3.1 Introduction

In recent years, with accelerated shrimp cultivation, microbial diseases have become increasingly prominent, and are a major setback restricting the development of aquaculture (Lightner, 2005; Akira et al., 2006). Commercially, shrimp farming has been growing dramatically on the world market over the last few decades. However, shrimp farmers have suffered significant economic losses due to viral diseases, of which the white spot syndrome virus (WSSV) is the major viral pathogen of penaeid shrimps (Escobedo-Bonilla et al., 2008; Sanchez-Paz, 2010; Lin et al., 2011). WSSV is a large, non-occluded, enveloped, rod or elliptical-shaped dsDNA virus, which cause massive death in cultured shrimp (Leu et al., 2009). Preventing and controlling the spread of disease has become a tough task for the shrimp industries. The main reason for the mammoth loss and the least recovery of cultivated animals is their immune system. The invertebrates lack an adaptive immune system and rely only on innate immunity to combat invading pathogens. Lacking an adaptive immunity compels shrimps to defend the invasion of pathogens solely depending on the innate immunity, which functions through a series of cellular and humoral responses (Lee and Soderhall, 2002; Li and Xiang, 2013; Tassanakajon et al., 2013). The major immune responses in invertebrates include (1) the generation of antimicrobial peptides mediated by toll-like receptors; (2) coagulation of hemolymph; (3) formation of melanin and (4) activation of a complementation-like system mediated by lectins. The toll-like receptor (TLR)/NF-kB signaling pathway plays a critical role in the innate immune system against microbial infection across a wide range of vertebrates and invertebrates (Akira et al., 2001). In mammals, many pathogen-associated molecular patterns (PAMPs), derived from various microbial community like the viruses, bacteria, fungi, and protozoa, can be detected by distinct pattern recognition receptors (PRRs) such as the Toll-like receptors (TLRs). These TLRs are the triggering molecule that lead to the subsequent activation of the nuclear factor NF-kB pathway, which regulates numerous genes that contribute to immune defense (Akira et al., 2006). Like the crustaceans, the much homologous insect Drosophila, that shares a
common ancestral root, also rely on the innate immune system. In Drosophila, Gram-positive bacteria, fungi, and certain viruses can activate the Toll pathway via the Toll/MyD88/Pelle/Tube/TRAF6 cascade, leading to the activation of Dorsal, an NF-κB family protein (Lemaitre and Hoffmann, 2007; Valanne et al., 2011). Activated Dorsal translocates to the nucleus to induce the expression of antimicrobial peptide genes (AMP) (Lemaitre and Hoffmann, 2007). Ten TLRs have been identified in humans and nine in Drosophila melanogaster, each representing a transmembrane protein with a cytoplasmic Toll/interleukin (IL)-1 receptor (TIR) domain and an ectodomain comprising leucine-rich repeats (Akira et al., 2001; Takeda and Akira, 2004; Roach et al., 2005; Blasius and Beutler, 2010; O’Neill et al., 2013). Though emerging research strategies including immunization with inactivated pathogens, immunoglobulins and DNA vaccines have been used to protect shrimps against WSSV (Witteveldt et al., 2004; 2006; Jha et al., 2006, Lu et al., 2009, Tharntada et al., 2009, Zhao et al., 2009), there is no effective antiviral treatment to control the outbreaks and prevalence of WSSV.

