CHAPTER 3

RESULTS

3.1 WSSV INFECTION IN SHRIMPS

In order to establish WSSV infection in lab conditions and test for the pathogenicity, shrimps were infected with WSSV intramuscularly. Infected shrimps did not exhibit any clinical sign till 12 hrs post infection. Visible signs of pathogenesis could be observed after 24hrs p.i., when the shrimps stopped feeding, showed disorientation in swimming and became lethargic. 36 hrs post infection, the dorsal side, appendages and telson became red due to expansion of chromatophores and the shrimps started swimming on one side. Subsequently, 48hrs post infection, the shrimps stopped moving, sunk to the bottom with dorsal side up and moved only their appendages. This condition of shrimp was called moribund. Hemolymph from moribund shrimps did not clot while in the uninfected, clotting was noticed.

3.2 SELECTION OF SHRIMP SPECIES FOR INFECTIVITY STUDIES

Once it was confirmed that the infection can be established in lab conditions, a comparative study was carried out with *P.indicus* and *P.monodon* to know which would be the ideal species of shrimp that would yield a high viral load. *P.indicus* died within 48hrs after intramuscular injection of the
virus, however it took 6 days for the virus to infect the shrimps by oral route. *P. monodon* survived only for 36hrs by intramuscular injection and 4 days by oral route. Both the shrimp species exhibited all the clinical signs of the WSSV infection. Since *P. indicus* survived for a longer time than *P. monodon*, both by the intramuscular infection and oral route of infection, *P. indicus* was used for all the studies described in this thesis (Table 3.1).

### 3.3 PURIFICATION OF VIRUS

The next step after identifying the ideal species for virus propagation was to confirm the authenticity of the virus as WSSV. The only way to authenticate is to compare it at the DNA level. For this the virus was purified from homogenized tissue sample and hemolymph of infected shrimps as a single band between 30 and 40% sucrose, by density gradient centrifugation. Though the purity and yield of the viral DNA extracted by both proteinase K and high salt method were similar, the high salt method was used for all further studies as it is non enzymatic, time saving and easy to perform (Table 3.1a). PCR analysis using primers designed by Takahashi et al., 1996 revealed that, the DNA obtained from both the methods amplified the expected 643 bp product (Fig.3.1). To further establish the authenticity of the WSSV, the 643 bp product was sequenced and upon comparison, it revealed 100% identity with the sequences previously published by other workers (Fig.3.2).

After authenticating the viral identity at the DNA level, the protein profile of the virus was studied. A comparison was made between the protein profiles of purified virus from the hemolymph and tissues of infected shrimps. Protein profiles of the virus from hemolymph of both uninfected and infected shrimps showed a very high concentration of 66 kDa protein, while this protein
Table 3.1 Comparison of WSSV infection by intramuscular and oral route with *P.indicus* and *P.monodon*

<table>
<thead>
<tr>
<th>Mortality</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra muscular injection</td>
<td></td>
</tr>
<tr>
<td><em>P.indicus</em> 100%</td>
<td>48-54 hours</td>
</tr>
<tr>
<td><em>P.monodon</em> 100%</td>
<td>38-42 hours</td>
</tr>
<tr>
<td>Oral route</td>
<td></td>
</tr>
<tr>
<td><em>P.indicus</em> 27%</td>
<td>5 days</td>
</tr>
<tr>
<td>67%</td>
<td>6 days</td>
</tr>
<tr>
<td>100%</td>
<td>7 days</td>
</tr>
<tr>
<td><em>P.monodon</em> 53%</td>
<td>3 days</td>
</tr>
<tr>
<td>73%</td>
<td>4 days</td>
</tr>
<tr>
<td>100%</td>
<td>5 days</td>
</tr>
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Table 3.1a Comparison of High Salt vs Enzymatic extraction

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<thead>
<tr>
<th></th>
<th>High Salt</th>
<th>Enzymatic</th>
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<tbody>
<tr>
<td>Yield</td>
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<td>Purity</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Cost</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Ease</td>
<td>Easy</td>
<td>Cumbersome</td>
</tr>
<tr>
<td>Time</td>
<td>Less</td>
<td>More</td>
</tr>
</tbody>
</table>
Fig. 3.1 1% Agarose gel electrophoresis showing the PCR amplification of 643bp product using the primers designed by Takahashi et al., (1996) from the WSSV DNA extracted by enzymatic and high salt method
Lane M. 100bp ladder 1. DNA extracted from uninfected tissue
2. DNA extracted by high salt method
3. DNA extracted by enzymatic method
Fig. 3.2 Clustal W alignment of the 643 bp PCR product (SatTakWSSV) amplified using primers designed by Takahashi et al., (1996) with sequences published by Tsai et al., (2002) (TsaiTakWSSV), VanHulten et al., (2001) (VanTakWSSV), Yang et al., (2001) (YangTakWSSV) and Takahashi et al., (1996) (TakWSSV) Solid boxes indicate nucleotides that are identical to those of SatTakWSSV at a given position. The numbers along the margin and at the end of the last line designate the positions of the nucleotide residues.
was seen in rather low concentrations in the tissues. Four proteins expected to be viral proteins with molecular weight 14 kDa, 18 kDa, 23 kDa and 30 kDa were more prominent in tissues than in the hemolymph (Fig.3.3A,B). Hence tissue samples from the infected shrimps were used for all the investigations in this study.

