A MULTIFACETED STUDY OF WHITE SPOT SYNDROME VIRUS (WSSV)

—A SHRIMP PATHOGEN

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By
Zerong You

Dissertation Committee:

Philip C. Loh, Chairperson
Paul Q. Patek
Roger S. Fujioka
James A. Brock
Yuanan Lu

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ABSTRACT

The white spot syndrome virus (WSSV), which has been associated with major viral epizootics, was first identified in China and Taiwan in 1992 and soon spread in shrimp farms all over the world. The mortality rates for shrimp population experiencing WSSV infections can reach 100% within three to ten days from the onset of the symptoms.

In this study, a WSSV strain was isolated from *P. chinesis* from Qindao, China. The morphology of this WSSV isolate was examined by electron microscope (EM) and compared with other WSSV isolates.

A cDNA library was constructed from white spot syndrome virus (WSSV) infected penaeid shrimp. Among cDNA clones with WSSV sequence insert, twelve clones were sequenced and analyzed. By comparing with the DNA sequence in the GenBank, cDNA clones containing sequence identical to that of WSSV envelope protein VP28 and nucleoprotein VP15 were identified. Poly(A) sites on the mRNAs of VP28 and VP15 were identified.

In the course of the study, a PCR strategy was developed for amplification of double-stranded cDNA and enrichment of abundant cDNA. A screening strategy was established to identify clones with viral cDNA insert from a cDNA library. The cDNA library approach does not require the isolation of virion, which usually is difficult, time-consuming and expensive. The cDNA library method is readily applicable to other viruses and the protocol used in this study can be used with little modification.

Genes encoding major viral structural proteins VP28, VP26, VP24, VP19 and VP15 from five WSSV isolates were sequenced and compared with that of other sequences of WSSV strains available in the GenBank. The genes encoding these major viral structural proteins are identical among WSSV strains isolated from different shrimp species and/or geographical areas. Gene probes developed based on the DNA sequences of viral structural proteins can be used for WSSV diagnostic/identification.

A truncated version of the white spot syndrome virus (WSSV) 27.5kDa envelope protein was expressed as a histidine tag fusion protein in *Escherichia coli*. The bacterial expression system allowed the production of up to 10 mg of purified recombinant protein per liter of bacterial culture. Antiserum from a rabbit immunized with the recombinant protein was found to recognize the 27.5kDa viral envelope protein of WSSV isolated from different geographic regions. The antiserum did not recognize any of the other known WSSV structural proteins. A sensitive immuno-dot assay for WSSV was developed using the specific rabbit polyclonal antiserum.

A wet-format dipstick model was developed for simple and rapid WSSV detection. Different types of membranes were tested as solid support for dipstick assay. The conditions for antibody and microparticle coupling reaction were optimized. The present study would be of value for the development of a dry-format dipstick with detector reagent (antibody and microparticle complex) dried on a conjugate pad, which would be more desirable for field use.

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INTRODUCTION

1.1 BACKGROUND AND MOTIVATION

1.1.1 Background

Modern shrimp farming started in 1930's in Japan. In 1960's and early 1970's, researchers and farmers developed ways to grow shrimp in semi-intensive and intensive ponds. Different penaeid shrimp species were tested for their farming potentials. In the process, the breeding and spawning techniques for most of the farmed species were developed. Grow-out technology, nutrition and disease control of the shrimp were also studied. These efforts laid the groundwork for the shrimp industry which then expanded in the next twenty years. From 1975 to 1985, the world cultured shrimp production grew from 50,000 to nearly 200,000 metric tons. In 1999, the total world production of farm-raised shrimp reached 814,250 metric tons (CP Group, 2000). Currently Thailand is the number one producer in the Eastern Hemisphere while Ecuador tops Western production. China, and Indonesia and India are also major contributors. At present, the USA is the largest consumer of shrimp which contributes to her trade deficit of $3.3 billion.

Several hundred species of shrimp inhabit the waters of the globe. All farm-raised and most of the shrimp caught by fishermen belong to the Penaeidae family of decapod crustaceans. The genus name is *Penaeus*, with the majority of the cultured animals including the following five species: *Penaeus monodon* (Giant tiger shrimp), *Penaeus vannamei* (Western white shrimp), *Penaeus chinensis* (Chinese white shrimp), *Penaeus stylirostris* (Western blue shrimp), *Penaeus japonicus* (Japanese Kuruma shrimp) (available at http://members.aol.com/brosenberr/Species.html).

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With the rapid increase in cultured shrimp production by aquaculture practices, a major problem created was infectious disease, especially of viral etiology. Viral epizootics have caused major "crashes" in Taiwan (1987-1988), China (1993-1994), Indonesia (1994-1995) and India (1994-1996). In the Western Hemisphere, Ecuador (1993-96) and Honduras (1994-97) have suffered significantly from viral diseases (available at http://members.aol.com/brosenberr/About.html). At present there is no effective chemotherapy available for shrimp viral diseases. While some 22 shrimp viruses have been described, only a few, such as White Spot Syndrome Virus (WSSV), Yellow Head Virus (YHV), Taura Syndrome Virus (TSV), and Infectious Hypodermal Hematopoietic Necrosis virus (IHHNV) have caused massive mortalities in cultured shrimp (available at www.oie.int/eng/normes/fmanual/A_00045.htm). TSV was the biggest "killer" in South America prior to the arrival of WSSV in 1999. WSSV and YHV emerged in the 1990s in Asia and now are present in shrimp farms worldwide. The estimated total loss due to wide spread viral epizootics has averaged about $1 billion (U.S.) annually since 1994. In the United States, outbreaks of shrimp viral diseases have caused some 50-95% loss of production in affected shrimp farms (Aquaculture 1999; available at www.aphis.usda.gov/vs/aqua/wssv.html).

1.1.2 Motivation

Today viral pathogens continue to pose a serious threat to the worldwide shrimp aquaculture industry. At present there are no simple solutions to the viral problem. Although the development of Specific-Pathogen-Free (SPF) shrimp broodstock has contributed to the reduction of the occurrence of viral diseases, such animals are still susceptible to shrimp viral pathogens, such as WSSV, YHV and TSV. The SPF

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broodstocks are animals determined by the laboratory to be free from any of the OIE/USMSFP specified pathogens (Office International Des Epizooties/the U.S. Marine Shrimp Farming Program) of which at least 3 are viruses.

For the effective control and prevention of shrimp viral diseases, a multifaceted approach is required. Parameters, such as water quality, healthy broodstock, nutrition, proper waste disposal, pollution control, *etc.* are all needed, but crucial to success is the requirement of a quality monitoring program for viral pathogens employing the most efficient detection/diagnostic technologies. These technologies would identify infected animals long before they show clinical disease, particularly the asymptomatic or inapparent carriers of viruses amongst the broodstocks. Furthermore, rapid detection of infectious outbreaks are very important for better management of diseases and also to prevent the dissemination of viral pathogens. While a number of effective nucleic acid - and immunologically - based detection/diagnostic technologies have been developed, they are still not cost-effective and are not practical for routine employment by the average hatchery and shrimp farm. Urgently needed are detection/diagnostic methodologies which are simple, rapid, specific, sensitive, reliable, require minimal equipment and of supreme importance, low in cost.

The present study is directed to the development and evaluation of a detection/diagnostic technology that will meet these criteria. In this study, the WSSV will be used as the model virus system since its epizootics are the current major plaque to the sustainability of the worldwide cultured shrimp industry. This study is divided into to two parts.

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Part I

a). The isolation and characterization of the genes encoding the major structural proteins of WSSV, such as vp27.5 kDa and the overexpression of the gene contained in a plasmid d vector construct in a host bacterial system, *E.coli*.

b). The purification and immunological characterization of the recombinant structural viral proteins, such as vp27.5 kDa of WSSV.

Part II

The employment of the recombinant structural viral protein vp27.5 to develop immunologically-based detection/diagnostic technologies for WSSV which would be simple, rapid, specific, sensitive, reliable, field-friendly and importantly affordable. The followings are the objectives of this study:

1. To isolate and identify the genes encoding the major structural proteins of WSSV, such as vp27.5kDa.

2. To construct a plasmid vector containing the gene and to overexpress the viral structural protein in *E. coli*.

3. To purify the vp27.5kDa recombinant protein.

4. To produce polyclonal antibody using the recombinant WSSV viral protein as antigen.

5. To initially develop and evaluate an immuno-dot assay for WSSV detection using the polyclonal antibody against vp27.5kDa protein.

6. To develop and optimize an immunochromtographic device or a membrane-based dipstick virus detection system using antibody-coated colored latex beads and nitrocellulose membrane.

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1.2 LITERATURE REVIEW

1.2.1 Shrimp viruses

Twenty-two shrimp viruses have been described, but only a few have been studied in detail. Penaeid shrimp viruses belong to at least six families. The viral families are *Parvoviridae, Baculoviridae, Rhabdoviridae, Reoviridae, Togaviridae,* and *Iridoviridae* (Loh *et al.*, 1997). There are also some ungrouped viral isolates (Loh *et al.*, 1997). This number is rising as the studies of penaeid shrimp viral diseases result in more viruses being isolated. Some viruses can infect all cultured and wild penaeid shrimp species while others infect only certain species. Certain viruses are especially virulent to penaeid shrimp at certain growth stages. Several viruses have been implicated in massive mortalities in cultured shrimp, such as White Spot Syndrome Virus (WSSV), Yellow Head Virus (YHV), Taura Syndrome Virus (TSV), and Infectious Hypodermal Hematopoietic Necrosis Virus (IHHNV).

Taura Syndrome (TS) was first identified in *Penaeus vannamei* in commercial shrimp farms located near the mouth of the Taura River in the Gulf of Guajaquil, Ecuador in June 1992 and TS was initially attributed to toxicity of a fungicide used in banana plantations adjacent of affected shrimp farms (Jimenez, 1992; Lightner *et al.*, 1994; Wigglesworth, 1994). The characteristics of the disease are necrosis and nuclear pyknosis of the cuticular epithelium of the general body surface, appendages, gills, mouth, esophagus, stomach and hindgut (Brock *et al.*, 1995; Lightner *et al.*, 1995; Lightner *et al.*, 1996). Subsequent studies identified that TS was caused by a virus – Taura Syndrome Virus (TSV) (Brock *et al.*, 1995; Hasson *et al.*, 1995). TSV has been classified as a member of the genus *Cricket paralysis-like viruses* (CrPV) (Mari *et al.*,

2002). TSV is a non-enveloped icosahedral virus. Its genome is a positive sense, linear, single-stranded RNA which is polyadenylated at the 3'end. The size of the genome is approximately 9 kb in length. The viral structural proteins include three major (55 kDa, 40 kDa and 24kDa) and one minor (58 kDa) protein. The site of viral replication is cytoplasm (Bonami et al., 1997). The disease has peracute and recovery or chronic phases. Peracutely affected animals usually die during the process of molting. Those that survive molting either recover or are chronically affected by TSV. Since the TS outbreak in 1992, TSV has been spread throughout the Americas (Lightner 1996). TSV is highly infectious and lethal to P. vannamei. In contrast, P. stylirostris and P. setiferus were found to be resistant to experimental challenge with TSV (Loh, 1999).

Yellow-head disease was first reported by Limsuwan (1991). The principle species affected is Penaeus monodon, which is commonly cultured in Thailand and other Southeast Asian countries. It has caused massive losses among shrimp farms in Thailand. The infectious agent was initially identified as a baculo-like virus called Yellow Head Virus (YHV) (Boonyaratpalin et al., 1993; Chantanachookin et al., 1993). The shrimp infected with YHV often show light yellow coloration of the cephalothorax and gills, and have a pale or bleached appearance (Limsuwan, 1991). The cumulative mortality rate is near 100% within 3-5 days after appearance of clinical signs (Boonyaratpalin et al., 1993). Later work showed that YHV is actually an RNA virus that resembles rhabdoviruses or coronavirses (Wongteerasupapy et al., 1995), rather than baculoviruses (Boonyaratpalin et al., 1993; Chantanachookin et al., 1993). YHV is an enveloped virus with bacilliform shape. The size of virion measures 150-200 nm x 40-50 nm. The site of viral replication is cytoplasm (Boonyaratpalin et al., 1993;

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Chantanachookin *et al.*, 1993). Genome analysis of YHV showed an unsegmented, single-stranded RNA. The RNA genome is ~22 kb (Nadala *et al.*, 1997a) with positive polarity (Tang *et al.*, 1999). SDS-PAGE analysis of YHV virion revealed four major proteins with the following estimated molecular weight: 170, 135, 67, and 22 kDa. The 135 kDa protein was determined to be glycosylated (Nadala *et al.*, 1997a). In addition to the original host, *P. monodon*, YHV has also been shown to infect and cause disease in other species of penaeid shrimp, such as *P. stylirostris* and *P. vannamei*, which are commonly cultured in Hawaii and elsewhere in the western hemisphere (Lu *et al.*, 1994; Lightner 1996).

Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) was first detected in juvenile *P. stylirostris* from Hawaii in 1981 (Lightner *et al.*, 1983a, b) and has subsequently been detected in cultured and wild penaeid shrimp worldwide (Lightner *et al.*, 1983b; Lightner *et al.*, 1996). IHHNV may infect all penaeid shrimp species (Lightner *et al.*, 1985). It only causes massive mortality of juveniles of cultured and wild-caught *P. stylirostris* (Weppe *et al.*, 1992; Morales-Covarrubias *et al.*, 1999; Pantoja *et al.*, 1999). Gross clinical signs of IHHNV are not specific. The juvenile *P. stylirostris* with acute IHHN show reduced food consumption, fouling of the cuticle, appendages, and gills. The infected juvenile *P. stylirostris* swim lethargically near the water surface, and then become motionless, roll over and sink slowly to the bottom. The mortality rate is about 90% (Bell *et al.*, 1984; Lightner *et al.*, 1983a). Some of the penaeid shrimp species may carry the virus without any apparent symptom. Individuals with asymptomatic IHHNV infections have been found among farm-raised *P. vannamei* (Lightner *et al.*, 1983b; Kalagayan *et al.*, 1991). IHHNV is a non-enveloped, very small

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(20-22 nm in diameter) icosahedral virus. It replicates in the nuclei of host cell. The genome of IHHNV is a linear, single-stranded DNA of either negative or positive polarity with estimated size of 4.1 kb (Bonami *et al.*, 1990; Mari *et al.*, 1993). There are four known structural proteins, VP1 to VP4, with molecular weights of 74, 47, 39, and 37.5 kDa. IHHNV belongs to the *Parvoviridae* family (Bonami *et al.*, 1990).

The WSSV, which has been associated with major viral epizootics, was first identified in China and Taiwan in 1992 (Chen, 1995). The virus is also known as Hypodermic and Hematopoietic Necrosis Baculovirus (HHNBV) (Huang *et al.*, 1995), Rod-shaped Nuclear Virus of *Penaeus japonicus* (RV-PJ) (Inouye *et al.*, 1994), Systemic Ectodermal Mesodermal Baculovirus (SEMBV) (Wongteerasupaya *et al.*, 1995; Hameed *et al.*, 1998), White Spot Baculovirus (WSBV) (Wang *et al.*, 1995), and Chinese Baculovirus (CBV) (Lu *et al.*, 1997; Nadala *et al.*, 1997b). The WSSV has spread to most shrimp-growing countries in Asia, such as Japan, Korea, Thailand, Malaysia, Indonesia, India, Vietnam, and the Philippines. (Inouye *et al.*, 1994; Nakano *et al.*, 1994; Cai *et al.*, 1995; Winaro, 1995; Flegel *et al.*, 1995; Park *et al.*, 1998; Heo *et al.*, 1998; Mohan *et al.*, 1998; Magbanua *et al.*, 2000; Corsin *et al.*, 2001, 2002). Although Asian in origin, the WSSV was reported in the U.S. in 1995 in shrimp farms in Texas and subsequently in South Carolina (Lightner *et al.*, 1997; Nadala *et al.*, 1998a). Since late January 1999, WSSV has been detected in tissue samples of cultured shrimp from Nicaragua, Honduras, Guatemala, and Panama (Aquaculture, 1999). To date, the WSSV can infect all species of cultured penaeid shrimp and prawns. It is an especially lethal disease of special concern because it has been detected in a number of crustacean species like amphipods, crabs (Lo *et al.*, 1996a) and crayfish (Richman *et al.*, 1997). The disease

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typically occurs in juvenile shrimp but sometimes manifests itself in adult stages. Clinical signs include shell spotting from abnormal deposits of calcium salts, a reddish discoloration due to expansion of cuticular chromatophores. When farmed-shrimp are infected, they become lethargic, stop feeding, swim slowly near the pond surface, and eventually sink to the bottom and die. The mortality rates for shrimp population experiencing WSSV infections can reach 100% within 3 to 10 days from the onset of the symptoms (Lightner, 1996).

The WSSV is a double-stranded DNA virus with a genome size of approximately 290 kb (Yang et al., 1997; van Hulten et al., 2001). The electron microscopic (EM) studies showed that WSSV is an enveloped virus with a rod-shaped nucleoprotein core measuring 316 to 350 nm in length and ~ 65nm in diameter (Nadala et al., 1998b). The route of transmission of WSSV appears to be the water-borne-oral feeding routes (Loh et al., 1997). The primary route of the viral entry is the gut and gill of the shrimp with the WSSV replicating primarily in the lymphoid organs and gill (Chang et al., 1996). The site of replication and maturation of the virion occurred in the nucleus (Loh et al., 1997) of the infected cell. The in vitro replication of WSSV in primary shrimp lymphoid cell cultures has been reported (Tapay et al., 1997). The WSSV virion contains at least four major viral structural proteins; these are comprised of three outer non-glycosylated membrane proteins (19, 27.5 and 75 kDa) and one a 23.5 kDa capsid protein (Nadala et al., 1998b). Employing western blot analysis the 27.5 kDa (vp27.5kDa) protein was found to appear as early as 41 hrs post infection (p.i.) in experimentally infected shrimp well before the appearance of any characteristic clinical symptoms (72 h p.i.) (Nadala et al., 1997b). In vitro studies employing primary shrimp lymphoid cell cultures reaffirmed

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the early appearance of WSSV vp27.5kDa. Also, the protein was found to be highly

immunogenic in mammals. The early appearance of vp27.5kDa protein before the

appearance of any clinical symptoms and also its ability to induce antibody in rabbit

indicated the applicability of this viral protein as an early effective bio-indicator of

WSSV infections.

1.2.2 Technologies developed for shrimp viruses detection

Earlier diagnostic procedures for virus infection of penaeid shrimp were largely

dependent upon clinical history and conventional histopathological examination using

light and transmission electron microscopy (Lightner *et al.*, 1998). The clinical history

includes an account of the disease at the farm or in the region, the source of brood stock,

type of feed, environmental conditions, and any relevant events. Shrimp infected by

virus may show a number of gross or clinical signs, such as visible lesions, abnormal

behavior and growth, loss of appetite, stop feeding, *etc*. Characteristic histological

changes have been used for diagnosing certain shrimp virus infections (Lightner *et al.*,

1995). In the mid-1970's, transmission electron microscopy (TEM) was applied as a

diagnostic tool by shrimp pathologist. By using TEM, Couch (1974a, b) determined that

the unusual tetrahedral bodies of the hepatopancreas of *P. duorarum* and *P. aztecus* were

due to the infection of the hepatopancreas by baculovius which formed occlusion body.

This penaeid shrimp virus was named *Baculovirus penaeid* (Couch 1974b). Electron

microscopy study provided the evidence of WSSV infection in Kuruma shrimp in Japan

(Takahashi *et al.*, 1994) and cultured shrimp in Taiwan (Chou *et al.*, 1995). The practical

application of these procedures was sometimes difficult and sensitivity was poor.

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In order to detect asymptomatic and subclinically-infected animals, labor intensive and non-cost effective bioassay using live shrimp were performed. For example, a bioassay was used to detect the IHHN virus in asymptomatically infected shrimp imported into Hawaii (Lightner *et al.*, 1983b, Bell *et al.*, 1984). Since the mid-1990's, such asymptomatically viral-infected animals have been detected by molecular-based methods, which include nucleic acid probe and the polymerase chain reaction (PCR). These methods are highly specific and sensitive. The application of non-radioactive labeling methods has made nucleic acid probe technology readily available for shrimp virus diagnosis. The nucleic acid probes have been widely used in *in situ* hybridization, Southern blot assay and DNA-dot blot assay. There are two systems to label the nucleic acid. In the direct method, the detectable molecule (fluorescein or other fluorochromes) was directly coupled to the nucleic acid probe so that probe-target hybrid can be visualized under a fluorescent microscope immediately after the hybridization reaction (Baumann *et al.*, 1980; Baumann *et al.*, 1984; Renz *et al.*, 1984). The indirect system requires the probe to contain a reporter molecule, which can be introduced chemically or enzymatically such as biotin (e.g. Langer *et al.*, 1981) and digoxigenin (DIG) (Boehringer Mannheim). In principle enzymatically-labeled nucleic acids with biotin-dUTP can be detected by anti-biotin antibodies. However, streptavidin is more frequently used because of its high binding capacity for biotin (Boehringer Mannheim). Hybridized DIG-11-dUTP labeled probe may be detected with high affinity anti-DIG antibodies that are conjugated to alkaline phosphatase, or horseradish peroxidase, *etc.* (Boehringer Mannheim). Detection sensitivity depends upon the method used to visualize the anti-DIG antibody conjugate.

In situ hybridization technique was originally developed by Pardue and Gall (1969) and (independently) by John *et al.* (1969). It allows specific nucleic acid sequence to be detected in morphologically preserved cells or tissue sections. It is used in the laboratories as one of the confirmatory diagnostic methods. Generally, the nucleic acid probe was generated by polymerase chain reaction (PCR) or cloning certain viral genomic fragment. The first nucleic acid probe for the diagnosis of the IHHNV was developed in 1993 (Mari *et al.*, 1993). As a single-stand (ss) DNA virus, large majority of minus-strand DNA is incorporated into the capsid of IHHNV. A small amount of re-annealed plus- and minus-strands obtained after nucleic acid extraction were blunt-ended and cloned into the pUC18/*Escherichia coli* strain DH5α cloning system. Selected clones were studied and characterized using restriction enzymes. One of them, BQ31, was used to construct different sized probes labeled with DIG-11-dUTP. The probes reacted strongly with dilutions of homogenized IHHNV-infected shrimp tissues and, conversely, did not react with uninfected shrimp tissues *in situ* (Mari *et al.*, 1993). DIG-labeled DNA probes have been used in *in situ* hybridization for detection of other DNA viruses, such as baculovirus penaeid (BP) (Bruce *et al.*, 1993), *Penaeus monodon*-type baculovirus (MBV) (Poulos *et al.*, 1994), WSSV (Chang *et al.*, 1996; Durand *et al.*, 1996; Nunan *et al.*, 1997, Wang *et al.*, 1998; Chen *et al.*, 2000) and hepatopancreatci pavovirus (HPV) (Mari *et al.*, 1995; Pantoja *et al.*, 2001). For RNA virus such as YHV and TSV, the cDNA has been synthesized and labeled as gene probe for viral detection (Tang *et al.*, 1999; Hasson *et al.*, 1997).

Southern blot assay and DNA-dot blot hybridization assay allow detection of the specific sequence of genomic DNA or DNA fragment which has been cross-linked to a

solid support, such as nitrocellulose or nylon membrane. In both assay, the genomic DNA of suspected viral infected tissue or viral genomic DNA need to be isolated. As in Southern blot assay, the genomic DNA will be digested by restriction enzyme(s). The DNA fragments will be separated by agarose gel electrophoresis, transferred to a nylon membrane, cross-linked to the membrane by UV, and denatured by NaOH. During hybridization, DIG-11dUTP labeled DNA probe will bind to complimentary DNA sequence on the membrane. Usually, anti-DIG antibody couple with alkaline phosphotase is used to detect the signal. The system is very sensitive. Theoretically, it could detect 1pg of target DNA. But Southern blot has not been widely used in shrimp viral detection. It only has been used to study the genetic difference among the different shrimp virus isolates. It has been applied to distinguish between the WSSV isolate from crayfish and the WSSV isolates from penaeid shrimp (Wang *et al.*, 2000). Dot blot hybridization assay using either tissue homogenate or extracted DNA has been developed for WSSV detection (Chang *et al.*, 1998; Lo *et al.*, 1999). The sample needs to be initially boiled for 10 min then quickly chilled on ice in order to denature the DNA and finally applied onto the nylon membrane. The nylon membrane will be allowed to air-dry and subjected to 2 min of UV cross-linking. The subsequent detection steps will be carried out as that of a Southern blot. Compared to *in situ* hybridization and Southern blot, the dot blot assay has similar sensitivity and specificity, but it is faster and more convenient.