The ideology of TLRs have now changed the focus of research from 'cure' to 'curb'. Hence, to trigger the TLRs, specific ligands are being used. Immunomodulators have now become the 'tool' to handle the state of pathogenic infections. The immunomodulators can be natural or synthetic. In this study, one natural compound, Curcuma longa extract (CLE) and one synthetic lead, CpG oligonucleotide was used. Also, the mechanism of innate action by these deliverables in the shrimp was analyzed. As cited in the previous chapter, traditional therapy in India with medicinal plant extracts has been in use from ancient times. Turmeric (Curcuma longa) is extensively used as a spice, food preservative and colouring material in India, China and South East Asia. It has been used in traditional medicine as a household remedy for various diseases, including biliary disorders, anorexia, cough, diabetic wounds, hepatic disorders, rheumatism and sinusitis (Ammon et al., 1992). Other than boosting the adaptive immunity of an organism, it is better to target and trigger the mechanism of innate response. Reports on the use of C. longa in shrimps are available, but the mechanism of the response upon administration of the lead remains unclear. Hence in this study,
the CLE and its nanoemulsion (CNE) were taken and their immunomodulatory potential in WSSV challenged farm reared *L. vannamei* and *P. monodon*. The synthetic immunomodulator is the CpG oligodeoxynucleotides (ODNs). CpG oligodeoxynucleotides (ODNs), containing the unmethylated CG dinucleotides, represent a classical type of conserved pathogen-associated molecular patterns (PAMPs) which can activate the immune system in many animal species (Krieg, 2002). Since CpG ODNs can induce apparent immune protection against various bacteria and virus, they are widely used as the adjuvant or immunostimulants in diseases control (Vollmer and Krieg, 2009; Bode *et al.*, 2011). In invertebrates, especially in the crustacean animals, the immune responses triggered by CpG ODNs are previously reported. The studies demonstrated that CpG ODNs can activate the innate immune system of shrimp and crab, and enhance the cellular and humoral immune responses. After the injection of multi-copy CpG motifs, the total hemocyte count of shrimps fluctuated, while the phagocytic activities and the levels of reactive oxygen species (ROS) inside of the haemocytes were increased (Sun *et al.*, 2013). The other non-specific parameters like the lysozyme and phenoloxidase activities were also enhanced in the hemocytes of shrimps after they were stimulated by CpG ODNs (Chuo *et al.*, 2005; Chen *et al.*, 2007). These studies that show powerful immune responses activated by CpG ODNs, strongly recommend CpG as a novel immunostimulant in aquaculture industry (Liu *et al.*, 2009; Zhang *et al.*, 2010; Lightner, 2011). Among crustaceans, the Toll pathway in the pacific white shrimp *Litopenaeus vannamei* has been well studied. Since 2007, three *L. vannamei* Toll receptors have been identified, and recently the homologues of MyD88, Cactus and Dorsal of *L. vannamei* (LvMyD88, LvCactus and LvDorsal) have also been reported, and they play functional roles during immune responses against bacterial and viral infections (Yang *et al.*, 2007; Huang *et al.*, 2010; Zhang *et al.*, 2012; Li *et al.*, 2012; Wang *et al.*, 2012). Dicer, an antiviral associated factor plays the role of dicing the viral RNA and regulates the viral replication directly (Alyari *et al.*, 2009). The anti-viral associated factors of the RNAi family like Dicer and Argonaute were elevated in *L. vannamei* when immunized with pUC57-CpG containing multi-copy CpG motifs (Zhang *et al.*, 2010). Elucidation of the regulatory mechanisms in the *L. vannamei*
Toll/NF-KB pathway can facilitate researches on the immune system of crustaceans and invertebrates and can help us fight against pathogens that threaten shrimp farming industry.

After the TLRs, the next molecule that gets a prime attraction downstream is the NF-κB. The nuclear factor-kappa B (NF-κB) emerged not only as a major regulator of inflammatory responses but also in cellular activities, including cell proliferation, cell death, etc. (Vallabhapurapu and Karin, 2009; Wan and Lenardo, 2010). Although in many cases, the infected host utilizes NF-κB against the pathogen, in some cases, it is clear that the pathogen benefits from hijacking NF-κB (Santoro et al., 2003). There are several reports on many viruses which have evolved to adopt different strategies by stimulating and utilizing NF-κB activation to favor their replication. Eg. Influenza virus, adenovirus, human immunodeficiency virus type 1 (HIV-1), hepatitis B virus as well as Epstein–Barr virus (Pahl and Baueerle, 1997; Sugano et al., 1997; Diao et al., 2001; Mogensen and Paludan, 2001; Surabhi and Gaynor, 2002). The innate immune system is a crucial first line of defense against pathogens in shrimps. Many signaling pathways are implicated in the cellular innate immune responses, such as Toll, IMD and JAK/STAT pathways (Li and Xiang, 2013). Previous studies have reported that in Litopenaeus vannamei JNK and ERK were involved in WSSV infection and WSSV could benefit from their activation (Shi et al., 2012 a,b). Also other studies have shown that L. vannamei NF-κB (LvNF-κB) might play important roles in WSSV replication (Huang et al., 2010; Wang et al., 2011). Labreuche et al., (2009) reported the lack of toll receptor in activation of antiviral response in L. vannamei.

