The susceptibility of *P. monodon* and *P. indicus* was compared and it was found that the former was more susceptible. Nevertheless, WSSV caused 100% mortality in both species of shrimp. The observation made by Sahul Hameed et al. (1998) is supportive of the finding in the present study. Similar differences in susceptibility to viral, bacterial and fungal infections among penaeid shrimps have been reported (Lu et al., 1994; Sahul Hameed 1995). To date, WSBV is the only known crustacean baculovirus that affects a wide range of tissues in the penaeid shrimps as well as other crustaceans, whereas MBV, (Monodon baculovirus) and PBV (Penaeid baculovirus) are tissue and host specific. WSSV (PmNOBIII) has been described from *P. monodon* (Wang et al., 1995; Chang et al., 1996; Karunasagar and Karunasagar 1997; Karunasagar et al., 1997). Woongterasupaya et al. (1996) reported the occurrence of the infection in ecto and mesoderm derived tissues from *P. monodon*, *P. chinensis*, *P. indicus*, *P. japonicus*, *P. merguensis* and *P. vannamei* obtained from China, Thailand, Indonesia and India. This suggests that WSSV is a serious pathogen for a range of crustaceans with a wide geographic distribution. It is worthwhile to note that *P. indicus* is a relatively refractory species to the infection caused by WSSV as in the case of Vibrio infection observed by Sahul Hameed (1995). Since *P. indicus* can survive for a longer time than *P. monodon*, it is possible to obtain a higher viral load for experimental studies. The mortality caused due to intramuscular injection was rapid when compared to oral or natural routes of
infection. Similar observations made by Sahul Hammed et al. (1998) and Rajan et al. (2000) indicated that intramuscular injection of purified virions caused 100% mortality within few days post infection (1-5 days). In contrast, natural routes of infection took longer to establish before causing mortality.

Only sequencing the genome or a fragment of the genome and comparing it with the already published sequence can authenticate the viral identity. For this the viral DNA was extracted by a non-enzymatic high salt method. Various protocols have been developed to extract DNA for PCR detection of WSSV in shrimp by different workers (Lo et al., 1996a; Yang et al., 1997; Nonaka et al., 1998). In all these methods, proteinase K and solvents like phenol, chloroform and iso-amyl alcohol were used for de-proteinizing cell digests. The present method of extraction eliminates completely the use of enzymes and solvents. This is achieved by salting out the cellular proteins by dehydration and precipitation with a saturated sodium chloride solution (Miller et al., 1988). The WSSV-DNA preparation was free of protein, host DNA and RNA, and degrading enzymes. The WSSV-DNA produced by this method was of good quality and was suitable for restriction enzyme studies and for different PCR protocols such as single step PCR, nested PCR and single tube semi-nested PCR. This method worked successfully for WSSV-DNA extraction from different organs (hemolymph, eyestalk, carapace, head muscle, heart, gills, appendages, heptopancreas, stomach, intestine, abdominal muscle and tail muscle) of infected shrimp. More importantly this procedure works equally well with fresh samples as well as, with those stored at 4°C and -70°C. Thus this technique eliminates the step of prolonged digestion of samples with Proteinase K, saving time and cost. The sequence of the 643bp PCR product of the viral DNA obtained showed 100% identity with sequences
published by Takahashi et al., 1996; VanHulten et al., 2001; Tsai et al., 2002 and Yang et al., 2001, indicating that all the viruses from different geographical locations were closely related.

A very simple methodology involving a few rounds of freezing and thawing and a brief spin was developed to enrich the virus. This technique was found to be very useful in obtaining virus samples for studies like DNA extraction ELISA, SDS PAGE, Western blot Dot blot and Latex agglutination. The conventional procedure for purifying the virus is by density gradient ultra centrifugation. There are various modifications reported by other researchers in the density gradient ultra centrifugation, based on the chemical used for setting up the gradient, sucrose (Sahul Hameed et al., 1998, Zhan et al., 1999), CsCl (Nadala and Loh 1998) Sodium bromide (Zhang et al., 2002a) and Renografin (Poulos et al., 2001). But all these gradient centrifugation need ultra centrifuge that can go up to 100,000 rpm which makes it difficult to perform routinely. A comparative study revealed nearly the same protein profile before and after ultracentrifugation. Similar procedure to enrich the virus from tissues was used by Nadala and Loh (2000) and Liu et al. (2002). The protein profiles of the enriched virus from pooled tissues and hemolymph revealed tissue as the ideal organ for purification of the virus. The possible reason for the higher prominence of viral protein in tissue samples than hemolymph could be because of the 66 kDa protein contamination, a major component of the hemolymph. Tissue samples from the infected shrimps only were used for all studies. WSSV is known to have wide host range and wide geographical distribution. A lot of reports are available on the use of tissues for the purification and identification of WSSV. Kasornchandra et al. (1998) used tissues for PCR, Wang et al. (2000a) used epidermis, stomach, appendages and gill for identifying genome
variation among geographical isolates using restriction and southern blot analysis. Chang et al. (2001) used tissues for ARFLP from crabs. Hence for all further studies tissue sample was used.