The highly sensitive PCR procedure has been adopted for shrimp viral detection since 1990s (Lo *et al.*, 1996b). PCR is accomplished by using oligonucleotide primers to target specific genomic segments of the virus. Different PCR methods have been

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developed to increase sensitivity and specificity. One-step PCR is to amplify the template in a single reaction. It has been used to detect and screen for shrimp viruses by several laboratories. In 1993, Chang *et al.* developed a set of oligonucleotide primers for the amplification of a DNA fragment of Penaeus monodon-type baculovirus (MBV). The method was applied to detect MBV-infected postlarval of *P. monodon* (Chang *et al.*, 1993, Hsu *et al.*, 2000). The same strategy has been used in the detection/diagnosis of other DNA viruses of shrimp. The WSSV has been detected in postlarval of *P. monodon* from Thailand (Withyachumnamkul, 1999), Taiwan (Hsu *et al.*, 1999; Peng *et al.*, 2001), Vietnam (Corsin *et al.*, 2001, 2002) and in many other areas from various shrimp species at different developmental stages. The PCR has also been used in studying experimental transmission of WSSV from crabs to shrimp *P. monodon* (Kanchanaphum *et al.*, 1998). One-step PCR has also been used in detection of HPV (Sukhumsirichart *et al.*, 1999; Pantoja *et al.*, 2000; Phromjai *et al.*, 2001) and IHHNV (Nunan *et al.*, 2000) in penaeid shrimp. Sometimes, two-step PCR was employed to increase detection sensitivity. In two-step PCR, the product of the first PCR will be used as the template for amplification. This method has been applied to screen WSSV in *P. monodon* brooders in Taiwan (Hsu *et al.*, 1999). A two-step PCR protocol has been used to detect at least four geographic isolates of WSSV from both experimentally- and naturally- infected shrimp (Tapay *et al.*, 1999). By combining two-step PCR and Western blot, researchers have reported for the first time the occurrence of WSSV in the Philippines (Magbanua *et al.*, 2000). Nested PCR can improve detection specificity by making a second round of amplification using primers placed internal of the first pairs of primers. These internal primers are called "nested" primers. The nested primers bind within the first PCR product and produce a

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second PCR product that will be shorter than the first one. The logic behind this method is that if the wrong sequence was amplified by mistake, it would be almost impossible to be amplified a second time by a second pair of primers. Nested PCR assay described by Belcher *et al.* (1998) can detect eight viral genome equivalents of MBV. A semi-nested PCR is a nested PCR with just one of the primers internal to the primers used in the first round. This method does not add as much specificity as nested-PCR, but it will add enough specificity to get a specific PCR product in certain applications. A semi-nested PCR method can detect as little as 5 fg of WSSV DNA, which is equavalant to 20 viral particles in crude extracts of postlarval samples or in extracts of pleopods and haemolyph from larger shrimp (Kiatpathomchai *et al.*, 2001). Reverse transcription polymerse chain reaction (RT-PCR) has been developed for the detection of RNA-containing shrimp viruses. RT-PCR uses either avian myeloblastosis virus (AMV) or Moloney murine leukemia virus (MMLV or MuLV) reverse transcriptases for the synthesis of first strand cDNA. Second strand cDNA synthesis and subsequent PCR amplification is then performed with *Taq* DNA polymerase. RT-PCR has been applied to the detection of TSV (Nunan *et al.*, 1998) and YHV (Tang *et al.*, 1999). By using reverse transcription followed by nested PCR, a highly sensitive test was developed to detect Australian gill-associated virus (GAV) and lymphoid organ virus (LOV) of *Penaeus monodon* (Cowley *et al.*, 2000).

PCR can be used not just for the detection of specific sequences, but also for their quantification. There is a quantitative relationship between the amount of starting target sequence and the amount of PCR product at any given cycle that falls within the reaction's exponential range. Real-time quantitative PCR is a powerful tool that can be

used for gene expression analysis, genotyping, pathogen detection/quantification, mutation screening and DNA quantification (Gibson *et al.*, 1996; Heid *et al.*, 1996). All real-time PCR systems rely upon the detection and quantification of a fluorescent reporter (Livak *et al.*, 1995), the signal of which increases in direct proportion to the amount of PCR product in a reaction. The signals are monitored as they are generated and are tracked after they rise above background but before the reaction reaches a plateau. Initial template amount can be calculated by analyzing the shape of the curve or by determining when the signal rises above some threshold value. In the simplest and most economical format, that reporter is the double-strand DNA-specific dye SYBR® Green (Molecular Probes, Eugene, Oregon). SYBR Green binds double-stranded DNA, and upon excitation emits light. Thus, as a PCR product accumulates, fluorescence increases. Recently, this technique has been applied in the detection and quantification of IHHNV and WSSV. When with genomic DNA as template, SYBR Green PCR was found to be 100- to 2000-fold more sensitive than conventional PCR (Dhar *et al.*, 2001; Tang *et al.*, 2001).

The advantages of PCR detection are the followings: the sample can be DNA extract or tissue homogenate; only very small amount of template is needed for the assay; when real-time PCR is applied, the number of viral genome in the sample can be quantified. It should be noted that PCR may occasionally yield false positive results. Care needs to be taken to prevent cross contamination.

Cell culture methods are well developed in mammalian systems and have been applied to isolate and identify many animal viruses. Cell culture systems have been used to study the biochemistry of animal virus replication and also employed as a diagnostic

tool. For the invertebrates, insect cells have been successfully cultured *in vitro* as continuous cell lines for decades and used to study insect viruses (Vago, 1971; Maramorosch *et al.*, 1982). Although numerous attempts have been undertaken to establish a continuous cell line from crustaceans, such as shrimp, crab, lobster, and crayfish, none have been succeeded. The current lack of continuous shrimp cell lines suitable for isolation and growth of shrimp viruses is an obstacle to diagnosis of viral diseases of shrimp. The first primary shrimp cell culture was developed from tissues of *Penaeus monodon* (Chen *et al.*, 1986). Since then, an increasing number of primary cell cultures obtained from various shrimp organs have been reported (Nadala *et al.*, 1993; Itami *et al.*, 1999; West *et al.*, 1999; Fraser *et al.*, 1999; Mulford *et al.*, 2000; Shimizu *et al.*, 2001). A primary cell culture was employed to isolate a new virus rhabdovirus of penaeid shrimp (RPS) from IHHNV infected penaeid shrimp (Lu *et al*, 1991). Primary shrimp lymphoid cell cultures were used for the first time in an *in vitro* assay to quantify YHV (Lu *et al.*, 1995a,b) and subsequently used to determine the titer of WSSV from naturally infected *Penaues japonicus* and experimentally infected *Penaeus stylirostris* (Tapay *et al.*, 1997). Monolayer cultures established from ovary, heart, lymphoid tissue and peripheral hemocytes of penaeid shrimps including *Penaeus monodon, P. japonicus* and *P. penicillatus* were used to study their susceptibility to WSSV and YHV (Chen *et al.*, 1999).

During the mid-90s, immunological-based methods also played an important role in shrimp virus detection. YHV was detected by nitrocellulose-enzyme immunoassay (Lu *et al.*, 1996) and Western blot using anti-YHV polyclonal antibody (Nadala *et al.*, 1997b) and monoclonal antibody (Sithigorngul *et al.*, 2000). Anti-WSSV polyclonal

antibodies were developed and used in Western blot (Nadala *et al.*, 1997b; Hameed *et al.*, 1998) and the immuno-dot assay (Nadala *et al.*, 2000) for the detection of virus. Although the commercial development and routine application of these methods for WSSV have lagged behind the development of nucleic acid-based detection methods, the use of immunological-based tests employing either polyclonal or monoclonal antibodies will become more common in the detection of WSSV and other shrimp viruses. Because of their speed, versatility, relatively low cost, simplicity and relatively good sensitivity, immunological methods, such as membrane-based dipstick test, are potentially very useful for the routine detection and diagnosis of shrimp viral infections.

1.2.3 The background and application of dipstick assay

Over the past ten years, many rapid membrane-based diagnostic test kits that utilize the principles of immunochromatography have been commercialized. The membrane-based tests have broad applications in clinical test, agriculture, environmental assessment, veterinary, drug abuse test, *etc.* Unlike the expensive instrumented methods, the low cost strip tests are simple, fast, and sensitive. The market demand continues to grow at a very quick pace as the applications of the tests increase. The immunochromatographic test industry is a multimillion-dollar industry world wide.

The immunochromatographic devices come in a wide variety of designs with a diverse assortment of housings. Most tests are based on one of two simple formats: flow-through and lateral-flow. The flow through or immunoconcentration format employs solid phase technology that was developed in the mid 1980's. Briefly, the antigen is immobilized on a porous membrane. The specimen flows through the membrane and is adsorbed onto an absorbent pad. A dot or a line visibly forms on the membrane when

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developed with a signal reagent. The signal reagent can be enzyme-linked colorimetric substrate or antibody conjugated with colored particles. The first developed test of this format is (human) Chroionic Gonadotropin (hCG) for the detection of pregnancy. This type of immunoassays is rapid, reasonably sensitive, but has some drawbacks. The flow-through tests require several steps for the addition of specimen, wash buffers, and signal reagent. The reagents used typically require refrigeration. These requirements may lead to potential error and are therefore usually restricted to professional use.

The most recent development is the lateral-flow format. It is also called immunochromatographic strip or dipstick test and the test requires only one step. It is widely used in physician-office assays as well as in over-the-counter tests (e.g. Unipath's Clear Blue pregnancy test). The ability to manufacture nitrocellulose (NC) membrane of large pore size makes it possible to develop the dipstick test. In dipstick test, the lateral flow of fluids through the NC membrane enable the application of various immunoreactant at different locations along a membrane strip. Different types of dipstick follow the same principle. A ligand specific for the analyte (normally an antibody) is immobilized to the membrane as a capture line. The detector reagent, typically an antibody coupled to latex or colloidal metal, is deposited into the conjugate pad. When specimen (urine, plasma, whole blood, *etc*) is added to the sample pad, it rapidly wets through the conjugate pad and the detector reagent is solubilized. The detector reagent begins to move with the sample flow front up the membrane strip. Analyte that is present in the sample will be bound by the antibody that is coupled to the detector reagent. As the specimen passes over the zone to which the capture reagent has been immobilized,

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← Capture line

antibody(Ab)   latex bead(Lb)   Ab-Lb conjugate

Ab-Lb conjugate   antigen(Ag)   Ag-Ab-Lb complex
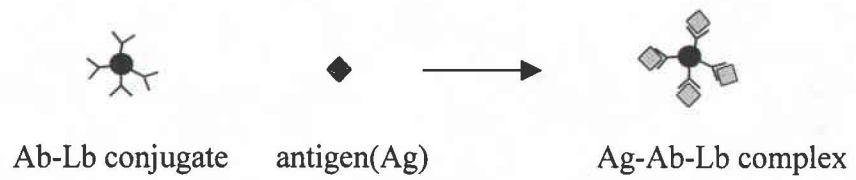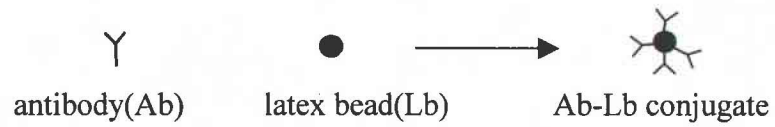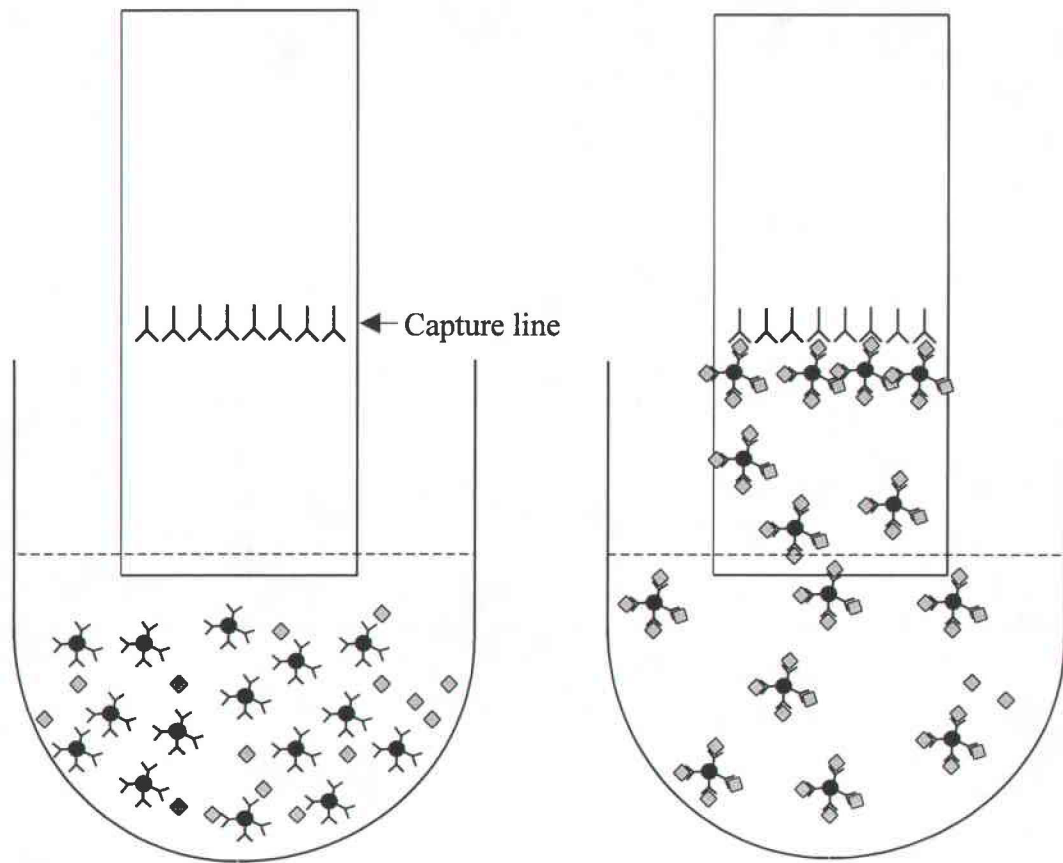
Figure 1. A dipstick assay using latex bead as detector agent.

the analyte detector reagent complex is trapped (Figure 1). A positive reaction results in a visual line on the membrane. The color develops in proportion to the amount of analyte present in the sample. Some of the test device also has a procedural control line. In this case, a visual line at test and control sites indicates a positive test result, a line only at control location indicates a negative test result. The absence of a line at the control site means the test is invalid. Most lateral-flow tests require no additional equipment or refrigeration. The test results can be obtained in less than 15 minutes. Some test strip is encased in a plastic cartridge. Others are usually found free of any plastic housing, such as pH test strips or environmental test strips.

The principle of the dipstick is immunochromatography. The following factors should be considered when developing the dipstick assay: sensitivity, specificity, assay time requirement, and the stability of the final product. Depending on the application of the dipstick, sometimes one of the factors has to be sacrificed in order to improve the other. The reagent required include nitrocellulose membrane, which is a solid phase support for the assay. The monoclonal or polyclonal antibody will be used as capture antibody. The microparticles, such as latex bead and colloidal gold, will be used as detector reagent.

A variety of polymeric microporous membranes have characteristics that meet some or all of the requirements to be used as a solid phase in immunoassay. Nitrocellulose membrane (NC), Nylon membrane, and polyvinylidene fluoride (PVDF) membrane all have protein-binding capacity in an acceptable range for immunoreaction. For dipstick assay, NC membrane is the best choice. NC membrane has a higher average protein binding capacity than PVDF. The use of the hydrophobic PVDF membrane in

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dipstick is problematical because the membrane must first be wetted with nonaqueous solvent e.g. methanol. This characteristic renders PVDF membranes unusable for dipstick assay. Even though Nylon membrane has the highest average protein binding capacity among three membranes, the higher background absorbance of the nylon membrane seriously compromises its use in dipstick assay. Finally, the immobilization of capture antibody of these two membranes is not as simple or rapid as the preparation of NC membranes. The binding of proteins to NC is extremely fast and therefore preparation of the membrane is very rapid. NC membranes are the best choice for the dipstick assay since they can be manufactured with a variety of pore sizes, yielding membranes with different flow properties and protein binding capacity. The membranes also may be cast on nonporous plastic.

In dipstick assay, antibody-coated dyed particles are employed to give a direct visual signal. This detection system eliminates the need for the addition of enzyme substrates. The antibody could be either monoclonal or polyclonal antibody. The antibody for conjugation and capture could be the same or two different antibodies. The dipstick test has the beauty of simplicity, sensitivity, and low cost. Dipstick tests have been used widely in the detection of bacterium, virus, parasite, hormone, and illegal drug use. The test specimens can be saliva, whole blood, plasma, urine, and environmental sample, *etc*. Numerous diagnostic and medical products makers manufacture different types of dipstick devices for the market. Among the manufactures, Becton Dickinson and Co (Franklin lakes, NJ) and Unilever (London and Rotterdam) are the two biggest suppliers.

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Dipstick tests have been widely used in the detection and diagnosis of infectious diseases. There are disptick tests on the market for *Streptococcus pyogenes*, *H. pylori*, Mycobacterium. There are also test kits developed or being developed for bacteria causing food contamination, such as *Salmonella*, *E.coli* O157. Among them, the Strep A dipstick test (e.g. BD Diagnostic Systems, Maryland) is well developed and widely used. *S. pyogenes* is the most common bacterial agent associated with upper respiratory tract infectious in humans. Early diagnosis and treatment has been shown to reduce the severity of symptoms and further complications such as rheumatic fever and glomerulonephritis (Wannamaker, 1983, Still, 1995). The conventional methods used for identification of group A strep depend on the isolation and identification of the organism on blood agar plates. These methods usually require 18-24 hours of incubation at 37°C. This delay in identifying group A strep often causes physicians to administer therapy without first knowing the etiological agents. With the development of immunological tests capable of detecting group A streptococcal antigen directly from throat swabs rapid test results can now be achieved for better patient treatment. The principle of the test employs a unique combination of anti-Strep A antibody using a colloidal gold dye conjugate (or colored latex bead) and solid phase anti-Strep A antibodies to selectively identify group A streptococcus with high degree of sensitivity and specificity. Becton Dickinson's LINK2™ StrepA rapid test kit for the detection of Group A strep is one of the many detection kits on the market. This test kit is a dipstick assay with a built-in procedural control. The overall accuracy is 95.8%. The test results can be ready in one min. The diagnosis of the infection should be evaluated with other clinical information.

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The dipstick assays for animal and human virus detection are based on one of the following two principles. First, the test kits may be designed by using the specific anti-viral antibody to detect the virus itself or viral protein in the specimen. The test kits for Rotavirus detection from the fecal samples of animal and human belong to this category (e.g. Adeno/Rota STAT-PAK). The Hepatitis B rapid test selectively identifies HbsAg associated with Hepatitis B viral (HBV) infection. HbsAg is a large complex of viral envelope protein and has become a useful diagnostic marker of HBV infection. Second, the test kits are developed to detect the antibody in the blood produced by viral infection. Dipstick tests have been designed to screen HIV infection by detecting the HIV antibody in the blood sample.

Probably 30-40% cases of infectious diarrhea are caused by viruses (Medical Encyclopedia, available at www.nlm.nih.gov/medlineplus/ency/article/000252.htm). Rotaviruses group A is the most common cause of acute and severe infectious diarrhea in children. It has been estimated that more than one million rotavirus-associated deaths among infants and young children occur annually worldwide. Beside Rotaviruses, a significant number of such cases are due to Adenovirus (types 40/41). An initial screen in such cases should be able to detect both viruses. The Adeno/Rota STAT-PAK is a dipstick screening test to detect Adenovirus and Rotavirus antigens in human feces. The test was developed by Chembio Diagnostic Systems, Inc. (Bedford, New York). Monoclonal antibodies against Adenovirus and Rotavirus are conjugated to the blue and red latex particles respectively. Capture antibodies to these viral antigens are immobilized in the TEST area of the dipstick. The dipstick is put in the test tube containing a sample. If the stool sample extract contains Adenovirus and/or Rotavirus,

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they will form an antigen-antibody complex with the dye particles. As this complex migrates along the dipstick to the TEST area, the colored lines (Blue line: positive Adenovirus; red line: positive Rotavirus) are formed indicating a positive test. The remaining conjugate migrates to the CONTROL area of the dipstick forming a dark blue line. This indicates proper performance of the test.

Rapid HIV tests are widely used in developing countries where laboratory resources are limited (Branson, 2000). In developed countries, immediate HIV test results are required in order to make treatment decisions and to assist with prevention strategies. The enzyme-linked immunoassay (ELISA) has been used for screening HIV antibodies in blood. With regard to false positives, Western blot or immunofluorescence assay has been recommended as a supplemental test to confirm a positive test for HIV. ELISA and Western blot require specialized equipment and technical expertise. They cannot be completed in less than 24 hours. ELISA and Western blot are not feasible for small laboratories in many developing countries where resources are limited. A number of simple, rapid assay emerged to meet the demand in such countries both for blood screening and voluntary testing. Numerous studies demonstrated that alternative confirmatory strategies using algorithms with combinations of screening tests produced reliable results, comparable to those of standard ELISA and Western blot (Mitchell *et al.*, 1991; Urassa *et al.*, 1992, Brattegaard *et al.*, 1993; Stetler *et al.*, 1997). World Health Organization (WHO) currently recommends the routine use of combinations of screening tests for HIV screening, surveillance, and diagnosis (UNAIDS, 1997 and 1998). Screening with combinations of rapid HIV tests proved to be less expensive than the ELISA/Western blot, and also made it possible to offer same-day test results. More than

60 rapid HIV tests have been developed and used in various countries (Branson, 2000). The dipstick test requires only one step and incorporates both antigen and signal reagent into a nitrocellulose strip. A few of the strip tests also deploy different antigens at different locations to allow differentiation of HIV-1 group M, HIV-1 group O, and HIV-2 antibodies (e.g. Determine HIV-1/2/O, Abbott Laboratories). The cost of the tests is usually less than $2.

Dipstick test has also been developed to detect protozoa and parasite infections. Generally, monoclonal antibodies (MAB) which are specific to the protein antigens of the parasite, are used as capture and detector agent. Advances have been made in rapid dipstick assays to detect malaria, leishmania and leptospirosis. No other single infectious disease has had the impact on humans that malaria has. It has been estimated that more than 100 million people are infected, and about one million die annually of malaria in Africa alone. Human malaria is caused by four species of *Plasmodium*. Microscopy of serial blood smears to detect the presence of the malaria parasites continues to be the "gold standard" for clinical diagnosis because it allows estimation of parasitemia, distinction between parasite growth stages and identification of the four different plasmodium species of malaria. Recently, several dipstick assays are available on the market for malaria detection (e.g. Parasight-F, Becton Dickinson Tropical Diagnostics, Sparks, MD). These assays have been used in various clinical settings in North America and endemic regions. The newer assay OptiMAL (Flow Inc. Portland, OR) can distinguish between *P. falciparum* and *P. vivax*. The assay can also distinguish between living (current infection) and dead parasites (recently treated infection) (Palmer *et al.*, 1998).

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Recently, researchers at The Scripps Research Institute have developed a dipstick assay, similar to a home pregnancy test, which can detect mercury contamination in fish (Brummer *et al.*, 2001).

The development and application of dipstick assay for WSSV detection will be highly beneficial to the shrimp industry in the following ways: the assay can be used in the field by farmers since there is no requirement for instruments and special training; the cost of assay is low so that large number of samples can be screened and thus the well being of the shrimp be monitored frequently; finally, the same principle could be applied to the detection of other shrimp viruses.

CHAPTER 2

MATERIALS AND METHODS

2.1 WSSV PROPAGATION AND PURIFICATION

2.1.1 Viral inoculum preparation

Infected shrimp gill tissue was collected and homogenized with a Polytron [Brinkmann Polytron 3000 (Kinematica AG) with 90/Polytron PT-DA 3012/2TS] (Brinkmann Instruments Inc., NY, USA) in phosphate-buffered saline (PBS, pH 7.4) to a final concentration of 3% (w/v). The tissue homogenate was centrifuged at 3000 g for 15 min at 4°C. The supernatant was collected and filtered through 0.45 µm membrane filter. The supernatant was used for inoculating shrimp or was aliquoted and stored at -80°C for future use.

2.1.2 WSSV propagation and purification

WSSV was propagated in specific pathogen-free (SPF) *Penaeus vannamei* (50-60 g) by intramuscular injection of 200 µl of inoculum. The shrimp were monitored after the injection. The shrimp which died within 24 hrs were discard since generally they died of trauma. Two to four days after the injection, hemolymph was collected from moribund shrimp, mixed with the same volume of sodium citrate (20% w/v), and stored at -80°C. The infected shrimp were frozen for future viral purification.

To purify the WSSV, gill and head soft tissue were harvested and pooled from frozen infected shrimps. Tissue homogenate (10% w/v) was made by suspending the tissue in TNE (50 mM Tris, 100 mM NaCl, 1.3 mM EDTA, pH7.4) and homogenizing

with a Polytron. The homogenate was centrifuged at 5,000 g for 15 min at 5°C to pellet the tissue debris. Four layers of cheesecloth were used to filter out any floating debris. The supernatant was centrifuged at 74,000 g for 30 min at 5°C using 1 ml of sucrose cushion (25% w/w sucrose in TNE) to pellet the virus. The viral pellet was resuspended in TNE-Triton (1% Triton X-100 in TNE) and centrifuged at 2000 g for 20 min at 5°C to further clear the tissue debris. The supernatant was collected and centrifuged at 113,000 g for 30 min at 5°C with 1 ml of sucrose cushion to pellet the virus. The viral pellet was suspended in TNE, layered on top of a continuous CsCl gradient (30-50% w/v), and centrifuged at 192,000 g for 2.5 hrs at 5°C. The virus band located right in the middle of the tube was collected, diluted in TNE (at least 1:5 dilution) and pelleted at 101,000 g for 30 min at 5°C using a 0.5 ml of sucrose cushion. The viral pellet was collected in PBS buffer, checked for virus under the electron microscope and stored at -80°C.

2.1.3 Negative staining and electron microscopy

The purity of the viral preparation was examined by electron microscope (EM) using negative staining. 5 μl of virus sample was applied to the carbon-coated copper EM grid (200 mesh) and incubated for 15 min at room temperature (r.t.). The grid was rinsed with distilled water and stained with 2% uranyl acetate (w/v in H_2O, adjust pH to 7.0 with 1 N NaOH and filtrate using 0.45 μm filter) for 30 seconds. The grid was rinsed with distilled water and allowed to dry for at least 15 min before viewing under the EM.

2.2 CONSTRUCTION OF WSSV CDNA LIBRARY AND GENE CLONING

2.2.1 Isolation of nucleic acid from virion and virus-infected shrimp tissue

2.2.1.1 Isolation of WSSV genomic DNA

The WSSV genomic DNA was isolated from CsCl banded virions by phenol-chloroform extraction (Sambrook *et al.* 1989). The virions were resuspended in 100 µl of PBS. Proteinase K was added to a final concentration of 100 µg/ml. The suspension was incubated for 1 hr at 37°C. An equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1, pH 8.0) (Sigma, MO) was added and gently mixed for 10 min. The two liquid phases were separated by centrifugation at 12,000 g for 15 min at r.t. The aqueous phase was transferred to a new tube. One-tenth volume of 3 M sodium acetate (pH 5.2) was added and gently mixed. An equal volume of isopropanol was added to the mixture and mixed well. The tube was kept at -20°C for 30 min. The pellet of DNA was recovered by centrifugation at 12,000 g for 10 min at -4°C. The pellet was washed twice with 70% ethanol and allowed air-dry. The DNA was dissolved in 50 µl of TE (10 mM Tris, 1mM EDTA, pH 8.0) and was analyzed by agarose gel electrophoresis. The quantity of DNA was estimated by spectrophotometric measurement of the amount of UV absorption at 260 nm (1 OD_{260} corresponds to ~50 µg/ml of double-stranded DNA). The DNA was stored at -20°C.

