CHAPTER-6

Conclusion and scope of future research

Aquaculture has become the fastest growing food producing segment and is one among the major contributors to National economic development, global food supply and nutritional security. The need of the hour is the development of new approaches to control diseases, which are cost-effective, ecologically sustainable, industrially durable and safe to administer. The continuing decline of marine fisheries and the increased demand for sea food by consumers have created a gap between demand and supply. To meet the demand up to 85%, shrimp/prawn production has to be stepped up to intensive cultivation practices typified by ultra high stocking densities and feed loading .Under such practices, as much as 40% of pond water have to be exchanged every few days to remove toxic waste metabolites. Discharge of nutrient-enriched waste water and bottom sediments from prawn pond in to adjacent coastal waters has frequently resulted in eutrophication, oxygen depletion, spread of diseases to wild population and "genetic pollution" as a result of farmed marine species mixing with wild stocks (Cognetti *et al*., 2006). Effective management of water quality in prawn pond is critical pre-requisite not only for maximizing the productivity but also for mitigating the adverse impact of discharging.

Several approaches have been proposed as alternatives to chemotherapy to increase aquaculture production, including improved animal husbandry practices, improvement in the nutritional quality of feed, the use of 'microbially matured' rearing water colonized by non-pathogenic bacteria (Skjermo *et al*., 1997), disinfection of fish eggs, biocontrol using autochthonous microbes to repress the growth if pathogens in rearing environment (Nogami and Maeda, 1992), treatment with UV, use of nonspecific immunostimulants or vaccination (Anderson, 1992), phage

therapy and probiotic bacteria to exclude or inhibit pathogens (Gatesoupe, 1999).

Vibrios are found in broad ranges of environment and are able to persist because of their ability to survive cycles of feast and famine. Starvation adaptive pathway protects vibrios against number of stresses and prepares themselves for subsequent overgrowth under favorable conditions. Biofilm formation to protect them from protozoan grazing, regulation of virulence, host colonization etc is the various strategic measure adapted by vibrios for their survival to varied environmental conditions. Vibrios harboring the external surface of marine zooplanktons have extensive Chitinolytic activity. They play a significant role in the mineralization of chitin in the aquatic systems by utilizing it as both carbon and nitrogen source. Proteinaceous bacteriocin-like inhibitory substance (BLIS) produced by *V.harveyi* inhibits *V.fischeri*, *V.gazogenes, V.parahaemolyticus* and *V. alginolyticus* as pathogens of shrimp, clam, seabreams etc.

Other agents responsible for Virulence

The capability of bacteriophages for the movement of genetic material amongst bacteria constitutes one type of vehicle for transferring important virulent factors (Payne *et al*., 2004). Virulence of *V. harveyi* may be controlled by quorum sensing in so far as it has been confirmed that this regulates type III secretion (Henke and Bassler, 2004a, b). The capability of the pathogen to attach to chitin by means of a specific protein-mediated mechanism may be of significance for the adhesion, colonization and subsequent infection of the host (Montgomery and Kirchman 1993, 1994). Interestingly, it has been suggested that the ability of the bacteria to bind iron could be an important virulence factors (Owens *et al*., 1996). Moreover, the persistence and survival of *V. harveyi* in shrimp hatcheries have been attributed to its ability to form biofilms with resistance to disinfectants and antibiotics (Karunasagar *et al*., 1994).

Management measures adapted for disease control

- Checking the pathogen entry into culture system through seed, feed, water and carriers.
- Stocking disease free and healthy hatchery seeds in well prepared ponds.
- Stocking density should be limited to 30,000/ ha water spread area.
- Consistent maintenance of optimal water parameters helps avoid stress factors throughout the crop.
- Adopting bio-secured systems including closed, reduced water exchange or increased water re-use and other bio-secure practices.
- Adopting better management practices such as disinfecting water, brood screening, seed screening, rinsing of eggs and nauplii of shrimps with clear water, significantly reduces water borne infections.
- Monitoring shrimp health conditions through rapid diagnostic techniques and adjustment of feed quality according to growth and days of culture.
- Preventing the use of antibiotics and pesticides during culture period.
- Avoiding the feed with trash fish or other by-products to cultured animals.
- Avoiding discharged water from ponds affected or suspected to be affected by pathogens into natural environment.
- Above strategies together with crop holiday, crop rotation, reservoir system and good management practices help in better management of disease control.