SDS PAGE profile of the infected tissue sample showed 7 major proteins of molecular weight 45, 35, 30, 28, 23, 18 and 14 kDa. Four more proteins of molecular weight 25 kDa, 52 kDa, 66 kDa and 72 kDa were also noticed. The concentration of the proteins of molecular weight 23 kDa and 52 kDa was found to be very less, while 66 kDa and 72 kDa proteins were observed in normal tissue sample as well and hence these four proteins were not analyzed further. Huang et al. (2001) identified 13 major proteins of nearly the same molecular weight from the infected crayfish *Cambrus clarki*. Similarly proteomic analysis of WSSV by Huang et al. (2002a) has revealed 24 major proteins, some of which matched with protein profile observed in the present study. Further VanHulten et al. (2000) have also reported 4 major proteins in the same size range such as 19, 24, 26 and 28 kDa and Wang et al. (2000) have found three major proteins with approximately similar size in six geographical isolates of WSSV from China, India, Thailand, as well as three locations in USA.

Among the five prominent proteins of molecular weight 14 kDa, 18 kDa, 23 kDa, 30 kDa and 35 kDa, 18 kDa antiserum was selected for diagnostic studies. One of the possible reasons for this is the predominance of 18 kDa protein in different geographical isolates as reported by Wang et al. (2000a).
A comparison between Western blot and PCR was made by Magbanua et al. (2000). It revealed that some samples that were only 2 step PCR positive were positive by Western blot. However PCR was found to be more sensitive than Western blot. This could vary depending on the antigen that is chosen for developing diagnostic test and also the titre of the antiserum. With the right choice of antigen and with high titre antiserum both Western blot and agglutination test can be as sensitive as PCR. Thus in all the ways this assay will be suitable to detect infection in field sample.

Using dot blot assay with anti 18 kDa antiserum it was possible to detect the WSSV infection in shrimps with protein concentration as low as 10pg. However same test carried out by Sahul Hameed et al. (1998) with antiserum against 27 kDa protein could detect the infection only with 800 ng of protein. Various other authors have demonstrated the use of polyclonal antiserum and monoclonal antibodies for the diagnosis of WSSV infection. Sahul Hameed et al. (1998) reported the use of polyclonal antiserum against 27 kDa protein and Poulos et al. (2001), Liu et al. (2002) and Anil et al. (2002) produced monoclonal antibody against the same 28 kDa protein and used it for diagnosis. You et al. (2002) and Zhang et al. (2002a) have used recombinant 28 kDa protein for raising polyclonal antiserum. In spite of the availability of numerous reports on the usage of antiserum based diagnosis, there are very few studies on the tissue distribution of the virus and the time course analysis of WSSV infection in infected shrimps.

A time course study was carried out using Western blot and ELISA to assess the appearance of viral antigen after WSSV infection in different infected tissues. Till 24hrs post infection the viral protein concentration was
found to be less using Western blot and ELISA, but it increased rapidly after 36hrs till the shrimps became moribund. A time course analysis carried out by Nadala et al. (1997) using antiserum against whole virus indicated that the infection could be detected only at 41hrs post infection in gill and 43hrs post infection in hemolymph. The finding of the present study suggests that the WSSV infection could be detected as early as 24hrs post infection in tail and head tissue using the anti 18 kDa antiserum.