2.2.1.2 <u>Isolation of total RNA from WSSV infected tissue</u>

Total RNA was isolated from shrimp gill tissue using TRI_{ZOL}®LS Reagent (GIBCOBRL, NY). Two grams of gill was collected from WSSV infected moribund shrimp and homogenized in 15 ml of TRI_{ZOL}®LS Reagent (0.75 ml of TRI_{ZOL} per 50-100 mg of tissue) using a Pyrex glass tissue homogenizer. The homogenized tissue was incubated for 5 min at r.t. to permit the complete dissociation of nucleoprotein

complexes. 4 ml of Chloroform (0.2 ml of Chloroform per 0.75 ml of TRI$_{ZOL}$) was added. The tube was shaken vigorously for 15 sec and incubated for 15 min at r.t. The sample was centrifuged at 12,000 g for 30 min at 4°C. The colorless upper aqueous phase was transferred to a clean new tube. The RNA was precipitated with 10 ml of (2/3 volume of TRI$_{ZOL}$ used) isopropyl alcohol by centrifuging at 12,000 g for 10 min at 4°C. The RNA pellet was washed with 75% ethanol and air-dried for 10 min. The RNA was dissolved in 200 µl of RNase-free water and analyzed by agarose gel electrophoresis. The RNA can be quantified as DNA at 260 nm by spectrophotometer (1 OD$_{260}$ corresponds to ~40 µg/ml of single-stranded RNA).

2.2.1.3 Isolation of mRNA from the total RNA

mRNA was isolated from the total RNA using Oligotex™ (Qiagen, CA). The amount of total RNA was determined by spectrophotometer. Total RNA, diethyl pyrocarbonate (DEPC) -treated water, 2 x Binding Buffer and Oligotex resin were mixed according to Oligotex™ mRNA handbook (Qiagen, CA). The mixture was incubated for 3 min at 65°C in a water bath in order to disrupt secondary structure in the total RNA. The sample was removed from the water bath and kept at r.t. for 10 min. In order to pellet the Oligotex resin containing mRNA, the sample was centrifuged for 2 min at 18,000 g. The Oligotex pellet was resuspended in 400 µl of Wash Buffer OW2 by vortexing. The mixture was transferred to a spin column (included in the kit) and centrifuged at 14,000 g for 30 sec. The column was washed with 400 µl of Wash Buffer OW2 and the flow through was discarded. The mRNA was eluted twice with 20 µl to

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100 µl of preheated (70°C) Elution buffer. The two elutions were pooled. The mRNA

was used for cDNA synthesis or stored at -70°C for future use.

2.2.2   Construction of WSSV cDNA library

cDNA was synthesized from mRNA. First-strand cDNA synthesis was catalyzed

by a reverse transcriptase using Oligo(dT)$_{12\text{-}18}$ or random hexamers as primer.

Oligo(dT)$_{12\text{-}18}$ would prime at 3' end of polyadenylated mRNAs. Random hexamer

would prime at random sites on all mRNAs. Second-strand cDNA was synthesized by

the action of both RNase H (which nicked the RNA strand of the RNA:cDNA hybrid) and

DNA polymerase (which used these nicks to replace RNA with DNA by nick translation).

By adding adaptors at the ends of cDNA, cDNA can be amplified by PCR to enrich the

abundant cDNA. The PCR amplified cDNA would be digested by restriction enzymes

and inserted into a vector by ligation. The ligation product would be used to transform

E.coli to set up a cDNA library.

2.2.2.1 Synthesis of cDNA

cDNA was synthesized using TimeSaver™ cDNA Synthesis Kit (Amersham

Pharmacia Biotech, NJ). 20 µl of mRNA (1-5 µg) was heated at 65°C for 10 min, then

chilled on ice. A First-Strand Reaction Mix (Murine Reverse Transcriptase and dNTPs),

1 µl of DTT solution (200 mM), and 1 µl of primer (Oligo(dT)$_{12\text{-}18}$ or random hexamer at

an appropriate concentration) was added. Generally, higher the concentration of the

primer, the shorter the cDNA synthesized. The mixture was incubated at 37°C for 1 hr.

The first-strand reaction mixture was transferred to a Second-Strand Reaction Mix

(RNase H, DNA Polymerase I, and dNTPs). The mixture was incubated at 12°C for 30

min and then at 22°C for 1 hr. The reaction was terminated by heating the reaction

mixture at 65°C for 10 min. cDNA was purified by phenol/chloroform extraction and

spin column to remove excess dNTPs and primers.

2.2.2.2 Oligo(dG) and Oligo(dT) tailing

Complementary homopolymeric tails were added to the cDNA. In a 20 µL of

reaction mixture, there were 10 µL of cDNA, 10 unit of terminal deoxynucleotide

transferase (TdT) (Promega, WI), buffer for TdT, 200 µM of dGTP or dATP. The

reaction mixture was incubated at 37°C for 20 min.

2.2.2.3 Polymerase chain reaction (PCR)

PCR is a powerful tool for DNA amplification. The PCR products were used for

cloning and generating probes for nucleic acid hybridization. PCR was also used to

screen for positive clones after transformation. "Hot Start" was used to reduce non-

specific DNA amplification. All of the PCR reactions in this study were done on

GeneApm® PCR System 2400 (Perkin-Elmer Corporation, Connecticut). In a total

volume of 50 µl reaction mixture: 1 µl of DNA (100 ng/µl), 0.2 µl of *Taq* DNA

Polymerase (5 unit/µl, Promega, WI), 1 µl of dNTP mix (10 mM each of dNTP), 5 µl of

$MgCl_2$ (25 mM), and 5 µl of 10X Mg^{2+} free reaction buffer was added. DNA templates

were denatured at 94°C, the annealing temperature and time were determined based on

Tm values the primers. Elongation temperature was 72°C, and elongation time varied

according to the size of PCR product. The longer the size of expected PCR product, the longer the elongation time. The size of PCR product was checked on 1.5% agarose gel.

2.2.2.4 Restriction enzyme digestion

Restriction enzymes usually recognize 4-8 base-pair long specific DNA sequences and cleave double-stranded DNA with the production of cohesive or blunt ends. The cleavage property of restriction enzymes provides molecular biologists with a powerful tool to manipulate DNA *in vitro*. All restriction enzymes and their corresponding buffers are normally stored at -20°C. In general, 1 unit of enzyme is required for digesting 1 µg of DNA in 20 µl of a total reaction volume at 37°C. The digested DNA was examined by agarose gel electrophoresis and subjected to further treatments.

2.2.2.5 Transformation of *E. coli*

Preparation of competent E. coli cell

Luria-Bertani (LB) medium (Sambrook *et al.*, 1989) was used for growing the *E. coli* strains. Tryptone (10.0 g), Yeast extract (5.0 g), and NaCl (5.0 g) were dissolved in 1 liter of deionized water and the pH was adjusted to 7.0 with 0.1 N NaOH. For LB agar plate, 18 g of agar was added per liter before autoclaving.

E. coli strain XL-1 blue or BL21 (DE3) pLysS was streaked directly from a frozen stock on LB plate. The plate was incubated at 37°C overnight. A single colony was transferred into 3 ml of LB broth and grown overnight. The culture was diluted in 300 ml of LB broth and incubated with shaking at 37°C for approximately 2 hrs until O.D.$_{600}$ reached 0.3–0.4. The culture was kept on ice for 10 min and spun at 1000 g for 10 min at

4°C to get the cell pellet. The pellet was resuspended in 20 ml of ice-cold 0.1 M CaCl$_2$

and kept on ice for 20 min. The cell was centrifuged at 1000 g for 10 min at 4°C and

finally resuspended in 3 ml of ice-cold 0.1M CaCl$_2$ with 15% glycerol. The cell

suspension was aliquoted in 50 µl in microcentrifuge tubes. The fresh competent cells

are ready to use. Alternatively, the competent cells can be frozen immediately in liquid

nitrogen and stored at -80°C for future use.

Transformation of E.coli

10 ng or more of pure plasmid DNA or 1µl of ligation mixture was added to 50 µl

of *E.coli* competent cells and mixed well. The mixture was kept on ice for 10-20 min,

then put into a 42°C waterbath for 1-1.5 min (heat shock). The mixture was transferred

to 2 ml of LB broth and the cell culture was allowed to grow at 37°C for 1 hr with

vigorous shaking. The cells were harvested by centrifugation at 4000 g for 5 min and the

culture was spread on LB agar plates. LB agar plate was pre-made and contained the

appropriate antibiotics, 40 µl of X-gal (20mg/ml in dimethylformamide) and 4 µl of a

solution of isopropylthio-β-D-galactoside (IPTG) (200mg/ml). The plates were incubated

at 37°C overnight. White colonies, which have inserted DNA fragment, were selected for

further analysis.

2.2.3   Screening of WSSV cDNA library

cDNA library was constructed by cloning cDNA fragment into plasmid vector

pUC19 by ligation. The ligation product was used to transform *E.coli* XL-1 blue. White

colonies were selected to grow in LB broth for plasmid purification by alkaline mini-

preparation. The purified plasmid DNA was denatured and spotted on Nylon membrane. The plasmid with insertion of WSSV DNA fragment was screened by nucleic acid dot-blot assay using Digoxigenin (DIG) labeled probes.

2.2.3.1 Alkaline mini-preparation of plasmid DNA

1.5 ml of *E.coli* culture was spun down in an Eppendrof tube to pellet the cells. The bacterial pellet was resuspended in 100 μl of Solution I (50 mM of Glucose, 25 mM of Tris-HCl, and 10 mM of EDTA; pH 8.0) by vortexing. 200 μl of freshly prepared Solution II (0.2 N NaOH, 1% SDS) was added. The tube was inverted several times to mix well and kept on ice for 5 min. 150 μl of ice-cold Solution III (3 M potassium acetate, 5 M acetic acid) was added, the tube was inverted several times to mix well, and kept on ice for 3-5 min. The tube was centrifuged at 12,000 g for 5 min. The supernatant was transferred to a new tube. An equal volume of phenol: chloroform (50:50) was added to extract protein contaminant. DNA was precipitated with two volumes of 100% ethanol at room temperature. The DNA pellet was washed with 70% ethanol, and air-dried. The plasmid DNA was dissolved in 50 μl of TE buffer (pH 8.0), and stored at -20°C.

2.2.3.2 Nucleic acid dot hybridization

Nucleic acid dot hybridization was carried out using the DIG system (Boehringer Mannheim Corporation, 1995). DNA can be labeled with DIG-11-dUTP by using such as PCR, nick translation, or random primed method, *etc*. After hybridization, the signal on the nucleic acid blot is detected according to the methods developed for western blots.

An anti-DIG antibody-alkaline phosphatase conjugate is allowed to bind to the hybridized probe. The signal is then detected with chemiluminescent alkaline phosphatase substrates on an X-ray film.

Labeling DNA probe

The probe was labeled by random-primed method using DIG DNA Labeling Kit (Boehringer Mannheim Corporation, IN). 10 ng - 3 μg of purified linear or plasmid DNA in a volume of 15 μl was denatured in a microcentrifuge tube by boiling in a waterbath for 10 minutes and was kept on ice. Then the following labeling reaction mixture was added: 2 μl of hexanucleotide mixture, 2 μl of dNTP labeling mixture, and 1 μl of Klenow enzyme (2 units/μl). The mixture was centrifuged briefly to mix well and incubated at 37°C overnight. The reaction was stopped by adding 2 μl of EDTA (0.2 M, pH8.0). The labeled probe can be used right away or can be stored at –20°C for future use.

Nucelic acid dot-blot assay

Plasmid DNA was denatured by boiling in a waterbath for 5 min and kept on ice. 1 μl of denatured DNA was spotted on nylon membrane (0.45 μm, MSI, MA). The DNA was fixed to the nylon membrane by UV cross-linking for 15 sec with a UV Crosslinker (Fisher Scientific). The hybridization was carried out in a Hybridization Incubator (Hybaid, National Labnet Company, NJ). The membrane was placed in a hybridization tube and blocked with 20 ml of prehybridization solution [5X SSC (750 mM NaCl, 75 mM sodium citrate, pH7.5), 0.02% SDS, 2% blocking reagent (Boehringer Mannheim

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Corporation, IN), and 50% Formamide] at 42°C for 1 h. The DIG-labeled DNA probe

was denatured by boiling for 10 min and added into the prehybridization solution.

Hybridization was carried out at 42°C in incubator for 12 hrs. After hybridization, the

membrane was washed 2x5 min in ample 2x wash solution (2X SSC, 0.1% SDS) at r.t.

with shaking. Then the membrane was washed 2x15 min in ample 0.5x wash solution

(0.5X SSC, 0.1% SDS) at 68°C with shaking. After the membrane was rinsed briefly (1-

5 min) in washing buffer [maleic acid buffer (0.1 M maleic acid, 0.15 m NaCl; pH7.5),

0.3% Tween® 20 (v/v)], it was blocked in blocking solution (2% blocking reagent in

maleic acid buffer) for 30 min at r.t.. Anti-Digoxigenin antibody conjugated with

alkaline phosphatase (Anti-DIG-AP) (Boehringer Mannheim Corporation, IN) was added

to the blocking solution in a 1:10,000 dilution. The membrane was incubated in antibody

solution for 30 min at r.t. with shaking. The membrane was washed 2x15 min in 100 ml

of washing buffer at r.t. after incubating in antibody solution. The membrane was

equilibrated in 20 ml detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, 50mM MgCl$_2$,

pH9.5) for 2-5 min at r.t. CSPD® (disodium 3-(4-methoxyspiro(1,2-dioxetane-3,2'-(5'-

chloro)tricyclo[3,3.1.1$^{3.7}$]decan}-4-yl) phenyl phosphate) ready-to-use solution was

applied to the membrane and the membrane was placed between two sheets of

transparency film. The damp membrane was incubated at r.t. for 5 min and was exposed

to X-ray film (Kodak X-Omat) at 37°C for 1 hr.

2.2.3.3 <u>Purification of DNA sample for sequencing</u>

Plasmid for DNA sequencing was purified by using Qiaprep Spin Plasmid Kit (Qiagen, CA). Briefly, 5 ml of overnight culture of *E.coli* (in LB medium) was pelleted by centrifugation. The pellet was resuspended in 250 μL of Buffer 1 in a microcentrifuge tube. 250 μl of Buffer P2 was added and gently mixed. 350 μl of Buffer N3 was added and mixed gently by inverting the tube 4-6 times. The mixture was centrifuged at 10,000 g for 10 min. The supernatant was transferred to a QIAprep column and centrifuged at 10,000 g for 30-60 sec. The flow-through was discarded. The column was washed by adding 0.75 ml of Buffer PE and centrifuged for 30-60 sec. The DNA was eluted with 50 μl of 10 mM Tris-HCl (pH 8.0) or H_2O.

PCR product was purified by using QIAquick PCR purification Kit (Qiagen, CA). 5 volumes of Buffer PB was mixed with 1 volume of the PCR product. The mixture was applied to the QIAquick column and centrifuged for 30-60 sec. The flow-through was discarded and the column was washed with 0.75 ml of Buffer PE. The DNA was eluted with 50 μl of 10 mM Tris-HCl (pH 8.0) or H_2O.

2.2.3.4 <u>DNA sequencing</u>

The concentration of purified plasmid DNA or PCR was determined by spectrometer. The sequencing was done at PBRC Molecular Biology Core Facility (University of Hawaii at Manoa). 1.0 μg of plasmid DNA template and 4.0 pmole of primer were included in 12 μl of reaction mixture. For PCR product, the amount of DNA

used for sequencing depends on the length of PCR product. 20ng/100bp template was used for sequencing.

2.2.3.5 Sequence analysis of cDNA clones

The WSSV cDNA sequences were compared with known sequences in Genbank using the BLAST® program. The sequences with homology were aligned using ClustralW (Thompson *et al.*, 1994) (http://clustalw.genome.ad.jp) and the aligned-multiple sequences were printed out by Boxshade program (http://bioweb.pasteur.fr/seqanal/interfaces/boxshade.html). The DNA sequences were translated into peptide sequences using the GCG® Wisconsin Package® (Genetic Computer Group). The deduced peptide sequences were compared with the protein sequences in the SWISS-PROT (http://www.ebi.ac.uk/swissprot/index.html), which provides a description of function, domain structure, post-translational modifications, variants, *etc* for each protein in the database. PROSITE© is a method to determine the function of uncharacterized proteins translated from genomic or cDNA sequence. It was used to help determine if the peptide had any pattern, motif, or other biological significant sites. The hydrophobicity characters of a protein or peptide was plotted using Kyte-Doolittle scale and GES scale in TopPred program (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html) (von Heijne, 1992; Claros *et al.*, 1994). The following parameters were used for the predication: GES-scale or Kyte-Doolittle scale; upper cutoff: 1.0; lower cutoff: 0.6; core window size: 5; wedge window: 1; critical loop length: 20; critical transmembrane spacer: 2.

2.3 EXPRESSION AND PURIFICATION OF RECOMBINANT PROTEIN

2.3.1 Expression of viral protein in *E.coli*

2.3.1.1 <u>TA cloning</u>

In order to create a *Nde*I restriction site at the start codon and a *Bam*HI site at the stop codon, the gene or gene fragment was amplified by PCR with custom-designed primers. The PCR product synthesized using *Taq* polymerase has a 3' adenine hangover. TA cloning vector was made in such a way that it has a 3' thimidine hangover so that the PCR product can be cloned directly with high efficiency. The PCR product was cloned into a TA cloning vector pCR2.1 from the Original TA Cloning Kit (Invitrogen, CA). The clone containing the insertion was transferred to *E. coli* strain INVαF' competent cell (Invitrogen, CA) and selected by kanamycin and *lacZ* reaction. The clone was analyzed by restriction enzyme digestion and PCR to confirm the insertion.

2.3.1.2 <u>Purification of DNA fragment from agarose gel</u>

DNA fragment of certain size or range can be obtained by separating DNA samples by agarose gel electrophoresis. The needed band was cut off from the agarose after staining it with ethidium bromide.(EtBr). The QIAquick Gel Extraction Kit (Qiagen, CA) was used to purify DNA. The agarose gel slice was dissolved in 3 volume of Buffer QG (100 mg ~ 100 μl) by incubating at 50°C for 10 min and vortexing the tube every 2-3 min during the incubation. After the gel slice was dissolved completely, 1 gel volume of isopropanol was added to the sample and mixed well. The sample was applied to the QIAquick column, and centrifuged at 10,000 g for 1 min. To wash the column, 0.75 ml of Buffer PE was added and centrifuged for 1 min at 10,000 g. The flow-through was discarded. The DNA was eluted with 50 μl of 10 mM Tris-HCl (pH 8.0) or H_2O.

2.3.1.3 Cloning the gene into a protein expression vector

The target gene was cloned in a pET vector under the control of T7 *lac* promoter

(Studier *et al.*, 1990) which was specifically recognized by T7 RNA polymerase. The

high-stringency expression host BL21(DE3)pLysS was used. BL21(DE3) carries a DNA

fragment containing the *lacI* gene, the *lacUV5* promoter, and the gene for T7 RNA

polymerase (Studier and Moffatt, 1986). Only when the inducer IPTG is present, T7

RNA polymerase can be synthesized and transcribe the target gene from the T7 promoter.

pLysS is a compatible plasmid containing a gene encoding the T7 lysozyme which is a

natural inhibitor of T7 RNA polymerase. The low level of lysozyme provided by pLysS

usually had little effect on expression of target genes following induction of T7 RNA

polymerase. pLysS also contains the chloramphenicol resistance gene as a selection

marker. The presence of pLysS increases the tolerance of BL21(DE3) for plasmids with

toxic inserts.

The *Nde*I-*Bam*HI fragment containing the gene from the TA cloning was cut off

by restriction enzyme digestion and purified. This fragment was ligated to the protein

expression vector pET-21b(+) (Novagen, WI) which was linearized by *Nde*I-*Bam*HI

digestion and purified from the agarose gel. The ligation mixture was then transferred to

E. coli strain DH5α competent cells (Bethesda Research Laboratories, MD). The

transformants were selected with ampicillin (or carbenicillin) and *LacZ* reaction. The

plasmid containing the desired *Nde*I-*Bam*HI fragment was examined by restriction

enzyme digestion and PCR.

2.3.1.4 Induction of viral protein in *E.coli*

100 ng of the protein expression plasmid with proper insertion was used to transform a special *E. coli* expression host strain BL21(DE3)pLysS. The transformants were selected by growing them on a 1.5% LB agar plate containing 100 µg/ml of carbenicillin (Car) and 34 µg/ml of chloramphenicol (Chl). Individual colonies were picked up and grown in 5 ml of LB broth containing carbenicillin and chloramphenicol at 37°C until OD_{600} reached 0.4-0.6. 100 µl of culture was transferred to 5 ml of LB broth containing the same amount of antibiotics. When the OD_{600} reached 0.6–0.8, IPTG was added to a final concentration of 1 mM. The culture was grown at 37°C for 3 h with vigorously shaking. The total proteins of induced and un-induced culture were isolated and used for sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis.

1 ml of cell culture ($O.D._{600}$=0.6–0.7) was harvested by centrifugation for 2 min at 12,000 g. The cells were washed twice with TE buffer. The cells were resuspended by adding 100 µl of 2x SDS-PAGE loading buffer [100 mM Tris·HCl, pH 6.8, 200 mM dithiothreitol (DTT), 4% SDS, 0.2% bromophenol blue, 20% glycerol] and mixed well. The sample was boiled for 3 min to denature the proteins. The protein sample was loaded to SDS-PAGE gel.

2.3.1.5 <u>SDS-PAGE analysis of the protein</u>

SDS-PAGE was performed according to Laemmli (1970) using Mini-PROTEAN II Electrophoresis Cell (BIO-RAD, CA). 12% and 15% separating gel was used depending the size of the protein sample to be analysized. To prepare 10 ml of 12% gel, the

following components were mixed: 3.3 ml of H_2O, 4 ml of 30% acrylamide mix, 2.5 ml of Tris (1.5 M, pH 8.8), 0.1 ml of 10% SDS, 0.1 ml of 10% ammonium persulfate (APS), and 4 μl of N,N,N',N',- tetramethylethylenediamine (TEMED). To prepare 10 ml of 15% gel, use 2.3 ml of H_2O and 5 ml of 30% acrylamide mix. The mixture was poured immediately into the gap between the glass plates. The separating gel was overlaid with H_2O. The gel was allowed to polymerize at r.t. for 30 min. The overlay was drained. 4 ml of stacking gel mixture was prepared by mixing the following components: 2.7 ml of H_2O, 0.67 ml of 30% acrylamide mix, 0.5 ml of Tris (1 M, pH 6.8), 0.04 ml of 10% SDS, 0.04 ml of 10% APS, and 4 μl of TEMED. The stacking gel solution was poured directly onto the surface of the polymerized separating gel. A clean Teflon comb was inserted immediately into the stacking gel. After polymerization was completed (30 min), the comb was removed and the wells were washed with running buffer (25 mM Tris, 250 mM glycine, 0.1% SDS, pH8.3). Then the gel was mounted in the electrophoresis apparatus and running buffer was added. After boiling the sample for 3 min in 1 x SDS loading buffer, up to 10 μl of the sample was loaded into each well. The gel was run at 200 V for 45 min. The gel was stained with SimpleBlue™ (Invitrogen, CA); alternatively, it could be used for western blot. After destaining in water, the gel was fixed in gel drying solution (5% v/v glycerol in H_2O) overnight and dried between two sheets of Cellophane Membrane (BIO–RAD, CA) using two plastic gel-drying frames.

2.3.2 Purification of recombinant protein

2.3.2.1 Large scale induction of recombinant protein

Single colony was picked from a freshly streaked plate to inoculate 50 ml of LB containing 50 ug/ml of carbenicillin (Car) and 34 ug/ml of chloramphenicol (Chl) in a 250 ml Erlenmeyer flask. The culture was incubated with shaking at 37°C until OD_{600} reaches about 0.4. This culture was used as starter culture to inoculate larger volume of media. 500ml of LB media (50 ug/ml Car, 34 ug/ml Chl) was inoculated with 3 ml of starter culture and incubated with shaking at 37°C until OD_{600} was approximately 0.5. To induce expression of target protein, IPTG was added into the culture to a final concentration of 1 mM. The culture was grown for ~ 3 hrs before harvesting the cells.

2.3.2.2 Purification of recombinant protein

Isolation of inclusion body from induced cells

The recombinant protein was purified by using His·Bind® Kit (Novagen, WI). The following procedure was for a 100 ml of culture and can be scaled up. The cells were harvested by centrifugation at 6,500 g for 15 min at 4°C. The cell pellet was resuspended in 40 ml of 1X Binding Buffer (50 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9) and sonicated briefly on ice to breakdown the cells and shear the DNA. The cell suspension was centrifuged at 5,000 g for 15 min to collect the inclusion bodies and cellular debris while leaving other proteins in solution. The supernatant was removed and the pellet was suspended in 20 ml of 1X Binding Buffer. The sonication was repeated twice to release more trapped proteins. Finally, the pellet was resuspended in 5 ml of 1X Binding Buffer containing 6 M guanidine. The mixture was incubated on ice

for 1 h to completely dissolve the protein. The insoluble material was removed by centrifugation at 16,000 g for 30 min. The supernatant was filtered through a 0.45 μm low protein binding filter before loading on a His·Bind column.

Resin preparation

His·Bind resin (in 20% ethanol) was resuspended well and transferred to a column to get 2.5 ml of settled resin. The settled resin was charged and equilibrated with the following: 3 volumes of sterile deionized water, 5 volumes of 1X Charge Buffer (50 mM NiSO₄), 3 volumes of 1X Binding Buffer (1 volume is equivalent to the settled bed volume of resin).

Column chromatography

The column chromatography was performed under denaturing conditions, the Binding, Wash and Elute Buffers were supplemented with 6 M guanidine-HCl. The prepared extract was loaded to the column and allowed to drain by gravity. The column was washed with 10 volume of 1X Binding Buffer and 6 volumes of 1X Wash Buffer (60 mM imidiazole, 500 mM NaCl, 20 mM Tris-HCl, pH7.9). The bound protein was eluted with 6 volume of 1X Elute Buffer (1M imidiazole, 500 mM NaCl, 20 mM NaCl, pH 7.9) in 1 ml fractions. The resin was stripped with 10-15 ml of Strip Buffer (100 mM EDTA, 500 mM NaCl, 20 mM KCl, pH 7.9) and stored in Strip Buffer at 4°C.