Role of Molecular tools in identification of vibrios

The principles of polyphasic taxonomy and the advent of new techniques such as DNA-DNA hybridization, nucleotide composition, measurements of amino acid sequence differences, screening of phenotypic characters, including various carbohydrates, proteins, lipids, aminoacids, and alcohols as source of carbon and/or energy, enzyme activity, salinity and temperature tolerance, luminescence, antibiograms and morphological features have proved as a firm basis for the current taxonomy of *Vibrio*.

The identification of *Vibrio* species requires the application of genomic analyses, including Amplified Fragment Length Polymorphism (AFLP), repetitive extragenic palindromic elements PCR (rep-PCR) and 16S rRNA gene sequencing (Thompson *et al*., 2001). *recA* has been suggested as a potential marker to unravel phylogenetic relationships among the higher taxonomic ranks such as families, classes and phyla because of its ubiquity and house-keeping function in bacteria (Ludwig and Klenk, 2001; Zeigler, 2003). Several highly powerful molecular tools, such as AFLP, FAFLP (fluorescent amplified fragment length polymorphism), IGS (intergenic spacer region), rep-PCR (Gurtler and Mayall, 2001), have become readily available for the identification of bacteria, including vibrios (Thompson *et al*., 2001; Sawabe *et al*., 2003). The phenotypic and genetic heterogeneity and the presence of mobile genetic elements in *V. harveyi* mean that species-specific marker common to all isolates would be extremely difficult, if not impossible, to locate.

Practical applications of molecular identification techniques are limited mostly to medically important strains. This reflects the need for rapid, easy and reliable identification systems for both clinical laboratories and aquaculture industries. The virulence genes appear to function as important candidates for identification of species and also for the differentiation of the pathogenic strain from its non-pathogenic counterparts.

Selection of suitable target genes and standardizing their detection conditions are the key criteria for development of sophisticated molecular identification systems like multiplex and real-time PCR.

Significance of the present Study

The present study focuses on vibrios especially *Vibrio harveyi* isolated from shrimp (*P. monodon*) larval production systems from both east and west coasts during times of mortality. A comprehensive approach has been made to work out their systematics through numerical taxonomy and group them based on RAPD profiling and to segregate the virulent from non- virulent isolates based on the presence of virulent genes as well as their phenotypic expression**.** The information gathered has helped to develop a simple scheme of identification based on phenotypic characters and segregate the virulent from non virulent strains of *V. harveyi*.

The subject matter in the thesis has been divided with the following heads:

- Numerical Taxonomy of vibrios based on un-weighted average linkage.
- Construction of its RAPD profile and analysis of amplicons of the house keeping genes in the *Vibrio* isolates (selected from Numerical taxonomy based on phenotypic characters).
- Detection of virulent and luminescent gene markers in the isolates of *V.harveyi* selected from the clusters obtained from Numerical taxonomy, based on phenotypic characters and RAPD fingerprinting.
- Evaluation of the extent of pathogenicity by determining the relation between phenotypic expressions based on *in vitro* assays.
- Determination of the correlation between the amplicons obtained using virulent and luminescent genes and the pathogenicity expressed in animal models.

Overall achievements of this work are summarized as given below:

- *Vibrio* spp. isolated from shrimp (*P. monodon*) larval production systems of both east and west coasts during times of mortality, and the type strains from BCCM/LMG (Belgium) and MTCC (IMTECH, Chandigargh, India) were subjected to phenotypic characterization and subsequent numerical taxonomy.
- Numerical taxonomy of 158 isolates was carried out (employing UPGMA method) by analyzing 135 phenotypic characters or operational taxonomic units (OTUs), based on which a dendrogram was constructed using the software NTSYS p.c. **17 Phena** defined at a Jaccards coefficient range of 0.55 to 0.988. The reproducibility of the unit characters was validated at a probability value $p \leq 0.05$ using Chi-square test.
- A dichotomous key was constructed based on the phenotypic traits of the isolates for identification of vibrios associated with shrimp hatchery systems. Based on the dichotomous key developed only 9 biochemical tests or phenotypical characters are sufficient enough for the identification of vibrios from shrimp larval rearing systems especially in the east and west coasts of India.
- Identification of the isolates based on sensitivity to antibiotics was employed in the dichotomous key of Alsina and Blanch (1994a, b). However, the key developed from this study for the identification of vibrios from shrimp larval rearing systems especially in the east and west coasts of India, does not employ antibiotic sensitivity. Several multidrug resistant forms are present in the environment especially in aquaculture systems where antibiotics are constantly being used as a control measure against bacterial pathogens. In this context, development of a dichotomous key with exclusion of antibiotic screening prevents error and/ or misidentification of the