Tissue distribution study using western blot revealed the order of reactivity of the 18 kDa protein as Tail> Headtissue > Eyestalk > Gill > Pleopod and periopod > muscle > heart > Hemolymph > stomach > hepatopancreas. Tail, head tissue and eye stalk were the early-infected organs (i.e) as early as 24hrs post infection harboring high viral load. However the infection was very less in hemolymph and heart, gill had moderate infection. Similar observation made by Tang and Lightner (2000) using competitive PCR showed that the quantities of WSSV genomes in hemolymph were generally much lower than in tissues. The viral load of the head and tail were more because both the head and tail of shrimp include large amount of the principal target tissues (cuticular epidermis, connective tissue and hemocytes) that are heavily infected in acute WSSV infection (Wang et al., 1999). Between these two organs tail had high viral load, which was evident from the band intensity. Durand et al. (2000) reported that on a per weight basis, peeled shells from the tail (with pleopods and tailfans) contain almost 10 times more WSSV than do whole heads or whole tails. In proportion of the total tail weight, the virus load of the peeled shell represents 55% of the total viral load in tail. Therefore when the shrimp are unshelled, 45% of the total viral load of the tail remains in the edible tail meat. Shrimp farmers have a false opinion that only the heads of
shrimps are infected, this led to the usage of tail as bait. Based on the observations made in the present study it can be conclusively said that using tail of infected shrimp as bait can transmit the disease.

Earlier Kou et al. (1998) reported gill to be the highly infected organ but they had not taken tail sample for their studies, but the present investigation showed that tail tissue and head tissue were the most infected organs. The possible reasons for this could be, the organs of ectodermal and mesodermal origin are the targets of the WSSV (Sahul Hameed et al., 1998). Among these ectodermal organs show a much higher prevalence of WSSV than the mesodermal organs. Tail is primarily derived from ectodermal and head tissue is from both ectodermal and mesodermal tissue and hence the difference. Wang et al. (1999) reported that the lack of infection in hepatopancreas could be due to the lack of appropriate viral receptors or due to lack of electrostatic interaction between virus and the host cell surface. Tang and Lightner (2000) have shown using competitive PCR that the quantities of WSSV genomes in hemolymph were generally much lower than that in tissues, specifically, 49 times less at 35hrs after infection. This is likely to be due to higher cell mass in the tissues relative to the hemolymph. Therefore, the use of infected head tissue and tail as a source for the production of large quantities of WSSV, is most suited compared to hemolymph.

This observation is of great concern, as the belief that while head is highly infected, tail poses a relatively low risk has led to the marketing of frozen tails in retail outlets (Durand et al., 2000). Likewise shrimps are cannibalistic animals, under intensive cultivation, the feelers, tail, tailfans, periopods, pleopods and even eyes of living shrimps are sometimes
cannibalized by other shrimps. This not only applies stress which could trigger multiplication of WSSV in lightly infected shrimp, but also provides a pathway whereby WSSV can spread quickly in the shrimp population so that an outbreak of WSS will likely be precipitated. So it's just a matter of few hours for the infection to proceed from a prepatent to patent stage. Shrimps are known to survive for months together even after being infected by WSSV. Tsai et al. (1999) reported that shrimps survive till adult stage even after being infected at nauplii stage. So it becomes imperative to have an easy diagnostic test that can detect the virus at an early stage so that quarantine measures can be taken and stocking densities can be reduced to minimise the stress on the shrimps and thereby the whole stock can be saved.

The development of simple, sensitive and rapid assay formats for the detection of shrimp viruses has been an elusive goal. Although the use of gene probes and DNA amplification techniques has provided valuable new testing formats, in general the technology is expensive, highly technical, not rapid and not amenable to field conditions (Poulos et al., 2001). A critical aspect of any diagnostic study is the nature of the diagnostic material used to determine the infection status. PCR detects only a fragment of the DNA. Whether the virus is viable or infectious cannot be known, a non-viable virus present on the sample surface can also contribute to a PCR positive result. Further different assays would offer different levels of sensitivity, depending on the preservation method, DNA extraction method, template concentration and the size of the amplicon (Cha and Thilly 1995). For instance a sample that tests positive at first amplification by one diagnostic PCR protocol may test positive only after reamplification by a less sensitive protocol. Similarly, a 2 step positive sample tested by a highly sensitive protocol may test falsely negative by a less sensitive
protocol. DNA extraction methods such as phenol chloroform extraction can be superior in terms of recovery of DNA and stability of DNA, but they are time consuming and expensive and this negates the benefit of rapid PCR or may not be feasible for large number of samples. However antibody based assays detects the intact virus (Thakur et al., 2002). The method based on agglutination allowed detection without any sophisticated equipment, since it can be viewed by naked eye or with the simple microscope to be certain.