Since the innate receptor for CpG ODNs in crustaceans is still unclear, the investigation of the interaction between these TLRs and CpG ODNs may shed new light to understand the mechanism of CpG ODNs triggering the immune responses in shrimps. The CpG interaction with the TLR trigger the innate immune response in the animals and helps to avert pathogenesis. CpG oligonucleotides (CpG ODNs) are effective immunostimulants that interact very specifically with the toll like receptor. Several researchers have studied the immunomodulating potential of CpG ODNs in aquatic animals. So far, the mechanism of CpG ODNs to trigger the immune responses
has been well studied in vertebrates, especially in mammals. However, the effect of CpG ODN and its mechanism of triggering the TLR mediated NF-κB activation in crustaceans is still unclear. Though there are many denied debates on the presence and absence of TLRs in *penaeids*, studies on the use of immunomodulators that stimulate the innate response are less. In this study, three different immunomodulators that regulate the immune response was used. The CLE, CNE and the CpG were administered in *L. vannamei* and the expression of the Toll/ MyD88/ Dorsal and the antiviral factor Dicer were analysed.

3.2 Materials and methods

3.2.1 Shrimps and Experimental setup

Healthy farm reared white leg shrimp, *Litopenaeus vannamei* (both 20 to 25 g of weight) were obtained from culture ponds of Agaram, Parangipettai and transported safely with the prerequisite aeration facility to the hatchery facility at CAS in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai, Tamil Nadu. The shrimps were acclimatized in the hatchery for 7 days before the start of study. During the acclimatization period, shrimps were fed with commercial pellet feed thrice a day. The feeding ratio was 3.0 % of the mean body weight. The intermoult stage of shrimp was used for the study. The molt stage was identified by examination of the uropoda in which partial retraction of the epidermis could be distinguished (Chan *et al.*, 1988). Before infection studies all the experimental animals were morphologically checked for any symptoms (Shrimp reddishness, antenna rot, spot in carapace, gill coloration, any wound and loose shells). Healthy (or) WSSV-free shrimps were used for experiments by randomly diagnosing them for WSSV infection by nested PCR analysis. Water quality parameters like temperature, pH and salinity conditions were recorded and it ranged from 25°C – 28°C, 7.4 - 8.0 and 27 – 30 ppt, respectively. Each experimental tank was accommodated with 20 animals in triplicate. The animals were starved for 24 h prior to the injection, and resumed 6 hours post injection. During the experiments, shrimp were fed three times a day with pellet feed.

3.2.2 WSSV inoculum
Live WSSV infected shrimps with prominent white spots on the carapace were collected from a local shrimp farm near Parangipetaltai. The hemolymph samples were drawn from the infected shrimps using sterile syringes (28 gauge) followed by centrifugation (3000 x g for 20 min at 4°C). The supernatant fluid was then re-centrifuged (8000 x g for 30 min at 4°C) and the final supernatant was filtered through a 0.45 μm filter (Citarasu et al., 2006). The filtrate was then stored at -20°C for infectivity studies.

3.2.3 Immunization of L. vannamei

The experimental animals were immunized with CLE, CNE and CpG as previously described in Chapter II (Section 2.2.3). Five separate experiment groups were set up for this study which are as follows:

1. Control – uninfected
2. Curcuma longa extract (CLE) (50μg/100 μl)
3. Curcumin nano-emulsion (CNE) (100μl)
4. CpG (20μg/100 μl)
5. WSSV

The shrimps were administered with the respective immunomodulators via intramuscular injection into the 3rd abdominal segment of the ventral side of the shrimps.