3.4 IDENTIFICATION OF VIRAL PROTEINS

Since the tissues contained high viral load the next attempt was to develop a method to obtain high yield of purified virus. For this, the protein profiles of the virus purified by the conventional density gradient ultra centrifugation was compared with the protein purified by mere freezing and thawing followed by few rounds of high speed centrifugation, without ultra centrifugation. Results indicated that the yield of the viral protein obtained after purification by ultra centrifugation was very low when compared to the yield obtained by purification without ultra centrifugation. Four viral proteins 14 kDa, 18 kDa, 23 kDa and 30 kDa were seen upon ultra centrifugation whereas the alternative method yielded 9 major viral proteins that were not observed in uninfected shrimp sample processed in the same way (Fig.3.4A,B). The results indicated that few rounds of mere freezing and thawing and centrifugation of the homogenized tissue sample was enough to release the virus into the supernatant.

3.5 ELECTRO ELUTION OF VIRAL PROTEINS FROM SDS PAGE AND PRODUCTION OF POLYCLONAL ANTISERUM

In order to develop a diagnostic test for WSSV the viral proteins were electro eluted and were used for raising antiserum. Seven of the nine viral
Fig. 3.3 12% SDS PAGE stained with coomassie brilliant blue showing the comparison of protein profile of WSSV in tissue and hemolymph of infected and uninfected shrimps

Fig. 3.3A Protein profile of infected and uninfected tissue
Lane M Marker 1 Uninfected tissue after purification
2,3,4 Infected tissue after purification
5 Infected tissue supernatant

Fig. 3.3B Protein profile of infected and uninfected hemolymph
Lane M Marker 1 Uninfected Hemolymph
2,3,4 Infected Hemolymph
Fig. 3.4 12% SDS PAGE stained with coomassie brilliant blue showing the comparative protein profile of uninfected and infected shrimp tissues; after density gradient centrifugation method and freeze thaw method

Fig 3.4 A Protein profile of the infected tissue after density gradient centrifugation. Lane M Marker 1. Virus purified by density gradient centrifugation 2. Uninfected shrimp tissue after density gradient centrifugation

Fig 3.4 B Protein profile of the infected tissue after freezing and thawing. Lane M. Marker Lane 1 uninfected shrimp tissue Lane 2 and 3 infected shrimp tissue
proteins were eluted from the gel by electro elution (Hunkapillar and Lujan 1986). The electro eluted proteins were resolved on a 12% SDS-PAGE which showed single protein bands at the respective molecular weight indicating that they did not contain any other contaminating proteins (Fig.3.5). Western blot analysis revealed that only 5 (14 kDa, 18 kDa, 23 kDa, 30 kDa and 35 kDa) out of the 7 proteins could be identified from the tissues using the antisera (Fig.3.6). Further analysis revealed the non-reactivity of 28 kDa and 46 kDa with pure proteins, hence only the remaining five antisera were analyzed.

### 3.6 IMMUNOBLOT ANALYSIS WITH ANTI 18KDA ANTISERUM

Infected tissues probed with the various antiserum showed prominently the presence of 18kDa compared to the other proteins. The uninfected shrimp tissue however did not exhibit reactivity with any of the antiserum. Therefore antiserum against 18 kDa protein was raised in rabbit for the diagnostic studies. This antiserum against 18 kDa protein used in the Western blot indicated that the reactivity with the 18 kDa protein could be detected until about 1/16000 dilution of the antiserum (Fig.3.7). Normal rabbit serum under similar conditions did not exhibit any reactivity (Fig.3.8). These results show that the antiserum specifically recognized the viral protein only. In order to identify the range of antigen concentration that the antiserum can identify, dot blot analysis was done. The results showed that the antiserum was able to detect antigen concentration as low as 10 picograms (Fig.3.9).
Fig 3.5 12% Coomassie brilliant blue stained SDS PAGE showing 7 viral proteins purified by gel elution Lane M Marker
1. 46kDa 2. 35kDa 3.30kDa 4.28kDa 5.23kDa 6.18kDa 7.14kDa