Hence an alternative to routine immunoassays, agglutination tests with Human RBCs, microspheres and Latex beads was developed. All the three agglutination tests performed very well for diagnosing WSSV infection. Red blood cells had the disadvantage of shorter shelf life and FITC coated microspheres can be visualized only using fluorescence microscope. Therefore, latex agglutination test was chosen for field evaluation because of the rapidity and simplicity.

Latex beads coated with anti 18 kDa antiserum agglutinated with infected tissue sample and it failed to agglutinate with normal tissue sample. Only minute amounts of crude infected shrimp tissue are required to perform the test. Using Latex agglutination it is possible to detect WSSV infection with protein concentration as low as 200 pg. Earlier reports by other authors showed that 400 pg of protein could be detected by dot blot (Anil et al., 2002) 400 pg by AcELISA, 300 pg by PCR, 375 ng by Western (Liu et al., 2002). Agglutination test was carried out with different organs of shrimp and compared with PCR. Reactivity with both the tests were comparable indicating that the test developed here can pick up infection from all the organs of shrimp. The latex agglutination assay procedure takes about 20 minutes in contrast to 4-
5 hrs for PCR (Tsai et al., 1999; Kou, 1996b), 8-10 hrs for Western blot (Nadala and Loh, 2000; Sahul Hameed et al., 1998), 5-6 hrs for real time PCR (Durand et al., 2003), 2-3 hrs for insitu hybridization (Chang et al., 1998), 16-20 hrs for histological identification and 3hrs for the immunodot test (Anil et al., 2002; You et al., 2002), 14hrs for colorimetric detection (Quere et al., 2002) and 6-8 hrs for ELISA (Chen et al., 2002). All these techniques are complicated and expensive for general use in hatchery and farms (Chang et al., 1998). But the latex agglutination is very fast and cheap when compared to all the above-mentioned techniques. This kind of agglutination test has not been used so far for diagnosing infection in shrimp. However, it has been extensively used for diagnosing infection in human Corynebacterium diphtheriae (Toma et al., 1997), Candida albicans and Candida krusei (Fregdiere et al., 1997), Clostridium difficile (Staneck et al., 1996), Streptococcus pneumoniae (Garcia et al., 1999), Mycoplasma capricolum (March et al., 2000). In order to make the test more authentic PCR and latex agglutination test was done for coded samples and 100% correlation was observed. This clearly indicates that the test developed here can detect the infection in the field samples to the same extent as the already available diagnostic technique like PCR and insitu hybridization, but in a much faster and rapid rate that the others. It should be pointed out that the agglutination test is in the process of being converted into a card test.

The only disadvantage of this technique is that it is not quantitative but it’s only qualitative. It can be said whether the given sample is infected or not using this test, but based on the agglutination the concentration of the viral protein cannot be obtained. Hence in order to look at the severity of infection, agglutination test alone may not be sufficient. Thus there is also a need to develop a molecular diagnostic test that can grade the infection for which,
N-terminal sequencing of the 18 kDa protein (named as Ws18) was done and primers were designed based on the protein sequence. The 18 kDa protein was chosen due to its presence in almost all the geographical isolates as reported by Wang et al. (2000). Hence it was used for developing diagnostic kit. The only way to confirm the authenticity of the 18 kDa protein was by N-terminal sequencing of the protein. But the protein sequencing data revealed that it was different from the already published vp19 sequence. It matched with ORF107 of VanHulten (2001) ORF 262 of Tsai et al. (2002) and ORF 207 of Yang et al. (2001). The 18 kDa protein was 262 amino acids in length and was coded by 828 bp gene.

The amino acid sequence of the ORF 107 was coded by 828bp, whereas 828 bp should actually code for 30 kDa protein. However the mobility of the protein was observed to be approximately 19 kDa. The possible reason for the loss of few amino acids and the shift in mobility could be because of N-terminal and C-terminal cleavage during posttranslational modification or proteolytic cleavage. The later could be a major possibility as the sequence was known to have 31 proteolytic cleavage sites that can yield fragments ranging from 16.7 kDa to 22 kDa protein. An observation made by Srivatsava and Srivatsava (2003) on the truncation of βB2 crystallin supported this view. The protein molecular weight was 19 kDa and had 14 cleavage sites and yielded fragments ranging from 4 kDa to 11 kDa. Little et al. (2002) reported the involvement of Matrix metalloproteinases in C-terminal cleavage of cartilage aggregans. Studies with the lassa virus revealed that glycoprotein was synthesized as precursor GP-C into the lumen of the endoplasmic reticulum and cleaved post translationally into the N-terminal subunit GP-1 and the C-terminal subunit GP-2 by subtilase KSJ-1/SIP (Eichler et al., 2003). Utsumi
et al. (2003) observed a C-terminal 15 kDa fragmentation of cytoskeletal actin. A 47 kDa protein was post translationally cleaved to release a 15 kDa active protein.