3.2.4 WSSV Challenging

All the experimental groups except the control group were challenged with WSSV on the 8th day of immunization. An aliquot of 100μl of the WSSV inoculum was intramuscularly injected into the 3rd abdominal segment of the ventral side of the shrimps. The gills, gut, hepatopancreas and ganglion were excised at 24h post challenging.

3.2.5 Quantitative PCR

Quantitative PCR (qPCR) was performed to investigate the gene expression of Toll, MyD88,
Dorsal and Dicer from the dissected tissue samples of the shrimps. For the qPCR analysis the tissues were collected on the 9th day. Total RNA was extracted from infected shrimp tissues by using TRIzol reagent with the total RNA isolation kit (Invitrogen, USA) according to the manufacturer's manual and stored at -80°C. The concentrations of total RNAs were determined by spectrophotometry at 260nm. To remove the genomic DNA, the RNAs were treated with DNase I (Sigma, USA) according to the manufacturer’s instructions.

One microgram of total RNA was reverse transcribed to cDNA using moloney murine leukemia virus (MMLV) reverse transcriptase (Sigma, USA) following the manufacturer’s instructions. The cDNA fragments obtained were subjected to qPCR with SYBR Green PCR mix (Applied Biosystems, USA). The primers used for PCR are given in Table 3.1. Amplification was carried out under the following conditions: pre-denaturation at 94°C for 2 min, 30 cycles of 94°C for 30s, 55°C for 30s, 72°C for 60s, followed by final extension at 72°C for 8 min. As an internal loading control, the shrimp β - actin cDNA fragment was amplified with primers using the same PCR conditions (Li-Shi Yang et al., 2007). Gene expression levels were normalised to the β – actin gene expression. The relative expression level of each gene was expressed as fold change and compared between control and treated groups.

3.3 Results

In this study quantitative PCR (qPCR) was performed to assess the effect of immunomodulators on the regulation of Toll/MyD88/Dorsal pathway and the expression of the antiviral factor, Dicer in different tissues.

3.3.1 Gills

Figure 3.1 illustrates the relative gene expression of the Toll/ MyD88/ Dorsal in the gills of the experimental animals. The control sample had no change in the expression level of all the genes. However, the expression of Toll gene was upregulated in WSSV group, while all the other genes were downregulated. There was no change in the level of expression all the three genes in the gills.
of CLE administered animals. In the experimental group treated with CNE, all the genes were upregulated relative to the expression of β – actin, of which maximum expression was observed for MyD88. However, the animals treated with CpG showed highest level of expression for all the genes as compared to other groups in the gill tissues.

3.3.2 Gut

The expression of genes associated with Toll/ MyD88/ Dorsal was investigated in the gut tissues of the experimental animals. Their relative expression levels normalized with β - actin is given in figure 3.2. The control gut showed upregulation in all the genes. In contrast, the wssv group showed downregulation in all genes. However, there was no relative change in the expression levels of all the genes in the gut of CLE and CpG treated animals. However, the gut of CNE treated animals exhibited increased expression of all the genes.

3.3.3 Hepatopancreas

Investigation of the relative expression levels of genes associated with the Toll/ MyD88/ Dorsal (Figure 3.3) revealed that the immunomodulators used in this study activated the innate immune response of *L. vannamei*. The control and WSSV showed downregulation in all the three genes. The CLE showed increased expression in Dorsal while the other two was downregulated. The CNE and CpG expression was positively regulated in all the three genes. Furthermore, all the genes were negatively regulated in the WSSV group as compared to control and immunized groups.

3.3.4 Ganglion

Figure 3.4 illustrates the relative expression of Toll/ MyD88/ Dorsal genes in the ganglion of experimental shrimps. All the three genes were upregulated in the control group and experimental groups treated with CLE and CNE. On the other hand, CpG administration decreased the expression levels. However, there is no relative change in the expression of these three genes in the WSSV group.

