Summary and Conclusions
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This thesis covers various aspects of viral diseases affecting shrimp aquaculture. The research component of this thesis can be divided into four areas. The areas covered are: 1) A study to determine the prevalence of WSSV among the crustaceans in the Vembanad estuary, the shrimp aquaculture farms surrounding the estuary, and the sea off Cochin coast, India using two sets of nested PCR primers. 2) An investigation to compare the sequence of six major structural proteins of WSSV; vp28, vp26, vp19, vp68, vp281, vp466 from different geographical locations with that of an isolate from India. 3) Simultaneous occurrence of HPV, IHHNV, MBV and WSSV in postlarvae of *P. monodon* from hatcheries in India was monitored by Polymerase Chain Reaction. 4) A real time PCR procedure was developed for the quantitative analysis of WSSV infection. The viral load of postlarvae from hatcheries in Kerala meant for aquaculture was also determined using the quantitative PCR.

5.1 Detection of WSSV in shrimps and other decapods from farm environment

A nested PCR method as described by Umesha et al., 2006 and Kimura et al., 1996 were employed in this study for the detection of WSSV in farmed and wild crustaceans. A total of three hundred and eight samples from farmed shrimp and other decapods from farm environment were analysed for presence of WSSV. The prevalence of WSSV in farmed *P. monodon* was 48% (54/112 samples) by first step PCR while it was 79% (88/112 samples) by nested PCR. The incidence of WSSV was 35% (29/84 samples) by first step PCR and 55% (46/84 samples) by nested PCR in *F. indicus*. Brown shrimp (*M. dobsoni*) had WSSV prevalence rates of 36% (8/22 samples) by first step PCR while it was 68% (15/22 samples) by nested PCR. The prevalence of WSSV in *M. monoceros* was 42% (13/31 samples) by first step PCR while it was 71% (22/31
samples) by nested PCR. WSSV was present in 2/15 (13%) *S. serrata* samples by first step PCR while it was 5/15 (33%) by nested PCR. The incidence of WSSV was 17% (3/18 samples) and 39% (7/18 samples) in *S. tranquebarica* by first step PCR and nested PCR respectively. The freshwater prawn, *Macrobrachium rosebergii* had WSSV prevalence of 35% (9/26 samples) by first step PCR and 92% (24/26 samples) by nested PCR.

5.2 Detection of WSSV in shrimps from Vembanad estuary

A total of 258 samples from four species of shrimp; Tiger prawn (*Penaeus monodon*)- 82 Nos, White prawn (*F. indicus*)- 43 Nos, Brown shrimp (*Metapenaeus dobsoni*)- 79 Nos and Speckled shrimp (*M. monoceros*)- 54 Nos collected from the Vembanad estuary (Kerala State) were analysed for the presence of WSSV. The prevalence of WSSV in *P. monodon* samples collected from Vembanad estuary was 44% (36/82 samples) by first step PCR while it was 72% (59/82 samples) by nested PCR. The incidence levels of WSSV in white prawn, *F. indicus* was 26% (11/43 samples) and 44% (19/43 samples) by first step PCR and nested PCR respectively. WSSV was present in 32% (25/79 samples) and 58% (46/79 samples) of *M. dobsoni* samples collected. The occurrence of WSSV in *M. monoceros* were 39% (21/54 samples) and 67% (36/54 samples) from Vembanad Estuary.