Determination of protein concentration

The protein concentration of each aliquot in Elution Buffer was determined by using BCA Protein Assay Kit (Cat.23225. Pierce, IL). Known concentration of BSA was used to plot a standard curve. 0.1 ml of serial dilution of protein samples were mixed

with 2 ml of the BCA (bicinchoninic acid) working reagent and incubated at 60°C for 30 min. The samples were cooled to r.t. and the absorbance at 562 nm was measured. The protein concentration of unknown sample was determined by using BCA assay and the standard curve.

Renaturation and concentration of recombinant protein

The elute aliquots which had a high protein concentration were pooled and dialysized against PBS with 4 M and 2 M Guanidine-HCI, and PBS only sequentially. Finally the protein sample was concentrated using Ultrafree 15 ml concentrator with 10 K nominal molecular weight limit (NMWL) (Millipore).

2.4 PRODUCTION OF POLYCLONAL ANTISERUM IN RABBIT AND IMMUNOASSAY

2.4.1 Production of polyclonal antiserum in rabbit

Polyclonal hyperimmune antiserum was prepared in New Zealand white rabbits (7-8 lb) according to the following immunization schedule: 1 mg of protein in 0.7 ml of PBS was emulsified with 0.7 ml of Freund's complete adjuvant (Sigma, MO) for about 15 min. The mixture was injected intrademerlly and subcutaneously on Day 0. Subsequent booster injections of 1mg protein in Freund's incomplete adjuvant were given on Day 24, 41, and 70. The rabbits were exsanguinated on Day 78. The sera were centrifuged at 1800 g at 4°C for 30 min to separate red blood cells from the serum. The serum was stored at -80°C.

2.4.2 Isolation of immunogloblin G (IgG) fragment from the antiserum

The IgG fragment was purified from the antiserum using ImmunoPure® (G) IgG

Purification Kit (Pierce, IL). Briefly, serum sample was diluted at least 1:1 with Binding

Buffer and centrifuged at 10,000 g for 20 min and the supernatant was applied to the

equilibrated protein G column. Up to 4 ml of the diluted sample was applied to the

protein G column and allowed to flow completely into the gel. The protein G column

was washed with 10 ml of Binding Buffer by applying 2 ml fractions for 5 times. The

bound IgG was eluted with 6 ml of Elution Buffer. The elute was collected in 1 ml

fractions and the pH of the fractions was immediately neutralized by addition of 100 µl of

1M phosphate buffer (pH7.2). The eluted immunoglobulin was desalted over an

Excellulose™ desalting column and concentrated by using Ultrafree®-15 Centrifugal

Filter Device (Millipore).

2.4.3 Isolation of F(ab')$_2$ fragment of IgG

The F(ab')$_2$ fragment of IgG was generated by pepsin digestion. IgG was dialyzed

into 0.1 M sodium acetate buffer (pH 4.5) for 4 h. Protein concentration was determined

after dialysis and pepsin (Sigma, MS) was added at an enzyme-antibody ratio of 1:50

(weight by weight). The reaction mixture was incubated for 6 hours at 37°C, and the

digestion was terminated by adding 1.5 M Tris buffer (pH8.8) until a pH of 8.0 was

attained. The reaction mixtures were dialyzed overnight into PBS. For removal of

residual intact molecules and Fc fragment, the reaction mixtures were passed through a

protein G column (Pierce, IL). The flow through was collected and combined with the wash fraction. The sample was analyzed by SDS-PAGE to determine its purity.

2.4.4 Western blot

The proteins were transferred from the SDS-PAGE gel to a pre-soaked nitrocellulose membrane (0.20 μm, Schleicher & Schuell, TN) by using a Mini Trans-Blot Electrophoretic Transfer Cell (BIO-RAD, CA) under a constant voltage of 100 V for 1 h at 4°C in transfer buffer (in one liter: 3.30 g Tris, 14.4 g glycine, 200 ml methanol). The efficiency of the transfer was checked by staining the gel with SimpleBlue™. Following transfer, the membrane was blocked in 5% milk PBS-T (PBS with 0.1% Tween 20) for 1 h at r.t. with shaking to reduce the background. Then the membrane was incubated with the primary antibody directly against the target protein (1:1000 antibody in PBS-T) for 1 h with shaking at r.t. The membrane was washed twice with PBS-T for 15 min to eliminate non-specific antibody binding. The membrane was soaked in secondary antibody solution (goat anti-rabbit IgG conjugated with horseradish peroxidase, BIO-RAD; 1:3000 in PBS-T) for 1 h with shaking at r.t. It was washed twice in PBS-T for 15 min and rinsed for 1 min in PBS before detection. ECL western blotting detection reagents (Amersham Pharmacia Biotech, NJ) was used for detection. An equal volume of Detection Solution 1 and Detection Solution 2 were mixed (the final volume required is 0.125 ml/cm² membrane). The membrane was incubated in the detection solution for 1 min at r.t. and exposed to X-ray film (Kodak X-Omat) for 15 sec to 2 min.

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2.4.5 Immunodot assay

Serial dilutions of 10% w/v of uninfected and infected gill homogenates were made in PBS (pH7.4) and samples were spotted onto nitrocellulose (NC) membrane (Schleicher & Schuell BA-S 83, 0.2μm, Keene, NH) at 1μl per spot and allowed to air-dry. The NC membranes were blocked with 5% skim milk in PBS, washed 3 x 5 min with PBS, and treated with anti-vp27.5 IgG (1:2000 dilution in PBS with 1% skim milk) for 1 hr. After washed 3 x 5 min with PBS, the membrane was incubated in goat anti-rabbit IgG conjugated with horseradish peroxidase (1:3000 dilution in PBS with 1% skim milk) (Bio-Rad, CA). The membrane was rinsed three times before adding the TMB (3, 3', 5, 5'-tetramethylbenzidine) (Kirkegaard & Perry Laboratories, MD) membrane peroxidase substrate for detection.

2.5 DIP STICK ASSAY

2.5.1 Coating of antibody to latex beads

Carboxylate-modified micro-particles (CM-MP) (0.279 μm, Seradyn, IN) were used for dipstick assay. For coating of antibody, the reaction was carried out in 500 μl buffer containing 50 μl of 10% (v/v) solid stock CM-MP (1% solid final), and affinity purified IgG fraction of the antibody. Different types of buffer and the suitable antibody concentration were examined to obtain the maximum antibody coating. The reaction mixtures were incubated at r.t. for 2 hrs with gentle shaking. To remove unbound antibody, the micro-particles were washed with the buffer and centrifuged at 9000 g for 10 min to remove the buffer for three times. Finally, the antibody coated CM-MPs were resuspended in buffer containing 0.1% fish skin gelatin (FSG) to prevent aggregations of

the antibody coated micro-particles. The amount of antibody bound to CM-MPs was determined by modified BCA assay (Griffin *et al.*, 1994).

2.5.2 Procedures for dipstick assay

100 ng of antibody was applied approximately 1 cm from the lower end of a 4x0.5 cm strip of polymer supported large pore size nitrocellulose membrane (Millipore). The immobilized NC were incubated at 37°C for 1 hr after air-drying. The dried NC strips can be stored in sealed plastic bag in a desiccator at r.t. The test was conducted in a 96-well plate. A total volume of 100 µl of sample, sample buffer, and antibody-coated CM-MP (0.1% v/v solid final) was added to the wells of the plate. The NC stripe was wetted by dipping it about 0.5 cm from the end into the sample. After incubation for 15 min, the strip was taken out to read the result.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 IDENTIFICATION OF A WSSV ISOLATE FROM *P. CHINESIS*

In this study, the WSSV was isolated from *P. chinesis* from Qindao, China. The morphology of this WSSV isolate was examined by electron microscope (EM) and compared with other WSSV isolates.

3.1.1 Virus isolation and purification

Frozen WSSV-infected shrimp, *Penaeid chinesis*, was obtained from Qingdao, the People's Republic of China in 1999. A 3% (w/v) of gill filtrate was prepared from the frozen shrimp and inoculated intramuscularly into specific pathogen free (SPF) *Penaeid vannamei* (50-60 g). It resulted in 100% mortality in the shrimp within 3-4 days of inoculation. The diseased shrimp exhibited red coloration on their appendages. But one of the characteristic clinical signs, white spots on the carapace (Figure 2), did not show up in the diseased shrimp. This WSSV isolate was named WSSV99C. Laboratory infection of SPF *P. vannamei* with WSSV isolated from *P. japonicus* (Dalian, China, 1995/WSSV95C), *P. monodon* (Indonesia, 1997/WSSV97In), *P. setiferus* (South Carolina, USA, 1997/WSSV97SC), and *P. japonicus* (Japan, 1998/WSSV98Jap) also lacked the symptom of the white spots on the carapace. WSSV infection may not be the only reason for the white spots to appear in naturally infected shrimp. Other factors, such as host animal species, the route of infection and the dose of the inoculum may contribute to the white spot formation.

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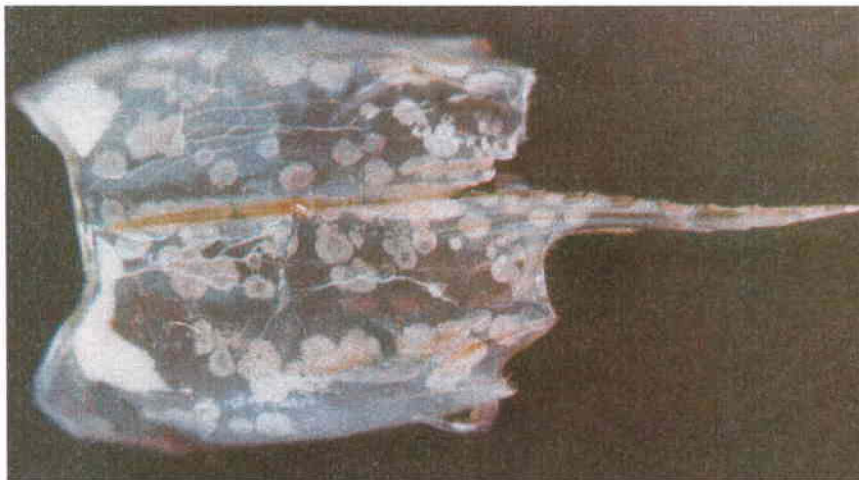
Figure 2. The white spots on diseased shrimp. A: The head of WSSV infected shrimp. B: White spot on the surface of the carapace. (Krishna *et al*, 1997).

3.1.2 Negative staining and EM examination of WSSV99C

Head soft tissue, gill, and hemolymph from moribund or dead shrimp were collected to use in virus purification. The purified virus was negatively stained and examined under the electron microscope (Figure 3). WSSV99C was a rod-shaped virus with an envelope. The size of its nucleocapsid was of approximately 330 nm in length and 65 nm in diameter. The virus did not show any morphological differences from other WSSV strains isolated from different shrimp species and from different geographical regions (Wongteerasupaya *et al.*, 1995; Wang *et al.*, 1995; Yang *et al.*, 1997; Hameed *et al.*, 1998; Nadala *et al.*, 1998a). However, genome sequencing and restriction fragment length polymorphism (RFLP) analysis have shown that there were differences among different WSSV isolates (Nadala *et al.*, 1998a; van Hulten *et al.*, 2001; Yang *et al.*, 2001). The genome size of WSSV isolated from viral infected *P. monodon* collected from Thailand was 292,967 bp (van Hulten *et al.*, 2001); that of virus isolated from *P. japonicus* shrimp collected from Xiamen, east China was 305,107 bp (Yang *et al.*, 2001); and that of WSSV isolated from Taiwan was 307,287 bp (Lo *et al.* GenBank access No. AF440570). RFLP analysis showed that WSSV95C could be distinguished from WSSV97In and WSSV97SC (Nadala *et al.*, 1998a) by restriction enzyme *Hind* III digestion. Additional genomic studies are needed in order to find genetic markers which can be used to distinguish different WSSV isolates. They would be valuable in tracking the origin of WSSV if there were outbreaks.
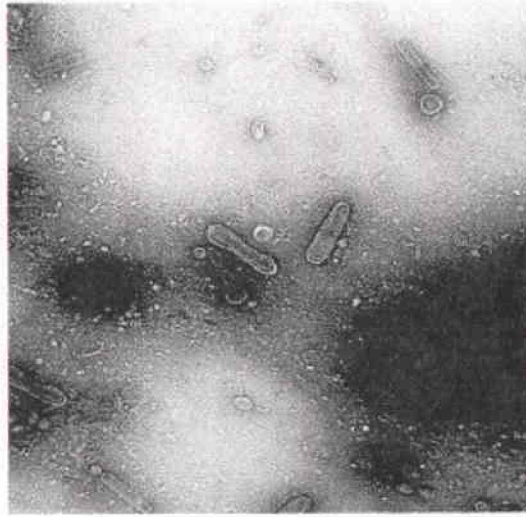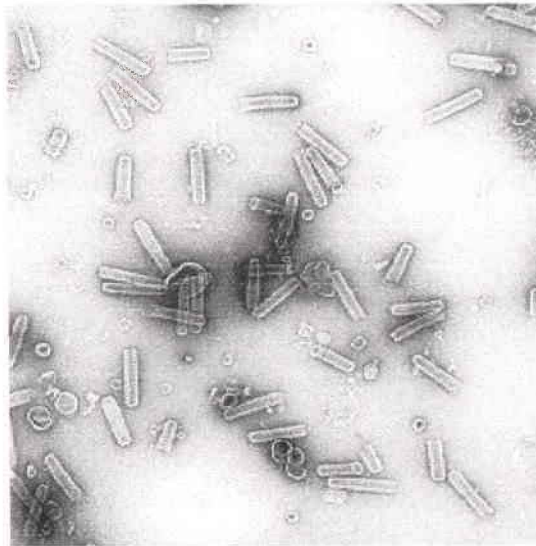
Figure 3. Electron microscope image of WSSV isolated from *P. chinesis* (Qindao, China). (A) WSSV virion. (B) WSSV nucleocapsid.

3.2 CONSTRUCTION AND SCREENING OF cDNA LIBRARY

White spot syndrome virus is a double-stranded DNA virus. WSSV95C genome size was estimated to be between 190 kb and 200 kb (Nadala *et al.*, 1998a), or ~200 kb (Yang *et al.*, 1997), by restriction enzyme digestions. The complete sequence of the WSSV genome was not available until 2001 (van Hulten *et al.*, 2001; Yang *et al.*, 2001).

In 1999, work was started on the cloning of the genes encoding major viral structural proteins beginning with the VP27.5 kDa component. We decided to set up a cDNA library instead of a genomic DNA library. Since one of the advantages of using cDNA library is that it generally contains only the coding regions of a genome with introns removed. With cDNA information, the coding regions on the genome can be correctly assigned. Sequencing of cDNA clones can also help us identify the polyadenylation (polyA) signal and polyA addition site on mRNA. A cDNA library can be used to study the viral gene expression and the abundance of viral mRNA in the tissues. Common to all screening processes, a well-designed strategy to enrich the target from the background is the key to success. Generally, viral structural proteins are expressed in large amounts during virus replication. There should be abundant amounts of corresponding viral mRNA molecules in the host cells. The cDNA library approach does not require the isolation of virion, which usually is difficult, time-consuming and expensive. The cDNA library method is readily applicable to other viruses and the protocol used in this study can be used with little modification.

3.2.1 Isolation of total RNA and mRNA from WSSV infected shrimp gill tissue

The transcription of DNA to RNA is the initial step in the gene expression. The amount and frequency distribution of RNA molecules reflects the developmental or physiological status of the cell. In addition, changes in the transcription pattern may occur in response to a disease. In a viral infected cell, the expression of viral genomes is in competition with that of the cellular genes. In order to synthesize abundant amounts of their proteins, viruses have evolved strategies that either confer competitive advantage to viral mRNA or abolish the synthesis or translation of cellular mRNAs. The actual amount of mRNA made also depends on the cell type and physiological state.

Previous studies had showed that WSSV had at least four major structural proteins (Nadala *et al.*, 1997b). Western blot analysis showed that one of the major WSSV structural proteins VP27.5 appeared at 41 hr post infection in experimentally infected shrimp (Nadala *et al.*, 1997b). *In vitro* quantal assay using primary shrimp lymphoid cell cultures showed that WSSV infected shrimp gill tissue had the highest viral titer among all tissues, e.g. hemolymph, head soft tissue, and muscles (Tapay *et al.*, 1997). Therefore, gill tissues from WSSV-infected shrimp were used for isolation of total RNA. The tissues were collected from moribund shrimp at 40 hr post infection. About 20 mg of total RNA was obtained from 1.7 gram of gill tissue. The total RNA was treated with DNase and polyadenylated mRNA was purified using Oligotex (Qiagen, CA). Agarose gel electrophoresis analysis of the synthesized cDNA was shown in Figure 4.
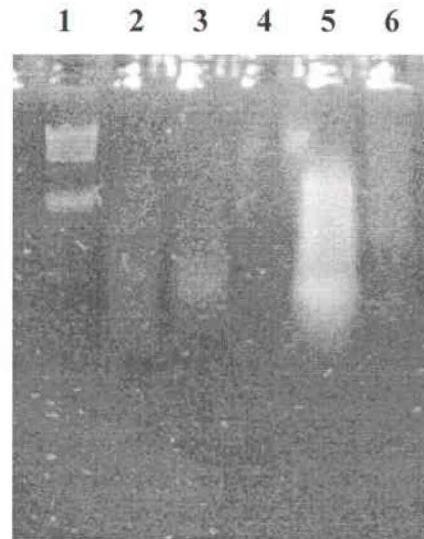
Figure 4. 1% agarose electrophoresis gel analysis of WSSV mRNA and cDNA. mRNA was isolated from WSSV infected shrimp gill. cDNA was synthesized using either random hexamer or oligo(dT)$_{12-18}$ as primers. Lane 1, λ DNA/*Hind* III molecular weight marker; lane 2, cDNA synthesized by using random hexamer as primer; lane 3, cDNA synthesized by using oligo(dT)$_{12-18}$ as primer; lane 4, blank; lane 5, RNA marker as control; lane 6, mRNA isolated from WSSV infected shrimp gill.

3.2.2 Construction and screening of cDNA library

3.2.2.1 Synthesis of cDNA

The cDNA can be synthesized by using either random hexamer or oligo(dT) as

primer. Random hexamers will prime at random sites on mRNA molecules. Oligo(dT)

will prime at the 3'-end of polyadenylated mRNAs. In this study, both primers were used

to synthesis cDNA. Agarose gel electrophoresis showed that the length of cDNA

synthesized by random hexamer method ranged from ~ 200 bp to 2 kb (Figure 4). The

length of cDNA synthesized by oligo(dT)$_{12-18}$ method ranged from ~400 bp to over 2 kb

(Figure 4). cDNA synthesized using random hexamer could increase the representation

of 5' ends of mRNA having significant secondary structure, or for mRNA lacking a

polyA tail, but the amount of large size cDNA fragments was small in the population.

With oligo(dT)$_{12-18}$ primer, the average size of the cDNA fragment was relatively large

and the size range was larger. The polyA site of mRNA could be determined by

sequencing of cDNA clones.

3.2.2.2 PCR amplification of cDNA

A PCR strategy was developed for amplification of double-stranded cDNA and

enrichment of abundant cDNA. A homopolymeric oligo(dC) or oligo(dA) tail was added

to the 3' end of cDNA by terminal deoxynucleotidyl transferase (TdT). A primer with a

poly(dG) or poly(dT) tail at its 3' end was used to amplify the cDNA with oligo(dC) or

oligo(dA) tail (Figure 5).

ds cDNA

5' ————————— A$_N$ 3'
3' ————————— T$_N$ 5'

Tailing

5' ————————— A$_N$A$_M$ 3'
3' A$_M$ ————————— T$_N$ 5'

1st PCR cycle

Template 5' ————— A$_N$ A$_M$ GGTACCGAATTCTGGATCCGC 3'
3' ————— T(T)$_{19}$CCATGGCTTAAGACCTAGGCG 5'
T-primer

T-primer
5'GCGGATCCAGAATTCGGTACC(T)$_{19}$————— A$_N$ 3'
3'CGCCTAGGTCTTAAGCCATGG A$_M$ ————— T$_N$ 5' Template

2nd PCR cycle

Template 5'GCGGATCCAGAATTCGGTACC(T)$_{19}$————— A$_N$ GGTACCGAATTCTGGATCCGC 3'
3'CGCCTAGGTCTTAAGCCATGG(A)$_{19}$————— (T)$_{19}$ CCATGGCTTAAGACCTAGGCG 5'
T-primer

Both strands will be used as template for further PCR amplification and will be amplified exponentially.

T-primer
5'GCGGATCCAGAATTCGGTACC(T)$_{19}$————— A$_N$ 3'
Template 3'CGCCTAGGTCTTAAGCCATGG A$_N$ ————— T$_N$ 5'

The amount of this product will grow linearly with PCR cycle number.

5'GCGGATCCAGAATTCGGTACC(T)$_{19}$
3'CGCCTAGGTCTTAAGCCATGG A$_n$

Short PCR products tend to form this inverted loop structure thus preventing being amplified.

Figure 5. A schematic diagram for PCR amplification of cDNA.
T-primer: 5'GCGGATCCAGAATTCGGTACC(T)$_{19}$ 3'

In order to determine whether oligo(dC) or oligo(dA) tail should be used for cDNA tailing, oligo(dC)-tailed cDNA was amplified by using G-primer: 5'GCGGATCCAGAATTC(G)$_9$ 3' (24 mer). Oligo(dA)-tailed cDNA was amplified by using T-primer: 5'GCGGATCCAGAATTCGGTACC(T)$_{19}$ 3' (40 mer). The 40 cycles of PCR were performed as follows: denaturation, 94°C for 10 sec; annealing, 60°C for 30 sec; elongation, 72°C for 60 sec. The concentration of the primers varied from 250 nM to 1000 nM. Agarose gel electrophoresis showed that there were several distinctive bands among the PCR product amplified by G-primer. This indicated that G-primer amplification resulted in preferential amplification of certain cDNA fragments. T-primer amplified cDNA PCR product showed a uniformed smear indicating that the amplification did not have any preference. Incidentally, PCR amplification of WSSV95C genomic DNA sheared by sonication and tailed by oligo(dC) or oligo(dA) showed similar results. Based on the above observations, T-primer was chosen for WSSV cDNA amplification.

Double-stranded cDNA was synthesized from mRNA isolated from WSSV infected shrimp gill tissue using TimeSaver® cDNA synthesis Kit. The excess random hexamer or oligo(dT)$_{12-18}$ primers were removed by using QIAquick PCR purification column. An oligo(dA) tail was added to cDNA by TdT and tailed cDNA was amplified by PCR using T-primer. Agarose gel electrophoresis showed that the average length of PCR product from cDNA synthesized using random hexamer was smaller than that of cDNA synthesized using oligo(dT)$_{12-18}$ (Figure 6A). It also showed that the average length of PCR product increased as the concentration of primer decreased (Figure 6A)

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from 1000 nM to 250 nM. The cDNA synthesized by using oligo(dT)$_{12-18}$ primer and amplified by 250 nM T-primer gave the largest average size of PCR product. This PCR product was size-fractioned by gel electrophoresis and the larger size fraction was used for second round PCR amplification. The PCR production synthesized by using 250 nM T- primer was purified and used for subsequent cloning (Figure 6B).

One serious problem of PCR amplification of heterogeneous cDNA molecules is that preferential amplification of relatively short cDNA sequences enriches short molecules in the PCR products. There are two approaches to addressing this problem. One approach is the re-amplification of size-fractionated amplified cDNA. The other approach was proposed by Lukyanov *et al*. (1995) that is based on insertion of inverted terminal repeats into amplified cDNAs. The inhibitory effect of self-annealing of the inverted terminal repeat on amplification efficiency depends on the length of amplified molecules: the shorter the molecule, the more effective is the inhibition due to self-annealing to its own ends. This can reduce the generation of the primer dimers and the average length of the amplified sequences could be controlled by varying the primer concentrations (Figure 6). In our case, each PCR reaction was carried out with one primer and the inverted terminal repeats consisted of the primers at both ends of the PCR products. The two approaches were combined in our study.
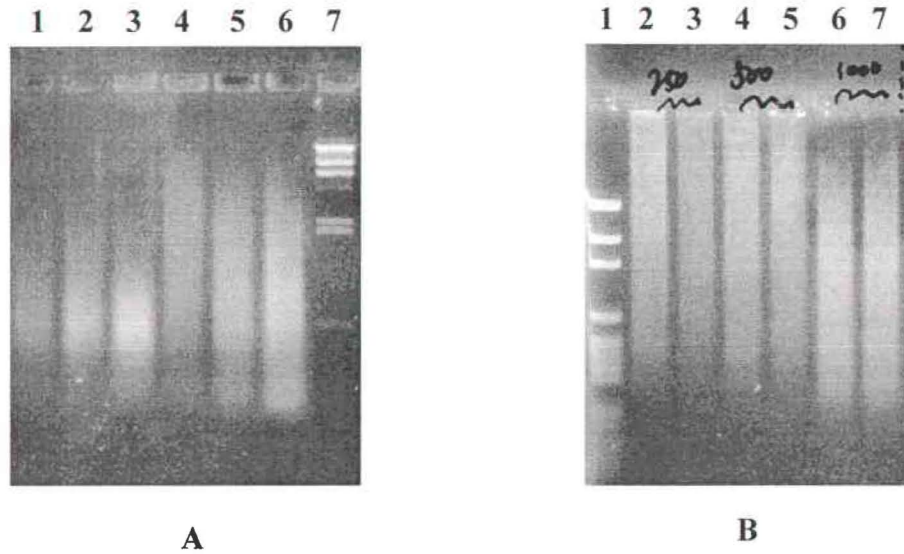
Figure 6. PCR amplified WSSV cDNA using different concentrations of T-primer.
(A) Lane 1-3, PCR product of oligo(dA) tailed cDNA (synthesized by using random
hexamer) amplified using 250, 500 and 1000 nM T-primer; lane 4-6, PCR product of
oligo(dA) tailed cDNA [synthesized by using oligo(dT)$_{12-18}$ primer] amplified using 250,
500 and 1000 nM T-primer; lane 7, λ DNA/*Hind* III molecular weight marker. (B) Lane
1, pGEM molecular weight marker (Promega, WI). Lane 2-7, 2nd round PCR
amplification using larger size fraction of 1st round PCR product as template (Figure
6A:lane 4) and T-primer. Lane 2 and 3, 250 nM T-primer; lane 4 and 5, 500 nM T-
primer; lane 6 and 7, 1000 nM T- primer.

