CHAPTER - 5

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The aim of this work was to develop biological control alternatives to chemotherapeutants such as antibiotics for use in penaeid larval rearing systems against vibrios with special reference to *V. harveyi*. Eighty seven luminescent bacterial isolates were obtained from shrimp hatchery systems of both East and West Coast of India. They were identified as *Vibrio harveyi* based on phenotypic characters. Source of *V. harveyi* was found to be the incoming nauplii, crabs used for feeding broodstock and sand around the raw sea water intake points. Luminescent bacteria could also be isolated from diseased larvae and larval rearing water and the hatchery drain off wastewater. The latter suggested the ineffectiveness of treatment of wastewater. Even though LB could not be detected in treated water, its presence in VBNC state could not be ruled out as the organisms were stressed during the treatment process of chlorination, dechlorination etc.

The antibiotic resistance among the isolates was extremely high. The similarity level ranged from 40 to 90 %. This implies a high heterogenous nature in the resistance profile and that the process of resistance acquisition is independent of the use and consequence of presence of the antibiotics in the system. However, the isolates had a very high MAR index at an average of 0.63 with little variation between those from different samples. This situation poses an additional threat of antimicrobial resistance to be acquired by human pathogenic bacteria in the environment. In the present study as antibiotic residues were not analyzed, the MAR index could not be linked to the widespread application of antibiotics in shrimp hatcheries in India. However, the study strongly suggests the need for developing biological control measures as alternatives for improving sustainability and productivity of shrimps in culture because antibiotic administration by and large is a futile exercise as demonstrated by the heterogenous nature of the resistance profile of *V. harveyi*. 
This justifies the evaluation of putative probionts and isolation of vibriophage for the control of vibrios as viable alternatives of antibiotics.

Four putative probiotics such as *Pseudomonas* MCCB102 and MCCB103, *Micrococcus* MCCB104, and *Bacillus* MCCB101 were evaluated for their probiotic potential against 87 isolates of *V. harveyi*. The two isolates of *Pseudomonas* and the isolate of *Micrococcus* were found to inhibit all isolates of *V. harveyi* tested by disc diffusion assay, but *Bacillus* MCCB101 was not. Additionally, the pseudomonads were inhibiting the *Bacillus*, but not *Micrococcus*. This led to the development of a combination of *Bacillus* MCCB101 and *Micrococcus* MCCB104 to evaluate their combination to inhibit *V. harveyi* in vivo.

In cocultures, *Pseudomonas* MCCB102 and MCCB103 could eliminate *V. harveyi* at high initial cell counts (10⁶ cfu ml⁻¹). But neither the *Bacillus* MCCB101 nor *Micrococcus* MCC104 could do so. This trend was seen in vivo also. However, the larval survival in these tanks was not related to the vibriocidal property of the pseudomonads as the survival in those tanks supplemented with them was 66.35 and 58.3 % respectively, while being more than 70% in tanks treated with *Micrococcus* MCCB104, *Bacillus* MCCB101 and their combination (Enterotrophotic), suggesting a complex relationship between *Vibrio* and probiotic population and larval survival. The observation could be confirmed by the challenge experiment where the highest RPS was in tanks supplemented with *Micrococcus* MCCB104, *Bacillus* MCCB101, *Pseudomonas* MCCB102 and MCCB103 and Enterotrophotic in the diminishing order of RPS at comparatively higher *Vibrio* count.

The observations pointed to the existence of a ‘probiotic effect’ exerted on the larvae of *P. monodon* by the four probionts when supplemented to the rearing water where their presence beneficially impacted larval survival. This implied that probiotics could favour the host animal in ways other than antagonism of pathogens as evidenced by *Bacillus* MCCB101 which improved larval survival even though it wasn’t exhibiting in vitro antagonism to *V. harveyi*. Probiotic effect is a subject worth unraveling.
The study restablished the importance of higher generic diversity in the rearing water and in larvae for better survival. Even in the control tanks (not treated with probiotics), the survival was reasonably higher and *V. harveyi* could not be recovered from the larvae after challenge.

Based on the study *Micrococcus MCCB104* can be reasonably recommended as the best probiotic in shrimp larval rearing system amongst the four studied when applied to the rearing water at a final density greater than $10^6$ cfu ml$^{-1}$. Trials in larger commercial systems have to be accomplished before commercialization.

During the in vivo studies recovery of the probiotics added could not be accomplished and whenever recovered, it was too close to the time of application and was found to taper off as the time from application lapsed. Therefore it is suggested that investigations on the fate of probiotics in larval rearing systems and in aquaculture as a whole have to be made an immediate requirement.

The second alternative to antibiotic treatment investigated was the vibriophages isolated from sediment of backwaters of Kochi. Based on their lytic efficiency five phages could be brought under study. They showed broad spectrum lytic activity when screened on 162 bacterial isolates including 87 *V. harveyi*.

Based on their morphology, the phages were placed in the family *Siphoviridae* of the order *Caudovirales*. All of them were double stranded DNA phages. Based on the banding pattern subsequent to digestion with restriction enzymes such as *EcoR1* and *Xba1* the phages were grouped into four distinct types.

The lytic efficiency of the five phages and in combination was similar. In all cases phages were able to arrest the growth of *V. harveyi* for about 12 hours after which phage resistant forms emerged and began to dominate. When phage lytic activity on
Vibrio harveyi LB31 (a strain found susceptible to all the phages during host range study) in plain seawater was tested and initial suppression of growth was noticed.

As there were reports of V. harveyi infective siphovirus-like phage (VHS1) enhancing virulence of V. harveyi strains to P. monodon, the similarity of the phages isolated here was compared with the former by checking for the presence of similar amplicons. The expected product size with the primer set PH102 was 2.5 kb but the amplified product size was only 1.5 kb and that too with only two of the phages. Even though this has apparently ruled out the similarity of these phages to VHS1, much work needs to be done in this direction.

Based on this study it can be categorically stated that sustainable shrimp larval culture can be maintained by the application of probiotics without resorting to any antibiotic treatment. The probionts such as Pseudomonas MCCB102 and MCCB103, Micrococcus MCCB104 and Bacillus MCCB101 are all potent candidates, but Micrococcus MCCB104 seems to be the best option. Vibriophage system remains an attractive alternative to antibiotics but it is too early to bring it into the field as two issues such as virulence mediation in V. harveyi and development of resistance forms need to be addressed. In fact the complex phage-bacterium relationship also needs to be elucidated.

To sum up probiotics and bacteriophages will take the shrimp larval rearing technology a long way to sustainability.