Chapter 6

CONCLUSION
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Aquaculture, is perceived as having the greatest potential to meet the growing demand for aquatic food as it accounts for almost 50 percent of the world’s food fish with its socio-economic and ecological impacts. Crustaceans form one of the main value added components in aquaculture and among them, shrimp aquaculture is the predominant one. The lucrative shrimp aquaculture suffered set backs in many countries mainly due to white spot syndrome virus (WSSV) (Genus – *Whispovirus*, Family – *Nimaviridae*), a rapidly replicating and extremely virulent shrimp pathogen, which has emerged globally as one of the most prevalent and widespread.

Prophylaxis include maintaining water quality of the environment, application of immunostimulants or vaccines to enhance the immune mechanism in shrimp, and/or selection of specific pathogen free animals.

Types of aquaculture shellfish vaccines for combating White Spot Disease (WSD) that have been experimented include administration of heat inactivated WSSV (Namikoshi et al., 2004); formalin inactivated WSSV (Namikoshi et al., 2004; Singh et al., 2005; Melena et al., 2006); recombinant viral proteins of WSSV - VP19, VP28, VP26, VP292, VP466 (Witteveldt et al., 2004a, 2004b; Namikoshi et al., 2004; Vaseeharan et al., 2006; Ha et al., 2008) and DNA vaccine for WSSV (Rout et al., 2007; Rajeshkumar et al., 2008; Li et al., 2010).

The protection seen in the above studies appears to be dependent on the shrimp species and on the time of application of the competing agents (Melena et al., 2006). The durations of protection (maximum seven weeks) and efficacies varied with viral proteins and between studies. Until now, most of the promising results obtained from experimental bioassays have been achieved largely on
empirical grounds as all the vaccine related studies in shrimp have used largely,
 survival as a parameter, mostly after a challenge (van de Braak, 2002). These
 studies have focused on the practical issues of vaccination, rather than targeting
 at the understanding of the mechanisms that are involved in this immune
 response (Johnson et al., 2008).

 In the present study, the following objectives were undertaken to gather
 information on the immune response of tiger shrimp, *Penaeus monodon*, against
 the administration of inactivated virus preparation (IVP):

 - Partial protein profiling of *Penaeus monodon* in response to IVP
   administration.
 - Non-specific immune response of *Penaeus monodon* to IVP administration.
 - Bio-defence genes in immunomodulation of IVP administered *Penaeus*
   *monodon*.
 - Viral gene expression of *Penaeus monodon* to IVP administration.

 The findings of the work are summarized below:

 ♦ The protein profile of IVP administered *P. monodon* before and after oral
   challenge was investigated employing 1 Dimensional Electrophoresis
   (1DE) and 2 Dimensional Electrophoresis (2DE).

 ♦ There were no remarkable variations in the 1D protein profile of gill and
   hepatopancreas of the IVP administered shrimp with the control which
   was maintained on normal diet.

 ♦ Protein profile of haemocyte lysate of IVP administered *P. monodon* by
   2DE was exhibiting variations from that of the control animals. This
   comparison was made before and after the challenge with WSSV, and in
   the latter the protein spots were lesser compared to those of the control
   suggesting the response of haemocyte to the IVP administration
indicating the down regulation of certain proteins under situations of IVP administration and challenge. This situation requires more investigations.

♦ In gill tissue, the 2D protein profile was following a pattern of disappearance of protein spots after challenge on the first day and fifth day of challenge in IVP administered animals compared to that of the control. Meanwhile on 10th day, the spots were comparatively higher than those of the control group. It was apparent that by 10th day of administration of IVP, its efficacy had been getting reduced reaching to a situation equivalent to that of the control group.

♦ The partial protein profile using 2DE has revealed that *P. monodon* haemocytes and gill tissue, exposed to a particular dose of IVP through oral route, respond to a WSSV challenge through a protein related mechanism.

♦ The reactive oxygen intermediates (ROI) and lipid peroxides were significantly higher in the haemolymph of the experimental animals (*P. monodon*) after the administration of IVP (before challenge).