environmental isolates, which are very likely to carry the antibiotic resistance gene transferred via R-plasmid.

- The isolates reproduced the results when subjected to the set of phenotypic characters according to the dichotomous key, suggesting that the present study could be useful in the routine identification of vibrios from shrimp larval rearing systems especially in the east and west coasts of India.
- Genotypic characterization of vibrios isolated from shrimp larval rearing systems in the east and west coasts of India was carried out employing molecular tools such as RAPD (Random Amplified Polymorphic DNA) and MLBPA (Multi-locus basepair analysis) using the primers for housekeeping genes.
- RAPD fingerprinting was carried out using 7 selected Operon primers which exhibited distinct and reproducible banding pattern ranging from 100- 4500 base pair and the results of amplification were scored and dendrograms were constructed using the softwares NTSYS p.c and PopGene 32.
- The interrelation between each isolates was examined based on similarity coefficient. The results suggested that the isolates of vibrio investigated diverged widely from the isolates which were grouped together as a cluster based on phenotypic characterisation. Divergence pattern exhibited by the isolates was highly different when analysed with each of the 7 different primers individually. This result suggests that a large number of heterogenic genotypes within the isolates of vibrios do exist.
- Considering the vibrio isolates obtained from similar sources as a population and analyzing the banding pattern obtained with all the 7 primers in total and processing the data of the 92 loci in PopGene32

software revealed that the isolates were grouped into 8 Clusters and were interrelated at ≥76%.

- Amplicons of 600, 400 and 200bp were found to be shared by most of the *Vibrio* isolates which were subjected to fingerprinting with 7 selected primers. The sharing of common bands indicated the presence of a highly conserved genomic region in diverse *Vibrio* strains. The presence of conserved region suggested that the isolates shared the same phylogenic lineage. This assumes significance as amplification of common fragments by RAPD-PCR with a particular primer has been shown to be useful in genetic amplifications and hybridization assays for diagnostic purpose.
- The dendrogram constructed exhibited a correlation between a given RAPD type and the geographical location or the source of the isolates. In this study isolates belonging to *V.mediteraneii* were grouped into the cluster of *V. harveyi, V.fluvialis* and *V.vulnificus*. Though the isolates were obtained from different sources, they exhibited high relatedness above 91% 86%S and 84.5%S respectively, suggesting that the isolates obtained from the same geographical area have a few genes in common which remains conserved, while the other genes have been acquired by the isolates through horizontal gene transfer or mutations.
- The representative isolates of vibrios (35 Nos. including the type stains) were selected from the dendrogram constructed based on phenotypic characterization were amplified with already reported housekeeping gene markers (*gapA*, *ftsZ*, *topA*, *mreB*, *gyrB*, *pyrH*, *recA* and *16S rRNA*).
- The results obtained with all the 8 different housekeeping gene primers were interpreted using the software PopGene 32. Analysis of house keeping genes showed that the representative isolates were grouped into three core groups, interrelated ≥ 79%S.