Fig 3.6 Western blot analysis of normal and infected crude lysates run on 12% SDS PAGE, transferred onto nitrocellulose membrane subsequently probed with antiserum raised against 5 major viral proteins in mice (1:1000), followed by incubation with secondary goat antimouse ALP(1:30,000), developed with BCIP and NBT; Lane M Marker 1.Normal crude lysate probed with all the five antisera 2.Infected crude lysate probed with 14kDa antiserum 3.probed with 18kDa antiserum 4.probed with 23kDa antiserum 5.probed with 30kDa antiserum 6.probed with 35kDa antiserum
Fig 3.7 Western blot analysis of infected crude lysate run on 12% SDS PAGE, transferred onto nitrocellulose membrane and subsequently probed with anti 18kDa antiserum at different dilutions, followed by incubation with goat anti rabbit ALP (1:30,000) developed with BCIP and NBT; Lane M.Marker 1:1:500 2:1:1000 3:1:2000 4:1:4000 5:1:8000 6:1:16000

Fig 3.8 Western blot analysis of infected crude lysate run on 12% SDS PAGE, transferred onto nitrocellulose membrane and subsequently probed with Normal rabbit serum (NRS) (1:4000) / rabbit anti 18kDa antiserum (1:4000) followed by incubation with secondary goat anti rabbit ALP (1:30,000), developed with BCIP and NBT; Lane M.Marker 1.Infected tail probed with NRS 2. Infected tail probed with anti 18kDa antiserum.
Fig 3.9 Dot blot analysis; Pooled suspension of tail tissue from infected shrimps at different concentrations were adsorbed onto nitrocellulose paper, followed by incubation with anti18kDa antiserum (1:4000) and goat anti rabbit ALP (1:30,000), developed with BCIP and NBT; Lane 1. PBS 2. Uninfected tail 100 μg 3. Infected tail 100 μg 4. 50 μg 5. 10 μg 6. 1 μg 7. 100 ng 8. 50 ng 9. 10 ng 10. 1 ng 11. 100 pg 12. 50 pg 13. 10 pg 14. 5 pg 15. 1 pg
3.7 DETECTION OF THE WSSV IN DIFFERENT TISSUES OF INFECTED SHRIMPS USING IMMUNOBLOT ANALYSIS WITH RABBIT ANTI 18KDA ANTISERUM

The next attempt was to identify the organs that were highly infected with WSSV for which Western blot analysis in different tissues was performed. Infected tail contained the highest viral load and hepatopancreas the least. The order of reactivity was tail > head tissue > eyestalk > gill > appendages > muscle > heart > Hemolymph > stomach > hepatopancreas (Fig.3.10A,B).

3.8 TIME COURSE STUDY OF WSSV INFECTION WITH DIFFERENT TISSUES BY ELISA WITH RABBIT ANTI18KDA ANTISERUM

A time course analysis using ELISA with tissue samples collected every 12 hrs post infection revealed that tail and head tissue were the organs that had the maximum viral load while heart and hemolymph contained moderate levels of virus. The 18 kDa antigen could be detected in head tissue, eyestalk and tail as early as 12 hrs p.i and it increased till 36 hrs. Antigen levels were maximal after 48 hrs at which time the shrimps became moribund. It was interesting to note that the antigen levels in heart and hemolymph did not increase appreciably even after 48 hrs p.i. when compared to other organs like tail, head tissue and eyestalk.(Fig. 3.11). Thus these findings support the observation of ELISA described above indicating the 18 kDa antigen was maximum in tail, head tissue and eye stalk.
Fig. 3.10 Western blot analysis of various infected organs to study the tissue distribution of the 18kDa antigen. Crude lysates of various infected organs run on 12% SDS PAGE, transferred onto nitrocellulose membrane, probed with anti 18kDa antiserum (1:4000), followed by incubation with goat anti rabbit ALP (1:30,000), developed with BCIP and NBT.

- **Fig. 3.10A**: Lane M. Marker 1. Infected tissue 2. Uninfected tissue 3. Head tissue 4. Eye stalk 5. Muscle 6. Appendages 7. Heart
- **Fig. 3.10B**: Lane M. Marker 1. Infected tissue 2. Uninfected tissue 3. Hepatopancreas 4. Tail 5. stomach 6. Hemolymph 7. Gill
Fig. 3.11 Time course study of WSSV infection in shrimps by ELISA, 200 μl of pooled tissue infected with WSSV at different time intervals containing 200 ng of protein coated onto the ELISA plate overnight, subsequently washed with PBS, blocked with 3% BSA, probed with rabbit anti 18 kDa antiserum (1:4000), followed by incubation with goat anti rabbit ALP (1:30,000) and developed with pNPP, OD measured at 405nm. Each tissue sample is a pooled suspension of tissues collected from five different shrimps.
3.9 TIME COURSE STUDY OF WSSV INFECTION WITH DIFFERENT TISSUES BY WESTERN BLOT

In order to examine the presence of 18 kDa antigen in the tissues of infected shrimps collected at various time periods of infection, Western blot analysis with rabbit anti 18 kDa antiserum was done. The results indicate 18 kDa antigen could be detected at 24 hrs post infection in infected tail, head tissue and eyestalk with tail showing a more prominent band than the other two organs. The 18kDa antigen could be detected only after 36 hrs of infection in the gill whereas heart and hemolymph showed the antigen only at 48 hrs p.i (Fig.3.12).