♦ The total haemocyte count (THC) and phenol oxidase activity were significantly higher in the group which was challenged on 5th day post administration of IVP. Significant difference was not observed in animals which were challenged on 10th day post administration.

♦ Among the non-specific immune indices analysed, only THC was found to show significant difference between the unchallenged and challenged animals with higher value for unchallenged group and the lower value for the group challenged on 5th and 10th day post administration. Possibly, a reduction in THC subsequent to challenge both in normal and
IVP administered group suggests that WSSV may be acting on the haemocytes inspite of IVP administration reducing their count.

♦ ROI and transglutaminase activity were found to be significantly higher in IVP administered group. In earlier studies wherever feed incorporated with β 1,3 glucan, certain Indian herbs, Vitamin C with its derivatives, copper and Vitamin E were applied, ROI was found to be higher. This has not been reported along with the application of WSSV vaccine in any form. Meanwhile, the higher transglutaminase activity in IVP administered group suggests that it has positively influenced the coagulation mechanism.

♦ All above observations indicate that the non-specific immune response of *P. monodon* on administering IVP and after the challenge differs from previous such studies where a high level of proPO, SOD, lysozyme and alkaline phosphatase activity in the haemolymph of vaccinated group (without WSSV challenge) was observed.

♦ Analysis of the bio-defence genes, related to the different immune related mechanisms such as proPO, peroxinectin, transglutaminase, haemocyanin, alpha 2-macroglobulin, caspase, PAP, C type lectin, astakine, crustin, SWD, penaeidin-3, lysozyme, superoxide dismutase, catalase, glutathione peroxidase, glutathione-s-transferase, Pm-argonaute, c-SPH, tropomyosin II, syntenin, eIF5A, cathepsin C, *PmA V*, PmRACK-1, Rab7, cyclophilin A, Hsc70 and chitinase were done.

♦ The higher number of amplified/ up-regulated genes was seen in gill tissue of IVP administered animals on 1DPA (before challenge) which indicated that the bio-defence response was elicited soon after the administration of IVP.
The amplification/ up-regulation of more number of genes observed in gill tissue of IVP administered animals on 5DPA (after challenge) and lesser number on 10DPA indicated that the bio-defence mechanisms were more active against WSSV on 5DPA compared to 10DPA.

The comparative analysis of the up-regulated genes in the gill tissue between the unchallenged and challenged group of animals on 1DPA, 5DPA and 10DPA showed that after the challenge, the genes which were up-regulated were different from those which were expressed in the unchallenged condition, except for astakine which was upregulated both in the unchallenged and challenged condition in the 1DPA.

The higher number of amplified/ up-regulated genes in the haemolymph of IVP administered animals when compared to control group on 1DPA, 5DPA and 10DPA (before challenge) showed that the bio-defence response was elicited primarily in the haemolymph than that was observed in the gill tissue.

The upregulation of different bio-defence genes in haemolymph in different groups showed that the immune response elicited against WSSV in IVP administered animals (after challenge) varied with time post administration of IVP such as 1st day, 5th day and 10th day post administration respectively where the highest activity was observed on 10th day. The decrease in the up-regulated genes on 1st DPA and 5th DPA, and subsequent increase in the up regulated genes on 10th DPA pointed that in the haemolymph, the bio-defence mechanisms were more active on 10th DPA unlike the condition observed in the gill tissue.

The comparative analysis of the up-regulated genes in the haemolymph between the unchallenged and challenged group of animals on 1DPA, 5DPA and 10DPA showed that after the challenge, the genes which were
up-regulated were different from those expressed in the unchallenged condition, except for the up-regulation of single whey domain (SWD) in the 1DPA group and (clip domain sereine protease homologue (c-SPH) in the 10DPA group.

♦ The amplification/ up-regulation of bio-defence genes in the hepatopancreas (before challenge) were PmAV on 1DPA and 5DPA and MnSOD on 5DPA which indicated poor expression of the bio-defence gene in hepatopancreas.

♦ However, after the challenge, amplification/ up-regulation of higher number of bio-defence gene in the hepatopancreas could be observed, five genes on 1DPA and three genes on 5DPA, which indicated that the tissue had elicited an up-regulatory mechanism of some of the bio-defence genes to combat the WSSV infection in these periods.