3.3.5 Expression of Dicer
The expression of the antiviral factor, Dicer in response to WSSV challenge in *L. vannamei* immunized with the immunomodulators was investigated. Figure 3.5 illustrates the relative expression levels of Dicer normalized to the expression of the reference gene, β – actin. The expression of Dicer was elevated in in gills of the shrimps immunized with CNE and CpG as compared to other groups. Maximum expression levels were observed in the hepatopancreas of CNE treated shrimps than that of other groups. In general, the expression of Dicer is upregulated in all the tissues of CNE treated shrimps indicating that CNE is a potential deliverable in positively regulating the expression of the antiviral factor, Dicer and enhancing the innate immune responses in penaeid shrimps against WSSV infection.

3.4 Discussion:

In this study, we attempted to tap the mechanism underlying the activation of innate immunity in the penaeid shrimps in response to natural and synthetic immunomodulators. Different tissues were taken to check the level of infection at various sites. The gills are the respiratory organ of the shrimp. The regulation of the transcription factors in the gills of control and the CLE treated animals showed no change in the level of expression. The values could be similar to the normalised values of the reference gene - β – actin. The upregulation of toll in the WSSV shows the response of the innate system to the invading host (Yang *et al.*, 2007). The CNE had an increased level of expression in all the genes. The upregulation of MyD88 clearly depicts the primary line of defence being triggered in the gills followed by the upregulation of the antiviral associated molecule Dicer indicating that an immune response is evident in the CNE treated animals. The expression level of all the genes in CpG immunized animals were increased. The upregulation in Toll, Dorsal and Dicer is the result of an immune defense in the respiratory tract of the animal.

The gut and ganglion play important roles in the defense mechanism of the shrimps. The gut digests most of the foreign particle when administered orally. This is relavent with the previous study reporting the recombinant protein construct of a viral epitope being degraded in the gut system of the shrimp (Alpers, 1994). Therefore, further study should be carried out on the
gastrointestinal absorption system and delivery system of recombinant protein vaccine in the gastrointestinal tract of the shrimp (Jerry et al., 2001). Therefore, the immune factors in the epithelial cells of the digestive track should have kept the animal safe from cross contamination with WSSV.

The hepatopancreas is the main tissue and first priority of any analysis in the shrimps. They are one of the lymphoid organs, which are the first line of defense. The Dicer alone showed minimal level of expression in the control. The ganglion also a part of the lymphoid had upregulation in control, CLE and CNE. The Toll expression in L. vannamei hepatopancreas control was low, high in the ganglion and this was similar to IToll being expressed in many tissues including hemocyte, gill, heart, brain, stomach, intestine, pyloric caecum, muscle, nerve, spermary and epidermis, with a lower expression level in the eyestalk and hepatopancreas (Inamori et al., 2004). When the cells are stimulated with a TLR ligand, adaptor proteins, such as myeloid differentiation factor 88 (MyD88), are recruited to the cytoplasmic portion of the TLRs through homophilic interaction of their TIR domains. This results in triggering of the down stream signaling cascades and production of proinflammatory cytokines and chemokines (Akira et al., 2006).

There are many viruses that activate and utilize the host NF-κB to favor their replications (Santoro et al., 2003; Hiscott et al., 2006). The dorsal in our study showed differential expression in all the tissue samples. The Toll pathway controls induction of antimicrobial peptides and a substantial number of other innate immune responsive genes through an intracellular signaling cascade resulting in the nuclear translocation of two NF-κB related transcriptional regulators, Dorsal and Dif (De Gregorio et al., 2002). However, the NF-κB proteins LvDorsal and LvRelish expressions were significantly activated by WSSV, and as a result they promote WSSV replication in L. vannamei (Qiu, 2014).