3.10 N-TERMINAL SEQUENCING OF THE 18 KDA PROTEIN

Since this 18 kDa (named as Ws18) protein was a potential candidate antigen for diagnosis, it was subjected to N-terminal sequencing to confirm its identity. The 18kDa protein was electro eluted and purified by RPHPLC. The purified protein was transferred onto PVDF membrane for N-terminal sequencing, which revealed a sequence LRGNEEMVGD. This sequence matched with 262 amino acid long ORF107 of the sequence published by VanHulten et al. (2001), ORF 207 of Yang et al. (2001) and ORF262 of Tsai et al. (2002). The sequence matched only from the 8th residue of the all three amino acid sequences (Fig. 3.13). The corresponding gene sequence in the complete genome was 828 bp long. This gene was PCR amplified from the genomic DNA of the WSSV and subjected to DNA sequencing for confirmation. Results indicated that there was 100% identity with the sequences that was reported earlier (Fig.3.14). Thus 18 kDa is a WSSV specific protein.
Fig. 3.12 Western blot analysis for crude lysate of different shrimp tissues infected with WSSV taken at different time intervals (every 12 hrs post infection p.i.) run on 12% SDS PAGE, transferred onto nitrocellulose membrane, subsequently probed with rabbit anti 18kDa antiserum (1:4000), followed by incubation with goat anti rabbit ALP (1:30,000), developed with NBT and BCIP; Lane M. Marker. 1. Infected shrimp tissue 2. Uninfected shrimp tissue 3. 12 hrs p.i 4. 24 hrs p.i 5. 36 hrs p.i 6. 48 hrs p.i. Each tissue sample is a pooled suspension of tissues collected from five normal and infected shrimps.
Fig. 3.13 Comparison of the N-terminal sequence data of the 18kDa protein with the consensus sequence already published

Published sequence

MSSNRFSQLRGNEEMVGDYSRWTTVKNRRNQQQYSHSFQPQQQ

N-terminal sequence

LRGNEEMVGD

QHQKRTSTNSPPAPPFIISWGALGYSMYRLDDQCRNCDETGYYNFHSYDRKREVRSLNTPSEGMRRTSRSSHPLNKKKDDEA

PPPQSNQHMYPLNYSFREYTPSSKLVNWDPYEQKQDKILQEEEA

RAPTPTPEKEFEVETKDDVIEETEPEPEPAPVDPDPDAITATT

TTTVATRHDSSSTVFLRNVISIVFVFLGVYSALFAKCIKSKKE
Clustal W alignment of the ws18 gene (SatWS18) amplified using primers designed based on the N-terminal sequence published by Tsai et al. (2005) (TsaiWS18), VanHulten et al., (2001) (VanWS18) and...
ELISA and Western blot described above can detect the viral antigen and thus the infection with WSSV. However it is no grade the severity of infection using immunological methods. Thus interesting to design a single tube semi nested PCR based on the DP of the 18 kDa protein which would facilitate the development based assay to detect the severity of the infection. In order to simulate condition, different concentrations of the viral genomic DNA template. Results indicated that WSSV DNA at a concentration of three PCR products of length 828 bp, 696 bp and 346 bp respectively, a moderate concentration of WSSV DNA, around 1 ng or 10 ng of WSSV DNA, and a low concentration of 10 pg or 1 pg or 100 fg gave only one product of 346 bp. PCR performed with concentrations less than 100 fg was negative (Fig. 3).

3.12 AGGLUTINATION TESTS

Since the 18 kDa antiserum showed specific reactivity in tissue and did not react with uninfected shrimp tissues, this rabbit antiserum was used for the development of a diagnostic test for V types of agglutination tests were developed to detect the WSSV shrimp. Human RBCs coated with rabbit anti 18 kDa anti conjugated gelatin micro spheres coated with anti 18 kDa antiserum beads coated with anti 18 kDa antiserum. In each test the same tissue evaluated
Human RBCs coated with rabbit anti 18 kDa antiserum with uninfected and infected tissue sample. Infected sample agglutinated RBCs but uninfected tissue failed to agglutinate (Fig. 3.16A).

Similarly FITC conjugated gelatin microspheres coated anti 18 kDa antiserum mixed with infected tissue samples agglutinated the uninfected tissue sample showed no reactivity (Fig. 3.16B).

Latex beads coated with rabbit anti 18 kDa antiserum with uninfected and infected tissue sample. Infected head tissue uninfected exhibited agglutination (Fig. 3.16C). Looking at the performance of the three tests, latex agglutination test was used for further evaluation. It was necessary to determine the lowest concentration of antigen that this test could detect, for this different concentration of tissue sample was added to latex beads. It was observed that agglutination was seen till 0.2 ng concentration of protein from infected tissue.