A single tube semi nested PCR was developed using the primers designed based on the N-terminal sequence of 18 kDa protein. Depending on the concentration of the DNA the number of amplified products varied between 3, 2 and 1 with high moderate and low concentration of DNA respectively. Using Single tube semi nested PCR it was possible to detect WSSV infection with DNA concentration of 10fg. Similar test developed by Kiatpomchai et al. (2002) could detect infection only from 100pg of DNA. This estimated level of sensitivity is comparable with that of the 2 step nested PCR technique reported by Lo et al. (1998). However this method is more advantageous because it is an uninterrupted, single tube reaction with reduced risk of contamination.

The reason for the appearance of single band when infection is very less using this technique can be explained by the basic principle involved in PCR. To amplify 828 bp only the viral genomic DNA serves as a template whereas for 646bp both the genomic DNA as well as the 828 bp product can act as templates, and likewise for 396 bp product genomic DNA, 828 bp and 646 bp all the three act as templates. Hence when the template DNA concentration is less then the concentration of 828 bp product formed will be very less to be viewed on a gel, and 646 bp will also be less but slightly more than 828 bp so as to be barely visible on a gel, whereas 396 bp is amplified to an appreciable amount, so that it can be clearly seen on a gel. The concentration of template DNA directly correlates with the number of virus particles.
One very important advantage of our technique is its ability to grade the severity of viral infection to 3 levels. Lo and Kou (1998) classified WSSV infection into three stages, the asymptomatic carrier state or prepatent or latent stage, the transition stage and the patent stage. So the condition where in we get only one amplified product of 396 bp by single tube semi nested PCR is called the prepatent or the asymptomatic carrier state (stage I), in this stage shrimps can survive for months together without stress, (stage II) or transition state where both the bands appear 396 bp and 696 bp, it can survive only for few hours or days, whereas the condition when all the three bands appear is called the patent or the highly infected stage which is sure to die in few hours (stage III). It is not possible for us to protect the shrimp in stage II and stage III, whereas shrimp in stage I can go through to harvest if proper quarantine measures are taken. Shrimps can remain alive indefinitely in its prepatent condition in the absence of stress, even an eyestalk ablation or periopod excision may lead to the conversion from prepatent stage to transition stage and from transition to patent stage (Peng et al., 1998).

Spawning is known to be a major stress for the animal and Lo et al. (1997) reported that only lightly infected and healthy brooders can spawn successfully. Before the WSSV outbreak, a female brooder in a hatchery was expected to spawn repeatedly from 2 to 4 times (some times up to 20 times or more) once every 2-4 days before death (Chen et al., 1990). However according to some hatchery operators in Taiwan, most female brooders have not managed to spawn repeatedly since the outbreak of WSS in 1992. Peng et al. (1998) reported that just the excision of periopod can trigger the transition of *P. monodon* from prepatent to patent infection state of WSSV. The importance of the above discussion in this study is, even minor stress can lead to mortality,
if the shrimp carries infection and if that minor stress is on a brooder it burdens
the shrimp exorbitantly. As a result the shrimp might die even without
spawning. Tsai et al. (1999) reported that brooders spawned successfully when
cultured under stress less condition even after being infected by WSSV.

Hence using this diagnostic technique the brooders can be
categorized into light, moderate and highly infected shrimps, and cultured in
very low stocking densities and stress less condition to reduce the burden on the
spawner. Thus millions of egg loss due to infection in spawners can be
prevented. It is important to know how WSSV is transmitted to other
crustaceans and whether the feed or the co-existing organisms contribute to the
spread of the disease. In order to investigate this *Artemia* (live feed for shrimp)
was checked for its carrier status. It was observed that *Artemia* cannot act as
carrier for WSSV.