5.3 Detection of WSSV in wild captured decapods from sea landings

A total of 504 samples from 15 species of decapods from wild were tested for the presence of WSSV. Of the fifteen species decapods tested, twelve species had WSSV incidence levels ranging from 6-23%. WSSV was not detected from the three species of deep sea decapods tested; *H. gibbosus, Plesionika spinipes* and *Puerulus spp*. The cultured species, *P. monodon* had the highest incidence of WSSV among the species from wild tested at 23%. The prevalence
of WSSV in *P. stylifera* collected from sea landings were 3% (2/78 samples) by first step PCR and 9% (7/78 samples) by nested PCR. The incidence of WSSV in Brown shrimp (*M. dobsoni*) from sea landings were 7% (4/54 samples) by first step PCR and 22% (12/54 samples) by nested PCR. Tiger prawn (*P. monodon*) had WSSV prevalence rates of 10% (5/52 samples) by first step PCR while it was 23% (12/52 samples) by nested PCR. The prevalence of WSSV in *F. indicus* was 7% (3/38 samples) by first step PCR while it was 18% (7/38 samples) by nested PCR. WSSV was present in 2/45 (4%) *M. affinis* samples by first step PCR while it was 5/45 (11%) by nested PCR. The incidence of WSSV was 4% (1/23 samples) and 13% (3/23 samples) in *H. woodmasoni* by first step PCR and nested PCR respectively. The prevalence of WSSV in *S. serrata* samples collected from sea landings were 7% (1/14 samples) by first step PCR while it was 21% (3/14 samples) by nested PCR. The incidence levels of WSSV in *S. tranquebarica* was nil (0/16 samples) and 13% (2/16 samples) by first step PCR and nested PCR respectively. WSSV was not detected in forty three samples of blood spotted crab; *P. sanguinolentus* by first step PCR while four samples (9%) was positive by nested PCR. The prevalence of WSSV in *P. pelagicus* was 6% (1/18 samples) by first step PCR while it was 17% (3/18 samples) by nested PCR. WSSV was present in 1/15 (6%) *P. homarus* samples by nested PCR while the virus was not detected in any of the samples by first step PCR. WSSV was present in 6% (1/18 samples) in scalloped spiny lobster, *Panulirus homarus* by nested PCR while the virus was not detected in any of the samples by first step PCR. The present study indicated the presence of WSSV in wild population of shrimp. The presence of WSSV in wild population of decapods is a matter of great concern as they may act as carriers of the infection.
5.4 *Insilico* analysis to compare variations in structural protein sequences of an Indian isolate of WSSV with sequences in databank

Six envelope protein genes of WSSV; vp28, vp26, vp19, vp68, vp281 and vp466 on PCR amplification from the viral genomic DNA gave amplified products of expected size 615bp, 615bp, 366bp, 207bp, 846bp and 1401bp respectively. These sequences were submitted to the GenBank were assigned accession numbers EF534254, EF534253, EU012447, EF534252, EF534251 and EF534255 respectively. The vp28 gene sequence of the Indian isolate of WSSV (GenBank Acc. No. EF534254) obtained in the present study was compared with sequences in GenBank database. In the present study, the sequences of six envelope proteins were compared with reported sequences of WSSV isolates. A common ancestor for the virus isolate could not be designated as there was variation in sequence with one envelope protein or other.

5.5 Comparison of VP28 gene sequence of the Indian isolate of WSSV obtained in the study with that available from the GenBank

The nucleotide sequence of the envelop protein; vp28 of the Indian isolate obtained in the study was compared with twenty-eight GenBank sequences from various geographical locations. Only one sequence reported from India (Accession No. DQ013883) had 100% homology with the isolate. There was an A→G nucleotide substitution at position 125 for all the other reported sequences, which translated into a substitution of Aspartic acid for Glycine. The nucleotide change at position 125 was present only in two WSSV isolates from India including the isolate reported in this thesis indicating that this mutation is restricted to India. Two sequences from China (AF502435 and AY249434) had an additional mutation at nucleotide position 119 from G→A which translated into an amino acid substitution from Arginine to Histidine. A Chinese isolate (Accession No. AY682926) and a Korean isolate (Accession No. AF380842) had a nucleotide substitution at position 234 and 444 respectively from T→C both of which did not translate to change of amino acid. A Thailand isolate (Accession No. EF194079), a Chinese
isolate (Accession No. DQ979320) and an Indian isolate (Accession No. AY422228) had three nucleotide substitutions including that at position 125. The Thailand isolate had nucleotide substitution at position 113 from T\(\rightarrow\)A and at 434 from T\(\rightarrow\)C which translated into change of amino acid from valine to glutamic acid and Leucine to proline respectively. The Chinese isolate had T\(\rightarrow\)C nucleotide substitutions at positions 306 and 536 with change of amino acid from valine at both positions to proline and alanine respectively. The Indian isolate had substitutions from T\(\rightarrow\)C at positions 483 and 485 with change of amino acid from phenylalanine to serine in the first position while the second position was conserved. There was nucleotide substitutions at ten different positions with the isolates studied, seven of which translated to a change of amino acid.