♦ The comparative analysis of the up-regulated genes in the hepatopancreas between the unchallenged and challenged group of animals on 1DPA, 5DPA and 10DPA showed that after the challenge, the genes which were up-regulated were different from those which were expressed in the unchallenged condition.

♦ From the analysis of amplified genes/ up-regulated genes/ genes without variation it could be observed that various cellular and humoral immune responses elicited on 1st day post administration, 5th day post administration and 10th day post administration in gills, haemolymph and hepatopancreas of IVP administered animals were varying from each other and were not comparable indicating that different bio-defence mechanisms were simultaneously getting activated in different tissues to modulate the WSSV succession in *P. monodon.*
Semiquantitative RT-PCR of WSSV genes (ie1, pk1, tk-tmk, rr1, dnapol, endonuclease, vp28, latency1 and icp11) in IVP administered P. monodon, before and after WSSV challenge were analyzed.

The animals which were stocked in the RAS and reared under biosecured condition were PCR negative for WSSV. However, prior to the experiment 76% of them were found to be nested PCR positive for WSSV. Nevertheless, histopathological examinations and immunohistochemistry did not show the presence of virions on the tissues which prompted us to go ahead with the study.

Among the tissues analyzed by RT-PCR, gills of the experimental animals (both challenged and unchallenged group) showed cumulative expression of 8 selected genes (ie1, tk-tmk, rr1, dnapol, endonuclease, vp28, icp11, latency1) followed by haemolymph (6 genes – ie1, pk1, tk-tmk, dnapol, vp28, icp11) and hepatopancreas (5 genes – ie1, rr1, dnapol, latency1, icp11).

The unchallenged group of (both IVP administered and normal feed administered) animals showed amplification of 3 genes (icp11, tk-tmk, vp28) in gills. Meanwhile, the haemolymph of unchallenged normal feed administered animals showed amplification of 3 genes (ie1, tk-tmk, vp28), and IVP administered animals 4 genes (icp11, ie1, tk-tmk, vp28). In the hepatopancreas of normal feed administered animals, viral genes were not amplified while in the IVP administered animals, 2 genes such as dnapol and latency1 were found to be expressed in the unchallenged animals.

This suggest viral gene integration with the host genome and their expression. However by histopathology and immunohistochemistry,
absence of virions was confirmed in experimental animals before challenge.

♦ In the WSSV challenged animals, 8 genes (\textit{ie1, tk-tmk, rr1, dnapol, endonuclease, vp28, icp11, latency1}) were found amplified in gill tissue and 6 (\textit{ie1, tk-tmk, dnapol, vp28, icp11, pk1}) in haemolymph, in both normal feed and IVP administered group. In the hepatopancreas of normal feed administered animals viral genes were not found to be expressed. However, in the IVP administered animals, 4 genes such as \textit{rr1, ie1, icp11} and \textit{latency1} were found to be expressed after the challenge.

♦ After the WSSV challenge, hypertrophied nuclei and VP28 were detected in gill tissues of the experimental animals by histopathology and immunohistochemistry respectively (both in control and IVP administered).

♦ The variations observed in different tissues, in different treatments and in different animal samples with respect to a WSSV challenge could not be directed to a temporal pattern of WSSV transcription as some of the late genes (eg: \textit{vp28} and \textit{icp11}) were already present without leading the animals to mortality during the time of experimentation.

♦ It was observed that \textit{P. monodon} when administered with IVP, adopted a unique immune mechanism with complexity in interplay of various pathways which warrants further studies.

♦ This complexity is seen in the protein profiling by 2DE, expression of non-specific immune parameters, immune gene expression, existence of viral gene integration without virion, and presence of hypertrophied nuclei and VP28 protein in IVP administered and challenged group with
out mortality during the experimental period both in the control and experimental group.

♦ Precisely this work opens up new avenues of research to explore the status of WSSV gene integration into the host genome without having the virion per se and the immune status of such animals in protecting themselves from a horizontal active virus invasion. In this context the ‘Vaccination’ has different dimensions.