Discussion
V. DISCUSSION

Though the mechanism of specific immune response in crustaceans is not understood, induction of protection by vaccination in shrimp has been reported for bacteria such as Vibrio spp (Itami et al., 1989; Teunissen et al., 1998; Alabi et al., 1999). Observation that shrimp surviving WSSV infection showed an increased survival compared to native shrimp after a re-challenge (Venegas et al., 2000; Wu et al., 2002) provided evidence for the presence of a quasi-immune response. Witteveldt et al., (2004a) demonstrated that *P. monodon* fed with pellets coated with bacteria expressing WSSV envelop protein VP28 were protected following challenge.

During the vaccination against WSSV the envelope proteins are targeted because these proteins are the first to interact with the host defense system and stimulate protective immune response. Therefore, researches have been focusing on using these envelope proteins with host defense components and resultant protective effect. However, the efficiency of different envelop protein vary. Studies by Witteveldt et al., (2004b) indicated that VP19 was effective in *P. monodon*. This has also been confirmed in crayfish, *Procambarus clarkia* by Jha et al., (2006). Yu-Mi et al., (2008) used VP19 and VP466 as vaccines and observed protection to the extent of about 50% when given by injection, but by oral route, only VP19 gave significant protection in *P. chinensis*. Satoh et al., (2008) noted that kuruma shrimp, *Marsupenaeus japonicus* orally vaccinated with VP26 and VP28 was protected when challenged by feeding but not when challenged by injection. Vaseeharan et al., (2006) noted protection in *P. monodon* following vaccination with VP292. In the present study four envelope proteins VP28, VP281, VP39 and VP466 of WSSV were used to vaccinate *P. monodon* and their
ability to protect shrimp against WSSV infection was evaluated. These four structural proteins might play a major role in the pathogenesis of WSSV infection (Nadala et al., 1997; Zhang et al., 2002a,b; van Hulten et al., 2000a, Wu et al., 2005). In this study the vaccination trials were divided into 2 types. The vaccine was delivered by injection or through oral route.

Vaccination by injection with four different envelope proteins yielded varying results. Vaccination with recombinant VP28 resulted in improved survival following challenge with WSSV with a relative percentage survival (RPS) of 59%. Another envelope protein VP39 showed a RPS of 48% the RPS in groups vaccinated with VP466 and VP281 were 35% and 25% respectively. Though protection by vaccination with VP28 has been reported by few investigators earlier, no reports are available for vaccination with VP39 and VP281. There is a single report (Yu-Mi et al., 2008) which showed 50% survival following vaccination with VP466. This study shows that VP 39 offers better protection compared to VP466. VP 281 was the least effective in this study.

Vaccination by injection is a straightforward method to establish the effectiveness of recombinant proteins in conferring protection against infection. But the oral vaccination is the only practical way to deliver potential vaccines to shrimp. In a natural situation, shrimp become infected through both oral and water-borne routes and the gills are thought to be a major point of viral entry. So in the study the effectivity of viral proteins in eliciting an immune response in shrimp, when fed orally with pelleted feed coated with bacterial cells expressing the proteins was evaluated. The shrimps were fed for two weeks with the vaccine feed and on the 13th day, shrimps were challenged by feeding with WSSV infected shrimp tissues. The results obtained for the oral vaccination with VP28, VP39, VP466 and VP281 resulted in RPS of 47%, 37%, 30% and 17% respectively. 100% mortality was observed in the
group fed with bacteria without vector construct. This indicated that protection obtained could be due to these proteins. It was observed that the recombinant protein vaccine with VP28 revealed highest protection of 47% when compared with other proteins. As in the case of vaccine injection, VP 39 and VP 466 were more effective than VP28. In the last group, high mortalities of 83% was observed suggesting that this protein provided low protection. The protection observed due to vaccination with recombinant envelope proteins can be considered solely due to recombinant proteins and does not involve synergistic effect of immunostimulation by bacterial cells expressing them. This is because the control groups vaccinated with non-recombinant bacteria showed high cumulative mortalities. Further, the difference in protection provided by different envelope proteins signifies the antigen specific immune response.

Though recombinant protein vaccines have been experimentally tried in shrimps, the mechanism of immunity has not been understood. Most studies are limited to use of one or two proteins, but this study compares the effectivity of four envelop proteins and the relative efficiency of these membrane proteins may help us to understand the mechanism of antiviral immunity of shrimp. Most studies have found VP28 to be the most effective (Witteveldt et al., 2004a; Jha et al., 2006; Satoh et al., 2008). This study also confirms this finding. Yi et al., (2004) suggested that VP28 is involved in attachment and penetration into shrimp cells. In this study, VP39 was next most effective protein. This protein was identified as an envelop protein by Zhu et al., (2006) using immunoelectron microscopy, but their western blot analysis indicated the presence of VP39 in both virions and viral envelop. Tsai et al., (2006) identified VP39 as an integument (layer between the envelope and nucleocapsid) protein. Thus the location of VP39 in the viral envelop may be different compared to VP28. The least effective
protein in this study, VP281 is also an envelope protein and Wu et al., (2005) showed that antisera against envelope proteins VP68, VP281 and VP466 could delay or neutralise WSSV infections. Further studies are required to understand the reasons for relative effectiveness of different envelope proteins in protecting *P. monodon* against WSSV infections.