5.6 Comparison of VP26 gene sequence of the Indian isolate of WSSV obtained in the study with that available from the GenBank

The vp26 gene sequence of an Indian isolate of WSSV obtained in this study (GenBank Acc. No. EF534253) obtained in the present study was compared with sequences in GenBank database. The nucleotide sequence of the gene encoding for envelope protein vp26 from an Indian isolate of WSSV obtained in this study was compared with 16 reported sequences and it was revealed that there was 100% similarity with fourteen of the reported sequences. The G\(\rightarrow\)A nucleotide substitution at the nucleotide position 575 for the Chinese isolate (Accession No. AY220746) resulted in a change of amino acid from Arginine to Lysine while a nucleotide change for the Vietnamese isolate (Accession No. AJ551446) at nucleotide position 345 from T\(\rightarrow\)C was conserved and did not translate to change of amino acid. The VP 26 gene was found to be highly conserved among isolates from different geographical locations with just two nucleotide variations from the sixteen isolates compared.
5.7 Comparison of VP19 gene sequence of the Indian isolate of WSSV obtained in the study with that available from the GenBank

The vp19 gene sequence of the Indian isolate of WSSV obtained in this study (GenBank Acc. No. EU012447) was compared with sequences in GenBank database. The vp19 sequence was compared with seventeen published sequences and it was found that there was 100% sequence similarity with four WSSV isolates reported from India, Vietnam, Taiwan and Mexico. Point mutations; T→C, T→A and C→T were found at six different nucleotide positions with 13 reported sequences. To ascertain whether the variation at nucleotide level resulted in change in at amino acid level, the protein sequences of vp19 gene were also compared. The T→C point mutation at position 57 (Accession No. AY316119 and AY873786) did not result in change in amino acid while a C→T mutation at position 196 (Accession No. AF332093 and AF402997) resulted in change amino acid from Proline to Serine. A T→A mutation at position 218 (Accession No. AY249444 to AY249448 and AF369029) had a change in amino acid from Valine to Aspartic acid. Two reported sequences from India, (Accession Nos. AY422227 and DQ902655), gave a T→C mutation at nucleotide positions 25 and 44 that translated into change of amino acids Serine to Proline and Valine to Alanine respectively. The WSSV isolate with Accession No. DQ902655 had an extra substitution at position 287 other than at nucleotide positions 25 and 44 that translated into Methionine, a change in the amino acid from Threonine.

5.8 Variation in sequence of vp281 of an Indian isolate of WSSV obtained in this study with that of sequences in GenBank

The nucleotide sequence of vp281 of an Indian isolate of WSSV (GenBank Acc. No. EF534251) obtained in this study was compared with sequences in GenBank database.
Comparison of vp281 sequence with six published sequences revealed A→T, C→A and G→A mutations at three different nucleotide positions. The A→T mutation at nucleotide position 807 present in all the reported sequences and the G→A mutation at position 843 (Accession No. AY517490) did not translate into change of amino acid. While the C→T mutation at position 841 (Accession No. DQ979321) translated into change in amino acid from Proline to Threonine.

5.9 Comparison of VP466 and vp68 gene sequence of the Indian isolate of WSSV obtained in the study with that available from the GenBank

The vp466 gene sequence of the Indian isolate of WSSV (GenBank Acc. No. EF534255) obtained in the present study was compared with sequences in GenBank database. Three reported nucleotide sequence of vp466 had 100% homology with the sequence reported in the present study. There was an A→T nucleotide substitution at position 1212 for a Thailand isolate (Accession No AF369029), which translated into a substitution of lysine to arginine.

The vp68 sequence of the Indian isolate of WSSV (GenBank Acc. No. EF534252) obtained in the study was 100% similar with the four reported sequences (AF332093*, AF369029*, AF440570*, AF4114664).