### 3.12.1 Latex agglutination test with different infected organs

Rabbit anti 18 kDa antiserum coated latex beads were used to test different tissues to assess the infectivity status. A clear agglutination was observed with all the infected tissues. The difference in the agglutination could not be detected between the organs. Further, samples, that were positive by latex agglutination, were subjected to PCR analysis for confirmation. The results of the latex agglutination were 100% correlated with the PCR analysis (Table 3.2).
Development of diagnostic test for WSSV using agglutination reaction; Human RBCs, FITC conjugated microspheres and Latex beads, were coated with rabbit anti18kDa antiserum and mixed with normal and infected head tissue.

Fig. 3.16
Fig. 3.16A Lane 1. RBCs coated with normal rabbit serum
2. RBCs coated with rabbit anti18kDa antiserum
Table 3.2 Comparison of PCR and Latex agglutination test with different organs of infected shrimp and coded samples

<table>
<thead>
<tr>
<th>Known samples</th>
<th>PCR</th>
<th>LATEX</th>
</tr>
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<tbody>
<tr>
<td>Tail</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Head tissue</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Gill</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Eye stalk</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Hemolymph</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Heart</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Coded samples</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAMPLE 1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SAMPLE 2</td>
<td>-</td>
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<tr>
<td>SAMPLE 3</td>
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</tr>
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<td>SAMPLE 4</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>SAMPLE 5</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>SAMPLE 6</td>
<td>++</td>
<td>++</td>
</tr>
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</table>

++ - Strongly positive
+ - Weakly positive
3.12.2 Comparison of DNA and protein based test for field sample infected with WSSV

Currently PCR with primers designed by various authors is being used for diagnosis of viral infection. In order to evaluate the applicability of the latex beads agglutination test in field samples, a comparative study was made between the already established techniques like PCR and Western blot with latex agglutination test. In this study the identification of the virus was done from coded unknown samples. Samples negative by PCR were negative by Western blot and Latex agglutination and the samples positive by PCR were also positive by Western blot and latex agglutination. Positive samples by PCR showed the 643 bp product (Fig. 3.17A) and the samples positive by Western blot showed the presence of 18 kDa antigen (Fig. 3.17B) (Table 3.2). In order to further establish the infectivity status of the sample, a bioassay was performed where all the 6 samples were injected into uninfected shrimps. As expected injection of samples 1 and 2 did not result in any infection in normal shrimps, while those that were infected with samples 3, 4, 5 and 6 exhibited all the symptoms of WSSV infection and died (Table 3.3). Together, these data suggests that the performance of latex agglutination is comparable to that of Western blot or PCR based analysis in identifying WSSV infection.

In order to evaluate the possible modes of transmission, live feed for shrimps (Artemia) and crustacean host (crabs) that coexist with prawns and feed for shrimps were tested for their carrier status.

3.13 INFECTIVITY STATUS OF ARTEMIA

Artemia is protein rich live feed for shrimps in the culture ponds. Different stages of Artemia were exposed to WSSV by immersion challenge
Fig 3.17 WSSV identification in coded samples of shrimps by Western blot and PCR

Fig. 3.17A Western blot analysis for crude lysate of tissues from 6 coded samples run on 12% SDS PAGE, transferred onto nitrocellulose membrane, subsequently probed with anti 18kDa antiserum (1:4000), followed by incubation with secondary goat anti rabbit ALP (1:30,000); developed with NBT and BCIP; Lane M Marker Lane 1-6 coded samples Lane 7 Positive control

Fig. 3.17B 1% agarose gel of the PCR product using primers designed by Takahashi et al. (1996) showing the PCR amplified products of the 6 coded samples Lane M Marker Lane 1-6 coded samples Lane 7 Positive control
Table 3.3 Bioassay with coded samples

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>% mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>0/5</td>
</tr>
<tr>
<td>Sample 2</td>
<td>0/5</td>
</tr>
<tr>
<td>Sample 3</td>
<td>5/5</td>
</tr>
<tr>
<td>Sample 4</td>
<td>4/5</td>
</tr>
<tr>
<td>Sample 5</td>
<td>5/5</td>
</tr>
<tr>
<td>Sample 6</td>
<td>5/5</td>
</tr>
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</table>
and were checked by PCR with primers designed by Takahashi et al. (1996) to assess the carrier status of *Artemia* for WSSV. Results showed that none of the developmental stages of *Artemia* were infected by WSSV (Fig. 3.18A). In order to further substantiate the above findings, the infectivity status experiment was carried out in the juvenile shrimps split into three groups. Group I consisted of the juvenile shrimps fed with *Artemia* exposed to WSSV, group II had juvenile shrimps fed with *Artemia* not exposed to virus and group III comprised of juvenile shrimps fed with infected shrimp meat. 100% mortality was observed in group III after 7 days while no mortality was seen in group I and II under similar conditions. Viral DNA was extracted from all the three groups and tested by PCR for the presence of WSSV. The juveniles fed with infected shrimp meat (group III), tested positive by PCR, while the other two groups were negative for WSSV (Fig.3.18B). Typical clinical signs of WSSV such as lethargy, anorexia and white spots were observed in-group III but not in other groups. These findings suggest that *Artemia* does not act as a carrier for WSSV.