- From this study, it was found that *recA*, *topA*, and *pyrH* genes among the 8 different housekeeping genes could be used as powerful markers for the identification of vibrios. As these three genes have potential sequences that are capable of creating phylogenetic trees with the highest resolution and consistent signal, and hence could be used for species discrimination.
- Genotypic analysis of 25 wild isolates of vibrios suggests that these isolates shared similarity at ≥95% with the isolates deposited in GenBank database. Identification of wild strains without studying their phenotypic profile of the may lead to erroneous identification, hence a detailed investigation of the phenotypic profile of the isolates is a prerequisite for identifying wild strains rather than completely depending on genotypic characterization such as analysis of 16S rRNA gene.
- The extent of virulence exhibited by the isolates of vibrios could be analysed by various *in vitro* assays including determining the hydrolytic potential, auto-agglutinating, self-pelleting, biofilm formation, cell surface hydrophobicity, adherence and cytotoxicity.
- All 158 isolates were positive for hydrolytic assays such as amylase, gelatinase, DNA-ase, chitinase, lecitinase, γ-hemolysin on human blood agar medium, and for auto agglutination test for self pelleting (SP+) and precipitation after boiling (PAB+). Of the 158 isolates examined, 125, 13 and 101 isolates were positive for aesculin hydrolysis, elastase, and lipase production respectively.
- Biofilm measurements at 570nm showed that of the 12 representative isolates, V3 and V45 possessed high degree of biofilm forming ability.
- *V. harveyi* V3, V28, V36, V57 and V71 were strongly hydrophobic and the remaining isolates and also the type strain of *V.harveyi* were

moderately hydrophobic and hence were more effective in eliciting pathogenicity in comparison with the reference isolates, *Bacillus* MCCB101, *Arthrobacter*MCCB104 and *V.cholerae* MTCC 3906 which were weakly hydrophobic.

- *V.harveyi* isolates were cytotoxic on HEp-2 cell line exhibiting CPE revealed by rounding, shrinkage of cytoplasm and dislodgement of cells which showed that the cell free supernatant harbored toxins which played an active role in pathogenesis.
- The isolates were also subjected to antibiotic susceptibility test using 81 different antibiotics and the MAR index was calculated. MAR index values above 0.2, suggested that majority of the isolates have originated from areas susceptible to constant antibiotics use.
- The sensitivity of the isolates to the different classes of antibiotics showed that the isolates were mostly resistant to Lincosamide, Peptides (Glycopeptides and Polypeptides), β-lactams, Steroids and Tetracycline class of antibiotics. Hence these antibiotics should not be used in aquaculture settings as a prophylactic measure targeting elimination of the pathogenic *Vibrio* population in shrimp hatchery systems.
- The extent of virulence exhibited by the selected 12 isolates of *Vibrio harveyi*, analysed based on the amplicons obtained with already reported virulent and luminescent gene markers was worked out.
- All 12 isolates of *V. harveyi* gave positive amplicons for *hlyA*, *rpoS*, *vopN*, *luxS*, *luxR* and *luxA* genes, suggesting that these genes were conserved within the species. However, the presence of *hlyA* gene in all the isolates suggested their capability to cause haemolysis in animals under stressed conditions.

- Presence of *luxS*, lux*R* and lux*A* genes in all the isolates suggested that the isolates mediated strong cell to cell communication by diffusible extracellular molecules or signals (auto inducers); enabling quorum sensing and indirectly favoring virulence expression. Also, majority of the isolates of *V.harveyi* except V54, V57 and V64 gave positive amplification to protease genes, suggesting that the presence of protease genes was linked with luminescence and virulence.
- The isolate V3 gave positive amplicons for all the 28 genes investigated while the type strain of *V.harveyi* (LMG 4044) showed only 17 positive amplicons and the isolate of *V. harveyi* V81 with 22 amplicons. Based on these observations, the isolate *V. harveyi* V3 (MCCB 111) could be ranked first as the most potent pathogen among the 11 representative isolates, while the type strain *V.harveyi* (LMG 4044) and isolate of *V. harveyi* V81 could be considered as the least potent ones .
- Pathogenicity assay of the 12 isolates *V.harveyi* was carried out on animal models such as gnotobiotic *Artemia* nauplii and post larvae of *Penaeus monodon*.
- The challenge study conducted on gnotobiotic *Artemia* nauplii with the 12 isolates of *V.harveyi* revealed that V3 was the most potent pathogen out of the 12 representative isolates and V81 along with *V.harveyi* (LMG 4044) were the least potent ones.
- The challenge experiments carried out on PL-5 showed that the isolates of *V.harveyi* V3 and V88 were the most potent pathogens amongst the 12 isolates of *V.harveyi* causing 100% mortality. VhL (LMG 4044) exhibited 27.6% mortality, while all other isolates exhibits very low or no mortality, behaving similar to that of the control.