5.10 PCR amplification to determine the prevalence of multiple viral infections in penaeid postlarvae

PCR examination 99 (95.2%) of the postlarval samples obtained from different hatcheries in Kerala gave positive result for at least one of the four viruses studied. Out of the 104 post larval P. monodon samples tested, 79 (76%) were positive for IHHNV, 65 (62.5%) were positive for HPV, 13 (12.5%) were positive for MBV and 13 (12.5%) were positive for WSSV. Dual to triple infection was present in 60.6% of the total postlarvae tested. Out of the 51 double positives
50 (98%) included either HPV or IHHNV infection. HPV or IHHNV was present in 11 (100%) post larval samples found positive for triple viral infection. A total of 99/104 (95.2%) post larval samples were positive for HPV and IHHNV alone or in combination with other viruses. HPV or IHHNV was present alone in 36 (34.6%) of the total post larval samples tested. Out of the 99 samples infected with virus, 79 (79.8%) of the samples had IHHNV alone or in combination with other viruses while HPV was present in 65 (65.6%) of the samples.

There is very little data on the simultaneous presence of WSSV, MBV, HPV and IHHNV in *P. monodon* postlarvae meant for stocking in aquaculture ponds. In this study, HPV and IHHNV alone or in combination was detected in 93.3% of the samples. It can be assumed that the very high rates of prevalence of HPV and IHHNV in samples are primarily due to lack of screening strategies for the presence of these viruses in India. Hence measures are yet to be initiated for control of HPV and IHHNV infection in shrimp. It is worth while to note that the percentage of hatchery reared postlarvae infected with WSSV and MBV is less. This is due to the stringent screening strategies initiated by hatcheries.

5.11 A Real-time SYBR Green PCR Assay for quantification of WSSV infection in postlarvae of *P. monodon*

A quantitative real time PCR method for detection of WSSV based on SYBR Green assay was developed. The primers were selected from ORF421 region of the WSSV genome. To determine the sensitivity of the real time PCR, a plasmid vector containing the cloned DNA fragment to be amplified was used as standard. Plasmid was serially diluted from 10^7 to 1.47 copies/ul. Strong linear correlation (r^2=0.9962 to 0.998) were obtained between the threshold
cycles (Ct) and the target plasmid standard with a slope of (-) 3.488 ranging from $2.35 \times 10^7$ to $1.47$ copies of DNA.

To determine the reproducibility of each reaction within each run (intra-assay) and between assays (inter-assay) $2.3 \times 10^7$ to $1.47$ WSSV copies were compared in independent reactions. The correlations of variability ranged between 1.71 and 0.49 indicating that there is very little variation between the runs. With an optimal PCR mixture, $10^7$ to $1.47$ copies of WSSV can be detected from a specimen.

5.12 WSSV quantification in postlarvae from four hatcheries

The postlarvae collected from 4 hatcheries had a range of 1.4 to $7.9 \times 10^5$ WSSV copies (mean=$1.2 \times 10^4$) mg$^{-1}$ tissue. Among the hatcheries screened, hatchery H1 showed the lowest degree of WSSV infection with mean copy number of $2.73 \times 10^2$ copies mg$^{-1}$ tissue followed by H2 with $3.55 \times 10^2$ copies mg$^{-1}$ tissue. The highest degree of WSSV infection was from H4 with $2.8 \times 10^3$ copies mg$^{-1}$ tissue. Out of the 119 post larval samples, 72% were infected with WSSV. Hatchery H2 has the lowest degree of WSSV infection with 53.6% (15/31) samples negative for the virus while hatchery H3 and H4 had the highest degree of WSSV infection with 83.3% (25/30) and 80.6% (25/31) infection rates respectively. The level of WSSV was less than 100 mg$^{-1}$ tissue in seventy samples (58.8%) and 12 (10.1%) samples showed higher levels of infection ranging from $8.67 \times 10^2$ to $7.9 \times 10^5$ WSSV copies mg$^{-1}$ tissue with three samples having more than $10^5$ copies, one from H4 and two from H3. The findings of this study indicates a very high level of WSSV infection (72%) among postlarvae of four hatcheries tested and hence proper screening strategies should be in place before stocking of postlarvae in rearing ponds.