The WSSV is known to be transmitted to cultured shrimp via
contaminated water and ingestion of WSSV infected shrimp meat
(Kasornchandra et al., 1998; Supamattaya and Boonyaratpalin 1996). There is
also some evidence of transmission of WSSV among the shrimp by
cohabitation in the shrimp-farming environment (Flegel 1997, 1998). Earlier
studies indicate that the most common marine and fresh water decapods
including non-cultured arthropods can be infected by WSSV and can act as a
reservoir or carriers for WSSV (Lo et al., 1996b; Chang et al., 1998; Lo and
Kou 1998; Maeda et al., 1998; Supamattaya et al., 1998 and Wang et al., 1998).
A detailed experimental study on the pathogenicity of WSSV on *Artemia* and
their possibility of acting as a reservoir or carriers of WSSV in the shrimp
hatcheries and farms is lacking from the above studies. The current study was
carried out to fill this gap, as *Artemia* is an important cohabitant and live feed for marine shrimp in the hatcheries and farms. The cumulative mortality data obtained from the pathogenicity experiments showed that the WSSV failed to infect the developmental stages of *Artemia* by the immersion challenge and oral route. The PCR results also confirmed the above observation. The exact mechanism of resistance to WSSV is not known at present. This is also the case for nodavirus, which failed to replicate in *Artemia* (Skliris and Richards, 1998).

A study carried out by (Chang et al., 2002) also supported the observation that *Artemia* cannot act as a carrier, white spot syndrome virus PCR positive *Artemia* cysts yielded PCR negative nauplii that failed to transmit WSSV when fed to shrimp post larvae. They suggested that the cyst could have been externally contaminated with WSSV particle, WSSV DNA or WSSV DNA fragment of unknown derivation and this were removed or diluted to undetectable levels, when the hatched nauplii were washed with clean, sterile sea water before being fed to the shrimp. This possibility for external contamination highlights the importance of the usual hatchery practice of washing *Artemia* cysts with chlorinated water before culturing.

The mortality data, PCR and Western blot analysis confirmed that WSSV can infect fresh water crabs and that WSSV maintains its infectivity in fresh water as reported by (Chang et al., 1998). Thus, these freshwater crabs should not be used as feed for brooders. A similar recommendation was made for the marine crabs (*Charybdis feriatus* and *Por. sanguinolentus*) that were often used as feed for brooders until they were proved to be hosts of WSSV (Lo and Kou, 1998). In order to avoid WSSV infection in grow-out ponds, shrimp farmers in some parts of Andhra Pradesh, India have started culturing
marine shrimp under freshwater conditions after acclimatizing the shrimp seeds to fresh water in the hatchery. The results show that this strategy may fail if WSSV infected freshwater crabs enters their ponds. Thus, it would be prudent for shrimp farmers to exclude these potential carriers from the farming systems and avoid using them to feed brooders.

Extensive investigation have been carried out on WSSV infection, however very little information is available on the mechanism of pathogenesis of infection. Though the mechanism could be examined using purified WSSV in experimental shrimps it is important to stress that WSSV protein may be contaminated with shrimp host protein. Hence it is necessary to obtain various purified proteins of the virus that could facilitate functional studies. In order to fulfill this mission rDNA technology was used to clone the structural genes. Due to concentration of the product obtained and also due to the ease of manipulating the protein for our study recombinant DNA technology have been used here. Primers were designed based on the sequence published by VanHulten et al. (2001) along with restriction enzyme sites to amplify and clone the full ORF of the viral structural genes. Viral protein genes vp14, vp19, vp24, vp26, vp28 were amplified from the WSSV genomic DNA. The observation that the primers amplified the same product length as that of the already published sequence is an indication that there are no major differences between various isolates. This finding is in concurrence with the data already published by Lo et al. (1999) and Wang et al. (2000a).