The CLE and CNE administration showed protection in the animals upon WSSV challenging. The CNE was seen to upregulate the expression in all the 3 genes and the antiviral molecule Dicer. This corresponded to the survival, PO, NBT and the histopathological analyses
showing possibility of activated immune response in the experimental animals. Though *C. longa* application has been reported in shrimp aquaculture, they are mainly used to treat bacterial infections (Ong-ard Lawhavinit, 2011; Malar and Charles, 2013). In the present study, curcumin extract and its nanoemulsion have been found to activate the innate immune response to alleviate WSSV infection in *L. vannamei*. They elevated the expression levels of genes associated with Toll pathway, which is an integral part of the shrimp innate immune system, thereby combating WSSV infection. The role of curcumin in activating the shrimp Toll pathway has not been reported so far. However, in mice curcumin administration has been found to improve the acute inflammation of microglia/macrophages and neuronal apoptosis through a mechanism involving the TLR4/MyD88/NF-κB (Hai-tao Zhu *et al.*, 2014).

CpG oligodeoxynucleotides (ODNs) represent a kind of PAMPs, which can activate host’s immune system. (Rui Sun *et al.*, 2014). CpG ODNs are found to regulate TLR pathways to produce various proinflammatory cytokines and other immune factors (Bode *et al.*, 2011). MyD88 is one of the essential adaptors mediating the downstream TLR pathways after sensing the PAMPs by TLRs (Akira and Takeda, 2004). So far, almost all the genes involved in the Toll pathways have been identified and the canonical Toll pathway is predicted and considered to be conserved in shrimp *L. vannamei* (Yang *et al.*, 2007; Wang *et al.*, 2011; Zhang *et al.*, 2012; Wang *et al.*, 2011a,b; Huang *et al.*, 2010). Although, the immunomodulatory potential of CpG ODNs in invertebrates have been well understood, their downstream signal cascades are unclear. In the present study, the downstream of Toll pathways involved in immune responses triggered by CpG ODNs 1826 were investigated in *L. vannamei*. The CpG (1826) immunized animals showed increased expression of genes related to innate immunity in the gills and hepatopancreas, but downregulated in the ganglion. The survival, PO and NBT values showed that the CpG showed a positive response to the invading viral pathogens. This is in accordance with the study of Zhang *et al.*, (2012) who showed that CpG ODN 2395 could enhance the expression of LvMyD88 in shrimp *L. vannamei*, suggesting that the Toll pathways in shrimp were activated by CpG ODNs. This is the first report so far to use CpG 1826 as
an immunomodulator to track the TLR-NFkB pathway in the *L. vannamei*.

Dicer is a member of the RNAse-III family, catalyzing the cleavage of dsRNA to siRNA and miRNA (Ketting *et al.*, 2001). Recently, it has been proved that there is a functional RNAi system in *penaeid* shrimps (Labreuche and Warr, 2012). Furthermore, it has been suggested that Dicer from both *P. monodon* and *L. vannamei* are involved in antiviral defense (Yao *et al.*, 2010). The level of Dicer expression is necessary to elucidate the immune response to pathogenesis. Dicer has been first identified in tiger shrimp, *P. monodon* and it was established that the knock down of Dicer-1 resulted in mortalities and higher viral load (Su *et al.*, 2008). However, reports on the effect of curcumin and CpG on the regulation of Dicer expression in WSSV challenged penaeid shrimps have not been done. In the present study, CNE and CpG were found to elevate the expression levels of Dicer in response to WSSV infection in *L. vannamei*. Similarly, Chen *et al.* (2011) also reported that Dicer was upregulated in shrimp immune responses when challenged with WSSV.

In conclusion, the relative expression of the Toll/MyD88/Dorsal and Dicer was upregulated immunized shrimps in response to viral infection. The response to the challenging was also evident from the results of the survival and non-specific immune assays. The CNE animals showed better response to others making it a potential deliverable to treat viral infections in shrimp culture. The efficacy of the nanoemulsion in improving the innate immune response in penaeid shrimps can be attributed to the increased bioavailability facilitated by the nanoemulsion as compared to the polyphenol extract of *C. longa* (CNE). The CpG results also revealed the mechanism of ODNs activating the innate immune system of shrimp. All encompassed, these results suggest that both the deliverables, CNE and CpG can be recommended for the aquaculture disease control.