RNAi technology is being increasingly used to silence genes and to investigate gene functions (Friedman and Perrimon, 2004; Vanhecke and Janitz, 2005). Although initial studies of RNAi focused on cellular mRNA targets, recent evidence suggests that it can also target sequence-specific viral RNAs, which have been well documented in many organisms (Randall et al., 2003). In invertebrates, RNAi studies are commonly performed with large dsRNA molecules, which are cleaved into smaller 21–25 bp siRNAs by the host enzyme Dicer (Hammond et al., 2000). In vertebrates, large dsRNA molecules were shown to induce apoptosis by the induction of the interferon (IFN)-inducible, double-stranded RNA-activated protein kinase PKR pathway (Manche et al., 1992) and therefore, most studies involved synthetic siRNAs. Genes homologous to those involved in this pathway are not found in the genomes of the invertebrate species *Caenorhabditis elegans, Drosophila melanogaster, Anopheles gambiae* and *Ciona intestinalis* (TCSC, 1998; Adams et al., 2000; Dehal et al., 2002; Holt et al., 2002). Sequence-independent side effects of large dsRNA molecules in these organisms have not been reported. However, in the shrimp *L. vannamei* large dsRNA molecules induced sequence-independent protection against viral infection (Robalino et al., 2004).

*In vivo* injection of dsRNA corresponding to the gonad inhibiting hormone (GIH) in the sand shrimp *Metapenaeus ensis* has been shown to lower the expression of GIH (Guan et al., 2004), indicating that dsRNA can be taken up in shrimps cells after injection. Also direct injection of dsRNA in other invertebrates such as *D. melanogaster* (Goto et al., 2003), *A.*
gambiae (Keene et al., 2004) and Tenebrio molitor (Valdes et al., 2003) led to successful and efficient gene silencing of either host genes or viral genes. Thus, dsRNA can act in invertebrate systems.

In present work it has been noted that RNAi directed against WSSV genome could effectively block expression of target genes and viral replication. Thus, it is possible to use RNAi to silence viral gene expression and to inhibit viral replication in shrimp. RNAi also provides a useful tool for further study of gene functions in shrimp and for prevention and treatment of viral diseases in aquatic animals. The delivery of dsRNA into shrimp was performed by intramuscular injection. This treatment enhanced the survival of the shrimp without any clinical signs of white spot syndrome.

VP28 is a major envelope protein of WSSV, which is also, required for the entry of this virus into the cell, also cell to cell infection and virus propagation (Van Hulten et al., 2001b; Zhang et al., 2002b). The intramuscular injection of dsRNA that are complimentary to the vp28 mRNA specifically degrades the VP28 mRNA, thereby preventing or reducing the expression of VP28 gene in WSSV injected shrimp.

The results obtained in this study, indicate that the prevention of the expression of the vp28 gene may hault the multiplication of WSSV in the cell. The 100% survival of the shrimps in the 1st group injected with vp28 dsRNA (Table 11) indicates the silencing of vp28 gene of WSSV is effective in protecting shrimp against WSSV infection. It has also been observed that there was a complete mortality in the shrimps of the group, which were injected with the non-specific dsRNA indicating that the gene silencing was specific. It was also noted that 100% mortality was observed in shrimps that were in the positive group which injected with only WSSV and 100% survival was noted in shrimps that were in negative control group.
where no WSSV was injected. The result obtained is almost similar to the work done by injection of *in vitro* synthesized dsRNA encoding VP28 and protein *kinase* in *P. monodon* (Kim *et al.*, 2007). They reported that almost complete protection was achieved by VP28 and protein *kinase* gene-specific long dsRNAs. Surprisingly even though the gene VP281 is an envelope protein, their results showed that the protection efficiency of VP281 dsRNA was slightly lower than that of control GFP dsRNA, suggesting that VP281 is not critical for WSSV infection.

The result of RT-PCR (Fig-29) suggests that the gene silencing was specific because the positive band can be seen in the shrimps that were injected with nonspecific dsRNA and also the shrimps in the group which were injected with only WSSV. No bands were detected in shrimp injected with VP28 dsRNA indicating that the action was specific and also in the shrimps in negative control group (buffer).

The results of western blot revealed that the VP28 protein specific for WSSV was expressed in the shrimps that were injected with nonspecific dsRNA and also in group of shrimps, which were injected with only WSSV (Fig-31). No protein was expressed in the shrimps that were in negative control group and also in the group of shrimp that were injected with VP28 dsRNA indicating that the expression of VP28 gene was suppressed which in turn halted the replication of WSSV in shrimps.

RNAi has been linked to viral resistance in eukaryotes raising the possibility that this phenomenon represents a form of sequence-directed immunity, which holds considerable promise as a therapeutic approach to silence genes of disease-causing viruses (Soutschek *et al.*, 2004; Robalino *et al.*, 2005). As indicated in the present study, the mortality of WSSV-
infected shrimp treated by VP28 dsRNA was nil when compared to positive control (WSSV only), showing that dsRNAs targeting genes involved in virus infection might be an efficient strategy for shrimp virus control.

Sarathi et al., (2008a) noted that oral administration of VP28 dsRNA resulted in 68% and 37% survival in shrimp fed with inactivated bacteria and chitosan dsRNA complex-coated feed, respectively. Xu et al., (2007) documented that suppression of transcription and expression of vp28 gene by vp28-siRNA resulted in the inhibition viral DNA replication, and he also suggested that the VP28 protein was involved in WSSV infection of shrimp. The results obtained in our study is similar to that of Sarathi et al., (2008b) who noted that P. monodon intramuscularly administrated with VP28 dsRNA showed 100% survival after WSSV challenge. Future work needs the study on the application of these results in the field.