### 3.14 INFECTIVITY STUDIES WITH FRESHWATER CRAB

The fresh water crabs, used as feed for shrimps were also infected with WSSV and showed similar clinical signs like the marine shrimps. WSSV caused 100% mortality in *P. pulvinata* and *P. hydrodomous*. However *P. hydrodomous* was able to survive for a longer time compared to *P. pulvinata* by both the routes of infection. 100% mortality was observed in *P. hydrodomous* after 84hrs and 10days by intramuscular injection and oral route respectively. While *P. pulvinata* showed 100% mortality in 64 hrs and 8 days by intramuscular injection and oral route respectively, suggesting that the infection caused by intramuscular injection was rapid when compared to the infection caused by oral route. To confirm the presence of WSSV, hemolymph obtained from the experimentally infected crabs was re-inoculated intramuscularly into
Fig. 3.18  1% agarose gel of the PCR product using primers designed by Takahashi et al (1996) showing the carrier status of Artemia using immersion method


Fig. 3.18B  Infectivity status studies with WSSV exposed Artemia as feed. Lane M: Marker 1. Negative control 2. Positive control 3. Group 1 Shrimp fed with Artemia exposed to WSSV 4. Group 2 Shrimp fed with Artemia not exposed to WSSV 5. Group 3 Shrimp fed with infected shrimp meat 6. Infected meat used for feeding shrimp 7. Artemia exposed to WSSV
fresh batches of uninfected freshwater crab. The inoculated animals showed all
the clinical signs of disease and severe mortality. PCR with Takahashi primers
with different infected tissues of moribund crab revealed that a 643 bp product
was higher in the head tissue than in other tissues like heart, hemolymph,
eyestalk and gill. No amplification was observed in the uninfected control
group (Fig.3.19A). The presence of WSSV was further supported by Western
blot analysis using rabbit anti 18 kDa antiserum. There was no reactivity with
the tissues of uninfected crab, while a single band at 18 kDa was observed with
pooled tissues from infected crab (Fig.3.19B). The cumulative percentage
mortalities for both the species of fresh water crabs (*P. pulvinata* and
*P. hydrodomous*) showed that they were highly susceptible to WSSV, like the
marine shrimps.

3.15 CLONING, EXPRESSION, PURIFICATION AND
CHARACTERISATION OF WSSV STRUCTURAL PROTEINS
VP19 AND VP28

In order to study the functional characteristics of the viral proteins of
WSSV, it would be necessary to produce them through rDNA technology. In
this way the uniform quality of the protein would be maintained and further,
reasonable amounts of highly purified proteins can be produced. To achieve
this goal two structural proteins, Vp19 and Vp28 have been produced as
recombinant proteins.

3.15.1 Amplification of viral structural genes from WSSV DNA

Viral structural protein genes *vp14, vp19, vp24, vp26, vp28* were
amplified from genomic DNA and the amplified products were of the expected
lengths 245 bp, 366 bp, 621 bp, 612 bp and 612 bp respectively (Fig.3.20).
Fig. 3.19  Infectivity studies with freshwater crab using PCR and Western blot

Fig. 3.19a  1% agarose gel of the PCR amplified products using primers designed by Takahashi et al. 1996 showing the infection in different organs of freshwater crab. Lane M. Marker 1. Positive control 2. Normal crab 3. Infected gill 4. Infected head tissue 5. Infected eye stalk 6. Infected heart 7. Infected hemolymph

Fig. 3.19b  Western blot analysis for crude lysate of tissues from freshwater crab run on 12% SDS PAGE, transferred onto nitrocellulose membrane, subsequently probed with anti 18kDa antiserum at 1:4000 dilution, followed by incubation with secondary at 1:30,000 dilution; developed with NBT and BCIP. 1. Infected tissue 2. Uninfected tissue
Fig. 3.20  1% agarose gel showing the amplification of viral structural genes vp19, 14, 24, 26 and 28 using gene specific primers Lane M. Marker
1. vp19 366bp  2. vp14 245bp  3. vp24 621 bp  
4. vp26 612bp  5. vp28 612bp
Sequence analysis revealed that the genes have a putative methionine start site in frame with the coding sequence. The genes had open reading frames of 245 bp for vpl4, 366 bp for vp19, 621 bp for vp24, 612 bp for vp26, 612 bp for vp28. The full length ORFs of all the 5 genes were compared with the sequence already published by VanHulten et al., 2001, Tsai et al., 2002, and Yang et al., 2001 a few substitutions were noted (Fig. 3.21 a, b, c, d, e). vpl9 gene sequence comparison revealed major C-T and T-C point mutations at two loci each. vp28 sequence comparison also showed C-T transition in 2 loci. vpl4, vp24 and vp26 did not show any point mutations. To examine whether this point mutation is also manifested at amino acid level, the genes were translated and compared. In Vp19, at position 9 proline was substituted by serine, at position 15 alanine was substituted by valine, at position 66 serine was substituted by proline and at position 96 methionine was substituted by threonine. In Vp 28 at position 18 alanine was substituted by cysteine and at position 162 serine was substituted by threonine (Table 3.4).