3.2.2.3 <u>cDNA cloning</u>

PCR amplified cDNA was purified by QIAquick PCR purification kit, digested

with restriction enzyme *EcoRI*, and ligated to a pUC19 vector which was also digested

with *Eco*RI and dephosphorylated with CIP. About 1×10^7 transformants were generated

from 100 ng of ligation product by transforming *E.coli* XL-1 blue competent cells. The

average length of inserts was between 500 bp and 1 kb as determined by isolation of

plasmid DNA and gel electrophoresis, even though the majority of cDNA population

used for cloning were large in length. It is known that transformation using Ca^{2+} treated

E.coli favors plasmids with smaller insert. Transformation efficiency is poor for clones

with larger insert. Delivering plasmid DNA to *E.coli* cell by electroporation will

overcome this problem. Electroporation relies on high-voltage pulses that cause cellular

membrane to break down, producing transient pores through which DNA can enter.

During electroporation, plasmid DNA with small or large insert has the same chance of

being delivered into the *E.coli*. cells.

3.2.2.4 <u>Screening of the cDNA library</u>

The cDNA library constructed using mRNA isolated from WSSV infected shrimp

was screened by using a set of probes in order to enrich and identify clones with viral

cDNA insert. DIG-labeled WSSV genomic DNA, DIG-labeled shrimp genomic DNA,

and DIG-labeled shrimp cDNA probes were used to screen the library. Shrimp genomic

DNA probe was used for screening shrimp cDNA clones. There might be false negative

due to the low sensitivity of this probe because for each cDNA species, the genomic

version is highly diluted by the genome size of shrimp. Healthy shrimp cDNA probe was

also used to identify false negative clones. WSSV genomic DNA probe was used to identify viral cDNA clones. But there might be false positive due to the possible contamination viral DNA by shrimp DNA. *In situ* colony hybridization was performed. The detection sensitivity was low and not enough to differentiate clones. Since each DIG-labeled probe used for screening was composed of many different types of DNA fragments, the concentration of each individual DNA fragment was very low among the population.

In order to increase the screening sensitivity, nucleic acid dot hybridization was performed. For nucleic acid dot hybridization, larger amount of purified DNA can be used. Plasmid DNA was isolated, denatured, spotted and fixed on a nylon membrane. The probe concentration and hybridization conditions were optimized (Figure 7). The WSSV cDNA clones identified by using above mentioned probes were confirmed by labeling the cDNA insert as a probe for hybridization. This gives the highest sensitivity but is practical only for the last step in screening as a labeled probe is prepared for each clone to be checked. DIG-labeled WSSV cDNA insert hybridized with WSSV genomic DNA, but did not hybridize with shrimp genomic DNA. Several WSSV cDNA clones were selected for sequencing and sequence analysis.
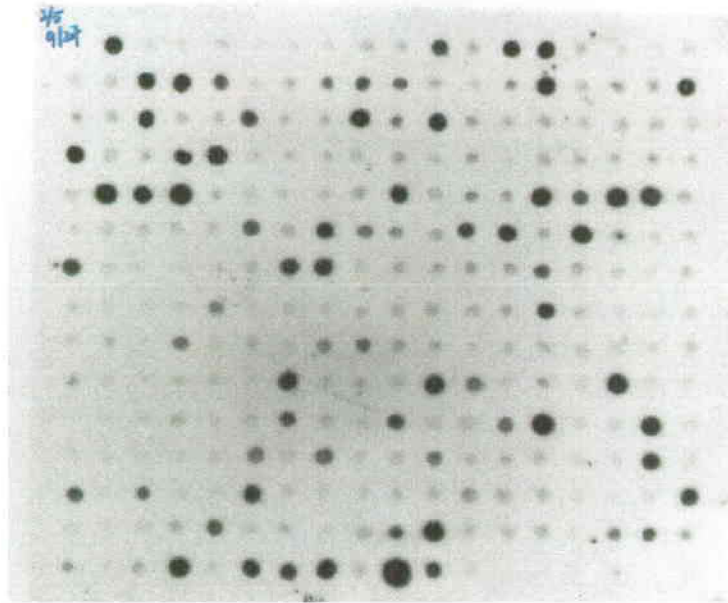
Figure 7. An example of DNA dot blot using different probes.
(A) a membrane with 260 cDNA clones was screened with DIG-labeled shrimp cDNA probe. (B) a replicate of the same membrane in A was screened with DIG-labeled WSSV genomic DNA. Clones marked in green were probably WSSV cDNA clones. Clones marked in black were probably shrimp DNA clones.

3.2.3 Sequence analysis of cDNA clones

The WSSV cDNA sequences were compared with known sequences in GenBank using BLAST® program. The deduced peptide sequences were compared with the protein sequences in the SWISS-PROT. Membrane proteins play key roles in biological systems as pores, ion channels, and receptors. Prediction that a protein has a membrane domain or domains is based on the hydrophobicity/hydropathy profile of the protein. Several scales were developed to determine the protein hydropathic profile (Engelman *et al.* 1986; Kyte *et al.*, 1982). The hydropathic profile of a protein or peptide is determined by assigning each amino acid a numerical value ("hydropathy index"). These values are then repetitively averaged along the peptide chain. The values assigned to each amino acid can either be the Hopp-Woods values (1981) or the Kyte-Doolittle values (1982). The Hopp-woods value of each amino acid is assigned based on the solubility of its side chain, while the Kyte-Doolittle values are determined based on empirical observation. Engelman *et al.* (1982) developed the Goldman-Engelman-Steitz (GES) hydrophobicity scale, based on experimental and theoretical considerations about how well each amino acid would enter the lipid bilayer from an aqueous environment. GES scale seems to predict known membrane helices better than other scales do, especially those that are known to contain some polar residues (Engelman *et al*, 1986). Engelman *et al.*'s method also accounts for the possibility of polar groups interacting in the bilayer, which would increase the chance of recognizing a transmembrane sequence containing a few polar groups. Analysis of solved membrane proteins reveals that the length range for a helix to stretch across an average bilayer is 14 to 36 amino acids (Bowie, 1997). Using different

hydrophobicity scale may result in variations in peptide hydrophobicity prediction. In

this study, the hydrophobicity characters of a protein or peptide was plotted using Kyte-

Doolittle scale and GES scale in TopPred program.

3.2.3.1 cDNA clone C6

Clone C6 had a insert of ~750 bp (GenBank access no. AY249434). An open

reading frame (ORF) which was 615 bp long was identified on this DNA fragment

(Figure 8). This ORF encoded a peptide with 204 amino acid (aa) of which the

theoretical molecular mass was 22 kDa. Nucleic acid sequence comparison showed that

it was identical to the following WSSV DNA sequences in GenBank: ORF wssv480 of

Taiwan isolate (AF440570) and ORF wsv421 of Xiamen, China isolate (AF332093).

The sequence of C6 clone was also identical to that of ORF1 of Thailand isolate

(AF369029), which encoded an envelope protein vp28 (van Hulten *et al.*, 2001). The

hydrophobic profile analysis using both Kyte-Dolittle and GES scales (Figure 9) revealed

the peptide encoded by the ORF on C6 had two hydrophobic segments. One of the

segments was at the N-terminal of the peptide, including approximate thirty amino acids.

The other hydrophobic segment was located at the C-terminal of the peptide and

encompassed about twenty amino acids. vp28 protein (van Hulten *et al.*, 2001) is the

same as VP27.5 outer membrane protein identified by Nadala *et al.* (1998b).

A putative polyadenylation (polyA) signal AATAAA was found 54 bp

downstream of the ORF stop codon TAA. The polyA tail was 16 bp downstream of the

polyA signal. The position of polyA site was confirmed by comparing this sequence with

the sequence of another cDNA clone C8 which contained 3' part of the sequence of the

ORF and the polyA tail. In eukaryotes, there is a consensus AAUAAA sequence located 10-30 nucleotides 5' of the polyA site. The putative polyA signal of this ORF was consistent with that of eukaryotes.

Zhang *et al.* (2002) isolated a cDNA clone C37 from a cDNA library of WSSV-infected *P. japonicus* tissue from Xiamen, China. Clone C37 was found to contain the gene encoding VP28 protein. Two putative poly(A) signals (54 bp and 201 bp downstream of the vp28 gene's stop codon, respectively) and a poly(A) site (221 bp downstream of the vp28 gene's stop codon) were identified on the insert (the sequence was named 421mRNA and deposited in the GenBank with access no. AF227911) of clone C37 by Zhang et al. (2002). However, we analyzed the genome sequence of WSSV isolated from viral infected *P. japonicus* tissue from Xiamen, China (Yang et al., 2001), and found that there is only one putative poly(A) signal (54 bp downstream of the vp28 gene's stop codon) for the vp28 gene (ORF wsv421) (Fig. 10); analysis of the genomic sequence downstream of vp28 genes of WSSV from Thailand (van Hulten et al., 2001) and Taiwan (Lo et al., 2001) also showed that there is only one putative poly(A) signal, which is consistent with our finding. Further study is needed to find out why the second poly(A) signal on clone C37 was not present on the WSSV genome. We have also noted that the 3' non-translational region of the vp28 mRNA by Zhang et al. (2002) showed significant base changes when compared to its genomic counterpart in the WSSV isolated from Xiamen, China (Yang et al., 2001) and other two WSSV isolates (van Hulten et al, 2001; Lo et al, 2001) (Fig. 10). Generally, precursor RNAs made in the nuclei of eukaryotic cells undergo three types of modifications. These include

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```
                                gttcgataaagaaaaaaactcgtc

atggatctttctttcactctttcggtcgtgtcggccatcctcgcc
M   D   L   S   F   T   L   S   V   V   S   A   I   L   A
atcactgctgtgattgctgtatttattgtgattttaggtatcac
I   T   A   V   I   A   V   F   I   V   I   F   R   Y   H
aacactgtgaccaagaccatcgaaacccgcacagacaatatcgag
N   T   V   T   K   T   I   E   T   R   T   D   N   I   E
acaaacatggatgaaaacctccgcattcctgtgactgctgaggtt
T   N   M   D   E   N   L   R   I   P   V   T   A   E   V
ggatcaggctacttcaagatgactgatgtgtcctttgacagcgac
G   S   G   Y   F   K   M   T   D   V   S   F   D   S   D
accttgggcaaaatcaagatccgcaatggaaagtctgatgcacag
T   L   G   K   I   K   I   R   N   G   K   S   D   A   Q
atgaaggaagaagatgcggatcttgtcatcactcccgtggagggc
M   K   E   E   D   A   D   L   V   I   T   P   V   E   G
cgagcactcgaagtgactgtggggcagaatctcacctttgaggga
R   A   L   E   V   T   V   G   Q   N   L   T   F   E   G
acattcaaggtgtggaacaacacatcaagaaagatcaacatcact
T   F   K   V   W   N   N   T   S   R   K   I   N   I   T
ggtatgcagatggtgccaaagattaacccatcaaaggcctttgtc
G   M   Q   M   V   P   K   I   N   P   S   K   A   F   V
ggtagctccaacacctcctccttcaccccccgtctctattgatgag
G   S   S   N   T   S   S   F   T   P   V   S   I   D   E
gatgaagttggcacctttgtgtgtggtaccacctttggcgcacca
D   E   V   G   T   F   V   C   G   T   T   F   G   A   P
attgcagctaccgccggtggaaatcttttcgacatgtacgtgcac
I   A   A   T   A   G   G   N   L   F   D   M   Y   V   H
gtcacctactctggcactgagaccgagtaaataaatcgtgctttt
V   T   Y   S   G   T   E   T   *
ttatatagatagggaattttaatattacaacaataagaaaataaa
                                                polyA signal
acaattgaggaaatttaaaaaaaaaaaaaaaaaaaaaa
            polyA tail
```

Figure 8. cDNA sequence of clone C6 and its corresponding amino acid sequence.

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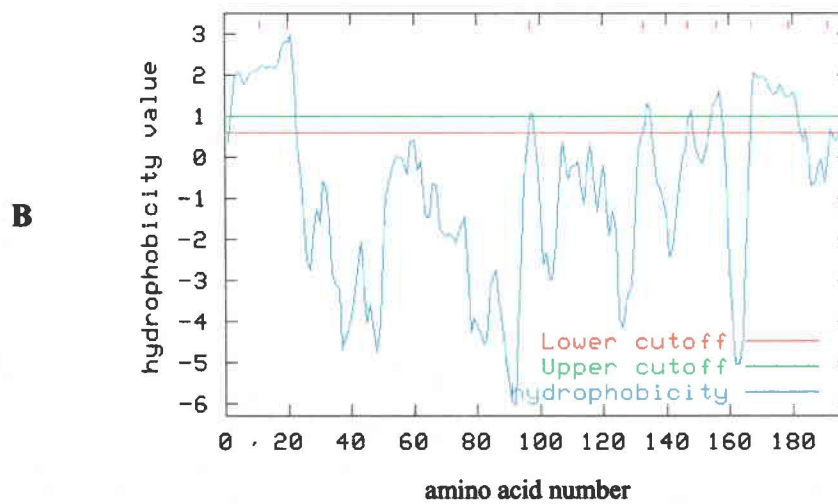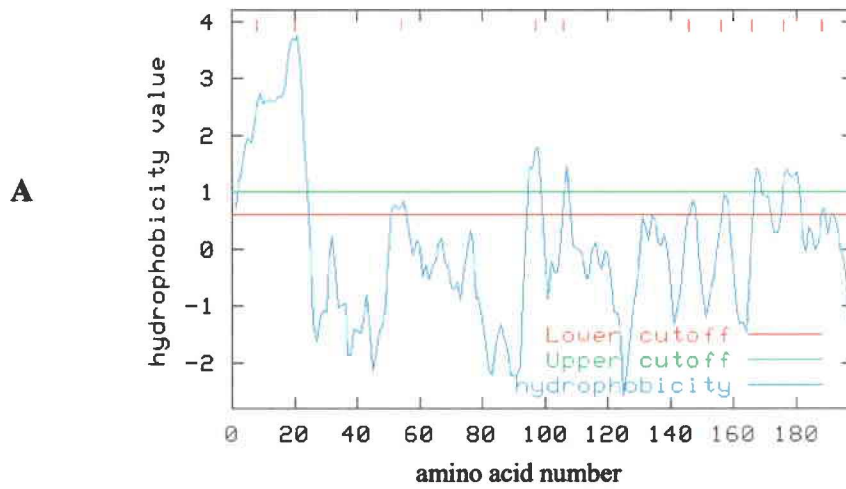
Figure 9. Hydrophobicity profiles of vp27.5 kDa protein using TopPred program. Hydrophobicity scales were Kyte-Dolittle scale (A) and GES scale (B).

```
ORF1     TAAATAAATCGTGCTTTTTTATATAGATAGGGAATTTTAATATTACAACAATAAGAAAATAAAACAATTG
wssv480  TAAATAAATCGTGCTTTTTTATATAGATAGGGAATTTTAATATTACAACAATAAGAAAATAAAACAATTG
wsv421   TAAATAAATCGTGCTTTTTTATATAGATAGGGAATTTTAATATTACAACAATAAGAAAATAAAACAATTG
421mRNA  TAAATAAATCGTGCTTTTTTATATAGATAGGGAATTTTAATATTACAACAATAAGAAAATAAAACAATTG
95C-C6   TAAATAAATCGTGCTTTTTTATATAGATAGGGAATTTTAATATTACAACAATAAGAAAATAAAACAATTG
         1........10........20........30........40........50........60.........
         Stop codon of vp28 gene                                        First Poly(A) signal


ORF1     AGGAAATTTATACCATATTTTATTGACCTACTTAACCTTCTTGCTATACAATGAATGTTTAGGTGACTGG
wssv480  AGGAAATTTATACCATATTTTATTGACCTACTTAACCTTCTTGCTATACAATGAATGTTTAGGTGACTGG
wsv421   AGGAAATTTATACCATATTTTATTGACCTACTTAACCTTCTTGCTATACAATGAATGTTTAGGTGACTGG
421mRNA  AGCGGATACCCTCTGGATCGTAAAGTTCCAGATGAACGTGTT----TTCGAGGATCTTCCTAACTTTGGC
95C-C6   AGGAAATTTAAAAAAAAAAAAAAAAAAAAAAAAAThe end of cDNA insert
              Poly (A) site on clone 95C-C6
         71.......80........90........100.......110.......120.......130........


ORF1     AAAAGTTTAGCAATATTATCCTTGAACGGGAAACATGCACCAATTACAGGCGCAATTTCATACGCTCTCG
wssv480  AAAAGTTTAGCAATATTATCCTTGAACGGGAAACATGCACCAATTACAGGCGCAATTTCATACGCTCTCG
wsv421   AAAAGTTTAGCAATATTATCCTTGAACGGGAAACATGCACCAATTACAGGCGCAATTTCATACGCTCTCG
421mRNA  CACATCCAAGTAAAGGTGTTCAATCACGGTGAACACATCCGCCATAATTAGGTAATACCGTTATATCAGA

         141......150.......160.......170.......180.......190.......200........


ORF1     GCCTATTGGTCTTTTCCTGGTCATACATTTTAGATACAATAGACAAAAATGGAATGTTTGTATAGATAGA
wssv480  GCCTATTGGTCTTTTCCTGGTCATACATTTTAGATACAATAGACAAAAATGGAATGTTTGTATAGATAGA
wsv421   GCCTATTGGTCTTTTCCTGGTCATACATTTTAGATACAATAGACAAAAATGGAATGTTTGTATAGATAGA
421mRNA  CACTGGTTATGATTGCCATTTTAGAGTCTGACCATTAATTCCGAGCAAAAGAAATA---AAATTAAAAAA
                                                             Second Poly(A) signal

         211......220.......230.......240.......250.......260.......270........


ORF1     ATTGGCAG
wssv480  ATTGGCAG
wsv421   ATTGGCAG
421mRNA  ATTGAC————————————————————————>
              Poly(A) site on 421 mRNA

         281.....
```

Figure 10. Boxshade of multiple-aligned WSSV genomic DNA and mRNA (cDNA) sequences, starting from the stop codon (TAA) of the gene encoding VP28 kDa protein.
ORF1: DNA sequence of WSSV Thailand isolate (van Hulten et al., 2001, GenBank access no. AF369029: from 613 bp to 900 bp);
wssv480: DNA sequence of WSSV Taiwan isolate (Lo et al., 2001, GenBank access no. AF440570: from 279477 bp to 279764 bp);
wsv421: DNA sequence of WSSV Xiamen, China isolate (Yang et al., 2001, GenBank access no. AF332093: from 244854 bp to 245141 bp);
421mRNA: mRNA sequence (clone C37) of WSSV Xiamen, China isolate (Zhang et al., 2002, GenBank access no. AF227911: from 1095 bp to 1373 bp);
95C-C6: cDNA clone sequence of WSSV95C (this study, GenBank access no. AY249434).
The black shading shows the differences between the sequence of 421mRNA and genomic DNA sequences of other WSSV isolates.

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methylation of the 2'- hydroxyl group of the ribose near the co-transcriptionally added cap, removal of introns and polyadenylation before transported into the cytoplasm for translation. Many genes, based on the cellular environment, have been characterized as having alternative polyadenylation site used at the 3'-end of their mRNAs. Multiple poly(A) sites have been observed in the genes of herpes simplex virus and adenovirus. In contrast to what Zhang et al. (2002) observed, significant base alterations in the 3'-nontranslational region have not been reported before.

3.2.3.2 <u>cDNA clone C603</u>

The sequence of C603 (GenBank access no. AY245790) (Figure 11) was identical to the DNA sequence of following ORFs in the GenBank: wssv473, wsv414, ORF182 from WSSV isolates of Taiwan, China, and Thailand respectively. The sequences of these ORFs were the same and encoded an envelope protein vp19 (van Hulten *et al.*, 2001; Nadala *et al.*, 1998b). PolyA site was 16 bp downstream of a putative polyA signal. vp19 was one of the WSSV structural proteins which was conserved among different WSSV isolates.

3.2.3.3 <u>cDNA clone C712</u>

cDNA clone C712 had an insert of ~950 bp. Sequence analysis of C712 showed that it was identical to a DNA fragment encoding ribonucelotide reductase small subunit (RR2) of WSSV Thailand isolate (van Hulten *et al.*, 2000) except that a 42 bp fragment was deleted in C712 (Figure12). The deletion may have occurred when the plasmid containing the cDNA insert was propagating in *E.coli* host strain XL-1 blue. Studies have shown that many segments of DNA cannot be cloned in conventional *E.coli* strains.

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These foreign inserts are capable of forming nonstandard secondary and tertiary structures, which are then deleted by the host. One of the structures that occurs frequently in eukaryotic DNA and is known to be highly unstable is cruciform which are caused by inverted repeats (Raleigh *et al.*, 1986; Kretz *et al.*, 1991). In C712, two identical invert repeats sequence (TTGAAC) flanked the 42 bp DNA fragment (Figure 12) which was deleted.

There was a putative polyA signal 14 bp upstream of polyA site according to the cDNA sequence of clone C712. Transcriptional analysis of the ribonucleotide reductase genes (Tsai *et al.*, 2000) showed that the mRNA RR2 ended 13 bp from the putative polyA signal. This discrepancy could be due to the technique used in transcriptional analysis.

RR2 is encoded by ORF wsv184 in Xiamen, China WSSV isolate (AF332093), and by ORF wssv243 in Taiwan WSSV isolate (AF440570). Ribonucleotide reductase has two different subunits: the large subunit (RR1) and the small subunit (RR2). It is an important enzyme which provides dNTP precursors for DNA replication.

```
                    taacgatgatgaggacaaatataagaac
                 N  D  D  E  D  K  Y  K  N
aggaccagggatatgatgcttctggctgggtccgctcttctgttc
 R  T  R  D  M  M  L  L  A  G  S  A  L  L  F
ctcgtttccgccgccaccgtttttatgtcttaccccaagaggagg
 L  V  S  A  A  T  V  F  M  S  Y  P  K  R  R
cagtaataaaaatatagaaaacacaacatcagggacaaaaattat
 Q  *
aattaaaacaccttttattataaaataaaatttatttgaaaactt
```

                        polyA signal

<u>aaaaaa</u>aaaaaaaaaaa➤
<u>polyA tail</u>

c603   --
wsv414   **ATG**GCCACCACGACTAACACTCTTCCTTTCGGCAGGACCGGAGCCCAGGCCGCTGGCCCT
wssv473 **ATG**GCCACCACGACTAACACTCTTCCTTTCGGCAGGACCGGAGCCCAGGCCGCTGGCCCT
ORF182   **ATG**GCCACCACGACTAACACTCTTCCTTTCGGCAGGACCGGAGCCCAGGCCGCTGGCCCT

c603   --
wsv414   TCTTACACCATGGAAGATCTTGAAGGCTCCATGTCTATGGCTCGCATGGGTCTCTTTTTG
wssv473 TCTTACACCATGGAAGATCTTGAAGGCTCCATGTCTATGGCTCGCATGGGTCTCTTTTTG
ORF182   TCTTACACCATGGAAGATCTTGAAGGCTCCATGTCTATGGCTCGCATGGGTCTCTTTTTG

c603   --
wsv414   ATCGTTGCTATCTCAATTGGTATCCTCGTCCTGGCCGTCATGAATGTATGGATGGGACCA
wssv473 ATCGTTGCTATCTCAATTGGTATCCTCGTCCTGGCCGTCATGAATGTATGGATGGGACCA
ORF182   ATCGTTGCTATCTCAATTGGTATCCTCGTCCTGGCCGTCATGAATGTATGGATGGGACCA

c603   --
wsv414   AAGAAGGACAGCGATCCTGACACTGATAAGGACACCGATGATGATGACGACACTGCCAAC
wssv473 AAGAAGGACAGCGATTCTGACACTGATAAGGACACCGATGATGATGACGACACTGCCAAC
ORF182   AAGAAGGACAGCGATTCTGACACTGATAAGGACACCGTTGATGATGACGACACTGCCAAC

c603   --TAACGATGATGAGGACAAATATAAGAACAGGACCAGGGATATGATGCTTCTGGCTGGG
wsv414   GATAACGATGATGAGGACAAATATAAGAACAGGACCAGGGATATGATGCTTCTGGCTGGG
wssv473 GATAACGATGATGAGGACAAATATAAGAACAGGACCAGGGATATGATGCTTCTGGCTGGG
ORF182   GATAACGATGATGAGGACAAATATAAGAACAGGACCAGGGATATGATGCTTCTGGCTGGG
           ****

c603   TCCGCTCTTCTGTTCCTCGTTTCCGCCGCCACCGTTTTTATGTCTTACCCCAAGAGGAGG
wsv414   TCCGCTCTTCTGTTCCTCGTTTCCGCCGCCACCGTTTTTATGTCTTACCCCAAGAGGAGG
wssv473 TCCGCTCTTCTGTTCCTCGTTTCCGCCGCCACCGTTTTTATGTCTTACCCCAAGAGGAGG
ORF182   TCCGCTCTTCTGTTCCTCGTTTCCGCCGCCACCGTTTTTATGTCTTACCCCAAGAGGAGG
         ****

c603   CAG**TAA**AAATATAGAAAACACAACATCAGGGACAAAAATTATAATTAAAACACCTTTTAT
wsv414   CAG**TAA**AAATATAGAAAACACAACATCAGGGACAAAAATTATAATTAAAACACCTTTTAT
wssv473 CAG**TAA**AAATATAGAAAACACAACATCAGGGACAAAAATTATAATTAAAACACCTTTTAT
ORF182   CAG**TAA**AAATATAGAAAACACAACATCAGGGACAAAAATTATAATTAAAACACCTTTTAT
         ****

                                    75
```

(continued)

```
                    polyA site                    polyA tail
c603      TATAAAATAAAATTTATTTGAAAACTTaaaaaaaaaaaa------------
wsv414    TATAAAATAAAATTTATTTGAAAACTTATGCATTGTTTGTATACACCCAAAAT
wssv473   TATAAAATAAAATTTATTTGAAAACTTATGCATTGTTTGTATACACCCAAAAT
ORF182    TATAAAATAAAATTTATTTGAAAACTTATGCATTGTTTGTATACACCCAAAAT
          *************************
```

Figure 11. Sequence of cDNA clone C603 and the sequence comparison with ORFs in the GeneBank of other WSSV isolates.

