyticus* (ATCC 17749) and *V.parahaemolyticus* (ATCC 17802) cultures and *ctx* negative *V.cholerae* (laboratory culture collection) cultures. *V.cholerae* (*ctx* negative) cultures yielded a single amplicon (248bp); *ctxAB* positive *V.cholerae* cultures yielded two amplicons (248bp and 777bp). Other *Vibrio spp.* did not yield these specific amplicons. The results indicate that the *V.cholerae*-duplex PCR was specific to *V.cholerae* and the PCR cycle conditions were adequate for obtaining the desired result. All the 42 *V.cholerae* isolates obtained from shrimp aquaculture system were subjected to *V.cholerae*-duplex PCR. Thirty nine *V.cholerae* cultures yielded a single amplicon of 248bp indicating that they are *V.cholerae* but non-choleratoxigenic. Three *V.cholerae* cultures yielded two amplicons viz., species specific 248bp and cholera toxin specific 777bp, thereby indicating that they were cholera toxin producing strains of *V.cholerae*. The result indicates that the majority of the *V.cholerae* isolates obtained from aquaculture farm water, soil and shrimp were non-toxigenic with respect to cholera toxin. The three *V.cholerae* isolates which were positive for the presence of cholera toxin genes were obtained from pond water. These three *V.cholerae* isolates agglutinated neither with polyclonal somatic O antiserum nor with O139 antiserum thereby grouping them as *V.cholerae* Non 1 and Non O139 serogroup. These three isolates failed to yield O1 specific or O139 specific amplicon when tested in PCR reconfirming that the isolates were indeed *V. cholerae* Non O1 and Non O139.

Cholera toxin production encoded by the *ctxAB* genes is the major factor in the pathogenesis of cholera and the presence of *ctxAB* is a prerequisite for full blown cholera disease (Cholera gravis) to occur. In this study a negative correlation was observed between total vibrio counts and *E.coli* ($r = -0.54$) in the shrimp culture system. The

incidence of *V.cholerae* observed in aquaculture ponds might also have been due to natural inhabitation. The structural genes for the ctx element reside on a filamentous phage ctx? (Waldor and Mekalanos, 1996). CTX? is found in all epidemic *V.cholerae* isolates but is rarely recovered from the non O1 non O139 VC environmental isolates. The spread of CT genes in the environment can be facilitated by the exposure of CTX? positive strains to sunlight (Faruque *et al.*, 2000). Genetic and phenotypic evidence strongly suggests that the O139 strain arose from a VC O1 strain by horizontal gene transfer. *V.cholerae* Non O1 and Non O139 strains can also acquire toxigenic genes for toxin production by transduction and therefore might be the source of new epidemics. Even though the existence of *ctx* carrying Non O1 and Non O139 *V.cholerae* isolates was very low in shrimp culture system, the ecological significance of *ctx* genes among these *V.cholerae* Non O1 and Non O139 isolates in the shrimp aquaculture environment needs to be further investigated. The detection of *ctx* positive isolates among Non O1 and Non O139 *V.cholerae* isolates from *P.monodon* shrimp aquaculture system is being reported for the first time in this study.

The autochthonous existence of *V.cholerae* especially Non O1 and Non O139 in aquatic environment has been reported from several areas world over. In such a scenario, rejection of fish/shrimp by the importing nations based on the presence of what appears to be autochthonous bacterial flora in the shrimp culture system appears to be stringent. It is proposed that the mere presence of *V.cholerae* Non O1 and Non O139 need not be the biohazard criterion for rejection of cultured *P.monodon* shrimp but as a safety measure *ctx* carrying *V.cholerae* Non O1 and Non O139 may be considered as potential public health risks. However, further studies are needed to establish *V.cholerae* Non O1 and O139 as native flora of black tiger shrimp culture system.

The sensitivity of *V.cholerae*-duplex PCR was determined. The *ctxAB* specfic primers yielded amplicon only when the concentration of *V.cholerae* was above 1000 cells/ml whereas the species specific sodB primers yielded amplicon at concentration of 100 cells/ml.

A SYBR Green I Real time PCR assay targeting the house keeping gene *gyrB* of *V.alginolyticus* was developed by Zhou et al (2007). However in this study, the real time PCR amplification conditions were employed in regular PCR. 24.3% (51 isolates out of

210 Vibrio cultures) vibrio isolated from hatcheries and 38.4% (96 isolates out of 250 Vibrio cultures) vibrios isolates from aquaculture farms were found to be *V.alginolyticus*. as they yielded the *V.alginolyticus* species specific 340bp amplicon. *V.alginolyticus* specific PCR could detect 90 cells/ml. A *V.alginolyticus*-duplex PCR method was developed by utilizing *V.alginolyticus* species specific primers (Zhou *et al.*, 2007) and Vibrio genus specific primers (Tarr *et al.*, 2007).