3.15.2 Cloning of vpl9 and vp28 genes in T7 expression system pRSET B

Due to the variation in the sequence, vpl9 and vp28 genes were selected for cloning. Both these genes were amplified by PCR and subjected to restriction and the purified product was ligated into a pRSET B vector and subsequently the ligated product was transformed into DH5α strains of E.coli.

Screening of the transformants was performed using lysate PCR 14 of the 15 transformants were positive for vpl9 (Fig. 3.22.A), while 13 of 15 were found to be positive for vp28 (Fig. 3.22.B). The recombinant plasmids were named pRSVP19 and pRSVP28. The sequencing of the insert revealed
### Table 3.4a Substitutions in the viral structural protein

<table>
<thead>
<tr>
<th>Sequence</th>
<th>No. of changes</th>
<th>Significance</th>
<th>Insig</th>
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</thead>
<tbody>
<tr>
<td>vp14</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vp19</td>
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<td>3</td>
<td></td>
</tr>
<tr>
<td>vp24</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vp26</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vp28</td>
<td>2 bases</td>
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</table>

### Table 3.4b Amino acid substitutions in Vp19 and Vp28

<table>
<thead>
<tr>
<th>Residue Number</th>
<th>Consensus</th>
<th>Change</th>
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</tr>
<tr>
<td>9</td>
<td>Proline</td>
<td>Serine</td>
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<tr>
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<tr>
<td>66</td>
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<tr>
<td>96</td>
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</tr>
<tr>
<td>Vp28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Alanine</td>
<td>Cystine</td>
</tr>
<tr>
<td>162</td>
<td>Serine</td>
<td>Threonine</td>
</tr>
</tbody>
</table>
Fig. 3.22 1% Agarose gel electrophoresis showing the PCR screening of vp19 and vp28 transformants after cloning into pRSET B vector

Fig. 3.22A Screening of vp19 transformants; 14/15 had the insert
Lane M.Marker Lane 1-15 PCR product of 15 clones

Fig. 3.22B Screening of vp28 transformants; 13/15 had the insert
Lane M.Marker Lane 1-15 PCR product of 15 clones
that the genes had a putative methionine start site in frame with the coding sequence of the vector and the cloning sites were verified to be Bam HI and EcoRI. The genes had open reading frames of 366 bp for vp 19 and 612 bp for vp 28 in continuation with vector ATG and His-tag. This 366 bp sequence of vp19 coded for a protein of molecular weight 18 kDa with the additional 3 kDa of the vector sequence the size of the protein was 21-21.5 kDa. Like wise for vp28, 612 bp sequence coded for a 23 kDa protein, with the additional 3 kDa from the vector the net molecular weight was 26 kDa.

3.15.3 Restriction digestion analysis of pRSVP19 and pRSVP28

In order to confirm the presence of the insert, both the recombinant plasmids pRSVP19 and pRSVP28 were subjected to restriction digestion analysis with BamHI and EcoRI, the insert was released from the vector to a size of approximately 366 bp and 612 bp respectively (Fig.3.23) confirming the presence of the viral genes in the vector.

3.15.4 Expression of the recombinant plasmids pRSVP19 and pRSVP28

Once the presence of the insert was confirmed by PCR and restriction, the plasmids were transformed into E.coli BL21 (DE3) an expression host. The induced and uninduced pRSVP19-BL21 (DE3) and pRSVP28-BL21(DE3) were analysed by SDS PAGE. A band corresponding to 21 kDa molecular weight was observed in the induced pRSVP19 (Fig. 3.24.A) and a band at 26 kDa was observed in the induced pRSVP28 (Fig. 3.24.B). No protein was found at the same position in uninduced pRSVP19 and pRSVP28, or in induced and uninduced BL21(DE3) harboring pRSET B vector.
Fig. 3.23 Restriction profile of the recombinant plasmids showing the inserts of vpl9 and vp28 gene
Approximately 1μg of pRSET B, the recombinant pRSVP19 and pRSVP28 plasmids were digested with BamHI and EcoRI and resolved on 1% agarose gel
Lane 1. Lambda HindIII marker
2. pRSET uncut
3. pRSET restricted with BamHI and EcoRI
4. pRSVP19 uncut
5. pRSVP19 restricted with BamHI and EcoRI
6. pRSVP28 uncut
7. pRSVP28 restricted with BamHI and EcoRI
M. 100bp ladder
Fig. 3.24 12% SDS PAGE stained with coomassie brilliant blue showing the expression of the recombinant proteins after induction with IPTG.