C603: cDNA clone No. 603 of WSSV95C

wsv414: ORF of WSSV genome (AF330293), Xiamen, China isolate

wssv473: ORF of WSSV genome (AF440570), Taiwan isolate

ORF182: ORF of WSSV genome (AF369029), Thailand isolate

```
RR2    ATGGAGTCAATCAAACTGTTCACCGTTGCTGGTCTGAATATGGAGCAAGCCAACCAAGTG
RR2    GCTGAAGAAATCAAGTCAGAATATAAAACCGAGGAGGAAAAGAGGATTGCCCAGGAAGTG
RR2    TTTGACAAATTCACCCAAAAAACTCATTATGCAAGTAGATACGTCTAAACACTTACTTACA
RR2    AGAGAAACCCCAACCGTTTTGTATCCCGCCCCATTGTCCATGAAGATCTCTGGGAAATG
RR2    TACAAAAAAGAGGTTGCCTGTTTTTGGACATTGGAAGAGATTGATTTCGAAAGGGATCCT

RR2    AAAGATTGGGAGAAACTCACTCAAGATGAGAAGGATTTCATTCTCCAGATTCTGGCGTTC
C712   --------------------------AAGGATTTCATTCTCCAGATTCTGGCGTTC
                                 ****************************

RR2    TTTGCATCCTCTGACGGAATTGTAATTGAAAATCTTACAACACGTCTTCGTCAAGTGGCG
C712   TTTGCATCCTCTGACGGAATTGTAATTGAAAATCTTACAACACGTCTTCGTCAAGTGGCG
       ************************************************************

RR2    CAGATTCCAGAAGCGAGGAGTTTCTTTGACTTCCAAGTTGGAATGGAGAGTATTCATGGC
C712   CAGATTCCAGAAGCGAGGAGTTTCTTTGACTTCCAAGTTGGAATGGAGAGTATTCATGGC
       ************************************************************

RR2    AACGTCTACGGAGAACTGATTGATAGACTGGTGCCCGACGAAAAAGACAAGGCTATCTTG
C712   AACGTCTACGGAGAACTGATTGATAGACTGGTGCCCGACGAAAAAGACAAGGCTATCTTG
       ************************************************************

RR2    TTTAACGCTGCACAACACTTCCCCGCCATCAAGAAGAAGGAGCAGTGGGCTATTAATTGG
C712   TTTAACGCTGCACAACACTTCCCCGCCATCAAGAAGAAGGAGCAGTGGGCTATTAATTGG
       ************************************************************

RR2    ATGCAAAGCAATAACGATTTGGCGGAACTAATTGTTGCCTTTGCTGCAGTTGAAGGAATC
C712   ATGCAAAGCAATAACGATTTGGCGGAACTAATTGTTGCCTTTGCTGCAGTTGAAGGAATC
       ************************************************************

RR2    TTCTTTAGTGGTGCATTCGCATCCATTTTCTGGATCAAGAACAGGGGTATTTTGCCTGGT
C712   TTCTTTAGTGGTGCATTCGCATCCATTTTCTGGATCAAGAACAGGGGTATTTTGCCTGGT
       ************************************************************

RR2    CTCACCTCCTCCAATGAGTTCATTTCTAGGGACGAAGGTCTTCATCGCGACTTTGCATGC
C712   CTCACCTCCTCCAATGAGTTCATTTCTAGGGACGAAGGTCTTCATCGCGACTTTGCATGC
       ************************************************************

RR2    ATGCTGTTGAAAAAGGGTTTTGTTGATACCCCATCAAGAGAAAGGATTCTTGAAATTGTC
C712   ATGCTGTTGAAAAAGGGTTTTGTTGATACCCCATCAAGAGAAAGGATTCTTGAAATTGTC
       ************************************************************
```

invert repeat

```
RR2    ACTGAAGCCGTCCGAATTGAACAAGAATTTCTCACAGTTTCCCTGCCTGTTAAATTAGTG
C712   ACTGAAGCCGTCCGAATTGAAC---------------------------------------
       **********************
```

invert repeat

```
RR2    GGAATGAACTGCAAGTTGATGAGCCAGTACATTGAATTTGTGGCAGATAAACTATTGGTT
C712   --------TGCAAGTTGATGAGCCAGTACATTGAATTTGTGGCAGATAAACTATTGGTT
               ***************************************************

RR2    GAAATGGGACTAGAAAAGCACTATAATGTTACCAACCCCTTCCCATTCATGGACAATATT
C712   GAAATGGGACTAGAAAAGCACTATAATGTTACCAACCCCTTCCCATTCATGGACAATATT
       ************************************************************

RR2    TCCCTCGAGAATAAGACCAACTTTTTTGAAAAGAGAGTCGCCGAGTATCAACGTGCCCAG
C712   TCCCTCGAGAATAAGACCAACTTTTTTGAAAAGAGAGTCGCCGAGTATCAACGTGCCCAG
       ************************************************************
```

(continue)

```
RR2    GTCATGGCTTCTATCAATAAGATCAAGAAGGACCAACAAACCCAAGAAACTGGTTCTCCT
C712   GTCATGGCTTCTATCAATAAGATCAAGAAGGACCAACAAACCCAAGAAACTGGTTCTCCT
       ************************************************************


RR2    CTCCCAATTCTGACTGCACCTCCTCCAGTCTCTTCCTCATCATCCGAACAAGAAGATGTT
C712   CTCCCAATTCTGACTGCACCTCCTCCAGTCTCTTCCTCATCATCCGAACAAGAAGATGTT
       ************************************************************
                                                       stop codon
RR2    GAAGACGGCGTCGGGGACTACATCAGTTATGACGATTTTTAGTTCCACTATTGTGTCAAT
C712   GAAGACGGCGTCGGGGACTACATCAGTTATGACGATTTTTAGTTCCACTATTGTGTCAAT
       ************************************************************


RR2    AGGTTGTGTATTGTATTATTATTGTTATAATATTTTTAAAAAATAAATGTTCTATAAGAC
C712   AGGTTGTGTATTGTATTATTATTGTTATAATATTTTTAAAAAATAAATGTTCTATAAGAC
       ************************************************************
                                                polyA signal

RR2    TAAAACAATGAATTTACTTCCAATATTCCTGACAACCTTTTTTGTTGCGGTAGATGCATG
C712   Taaaaaaaaaaaaaaaaaaaaaa
       *
         polyA site
```

Figure 12. Sequence comparison of cDNA clone C712 with that of WSSV
ribonucleotide reductase small subunit (RR2) gene (Lo *et al.*, 2001 GenBank access no.
AF267731).

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3.2.3.4 cDNA clone C738

C738 (GenBank access no. AY245789) had approximately a 500 bp insert (Figure 13). This sequence was present in WSSV isolates from both China and Taiwan, but not in WSSV isolate from Thailand (*P. monodon*). C738 was almost identical to part of ORF wsv489 sequence of WSSV China isolate and ORF wssv016 sequence of WSSV Taiwan isolate. There were nucleotide differences resulting in codon differences (shown in red in Figure 13). In C738, one codon in question was tac (encoding tyrosine or Y); while in ORF wssv016, it was ttc (encoding phenyalanine or F). In C738, another codon in question was atg (encoding methionine or M); in both ORF wsv489 and wssv016, it was ata (encoding isoleucine or I). The putative polyA signal was 163 bp down stream of the stop codon. The polyA site was 16 bp from the putative polyA signal. The protein encoded by this 738 bp nucleotide did not share any sequence homology with any protein with known function when compared with the protein sequences in the SWISS-PROT database. This protein did not have any pattern or motif with significant function according to PROSITE analysis. Hydrophobicity analysis revealed that this protein probably has no transmembrane segments (Figure 14). The function of this protein during WSSV infection needs to be investigated.

```
                        gaattccctatactgactgatattgctttcctt
                        F  P  I  L  T  D  I  A  F  L
            gcccgtgaaaaatcacgtgtcgagggttcgagattctacaacgat
            A  R  E  K  S  R  V  E  G  S  R  F  Y  N  D
            atgaagattggacctataacagcctacaaattgaatttgatgtgt
            M  K  I  G  P  I  T  A  Y  K  L  N  L  M  C
            aataaattcatggagtctgttgtgcaaaaggtgaaggcagaaata
            N  K  F  M  E  S  V  V  Q  K  V  K  A  E  I
            tccccatttgttgaagttagtgtatcaagtgaacttgaagggtca
            S  P  F  V  E  V  S  V  S  S  E  L  E  G  S
            ccttttgggatttcaagcaaagaatagtaaaacacacctagaat
            P  F  W  D  F  K  Q  R  I  V  K  H  T  *
            attgtaccatttttatgttgttgaataaccctttatcgggcaaga
            gttgatataccacaatgaccgtaaggtcgaaaatagcatacttct
            catccttcagggctacgccactatttcactagtgacacgagtttt
            ttacatcctattctcaggcaactcgaataaagtgatacttcaacg
                                       polyA signal

        gcaaaaaaaaaagaaaaaaaaaaaa
            polyA tail
```

```
wsv489    MVSTRSMEAKAAAAAKAKEVSPTTSKRKAEDLTEGTEEEEESVETHPPSKLPRVDEDEVY
wssv016   MVSTRSMEAKAAAAAKAKEVSPTTSKRKAEDLTEGTEEEEESVETHPPSKLPRVDEDEVY
C738      ------------------------------------------------------------


wsv489    IDENVDGDVQILASSIEVARMERERLAEAMVRDIKIEEEKAATEARKEIASRLIYKEMVY
wssv016   IDENVDGDVQILASSIEVARMERERLAEAMVRDIKIEEEKAATEARKEIASRLIYKEMVY
C738      ------------------------------------------------------------


wsv489    LLPQLENMTNRLRPRSLLRHNEMTITDRTFSDLQIFNKVTFEFPILTDIAFLAREKSRVE
wssv016   LLPQLENMTNRLRPRSLLRHNEMTITDRTFSDLQIFNKVTFEFPILTDIAFLAREKSRVE
C738      ------------------------------------------FPILTDIAFLAREKSRVE
                                                    *****************


wsv489    GSRFYNDMKIGPITAYKLNLMCNKFIESVVQKVKAEISPFVEVSVSSELEGSPFWDFKQR
wssv016   GSRFYNDMKIGPITAFKLNLMCNKFIESVVQKVKAEISPFVEVSVSSELEGSPFWDFKQR
C738      GSRFYNDMKIGPITAYKLNLMCNKFMESVVQKVKAEISPFVEVSVSSELEGSPFWDFKQR
          **************:*********:***********************************


wsv489    IVKHT
wssv016   IVKHT
C738      IVKHT
          *****
```

Figure 13. Nucleotide sequence of cDNA clone C738 and its sequence comparison with sequences of other WSSV isolates. The altered nucleotide and amino acid residues are shown in red.

wsv489: ORF of WSSV genome (AF330293), Xiamen, China isolate
wssv016: ORF of WSSV genome (AF440570), Taiwan isolate
C738: cDNA clone No. 738 of WSSV95C

Figure 14. Hydrophobicity profiles of a 245 aa peptide using TopPred program. Hydrophobicity scales were Kyte-Dolittle scale (A) and GES scale (B). The C-terminal region of this peptide was encoded by cDNA clone C738.

3.2.3.5 cDNA clone C772

cDNA clone C772 (GenBank access no.AY245788) had an insert of ~ 450 bp

(Figure 15). This sequence was identical to part of ORF126 of Thailand isolate (van

Hulten et al., 2001), ORF wssv307 of Taiwan isolate (Lo et al., 2001) and ORF wsv252

of China isolate (Yang et al., 2001). This cDNA clone revealed that the mRNA of the

ORFs encoding a 501 aa peptide was transcribed during WSSV infection. A polyA site

of the mRNA was 13 bp downstream of a putative polyA signal. This peptide did not

contain special biological motif or site according to PROSITE analysis. Hydrophobicity

analysis showed that this peptide had several small hydrophobic segments (Figure 16),

but the length of these segments was not long enough to across an average lipid bilayer

(requires 14 to 36 aa). The function of this protein with a theoretical molecular weight of

54 kDa (501 aa) remains unknown.

```
                                          gaattcgcg
                                          E   F   A
atgagtgtttctggtgccggggagaggattttttgttaacggtttg
M   S   V   S   G   A   G   E   R   I   F   V   N   G   L
gggatgttccagaaccgtaaaatgatacctgtaattgatcctctc
G   M   F   Q   N   R   K   M   I   P   V   I   D   P   L
acatatgaaaatgttgtatgtggtgagcatgatatacaaaaagaa
T   Y   E   N   V   V   C   G   E   H   D   I   Q   K   E
gatgctattctttctgtaaggagagctattgcagactataatgac
D   A   I   L   S   V   R   R   A   I   A   D   Y   N   D
tttgtaagtaagaacaagagagggaagaaacgcagcgcagaagaa
F   V   S   K   N   K   R   G   K   K   R   S   A   E   E
gaaaatgaagatgaagatgcagacgctagcagcagcagcagcagc
E   N   E   D   E   D   A   D   A   S   S   S   S   S
agtcctcctccttcttctcctcctgcacataaaaaatcacgtctt
S   P   P   P   S   S   P   P   A   H   K   K   S   R   L
Ccggatgaaggcgaaaaatgtacactctgttaattttttcaaaca
P   D   E   G   E   K   C   T   L   C   *

ataaactaaccccccttgtaaaaaaaaaaaaaaaaa
polyA signal                    polyA site
```

Figure 15. Nucleotide sequence and deduced amino acid sequence of cDNA clone C772.

Figure 16. Hydrophobicity profiles of a 501 aa peptide using TopPred program.
Hydrophobicity scales were Kyte-Dolittle scale (A) and GES scale (B). C-terminal
region of this peptide was encoded by cDNA clone C772.

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3.2.3.6 cDNA clone C794

Clone C794 (GenBank access no. AF376806) had an insert of ~700 bp (Figure

17). The peptide sequence deduced from cDNA was almost identical to the C-terminal

sequence of a protein with 1936 aa (theoretical size ~207 kDa) from three WSSV isolates

of Thailand (ORF 9), China (wsv447) (Yang *et al.*, 2001) and Taiwan (wssv705) (Lo *et*

al., 2001). A nucleotide c (C794) to t (the other WSSV isolates) change resulted in an

amino acid change (alanine to valine). A putative polyA signal was present 12 bp

upstream of polyA site. The hydrophobicity analysis showed that this protein was not a

membrane protein (Figure 18). The 207 kDa protein did not share any homology with

other proteins. This ~207 kDa protein had a G-LPGA-G-K-T motif near its N-terminus,

which agreed with the consensus pattern. The consensus pattern of ATP/GTP-binding

site motif A (or P-loop) is [A or G]-x (4)-G-K-[S or T] (Walker *et al.*, 1982; Saraste *et*

al., 1990). A signature amino acid sequence Asp-Thr-Gly (DTG) for the active site of

eukaryotic and viral aspartyl proteases was found (van Hulten *et al.*, 2001). The aspartic

acid was the active residue. A prenyl group binding site Cys-Ala-Ile-His (CAIH) was

also identified at the C-terminal extremity. Some of eukaryotic proteins are post-

translationally modified by the attachment of either a farnesyl or a geranyl-geranyl group

to a cysteine residue (Lowy *et al.*, 1989; Glomset *et al.*, 1990). The modification occurs

on cysteine residues that are three residues away from the C-terminal extremity; the two

residues that separate this cysteine from the C-terminal residue are generally aliphatic. In

this case, they were alanine and isoleucine. This Cys-Ali-Ali-X pattern is generally

known as the CAAX box. The function of this WSSV protein needs to be investigated.

```
                                       cccattaaggacaaaagtatt
                                       P   I   K   D   K   S   I
cttgaaagtaacccagaaacaccatctgatacgattagtcttttg
L   E   S   N   P   E   T   P   S   D   T   I   S   L   L
gcaccaagaaagacaatttcacctactaacaatctccacttttca
A   P   R   K   T   I   S   P   T   N   N   L   H   F   S
atgtctgaagatgtcctgttctgtggacaagttcatccaatgaaa
M   S   E   D   V   L   F   C   G   Q   V   H   P   M   K
agggtacaattttctttgcatgtaaagagaactggaggagcactg
R   V   Q   F   S   L   H   V   K   R   T   G   G   A   L
aaaagtacttttgaggaagaagaaggtcttcccacaaagatattc
K   S   T   F   E   E   E   G   L   P   T   K   I   F
tctcccaactttgccacatatcctctattcaaaaaatgtaaaatg
S   P   N   F   A   T   Y   P   L   F   K   K   C   K   M
tatggtgctataatcattgccatgactgaaatgcaagggcatgaa
Y   G   A   I   I   I   A   M   T   E   M   Q   G   H   E
tttgccaagtattcaacattggatatcagaaagagcatgtttact
F   A   K   Y   S   T   L   D   I   R   K   S   M   F   T
ggtgttggaactgtggtggatttggaaaagatatcaggagaaggt
G   V   G   T   V   V   D   L   E   K   I   S   G   E   G
aatgaagtaatggataaagttgacaaatttattgtgaaaaatgta
N   E   V   M   D   K   V   D   K   F   I   V   K   N   V
tcaaacatattgtttaaggaacaagggaagcgtgtctctttcttt
S   N   I   L   F   K   E   Q   G   K   R   V   S   F   F
gcttcatgtgctatacactagtagtaggaataaggaatgaataaa
A   S   C   A   I   H   *
ccattgtattagaatttaggcatttttattttgtaatatattaca

attgaaaatatagaagtaactgttactgttatacaaacaatacct

gaaataaacatagcaactccaaaaaaaaaaaaaaaaaaaa
    poly A signal                polyA tail
```

Figure 17. Nucleotide sequence and deduced amino acid sequence of cDNA clone C794. The altered nucleotide and amino acid residues are shown in red. **CAIH**: prenly group binding site.

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Figure 18. Hydrophobicity profiles of a 207 kDa protein using TopPred program. Hydrophobicity scales were Kyte-Dolittle scale (A) and GES scale (B). (core window size 10; wedge window 5). The C-terminal domain of this protein was encoded by C794.

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3.2.3.7 cDNA clone C806

C806 (GenBank access no. AY245786) had an insert of about ~ 800 bp (Figure 19). The peptide sequence deduced from cDNA was almost identical to a 275 aa peptide found in the three WSSV isolates except for two amino acid differences. This peptide did not share homology with any other known function proteins in SWISS-PROT database. Hydrophobicity analysis showed that the protein had three hydrophobic segments. The hydrophobic segment at the c-terminus was larger (spanning between 20 to 30 aa) and had a higher hydrophobic value (Figure 20), which might form a transmembrane domain. The function of this protein remains unknown.

3.2.3.8 cDNA clone C833

C833 (GenBank access no. AY245787) had an insert of ~700 bp. The cDNA sequence was identical to part of DNA sequence of a 894 bp ORF (ORF58, wsv077, and wssv134) from the three WSSV isolates (van Hulten *et al.*, 2001; Yang *et al.*, 2001; Lo *et al.*, 2001). This 894 bp ORF encoded a peptide with 298 aa. A polyA site was 11 bp down from a putative polyA signal (Figure 21). Hydrophobicity analysis showed that this peptide had several hydrophobic segments (Figure 22). This protein did not share any sequence homology with other proteins. PROSITE analysis showed it had a cell attachment sequence arginine-glycine-aspartate (RGD) close to its hydrophobic domain. The tripeptide RGD was originally identified as the sequence of fibronectin that mediated cell attachment. This motif can be found in the sequence of a number of proteins, where it has been shown to play a role in cell adhesion (Ruoslahti *et al.*, 1986; d'Souza *et al.*, 1991).

```
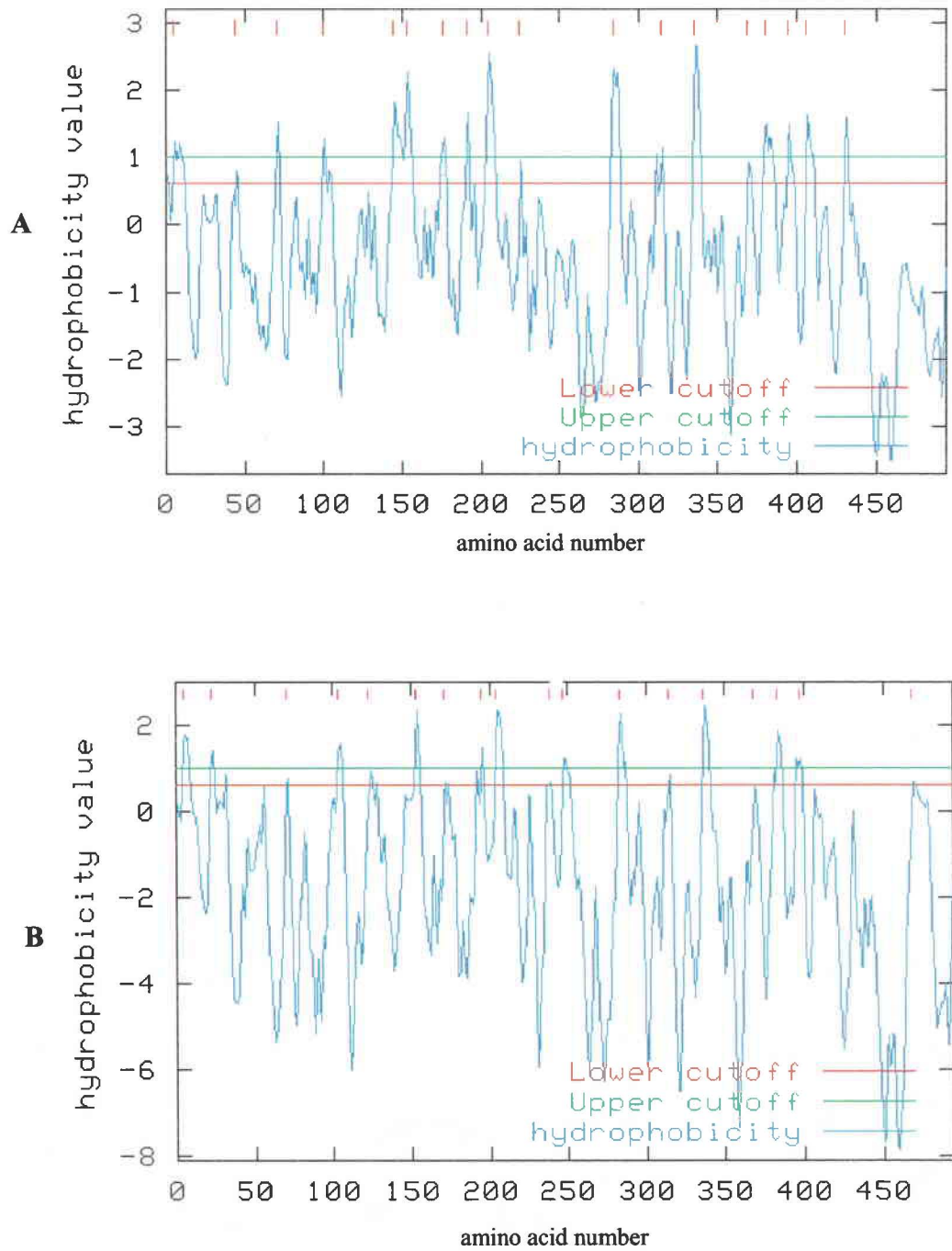                                          caacgaggagatg
                                           N   E   E   M
gttggggactattcaagatggacaactgtcaagaacaggaggaac
V   G   D   Y   S   R   W   T   T   V   K   N   R   R   N
agacagcaacagtattcccatagtttccgtccccaacaacaacaa
R   Q   Q   Q   Y   S   H   S   F   R   P   Q   Q   Q   Q
aacatcaaaaaagaacatcaaccaattctcctcctgctccacct
Q   H   Q   K   R   T   S   T   N   S   P   P   A   P   P
cctccattccccatcattagttggggagccctcggcagctactca
P   P   F   P   I   I   S   W   G   A   L   G   S   Y   S
atgtatcgactggatgaccagtgcagaaattgcgatgaaactggc
M   Y   R   L   D   D   Q   C   R   N   C   D   E   T   G
tattacaatttccactcttatgatagaaagagggaaagagttcgc
Y   Y   N   F   H   S   Y   D   R   K   R   E   R   V   R
tcattaaacaacactccaagtgaaggcatgtggcggcgcacaagt
S   L   N   N   T   P   S   E   G   M   W   R   R   T   S
agatcttccccccttccttaataagaagaaggacgttgacgaagct
R   S   S   P   F   L   N   K   K   K   D   V   D   E   A
ccacctcctcaatcaaaccaacacatgtacccccctcaacaagtac
P   P   P   Q   S   N   Q   H   M   Y   P   L   N   K   Y
agttcccgtgaatatactccttcatcaaagcttgtgaattggcga
S   S   R   E   Y   T   P   S   S   K   L   V   N   W   R
gacccttcacaagaaaaacaggacaagatcttacaagagggagaa
D   P   S   Q   E   K   Q   D   K   I   L   Q   E   G   E
gctcgcgcccctacacccactccccaagaaaaggaaccagaagta
A   R   A   P   T   P   T   P   Q   E   K   E   P   E   V
gaaactaaagatgatgttgtcatcgaggaagaaactgcaccagaa
E   T   K   D   D   V   V   I   E   E   E   T   A   P   E
ccagaaccagaaccagccccagttccagacccagatattcccgca
P   E   P   E   P   A   P   V   P   D   P   D   I   P   A
ataactgcaactactactactactacagttgcaacacgtcacgac
I   T   A   T   T   T   T   T   V   A   T   R   H   D
gattcttctacagtatttctcagaaatgttattctgagtatcgtg
D   S   S   T   V   F   L   R   N   V   I   L   S   I   V
ttttggtttctgggtgtttattctgcattatttgcaaaatgtatt
F   W   F   L   G   V   Y   S   A   L   F   A   K   C   I
Agatctaagaaggaataaataaatggtatatgaaatttaaaaaaa
R   S   K   K   E   *   polyA signal                polyA tail
aaaaaaaaaaaaa
```