- The 12 isolates of *V.harveyi* could be differentiated into three groups as the one associated with highest mortality, the ones with lowest mortality comparable to that in the controls and the ones with moderate mortality. Therefore it is unscientific to declare the entire V. *harveyi* clade as pathogenic to shrimp larvae.
- The type strain of *V.harveyi* (LMG 4044) gave no amplification for 11 marker gene but was still capable of causing 28% mortality in *P.monodon* PL. This result suggests that the 11 genes which were absent failed to play a major role in expression of pathogenecity. However, their presence might complement the virulence expression.
- The presence or absence of a single virulence factor is not critical for virulence of the isolate because the bacteria produce many different virulence factors. Hence, it could not be proved that the presence of specific virulence factor was the key factor associated with virulence to the host. However, it does not exclude the possibility that these virulence factors may be essential for virulence towards different host.
- From this study it can be concluded that the mere presence of the virulent genes will not elicit 100% mortality and that the expression of these genes, variation in the environmental conditions and induction of stress to the host animal, make them susceptible to pathogenic invasions.

Scope of future works

From the present study an excellent foundation on the characteristics of the 147 wild strains of vibrios isolated from larval rearing system could be obtained. Also the extent of pathogenicity expressed by the strains could be categorised into three different levels as highly, moderately potent and non pathogenic forms. This point to the fact that the entire *V. harveyi* clade is not pathogenic to shrimp larvae and the beneficial forms needs to be

retained as the natural flora for the proper functioning of the aquaculture settings.

- The difference in the banding pattern within *V.harveyi* isolates when subjected to RAPD fingerprinting and the divergence of the isolates which were clustered together based on phenotypic characterization is an area that can be taken up for further research. By sequencing the bands which are not conserved and comparing the sequences with the database will provide a better idea regarding the causes for heterogeneity and variation in virulence among *V.harveyi* isolates.

- Future works focuses on the expression of virulence and the factors responsible for the activation of the virulent factor or factors that remains suppressed in normal conditions and are expressed during adverse conditions needs to be carried out. Hence expression studies using mRNA is required which will provide further information on the virulent genes that are expressed during pathogenesis.

- For preventing large scale mortality caused in the larval production systems, the pathogenic forms should be clearly differentiated from the non-pathogenic forms at the earliest. Further works can be carried out for screening the pathogenic ones by developing a multiplex PCR as a diagnostic tool enabling the segregation of potent pathogenic strains from their non-pathogenic counterparts.

- The mechanism by which mobile elements and *V.harveyi* phages mediated virulence in a non-pathogenic *V.harveyi* strain is a vital area for further study.

- *V.harveyi* (V3) is found to be the most potent strain in this study. Hence by using this isolate as antigen, monoclonal antibody can be developed. These MAbs can be fused with fluorescent dyes and can be used for immunofluorescence and immunohistochemical studies. Based on histopathological and immunochemical methods the presence or

absence of *V.harveyi* isolates at the site of infection such as in the gills or hepatopancrease could be determined. A quantitative assay to enumerate *V. harveyi* in water and in larval body can be formulated.

The ban on the use of antibiotics in aquaculture settings, due to the development of multiple drug resistance strains necessitates the development of alternative methods to control vibriosis. In this context research has to be focused on evaluation of putative probiotics and vibriopahge therapy. These two methods are sustainable and will improve shrimp productivity without resorting to any antibiotic treatment.

- Another mechanism that can be targeted towards suppression of the virulence expression of *V.harveyi* without any impact on the bacterial growth is by disrupting the QS mechanism at different check points. Recently researchers are keen on identifying the checkpoints targeting inhibition at three different levels such as 1. Signal generation, 2. AHL signal dissemination and 3. Signal receptor.

Precisely this work opens up new avenues of research to further examine the genetic heterogeneity of *V.harveyi*, explore the conditions at which pathogenicity is expressed by them and development of multiplex PCR for the detection and segregation of virulent from non virulent isolates. Various alternative methods for preventing vibriosis in lieu of antibiotic are other strategies for sustainable shrimp larval production technologies.