Fig. 3.24A Expression profile of 19kDa recombinant protein
Lane M Marker 1. BL21(DE3) 2. BL21(DE3) with vector induced 3. 19clone uninduced, 4. 19clone 1hr induced 5. 19clone 2hrs induced 6. 19clone 3hrs induced

Fig. 3.24B Expression profile of 28kDa recombinant protein
Lane M Marker 1. BL21(DE3) 2. BL21(DE3) with vector induced 3. 28clone uninduced 4. 28clone 1hr induced 5. 28clone 2hrs induced 6. 28clone 3hrs induced
Expression level of 28 kDa protein was appreciably more than 19 kDa. However the expression levels of both the recombinant proteins were significantly higher when compared to the uninduced recombinant construct.

3.15.5 Confirmation of the expression of the recombinant proteins by anti-His antibody

The expression of the recombinant proteins pRSVP19 and pRSVP28 were confirmed by Western blot analysis with anti-His antibody. A band corresponding to 21 kDa molecular weight was observed in the induced pRSVP19 (Fig. 3.25A) and a band at 26 kDa was observed in the induced pRSVP28 (Fig. 3.25B). There was no reactivity with either the host control or the vector control.

3.15.6 Purification of 28kDa and 19kDa protein expressed in pRSET B

The expression of proteins in pRSET B expression system facilitates an easy one step purification on Ni$^{2+}$ immobilized columns. Purification by immobilised metal affinity chromatography yielded a single band in 150 mM immidazole fraction when run on a SDS PAGE. The protein was dialysed for removal of immidazole and the protein concentration was approximately 0.8 μg/μl. The purified protein showed the same mobility as the induced recombinant proteins from the crude cultures on an SDS PAGE (Fig. 3.26 A,B), thus confirming the authenticity of the protein.
Fig. 3.25 Western blot analysis to check the expression of recombinant proteins with monoclonal anti-His antibody; total protein extracts from recombinant clones and control pRSET B were separated on 12% SDS PAGE, transferred to nitrocellulose membrane and probed with mouse monoclonal anti-His antibody (1:3000), followed by incubation with goat anti mouse ALP (1:30,000), developed with BCIP and NBT.

Fig. 3.25A Western blot analysis for 19kDa recombinant protein using anti-His antibody Lane M Marker 1 BL21 (DE3) induced 2. pRSET-B induced 3. 19kDa uninduced 4&5. 19kDa induced

Fig. 3.25B Western blot analysis for 28kDa recombinant protein using anti-His antibody Lane M Marker 1 BL21 (DE3) induced 2. pRSET-B induced 3. 28kDa uninduced 4&5. 28kDa induced
**Fig. 3.26** SDS PAGE profile showing the recombinant proteins purified by IMAC; total and purified recombinant proteins were separated on a 12% SDS PAGE and stained with coomassie brilliant blue.

**Fig. 3.26A** Protein profile of purified recombinant 19kDa protein
Lane M Marker 1. Uninduced BL21(DE3)
2. Induced 19kDa in BL21(DE3) 3. IMAC purified 19kDa protein

**Fig. 3.26B** Protein profile of purified recombinant 28kDa protein
Lane M Marker 1. Uninduced BL21(DE3)
2. Induced 28kDa in BL21(DE3) 3. IMAC purified 28kDa protein
Characterisation of the recombinant 19 kDa and 28 kDa proteins

When the mouse anti 19 kDa antiserum was checked for its specificity using Western blot it reacted with a single protein of molecular weight 21 kDa in the induced sample alone. The reactivity with infected tissue sample was at around 19 kDa; it was approximately 3 kDa less than the recombinant antigen reactivity. The antiserum failed to react with uninfected tissue sample (Fig. 3.27A). Similarly, the 28 kDa antiserum was also found to be very specific without any cross reactivity with either the host alone or the vector without the insert (Fig. 3.27B). The results of the above studies clearly demonstrated that it has been possible to produce recombinant structural proteins of WSSV. This can now be used for functional studies.
Fig. 3.27 Western blot analysis to assess the reactivity of antiserum raised against the recombinant proteins; total protein extracts from recombinant clones, control pRSET B, normal shrimp tissue and infected shrimp tissues were separated on 12% SDS PAGE gel, transferred to nitrocellulose membrane and probed with 1:1000 mouse anti-Vp19/anti-Vp28 antiserum, followed by incubation with goat anti-mouse ALP (1:30,000), developed with BCIP and NBT.

Fig. 3.27A Western blot analysis with antiserum raised against recombinant 19kDa protein Lane M. Marker 1. BL21 (DE3) 2. BL21 (DE3) with vector induced 3. 19 clone uninduced 4. 19 clone induced 5. Normal tail 6. Infected tail.

Fig. 3.27B Western blot analysis with antiserum raised against recombinant 28kDa protein Lane M. Marker 1. BL21 (DE3) 2. BL21 (DE3) with vector induced 3. 28 clone uninduced 4. 28 clone induced 5. Normal tail 6. Infected tail.