89

```
wsv207     MSSNRFSQLRGNEEMVGDYSRWTTVKNRRNRQQQYSHSFRPQQQQQHQKRTSTNSPPAPP
wssv262    MSSNRFSQLRGNEEMVGDYSRWTTVKNRRNRQQQYSHSFRPQQQQQHQKRTSTNSPPAPP
ORF107     MSSNRFSQLRGNEEMVGDYSRWTTVKNRRNRQQQYSHSFRPQQQQQHQKRTSTNSPPAPP
C806       ----------NEEMVGDYSRWTTVKNRRNRQQQYSHSFRPQQQQQHQKRTSTNSPPAPP
                     *********************************************************

wsv207     PPFPIISWGALGSYSMYRLDDQCRNCDETGYYNFHSYDRKRERVRSLNNTPSEGMWRRTS
wssv262    PPFPIISWGALGSYSMYRLDDQCRNCDETGYYNFHSYDRKRERVRSLNNTPSEGMWRRTS
ORF107     PPFPIISWGALGSYSMYRLDDQCRNCDETGYYNFHSYDRKRERVRSLNNTPSEGMWRRTS
C806       PPFPIISWGALGSYSMYRLDDQCRNCDETGYYNFHSYDRKRERVRSLNNTPSEGMWRRTS
           ***********************************************************

wsv207     RSSPFLNKKKDVDEAPPPQSNQHMYPLNKYSFREYTPSSKLVNWRDPSQEKQDKILQEEE
wssv262    RSSPFLNKKKDVDEAPPPQSNQHMYPLNKYSFREYTPSSKLVNWRDPSQEKQDKILQEEE
ORF107     RSSPFLNKKKDVDEAPPPQSNQHMYPLNKYSFREYTPSSKLVNWRDPSQEKQDKILQEEE
C806       RSSPFLNKKKDVDEAPPPQSNQHMYPLNKYSSREYTPSSKLVNWRDPSQEKQDKILQEGE
           *****************************   ************************* *

wsv207     ARAPTPTPQEKEPEVETKDDVVIEEETAPEPEPEPAPVPDPDIPAITATTTTTTVATRHD
wssv262    ARAPTPTPQEKEPEVETKDDVVIEEETAPEPEPEPAPVPDPDIPAITATTTTTTVATRHD
ORF107     ARAPTPTPQEKEPEVETKDDVVIEEETAPEPEPEPAPVPDPDIPAITATTTTTTVATRHD
C806       ARAPTPTPQEKEPEVETKDDVVIEEETAPEPEPEPAPVPDPDIPAITATTTTTTVATRHD
           ***********************************************************

wsv207     DSSTVFLRNVILSIVFWFLGVYSALFAKCIRSKKE
wssv262    DSSTVFLRNVILSIVFWFLGVYSALFAKCIRSKKE
ORF107     DSSTVFLRNVILSIVFWFLGVYSALFAKCIRSKKE
C806       DSSTVFLRNVILSIVFWFLGVYSALFAKCIRSKKE
           ***********************************
```

Figure 19. Nucleotide sequence of cDNA clone C806 and comparison of amino acid encoded by C806 with peptide sequences of other WSSV isolates. The altered nucleotide and amino acid residues are shown in red.

wsv207: ORF of WSSV genome (AF330293), Xiamen, China isolate
wssv262: ORF of WSSV genome (AF440570), Taiwan isolate
ORF107: ORF of WSSV genome (AF369029), Thailand isolate
C806: cDNA clone of WSSV95C

Figure 20. Hydrophobicity profiles of protein encoded by C806 using TopPred program.
Hydrophobicity scales were Kyte-Dolittle scale (A) and GES scale (B).

```
                                atcttatctagacagcgagatttt
                                I  L  S  R  Q  R  D  F
gttgcagaaagtataacaggaacaaaggactggctcaaggctata
V  A  E  S  I  T  G  T  K  D  W  L  K  A  I
atgggttgtggtattataaggtatactgtatttgtcaataacctt
M  G  C  G  I  I  R  Y  T  V  F  V  N  N  L
gcaagatcaacactcgataatgatgatgacaaggcagcaacctat
A  R  S  T  L  D  N  D  D  D  K  A  A  T  Y
tataacacccctatatatggcgggtattgtaaaatggctataaag
Y  N  T  P  I  Y  G  G  Y  C  K  M  A  I  K
gactatgaaataccagattcgtacagcaaggtcgaagcggaacat
D  Y  E  I  P  D  S  Y  S  K  V  E  A  E  H
acagttgaaggaagaaagatgacctttaatataaaatggagaggc
T  V  E  G  R  K  M  T  F  N  I  K  W  **R  G**
gataccataaacaacctaataacaatcatccttcagtgacaggt
**D**  T  I  N  N  L  I  T  I  I  P  S  V  T  G
tatcttgcttccatctctgaagacgcagatgtgcaggcgccatta
Y  L  A  S  I  S  E  D  A  D  V  Q  A  P  L
cttttaaactgcaacaactgttttatagaggcagatatgagtagc
L  L  N  C  N  N  C  F  I  E  A  D  M  S  S
ctctacatggatgagaaaaaaacagaggcatcatttaccctcaac
L  Y  M  D  E  K  K  T  E  A  S  F  T  L  N
ttaccggaaatcgaaggagctgatgcgaatgcagtctatgaaata
L  P  E  I  E  G  A  D  A  N  A  V  Y  E  I
tgtatagtagtagtttgaggaggacaaataaaaaattggatccaa
C  I  V  V  V  *              polyA signal
```

aaaaaaaaaa
⟶
polyA tail

Figure 21. Nucleotide sequence and deduced amino acid sequence of cDNA clone C833. **RGD**: cell attachment sequence.

Figure 22. Hydrophobicity profiles of the peptide encoded by cDNA C833 using TopPred program. Hydrophobicity scales were Kyte-Dolittle scale (A) and GES scale (B).

3.2.3.9 cDNA clone C899

Clone C899 (GenBank access no. AF376807) had a 591 bp ORF. This ORF

encoded a 196 aa peptide. The theoretical molecule weight of this peptide is 21 kDa. A

putative polyA signal was located 14 bp upstream of a polyA signal. There was a three

base difference between the nucleotide sequence of C899 and that of the other WSSV

isolates. Only one of the base changes (a→g) resulted in amino acid change from

asparagine (N) to serine (S) in C899 (Figure 23). This protein did not share any

homology with proteins with any known function. No motif with biological significance

was identified by PROSITE analysis. The hydrophobicity analysis revealed that this

protein might have any two to three hydrophobic segments but they were not long enough

to form transmembrane domains (Figure 24). The function of this protein is still

unknown.

3.2.3.10 cDNA clone C913

C913 (GenBank access no. AY245785) had an insert of ~ 550 bp. C913 was

identical to part of sequence of ORF wsv497 of China isolate (AF332093). ORF wsv497

encoded a 565 aa peptide. C913 was identical to part of sequence of ORF wssv023 from

Taiwan isolate (AF440570) except for one base difference which caused an amino acid

change (Figure 25). ORF wssv023 encoded a peptide with 433 aa, which was shorter

than that of ORF wsv497 (Figure 25). The DNA sequence was not present in WSSV

Thailand isolate (AF369029). The 565 aa peptide did not share any homology with any

other proteins in the database in SWISS-PROT. This peptide had one small hydrophobic

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gcaaacaaagttgggagaaa

atggacgtttcttcctataagagcactattgactaccacaacatt
 M   D   V   S   S   Y   K   S   T   I   D   Y   H   N   I
gaagatatggacgatctccagcgcgccacctacaaggatcgtatg
 E   D   M   D   D   L   Q   R   A   T   Y   K   D   R   M
gagacggaattggtcctcgagatggctaagaaggagggaaggtac
 E   T   E   L   V   L   E   M   A   K   K   E   G   R   Y
gtccgatcgttggccaccatggacgaattggaggtacctgaagaa
 V   R   S   L   A   T   M   D   E   L   E   V   P   E   E
ccagccacttgctacacttgcggctacacctttattagacgcagg
 P   A   T   C   Y   T   C   G   Y   T   F   I   R   R   R
gcacccccaccaaaacgcaagtcaatattcagagagccttgcgct
 A   P   P   P   K   R   K   S   I   F   R   E   P   C   A
tacccagaacttctccccgatgcaccatcccccgtccgtttagaa
 Y   P   E   L   L   P   D   A   P   S   P   V   R   L   E
gagcttgtcgacgtgccagaaggagcgagttttttcacctaccct
 E   L   V   D   V   P   E   G   A   S   F   F   T   Y   P
ccctacgacgacggatcttctacatcgtcttcacaagccgaatgt
 P   Y   D   D   G   S   S   T   S   S   S   Q   A   E   C
gaagatgattatcctccaccgtacgacccatcagaagtccacag
 E   D   D   Y   P   P   P   Y   D   P   S   E   S   P   Q
aggtcccaagtgtgtgattattgtaccacacgtcaagtcctcagt
 R   S   Q   V   C   D   Y   C   T   T   R   Q   V   L   S
tctatgacggatcacgccagggccaacctcataaaaaatctgaag
 S   M   T   D   H   A   R   A   N   L   I   K   N   L   K
agggagaagaaggccctgggtctaggccgtcgcaacaactttagc
 R   E   K   K   A   L   G   L   G   R   R   N   N   F   S
tactagggttgtacaagaagaaaataggattgattgtatcgatga
 Y   *

cgaactttgcatgttttttgaccattcttttgcttgctgagtgtac

atagctttatatttgccaataaaacagaccccgaatgtaaaaaaa
                    polyA signal   polyA tail

```
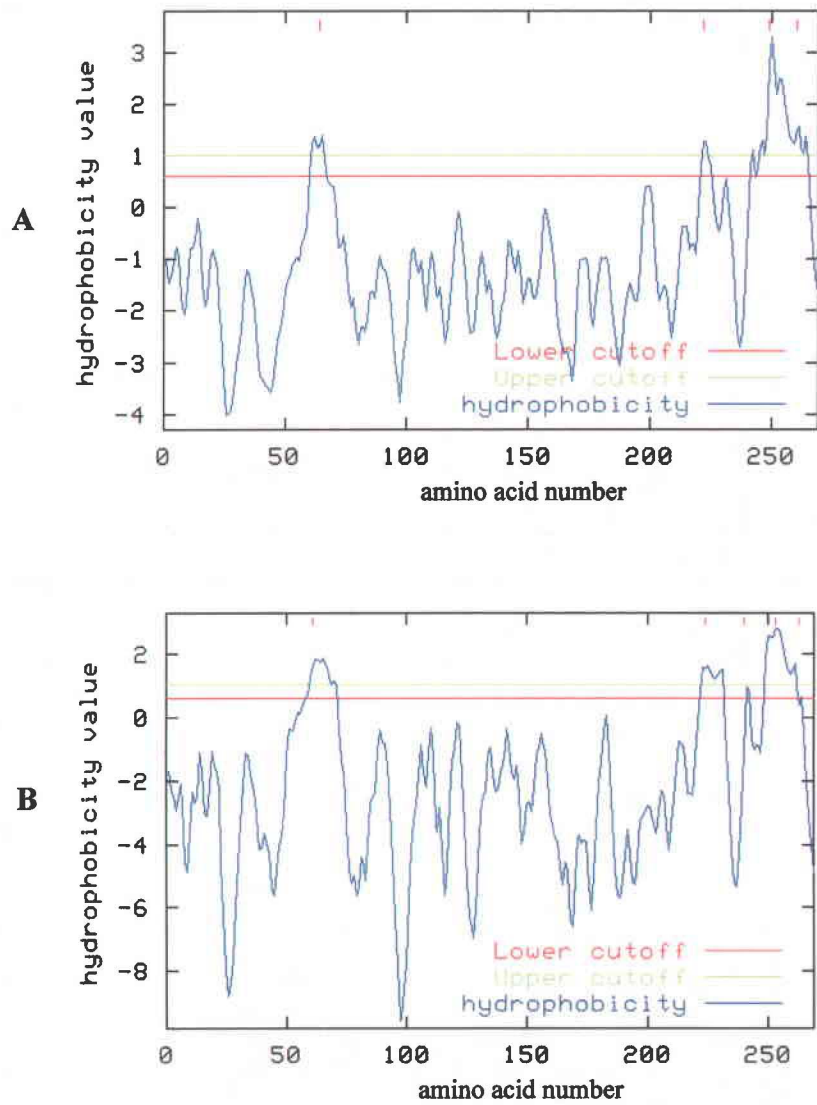wssv108    MDVSSYKSTIDYHNIEDMDDLQRATYKDRMETELVLEMAKKEGRYVRSLATMDELEVPEE
wsv051     MDVSSYKSTIDYHNIEDMDDLQRATYKDRMETELVLEMAKKEGRYVRSLATMDELEVPEE
ORF44      MDVSSYKSTIDYHNIEDMDDLQRATYKDRMETELVLEMAKKEGRYVRSLATMDELEVPEE
C899       MDVSSYKSTIDYHNIEDMDDLQRATYKDRMETELVLEMAKKEGRYVRSLATMDELEVPEE
           ***********************************************************


wssv108    PATCYTCGYTFIRRRAPPPKRKSIFREPCAYPELLPDAPSPVRLEELVDVPEGASFFTYP
wsv051     PATCYTCGYTFIRRRAPPPKRKSIFREPCAYPELLPDAPSPVRLEELVDVPEGASFFTYP
ORF44      PATCYTCGYTFIRRRAPPPKRKSIFREPCAYPELLPDAPSPVRLEELVDVPEGASFFTYP
C899       PATCYTCGYTFIRRRAPPPKRKSIFREPCAYPELLPDAPSPVRLEELVDVPEGASFFTYP
           ***********************************************************


wssv108    PYDDGSSTSSSQAECEDDYPPPYDPSENPQRSQVCDYCTTRQVLSSMTDHARANLIKNLK
wsv051     PYDDGSSTSSSQAECEDDYPPPYDPSENPQRSQVCDYCTTRQVLSSMTDHARANLIKNLK
ORF44      PYDDGSSTSSSQAECEDDYPPPYDPSENPQRSQVCDYCTTRQVLSSMTDHARANLIKNLK
C899       PYDDGSSTSSSQAECEDDYPPPYDPSESPQRSQVCDYCTTRQVLSSMTDHARANLIKNLK
           *************************.*******************************


wssv108    REKKALGLGRRNNFSY
wsv051     REKKALGLGRRNNFSY
ORF44      REKKALGLGRRNNFSY
C899       REKKALGLGRRNNFSY
           ***************
```

Figure 23. Nucleotide sequence of cDNA clone C899. The peptide encoded by C899 was compared with ORFs in other WSSV isolates. The altered nucleotide and amino acid residues are shown in red.

wssv108: ORF of WSSV genome (AF440570), Taiwan isolate
wsv051: ORF of WSSV genome (AF330293), Xiamen, China isolate
ORF44: ORF of WSSV genome (AF369029), Thailand isolate
C899: cDNA clone of WSSV95C

Figure 24. Hydrophobicity profiles of C899 protein using TopPred program. Hydrophobicity scales were Kyte-Dolittle scale (A) and GES scale (B).

```
                                                      ggac
                                                      D
Agtgtacgaaaccacttcaagacgagacttttctcccgcaccagt
S   V   R   N   H   F   K   T   R   L   F   S   R   T   S
ggcaatagtataacctttgcagtccctccaggagaaagggaacta
G   N   S   I   T   F   A   V   P   P   G   E   R   E   L
atggaaatggtcagagaggtaacaggaacagacatcaaaattttc
M   E   M   V   R   E   V   T   G   T   D   I   K   I   F
atggataatggaaaagtatatcaaaatggtgccgaaatcaatgtg
M   D   N   G   K   V   Y   Q   N   G   A   E   I   N   V
attgatccgacatctaaggaatacaaagagttactcaaaagggaa
I   D   P   T   S   K   E   Y   K   E   L   L   K   R   E
gaaaacttgcccgaagacgaacggaagcgtttgcggcgagaacga
E   N   L   P   E   D   E   R   K   R   L   R   R   E   R
cgcatgattttcaatacgtcaagggcaatttccatgtataatgaa
R   M   I   F   N   T   S   R   A   I   S   M   Y   N   E
gaacgtggagatggaggaagtggaggagaaacttcagaagatgga
E   R   G   D   G   G   S   G   G   E   T   S   E   D   G
gatggaaacggcagcaccagcagtaaaggagagaaaggaaaaga
D   G   N   G   S   T   S   S   K   G   E   K   R   K   R
gaagaaatgaagggaatgaatatgtagtccttttgaacaaggct
E   E   N   E   G   N   E   Y   V   V   L   L   N   K   A
tgtaaagacataaaagtttgctaaccatgcatttattttaaatggg
C   K   D   I   K   V   C   *
tttgggcc
```

```
wsv497    MARSVGLLSVTPEYDTFKYIKMEEFKTLKVKNGFTISGENPDKYEHILLSFKSVDRVTKS
C913      -----------------------------------------------------------
wssv023   -----------------------------------------------------------
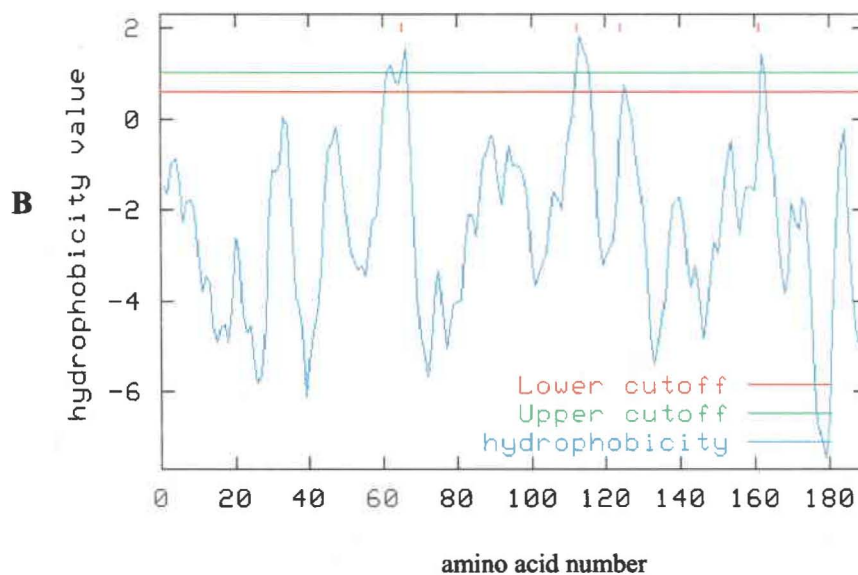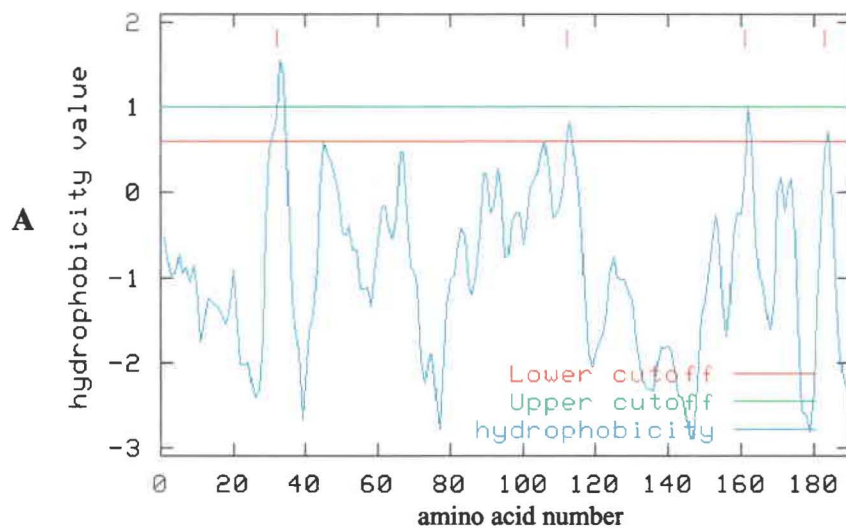
wsv497    ELRDGLYIVRLKDKEVLHIKNGVHRLRQLTGDNTLQVGLKYTHNLPRLGSLLQDDGCEDY
C913      -----------------------------------------------------------
wssv023   -----------------------------------------------------------

wsv497    GEKWNESLPIDMQNINKIVKEKALLSDKNFKFSPLYRLLHERLSNAAVKKCDYMIITTDF
C913      -----------------------------------------------------------
wssv023   -----------MQNINKIVKEKALLSDKNFKFSPLYRLLHERLSNAAVKKCDYMIITTDF

wsv497    LVGCGYTPSHCPRTLRNMEQLLVEQCGFSSRISVYDICDRLTYKGAYIANPITGSYSNMC
C913      -----------------------------------------------------------
wssv023   LVGCGYTPSHCPRTLRNMEQLLVEQCGFSSRISVYDICDRLTYKGAYIANPITGSYSNMC

wsv497    LIVPMDKLGLIFYNSTHPSAKSIGNYMSSLFNATVIYANERDNLQMDNFRREIKFAENEV
C913      -----------------------------------------------------------
wssv023   LIVPMDKLGLIFYNSTHPSAKSIGNYMSSLFNATVIYANERDNLQMDNFRREIKFAENEV

wsv497    NMKEEELKELRKRCAVSEEQRISLRDVHKKSSIATSRYDGGACLVFAFSDRDFSLLCRTN
C913      -----------------------------------------------------------
wssv023   NMKEEELKELRKRCAVSEEQRISLRDVHKKSSIATSRYDGGACLVFAFSDRDFSLLCRTN

wsv497    GNGSFYSATEEGIRYVSSPEYKKRDVGERRPRLIMSITGSDAPICIRDSVRNHFKTRLFS
C913      -----------------------------------------------------------
wssv023   GNGSFYSATEEGIRYVSSPEYKKRDVGERRPRLIMSITGSDAPICIRDSVRNHFKTRLFS

wsv497    RTSGNSITFAVPPGERELMEMVREVTGTDIKIFMDNGKVYQNGAEINVIDPTSKEYKELL
C913      ------------------MEMVREVTGTDIKIFMDNGKVYQNGAEINVIDPTSKEYKELL
wssv023   RTSGNSITFAVPPGERELMEMVREVTGTDIKIFMDNGKVYQNGAEINVIDPTSKEYKELL

wsv497    KREENLPEDERKRLRRERRMIFNTSRAISMYNEERGDGGSGGETSEDGDGNGSTSSKGEK
C913      KREENLPEDERKRLRRERRMIFNTSRAISMYNEERGDGGSGGETSEDGDGNGSTSSKGEK
wssv023   KREENLPEDERKRLRRERRMIFNTSRAISMYNEERGDGGSGGETSEDGDGNGSTSSKGEK

wsv497    RKREENEGNEYVVLLNKACKDIKVC
C913      RKREENEGNEYVVLLNKACKDIKVC
wssv023   RKREENEGNEYVVLLNKACKDRKVC
          ********************* ***
```

Figure 25. Nucleotide sequence of cDNA clone C913 and amino acid sequence comparison of peptide identified from WSSV isolates of China and Taiwan. The altered nucleotide and amino acid residues are shown in red.

wssv023: ORF of WSSV genome (AF440570), Taiwan isolate
wsv497: ORF of WSSV genome (AF330293), Xiamen, China isolate
C913: cDNA clone of WSSV95C

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Figure 26. Hydrophobicity profiles of 516 aa peptide which was partly encoded on C913 using TopPred program. Hydrophobicity scales were Kyte-Dolittle scale (A) and GES scale (B). (core window size 10; wedge window size 5)

segment with low hydrophobic value (Figure 26) and did not have any hydrophobic

domain (Figure 26). It did not show any pattern or motif with biological significance.

3.2.3.11 cDNA clone C958

A 249 bp ORF identified on C958 (GenBank access no AY245783) encoded a

small peptide of 82 aa. The polyA site of mRNA is 12 bp downstream of a putative

polyA signal (Figure 27). The cDNA sequence of C958 was 99% identical with genomic

DNA sequence of the following ORFs: wssv285, wsv230 and ORF155 of three different

WSSV isolates (Figure 27). One base difference (t to g) resulted in an amino acid change

from tyrosine to cysteine (in C958). This protein did no share sequence homology with

any other known function protein in SWISS-PROT.