The five genes that were amplified in this study were major structural proteins, which might play a main role in the pathogenesis of WSSV infection (Nadala et al., 1997; Zhang et al., 2000a, 2000b; VanHulten 2000). The genes
were subjected to sequencing to know whether there were any point mutations. The sequence that was obtained was compared with the already published sequences of VanHulten et al., 2000; Tsai et al., 2002; Yang et al., 2001) using Clustal W analysis. There were no difference between the sequences for the genes vp24, vp26, vp14, whereas there were C-T and A-T point mutations in vp19 and vp28 gene sequences. Previous reports indicate that single base change might affect the virulence of the virus. Chang et al. (2002) observed similar C-T and C-G point mutations and attributed it to non-infectious nature of the viral isolate due to the point mutation. Similar observation on C-T point mutation was also reported earlier by Chang et al., 2002 in RR1 gene from New Jersey WSSV isolate. Moon et al. (2003) reported that vp24 and vp26 showed 100% identity with the sequence already published, whereas in vp28 there was a single base substitution from C to T. This was identical to the observation made in the current study which clearly indicates that, the possibility of variation among geographical isolates cannot be ruled out. Lan et al. (2002) demonstrated that there was a sequence deletion at hot spots in the WSSV genome. There was a 4.6, 4.8, or 8.1 kbp deletion depending on the species of prawn. These deletions accounted for the virulence of the isolates. This observation has clearly indicated that there can be a difference in virulence due to mutations in the sequences. Hence it is logical to conclude that the high virulence shown by the Indian isolate is due to the point mutations in the vp19 and vp28 genes. Sequence variation among isolates of IHHNV originating from Hawaii and Americas was reported by Tang and Lightner (2002). Although IHHNV was reported to be extremely virulent towards *Penaeus stylirostris* during the early 1980s in recent years there had been an apparent decrease in IHHNV related mortality in stock of *P. stylirostris* (Morales Covarrubias et al., 1999). One possible reason for this reduction in virulence was attributed to the
change in IHHNV genome. The sequence within 2.9 kb region among these isolates showed 25 nucleotide substitutions; 14 of these resulted in amino acid changes, no deletions or insertions were detected. Change in pathogenicity of some paroviruses has been associated with amino acid change in capsid proteins (Tijssen et al., 1995, Fox et al., 1999), although in other cases no such association was found (Gallionella et al., 1995, Erdman et al., 1996). Tang and Lightner (2002) compared 14 cases of IHHNV infection and found that, cases 1 and 6 were IHHNV infected P. stylirostris; the virus from case 6 did not cause mortality in the infected shrimp whereas virus from case 1 caused mortality. There was single amino acid variation between the two isolates in ORF3 coding for a 47 kDa protein. The change was from leucine to isoleucine, conservative because both are non-polar amino acids with identical molecular weight. Similar argument can be made for the amino acid change that was noticed in the current study, in Vp19 there were 4 amino acid changes and 2 amino acid changes were noticed in Vp28, these might contribute to the virulence of the strain and might result in higher pathogenicity of the Indian isolate. Three out of four substitutions in Vp19 and both the substitutions in Vp28 were not conserved. Hence this could alter the function of the protein, investigating it further might account for the replacements. The variation observed here was not very high; Vp19 (1.08%) and Vp28 (0.21%) when compared to that reported for human parovirus B19 up to 4% (Erdman et al., 1996). Nevertheless it was more than the variation reported for IHHNV in shrimp (0.8%). Screening of few more isolates might give further substantiating evidence supporting this observation.

Sequence comparison have also revealed that the Indian isolate was more closely related to the Taiwan isolate, as there were 4-5 base differences
with the sequence reported by VanHulten et al. (2001). This throws light on the possible origin of this disease in India. For decades, shrimp seeds have been imported from China. Taiwan being the major exporters of shrimps served as a main source of seed for both developing and developed countries. So it is possible that the shrimp seeds obtained from Taiwan would have led to the introduction of this disease in India. There is no concrete evidence so far for this view, the reason for this being the lack of sequence data on the Indian isolate. So far there has been no report on the sequence of Indian isolate. For the first time in India a comparison was made between the foreign and Indian isolates, based on the sequence of 5 different structural genes. It is very clear that the Indian and the Taiwan isolates are more closely related and they share a common ancestor.

This finding is very important in the shrimp culture point of view. It proves beyond doubt that importing seeds from other countries without proper screening may lead to the spread of WSSV. Hence it is advisable to avoid the import of shrimps from foreign countries without proper screening. When we import seeds we are prone to introduce new diseases apart from the existing ones. This might prove deleterious because a seed from the foreign environment may be tolerant to a particular disease but when introduced into the Indian farms it might transmit the disease to the native isolate which might be susceptible to that disease. This might affect the whole crop and spread throughout the area.

After having identified the variation the next objective was to clone and express the structural protein genes. For this vp19 and vp28 were selected, as both these genes code for envelope protein (VanHulten et al., 2002; Zhang
et al., 2002b) and they showed few base changes from the sequences that were already reported. The main idea of purifying the 18 kDa protein from the virus was, it was reported in 5 geographical isolates hence a diagnostic test developed would detect the virus from different geographical locations. But N-terminal sequencing data revealed the protein purified in our lab was different from the already published sequence. Hence an attempt was made to clone and express the vp19 and vp28 genes obtained from the WSSV genomic DNA.