V.vulnificus-PCR uses species-specific primers that target the house keeping gene *hsp60* of *V.vulnificus* (Tarr *et al.*, 2007). Only the *V.vulnificus* cultures yielded the specific amplicon of 410bp. 14 *V.vulnificus* isolates were obtained from hatchery waters and 4 *V.vulnificus* isolates were obtained from post-larvae. *V.vulnificus* was not detected in aquaculture farm samples.

V.parahaemolyticus-PCR uses species-specific primers that target the *flaE* sequence in the flagellin gene of *V.parahaemolyticus* (Tarr *et al.*, 2007). Only the *V.parahaemolyticus* cultures yielded the specific amplicon of 897bp. 18 vibrio isolates (15 from water, 3 from PL) were found to be *V.parahaemolyticus*.

A pathogenic Vibrio-multiplex PCR method was developed to detect common pathogenic Vibrios. The pathogenic Vibrio-multiplex PCR utilized *V.cholerae* species specific primers (Tarr *et al.*, 2007) and *V.cholerae ctxAB* genes specific primers (Bacteriological Analytical Manual, 2001), *V.alginolyticus* specific *gyrB* primers (Zhou *et al.*, 2007), *V.vulnificus* specific *hsp60* primers (Tarr *et al.*, 2007) and *V.parahaemolyticus* specific *flaE* primers (Tarr *et al.*, 2007). Multiplex PCR was performed using a single PCR reaction mix which contained 5 sets of primers. The multiplex PCR can help in identifying the above mentioned human pathogenic Vibrios from an unknown colony on TCBS agar. When specific DNA was added as template to the PCR mix, only the corresponding primers specifically reacted and yielded that particular amplicon (single amplicon in the presence of multiple primers). Non cholera toxin producing *V.cholerae* cultures yielded an amplicon of 248bp; *ctxAB* positive *V.cholerae* cultures yielded two amplicons viz., 248bp and 777bp; *V.alginolyticus* cultures yielded an amplicon of 340bp; *V.vulnificus* cultures yielded an amplicon of 410bp and *V.parahaemolyticus* cultures yielded an amplicon of 897bp.

Real time PCR was performed for the detection and quantification of *V.cholerae*. The *V.cholerae* species specific (*sodB*) primers used in regular PCR were tried in the Real Time PCR method. Different concentrations of DNA (105ng to 0.001ng) extracted from *V.cholerae* was used in Real Time PCR. The C_T (Threshold cycle) values decreased proportionally with the increase in the specific DNA concentration. The Real time PCR method provided an extremely faster result when compared to conventional PCR. The result of the melting curve analysis confirmed the specificity of the *V.cholerae* species specific primers and their usefulness in the Real time PCR assay as it showed a single melting maximum (85°C) for different concentrations of *V.cholerae* type culture and a field isolate of *V.cholerae*. The Real time PCR method provided an extremely faster result when compared to conventional PCR and the species specific *sodB* primers were suitable for both conventional and Real time PCR detection of *V. cholerae*.

PCR fingerprinting of *V.cholerae* isolates was performed using RS-PCR, REP-PCR and ERIC-PCR methods. RS-PCR yielded fewer bands (maximum 4) when compared to REP-PCR (maximum 10 bands) and ERIC-PCR (maximum 12 bands). The dendogram of RS-PCR for 22 *V.cholerae* isolates showed 10 fingerprint patterns whereas the dendogram of REP-PCR for 25 isolates and dendogram of ERIC-PCR for 24 isolates showed, 25 fingerprint patterns and 24 fingerprints, respectively. 100% similarity between *V.cholerae* isolates obtained from shrimp aquaculture was noticed only in RS-PCR. None of the isolates showed 100% similarity either in REP-PCR or ERIC-PCR. From this PCR fingerprinting study on *V.cholerae* isolated from *P.monodon* shrimp farms, it can be concluded that REP-PCR and ERIC-PCR are best suitable to study the genetic variation amongst *V.cholerae* at a higher sensitivity level. On the other hand, the RS-PCR will be helpful in comparing the genetic similarity among *V.cholerae*. The isolates that were less genetically apart will be shown as similar in RS-PCR but will be shown as different in REP-PCR and ERIC-PCR. The presence of 470bp size amplification band in almost all the isolates of *V.cholerae* using ERIC-PCR analysis suggests it's potential use as a marker for the identification of *V.cholerae*. The greater similarity of *ctx* positive *V.cholerae* with ctx –ve *V.cholerae* isolates indicates that the *ctx* positive strains (Non O1 and Non O139) might have originated from autochthonous *V.cholerae* in the aquatic niche.