Although previous studies have shown that the WSSV virion is a very effective immunogen (Nadala et al., 1997), such antiserum has several limitations. Among these are the high costs and labor intensive processes of virus propagation and purification. In addition, even exhaustive purification does not completely remove trace amounts of shrimp proteins, which may be immunogenic. The bacterial expression system overcomes these obstacles and the recombinant protein is readily purified through a simple histidine tag purification process. The difference is the ease with which the viral proteins and the recombinant protein are purified is appreciably in favor of recombinant protein. Hence both vp19 and vp28 genes were cloned in pRSET B, T7 expression vector and purified by IMAC.

When the antiserums were checked for its specificity using Western blot it reacted with a protein of molecular weight 19 kDa and 28 kDa respectively in the virus. There was no cross reactivity of the antiserum with any of the uninfected shrimp proteins. There are earlier reports on production of recombinant proteins like the TK TMK in baculoviruses by (Tzeng et al., 2002). But it was a functional protein, among the structural proteins only 24, 26, 28 kDa proteins have been cloned and expressed (VanHulten et al., 2000a).
Functional proteins might not play any role in adhesion of the virus and entry of the virus into the host and it would be advantageous to develop a diagnostic technique based on the structural proteins, as it is only these proteins that are exposed and can be easily identified in an intact virus. (Zhang et al., 2001) reported the development of an immunoassay with recombinant antigen P204, a functional protein. Likewise various other investigators (Tsai et al., 2000b; Liu et al., 2001; Chen et al., 2002; VanHulten et al., 2000a; 2001a) have reported the cloning and expression of viral functional proteins using various vector system and host. (Zhang et al., 2002a; Zhang et al., 2002b) cloned the vp22 and vp28 gene and studied their localization and noted that these were envelope proteins. You et al. (2002) developed a polyclonal antiserum against 27.5 kDa and used it for developing dot blot test to detect WSSV. Poulos et al. (2001) developed monoclonal antibody against 28 kDa protein. But there are reports saying that the use of monoclonal antibody cannot detect the infection to the same extent, as the polyclonal as the lectins in the frozen shrimp samples are known to interfere with the detection (Zhan et al., 1999).

Studies carried out by VanHulten et al. (2001a) showed that it was possible to neutralize the virus using the antiserum raised against the recombinant protein Vp28. Similar reports on the vaccination of shrimps with recombinant rVp26 and rVp28 by Namikoshi et al. (2003) revealed that it was feasible to induce higher resistance in shrimps against WSSV by injecting the recombinant proteins. Hence the recombinant proteins produced in the current study can also be used as a vaccine candidate if vaccination proves to be effective against WSSV.
In this study for the first time in India the WSS viral proteins were cloned and expressed. Both the proteins that has been cloned and expressed in our present study are envelope proteins from the Indian isolate of the WSSV. A diagnostic technique developed using both the antisera might prove effective in identifying the virus with out any false negative results.

CONCLUSION

The aim of this study is to purify the WSSV proteins, raise antiserum against these proteins and develop protein and DNA based diagnostic tests. The WSSV virus was purified by density gradient centrifugation and the DNA was extracted by a non enzymatic method. Sequencing of the PCR product obtained using already established primers, confirmed the authenticity of the virus.

A comparison of the protein profiles of hemolymph and pooled tissue revealed tissue as the ideal organ to get high viral load. The virus was released from the tissue by mere freeze thaw method. Seven major viral proteins were identified by SDS PAGE and the prominent 18 kDa protein was selected for development of diagnostic assay. Antiserum raised against this protein was evaluated by dot blot, Western blot and ELISA. Time course analysis using ELISA and Western blot revealed tail to be the highly infected organ and the virus could be detected as early as 24 hrs post infection. Latex agglutination assay was developed for easy diagnosis of the WSSV infection. The results obtained using latex agglutination was found to be comparable with the PCR indicating that former could be an alternative to PCR because of its simplicity and rapidity. Primers were designed based on the N-terminal sequence data of
the 18 kDa protein. A non stop single tube seminested PCR was developed using these primers to grade the severity of the infection.

It is possible to detect the infection without any ambiguity using these two diagnostic techniques.

*Artemia* and Freshwater crabs were assessed for their carrier status and it was concluded that Freshwater crabs acted as a carrier while *Artemia* did not. Hence *Artemia* can be used as feed for shrimps and not fresh water crabs.

Five structural genes were amplified from the genomic DNA and sequenced. vp19 and vp28 genes showed variation and hence these 2 genes were cloned, expressed and characterized by Western blot. The molecular mechanism of WSSV infection in shrimps is being studied using these recombinant proteins.