3.2.3.12 cDNA clone C958-2

cDNA of C958-2 (GenBank access no. AY245784) encoded a 62 aa peptide

(Figure 28). This peptide was identical to the peptide sequence of a WSSV structural

protein VP15 (van Hulten et al., 2002) which was present and identical in several of

WSSV isolates of which genomic DNA sequences were available. A polyA site was

found 16 bp downstream of a putative polyA signal (Figure 28). VP15 did not have any

transmembrane domain and was a nucelocapsid protein. The VP15 protein also had the

DNA-binding activity (Zhang et al., 2001a).

```
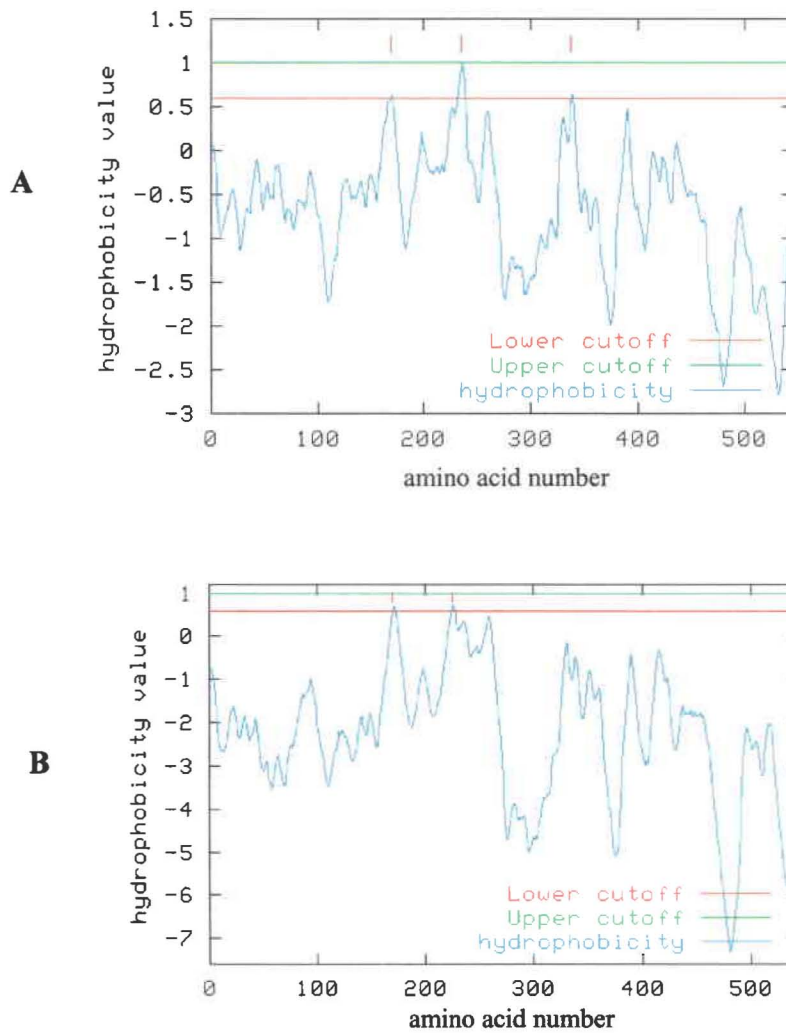            gaatattgaaagaagacttcttgaagaggaccgataaaaaa

atggccaccttccagactgacgccgatttcttgctggtgggggat
M   A   T   F   Q   T   D   A   D   F   L   L   V   G   D
gatactagtagatgtgaagaagtgatgaagacttttgatactgtt
D   T   S   R   C   E   E   V   M   K   T   F   D   T   V
gaggcagtcaggaagagtgatctagatgaccgtgtttacatggtg
E   A   V   R   K   S   D   L   D   D   R   V   Y   M   V
tgcctaaagcagggatctacttttgtcctcaatggaggcatcgaa
C   L   K   Q   G   S   T   F   V   L   N   G   G   I   E
gaattgcgtcttttgactggagattcaacgctggagattcaaccc
E   L   R   L   L   T   G   D   S   T   L   E   I   Q   P
agattgtgccaacaacagaataaataaagacggtgacgggagac
M   I   V   P   T   T   E   *
taatatctttcttagtttcccgtaacggtgaaatgttggtttatt

tcttccctatgtttaaaaatttgtcttggttaaaaaaataaaacg
                                        polyA signal

aaaactgtcaaaaaaaaaaaaaaaaaaaa
        polyA tail
```

```
wsv230    MATFQTDADFLLVGDDTSRYEEVMKTFDTVEAVRKSDLDDRVYMVCLKQGSTFVLNGGIE
wssv285   MATFQTDADFLLVGDDTSRYEEVMKTFDTVEAVRKSDLDDRVYMVCLKQGSTFVLNGGIE
ORF115    MATFQTDADFLLVGDDTSRYEEVMKTFDTVEAVRKSDLDDRVYMVCLKQGSTFVLNGGIE
C958      MATFQTDADFLLVGDDTSRCEEVMKTFDTVEAVRKSDLDDRVYMVCLKQGSTFVLNGGIE
          * * * * * * * * * * * * * * * * * *  * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

wsv230    ELRLLTGDSTLEIQPMIVPTTE
wssv285   ELRLLTGDSTLEIQPMIVPTTE
ORF115    ELRLLTGDSTLEIQPMIVPTTE
C958      ELRLLTGDSTLEIQPMIVPTTE
          * * * * * * * * * * * * * * * * * * * * *
```

Figure 27. Nucleotide sequence of cDNA clone C958 and the deduced amino acid
sequence was compared with other WSSV isolates. The altered nucleotide and amino
acid residues are shown in red.

wssv285: ORF of WSSV genome (AF440570), Taiwan isolate
wsv230: ORF of WSSV genome (AF330293), Xiamen, China isolate
ORF115: ORF of WSSV genome (AF369029), Thailand isolate
C958: cDNA clone of WSSV95C

```
                    gaaacaaagatgttgttagaacaaagaacattaaa

atggttgcccgaagctccaagaccaaatcccgccgtggaagcaag
M   V   A   R   S   S   K   T   K   S   R   R   G   S   K
aagaggtccaccactgctggacgcatctccaagcggaggagccca
K   R   S   T   T   A   G   R   I   S   K   R   R   S   P
tcaatgaagaagcgtgcaggaaagaagagctccactgtccgtcgc
S   M   K   K   R   A   G   K   K   S   S   T   V  . R   R
cgttcctcaaagagcggaaagaagtctggagcccgcaagtcaagg
R   S   S   K   S   G   K   K   S   G   A   R   K   S   R
cgttaattcttccctgtacaacaactatgttatttaattgattttt
R   *
tttcttctgaataattggaaataataaacatccattgaaacttaa
                        ‾‾‾‾‾‾‾‾                 ‾‾
                        polyA signal           polyA site
aaaaaaaa
‾‾‾‾‾‾‾‾▶
```

Figure 28. Nucleotide sequence of cDNA clone C958-2.

3.2.4 Sequence comparison of genes encoding major WSSV structural proteins

Genes encoding five viral structural proteins (VP15, 19, 24, 26 and 28) of five different geographical WSSV isolates (Table 1) were sequenced. The DNAs were amplified by PCR using WSSV genomic DNA as template and using different primers (Table 2). The PCR products were sequenced and compared. Instead of using *Taq* DNA polymerase, *Pfu* DNA polymerase (Stratagene, CA) was used in PCR to obtain high-fidelity amplification. The 3' to 5' exonuclease proofreading activity of *Pfu* DNA polymerase enables the polymerase to correct nucleotide-misincorporation errors. Sequence comparison indicated that as in four other WSSV isolates reported (Table 1), the sequences of each gene were all identical among the nine WSSV isolates (Table 1) except that the vp28 gene of Korean isolate has one base difference from that of other WSSV isolates, but this one base alteration did not cause amino acid sequence change (Moon et al., 2003). These results strongly suggest that the genes of WSSV structural proteins are highly conserved.

Studies have shown that there were genetic variations among different geographical WSSV isolates. Restriction fragment length polymorphism (RFLP) analyses using restriction enzyme *Hind* III can distinguish WSSV 95C from WSSV 97Indo and WSSV 98SC (Nadala et al., 1998). Lo *et al* (1999) combined PCR and RFLP to distinguish a series of Texas WSSV samples from the other geographic WSSV samples. Recently, the genomes of three different geographical WSSV isolates were sequenced and found to differ. The genome size of WSSV isolated from viral infected *P. monodon* collected from Thailand was 292,967 bp (van Hulten et al., 2001); the genome

size of virus isolated from *P. japonicus* shrimp collected from Xiamen, China was

305,107 bp (Yang et al., 2001); and the genome size of WSSV isolated from Taiwan was

307,287 bp (Lo et al., 2001). Though highly conserved, variations have been observed in

WSSV structural protein sequences. Wang *et al* (1999) sequenced the NH_2 terminal

sequence of a 14.5 kDa nucleocore protein of WSSV isolated from *Litopenaeus setiferus*

from South Caroline, US in 1998 as VARGGKTKGRRG (SWISS-PORT Protein Data

Bank Access No. P82006). Assuming the 14.5kDa protein is equivalent to the VP15

protein, it is only 74% identical to the VP15 protein (VARSSKTKSRRG) in other eight

WSSV isolates. Wang *et al* (1999) also showed that the 19kDa envelope protein of

WSSV isolated from crayfish appeared slightly larger in size than that of WSSV isolated

from shrimp. Protein sequencing is needed to confirm this difference.

Table 1. WSSV isolates which structural protein genes were compared in this study.

Viral isolate	Origin	Host animal	Reference
[1]WSSV 95C	Dalian, China	Penaeus japonicus	this study
[1]WSSV 97Indo	Indonesia	*Penaeus monodon*	this study
[1]WSSV 98Japan	Japan	*Penaeus japonicus*	this study
[1]WSSV 98SC	South Carolina, US	*Penaeus setiferus*	this study
[1]WSSV 99C	Qingdao, China	*Penaeus chinesis*	this study
WSSV Xiamen	Xiamen, China	*Penaeus japonicus*	Yang et al., 2001
WSSV Thailand	Thailand	*Penaeus monodon*	Van Hulten et al., 2001
WSSV Taiwan	Taiwan	not specified	Lo et al., 2001
[2]WSSV Korea	Korea	*Penaeus chinesis*	Moon et al., 2003

1. The structural protein genes (vp15, 19, 23.5, 26, and 27.5) of these isolates were sequenced in this study. The nucleotide sequences were deposited in the GenBank (access number: AY249434 to AY249458).
2. Moon et al (2003) sequenced the genes encoding VP28, 26 and 24 proteins for this Korean WSSV isolate.

Table 2. Primers used for PCR and sequencing of genes encoding major WSSV structural proteins.

WSSV structure proteins	Primers	
vp27.5	vp27.5F	5' CGA CAT CTT AAT AAC CAA GCA ACG 3'
	vp27.5R	5' AAA AGC ACG ATT TAT TTA CTC GG 3'
vp26	vp26F	5' ATC CAA CCA ACA CGT AAA GG 3'
	vp26R	5' CAA TTC CCA CTT TAC TTC TTC TTG 3'
vp23.5	vp23.5F	5' AAT AAA TCT CTC CCT AAC AAT GAA AGG 3'
	vp23.5R	5' TTT TCT CTC ATG ACC TTT GTA CAA CTT 3'
vp19	vp19F	5' GTC TTT ACG TTA CAT TGA CGT AC 3'
	vp19R	5' GTT TTA ATT ATA ATT TTT GTC CC 3'
vp15	vp15F	5' CTT AAC CAC AGT TGC ACT CAC 3'
	vp15R	5' GTA CCC TTA AAC TTT TAT ACC AC 3'

3.3 OVEREXPRESSION OF VP27.5 PROTEIN IN *E.COLI* AND THE PRODUCTION OF POLYCLONAL ANTIBODY

The WSSV virion contains at least four major viral structural proteins; these are comprised of three outer non-glycosylated membrane proteins (19, 27.5 and 75kDa) and one 23.5 kDa capsid protein (Nadala *et al.*, 1998b). Western blot analysis showed that VP27.5 kDa protein appeared as early as 41 hr post infection (p.i.) in experimentally infected shrimp well before the appearance of any characteristic clinical symptoms (72 hr p.i.) (Nadala *et al.*, 1997b). *In vitro* studies employing primary shrimp lymphoid cell cultures reaffirmed the early appearance of WSSV VP27.5 kDa. Also, the protein was found to be highly immunogenic in mammalian such as rabbit. The early appearance of VP27.5 protein well before the appearance of any clinical symptoms and its immunogenicity in rabbit strongly indicated the applicability of the viral protein as an early bio-indicator of infections by WSSV.

Cloning the gene of interest in a high expression vector often produces a substantial amount of the encoded protein, which can be used for various biochemical and immunological studies. The WSSV gene encoding the *vp28* protein was overexpressed in the baculovirus insect cell system (van Hulten *et al.* 2000). The eukaryotic proteins expressed by insect expression system may retain the immunologic property, tertiary structure, and other features, but the use of this system is not as simple as that of prokaryotic expression system.

Our purpose for overexpessing VP27.5 protein was to use it as antigen to produce antibody which would be used for WSSV detection. We chose to use one of the pET-

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based vectors (Figure 29, Novagen, WI), which was very easy to manipulate. pET vectors utilized the T7 RNA polymerase-based expression vector developed by Studier *et al.* (1986) to achieve very high levels of protein expression. The advantage of the pET system was that the T7 RNA polymerase was specific for its own promoter, which was found only on the expression plasmid. The T7 promoter was under the tight control of the lacIq gene during the culture growth. This prevented any expression that might adversely affect bacterial growth. When induced with IPTG, a T7 RNA polymerase was expressed in host *E. coli*, allowing transcription from the T7 promoter. Transcription and translation can be accomplished in just a few hours. *E.coli* strain BL21(DE3)pLysS (Novagen, WI) was used as host for protein expression. The pET vector used to express VP27.5 protein was pET21b(+). This vector fused a six histidine-peptide to the recombinant protein. This small addition rarely affected protein structure to a significant degree and therefore usually did not require removal following purification of the protein. The 6-His residues has a remarkable affinity for matrices containing nickel (Ni). The binding can occur under native as well as under denaturing conditions during purification process. Recombinant proteins, frequently encountered in inclusion bodies in bacterial expression systems, can be solubilized under denaturing conditions using either urea or guanidine hydrochloride. The solubilized 6xHis-tagged proteins can be purified by binding to nickel ions on the matrix. The strong affinity of the 6xHis tag tolerates denaturing conditions that facilitated the removal of nonspecific contaminants often associated with recombinant proteins expressed in bacteria. Elution was accomplished under mild conditions by either reducing the pH or adding imidazole as a competitor.

pET-21a-d(+) cloning/expression region

Figure 29. Construction of the WSSV structure protein expression plasmid. The PCR amplified WSSV gene was cloned in *Nde*I–*Xho*I restriction sites of pET-21b(+) vector.

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3.3.1 Overexpression of VP27.5 protein in *E.coli*.

Using PCR technique, *Nde*I and *Xho*I restriction sites were created at the "start" codon and "stop" codon of vp27.5 gene, respectively. The two primers used for PCR were: vp27.5FNED 5'CCGCATATGGATCTTTCTTTCACTC3' (25 mer) and vp27.5RXHO 5'CCTCGAGTCTCGGTCTCAGTGCC3' (23 mer). WSSV95C genomic DNA was used as template for PCR amplification. The PCR reaction was performed as followings: denaturation at 94°C for 5 min; 30 cycles of 94°C for 30 sec; 60°C for30 sec; 72°C for 30 sec; elongation at 72°C for 7 min. The PCR product containing the entire vp27.5 gene was first cloned into a TA cloning vector pCR2.1. The clone was digested with *Nde*I and *Xho*I restriction enzymes, and the insert was isolated and ligated to the pET-21b(+) expression vector which had been treated with same restriction enzymes. The resultant pET-27.5 construct was used to transform *E.coli* BL21(DE3)pLysS. The transformants were grown in LB medium containing carbenicillin and chloramphenicol. IPTG was used to induce expression of vp27.5 gene.

Induction of expression resulted in the synthesis of a recombinant protein with size of ~28 kDa (Figure 31), which was similar to size of VP27.5 protein from virus. The recombinant protein formed insoluble inclusion bodies.

Figure 30. Simple Blue™ stained 15% SDS-PAGE of vp27.5 protein overexpressed in *E.coli*. Lane 1, molecular weight marker; lane 2 & 3, sample with IPTG induction; lane 4, sample without IPTG induction.

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3.3.2 Overexpression of truncated VP27.5 protein in *E.coli*

3.3.2.1 Antigenicity and western blot analysis of VP27.5 protein

Study of WSSV structural protein indicated that VP27.5 protein was an envelope

protein (Nadala *et al.*, 1998b). The membrane protein has hydrophobic domain(s)

embedded in the lipid layer. The hydrophobic and antigenic profiles of the VP27.5

protein analyzed using the GCG® program indicated that it had a N-terminal hydrophobic

region spanning about 30 amino acids (Figure 9). The Antigenic Index method was

developed by Jameson and Wolf (1988). The antigencity represents the likelihood that

the stretch of amino acids would prove to be antigenic and elicit an immune response in

an organism into which it is injected. The antigenicity plot showed that this N-terminal

region had an antigenic index < 1.3 suggesting that it was unlikely to be antigenic (Figure

31). The rest of the polypeptide sequence had several putative antigenic regions with

high hydrophilicity, surface probability and antigenicity indices. This and previous data

on the VP27.5 kDa protein (Nadala *et al.* 1998b) suggest that it is membrane protein.

In order to avoid the problem of low yield commonly experienced with membrane

proteins expressed in *E.coli*, a truncated version of the protein lacking the N-terminal

hydrophobic region was used. The 117 nucleotides (nt) encoding 39 amino acid residues

comprising the N-terminal hydrophobic region were eliminated by PCR to give a 495 nt

fragment predicted to code for a 19 kDa polypeptide product for protein expression. The

truncated protein was expressed in *E.coli* in the form of a recombinant fusion protein

with a six-histidine tag at the C-terminus to aid in purification using IMAC method.

SDS-PAGE indicated that the size of the truncated protein product was ~24 kDa (Figure

32A). Even with the large hydrophobic region removed, the truncated recombinant

protein was insoluble when expressed in *E.coli*. Approximately 10 mg of recombinant

protein was purified from one liter of bacterial culture.

Western blot analysis of the truncated recombinant protein showed that it was

recognized by an antiserum generated against purified WSSV (Figure 32B). This

indicated that the bacterial expressed recombinant viral protein was not only antigenic, it

also reacted with antiserum made against WSSV.

3.3.2.2 Production of hyperimmune antiserum in rabbit

Although a previous study has shown that the WSSV virion is a very effective

immunogen (Nadala *et al*. 1997), such antisera have several limitations. Among these are

the high cost and labor-intensive processes of virus propagation and purification. In

addition, even exhaustive purification of the virus did not completely remove trace

amounts of shrimp proteins which may be immunogenic. In contrast, the bacterial

expression system overcomes these obstacles and the truncated VP27.5 recombinant

protein is readily purified through a simple histidine tag purification process.

3.3.2.3 Western blot analysis using polyclonal antibody

The sensitivity and specificity of the rabbit antiserum generated against the

truncated VP27.5 recombinant protein was analyzed. A 1:5000 dilution of the antiserum

was able to detect the native VP27.5 kDa protein from a 10% (w/v) of WSSV-infected

shrimp gill homogenate. In Western blots, the antiserum also detected the VP27.5

protein of WSSV-infected shrimp from different geographic regions (Figure 33).

However, it cross-reacted with some shrimp proteins, the two most prominent of which

were ≥80kDa. Adsorption of the antiserum to normal shrimp tissue removed the cross-reacting antibodies (Figure 33C). Compared to antiserum generated against purified WSSV (Figure 33A), the antiserum against the recombinant protein had reduced cross-reactivity (in terms of number and intensity) with shrimp proteins. The rabbit hyperimmune antiserum produced by immunization with the recombinant protein also had a high titer. It was relatively specific for WSSV VP27.5 protein and did not cross-reacted with other WSSV structural proteins.

3.3.2.4 Developing an immuno-dot assay for WSSV detection

A sensitive, specific and simple immuno-dot assay was developed for the rapid detection of WSSV using the IgG fraction of the hyperimmune rabbit antiserum absorbed with acetone-dried gill tissue. The immuno-dot assay detected WSSV in a 1:1000 dilution of a 10% (w/v) gill homogenate (Figure 34). This is the approximately equivalent of 5ng total protein. Hameed *et al.* (1998) previously reported a polyclonal WSSV antibody preparation which had a detection limit of 800ng of total hemolymph protein. This is about 150-fold less sensitive than the assay developed in this study. However, previous studies on the pathogenesis of WSSV indicated that the highest concentration of WSSV was generally found in gill tissue (Tapay *et al.* 1997).

Figure 31. The antigenicity plotstructure of vp27.5 kDa protein using GCG program.
Red circles: antigenicity index >= 1.3.

Figure 32. Simple Blue™ stained SDS-PAGE and Western blot of truncated recombinant vp27.5 protein. (A) Simple Blue™ stained 15% SDS-PAGE of IMAC purified truncated recombinant vp27.5 protein and (B) Western blot analysis of the truncated recombinant protein with an antiserum generated against WSSV. Molecular weight marker (lane1); IMAC-purified protein (lane2).

A

1 2 3 4 5 6

← 80 kDa

← 27.5 kDa

B

1 2 3 4 5 6

← 80 kDa

← 27.5 kDa

C

1 2 3 4 5 6 7

← 80 kDa

← 27.5 kDa

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Figure 33. Western blot of native WSSV VP27.5 protein in 10% gill homogenates collected from WSSV infected shrimp of different geographic regions. SPF *P. vannamei*. (Lane 1); WSSV China (Qingdao / *P. chinensis*) (Lane 2); WSSV US (South Carolina / *P. setiferus*) (Lane 3); WSSV Indonesia (*P. monodon*) (Lane 4); WSSV Japan (*P. japonicus*) (Lane 5); WSSV China (Dalian /*P. japonicus*) (Lane 6). The following primary antibodies were used in the Western blot: (A) antiserum against WSSV; (B) antiserum against the truncated VP27.5 recombinant protein; (C) antiserum against the truncated VP27.5 recombinant protein absorbed with acetone-dried shrimp gill tissue.

Figure 34. Immuno-dot assay of 10% w/v gill homogenates (equivalent to ~5μg/μl total protein) prepared from uninfected (control) and WSSV-infected shrimp. U, undiluted

3.3.2.5 Specificity of F(ab')₂ fragment of polyclonal antibody

Rabbit anti-VP27.5 antibody cross-reacted with two of the shrimp proteins in Western blot. It was also reported that there was non-specific reaction between shrimp tissue and mouse anti-vp28 antibody (Zhang *et al.*, 2001b). In order to investigate whether the non-specific reaction was caused by non-specific binding of the Fc portion of IgG, F(ab')₂ was obtained by enzyme pepsin digestion and used in western blot (Figure 35). Compared to IgG, F(ab')₂ still cross-reacted with the same two of the shrimp proteins, but the intensity of the reaction was markedly less (Figure 36). This indicated that Fc might contribute to some of the cross-reaction. The weak cross-reaction of the purified antibody preparation with two normal shrimp proteins may be due to shared epitopes between viral and shrimp proteins. The phenomenon of molecular mimicry between unrelated species has been described before (Oldstone 1998) and needs to be further analyzed in the present system.

Figure 35. Simple Blue™ stained 12% SDS-PAGE of IgG and F(ab')$_2$ fragment. Lane 1, low molecular range marker; lane 2, IgG fraction of anti-vp27.5 antibody; lane 3, F(ab')$_2$ of anti-vp27.5 IgG; lane 4, IgG fraction of anti-human serum albumin (HSA) antibody as control (Sigma Cat. No. A0433); lane 5, F(ab')$_2$ fragment of anti-HSA IgG as control (Sigma Cat. No. R9130); lane 6, low molecular range marker.

Figure 36. Western blot using IgG fraction of anti-vp27.5 antibody (A) and F(ab')$_2$ fragment of IgG (B). Lane 1, low molecular range marker; lane 2, 10% gill tissue of SPF shrimp; lane 3, 10% gill tissue of WSSV infected SPF shrimp.

3.4 DEVELOPING A DIP-STICK ASSAY

The principle of dipstick assay is immunochromatography. The reagents required include a kind of membrane (e.g. nitrocellulose), detector reagent (e.g. latex bead), and affinity purified IgG fractions of antibodies. Membrane is the solid support for the assay and has a significant effect on protein binding, which will have an impact on the overall performance of the assay. Colored latex beads conjugated with IgG serve as visual signals. Maximizing the amount of IgG conjugated to the latex bead will increase the sensitivity of the assay.

3.4.1 Picking a membrane

Three factors need to be considered when choosing a membrane for dipstick assay: the membrane type, posttreatments, and pore size. Three types of membranes have the ability to bind proteins: nitrocellulose (NC), polyvinylidene fluoride (PVDF), and nylon membrane. Only NC membrane has been used in dipstick assay. PVDF membrane is hydrophobic, and is difficult to be rewetted in aqueous sample. Nylon membrane has a high background absorbance which compromises its use in dipstick assay. NC membranes are the choice for the dipstick assay, since they have high protein binding capacities and the binding of proteins to the membranes is very rapid. NC membranes have been manufactured with different pore sizes and can be cast on nonporous plastic support. Following manufacture, NC membranes receive posttreatments to remove dust or to modify their rewetting characteristics. Trace chemicals may be introduced into NC membranes and affecting their performance in the dipstick assay. The measured pore size of a membrane depends on the method used to

measure it. Nominal pore size can be used with caution to differentiate membranes from a single manufacture, but cannot to be used to compare membranes from different manufactures (Millipore, 1999). It is important to know that as a membrane's pore size decreases, the lateral flow (or wicking) rate of the membrane also decreases. The flow rate of a membrane relates to the speed with which liquid can be filtered. The lateral (capillary) flow rate is one of the most important parameters for a membrane used in dipstick assay. The lateral flow rate is expressed as sec/cm (the larger this value, the smaller the flow rate). Generally, manufacturers specify capillary flow rate of a membrane [the time (sec) needed for deionized water to move up 4 cm along the membrane], but do not specify the pore size of a membrane.

Among well-known NC membrane manufacturers in the US, only Schleicher & Schuell (S&S) and Millipore were found to offer a range of large pore size NC membranes with product specification, which could be purchased and tested in this study. The membranes were tested for rewetting with deionized H_2O. Uneven rewetting (e.g. at capture line, or at sample front) can have serious consequences for dipstick assay. The sample may run along the plastic support of the membrane and results in a capture line that has no or little sample penetration. This will significantly reduce the sensitivity and selectivity of the assay (Jones, 1999). The S&S NC membranes tested were: large pore unsupported NC membranes AE98 (160-210 sec/4cm dH_2O) and AE100 (90-120 sec/4cm dH_2O); large pore polyester backing NC membranes FF170 (150-190 sec/4cm dH_2O), FF125 (100-150 sec/4cm dH_2O), and FF60 (40-80 sec/4cm dH_2O). There was a problem of rewetting with these membranes. When S&S membranes were dipped into

deionized H_2O, the water-front moved up evenly. However, when the capture antibody was applied to the membrane, the narrow area at the capture line on the membrane did not evenly wet or rewet. The uneven rewetting might due to the manufacture defects of the membranes. The rewetting of a NC membrane is usually dependent on the drying process. If the relative rates of drying vary across a membrane, the rate of rewetting will vary across the membrane. The application of an aqueous sample may wash water-soluble residues away from the point of application, which may affect the rewetting rate of the membrane. The uneven hydrophobicity of the membrane may also cause uneven rewetting. Hydrophobicity variation can be caused by hydrophobic or hydrophilic residues introduced by membrane posttreatments. The uneven rewetting can also be caused by inconsistency of membrane pore structure. Millipore Hi-Flow Plus membranes (HF75, HF90, HF120, HF135, and HF180) were tested the same way as S&S NC membranes and did not show any sign of rewetting problems. Therefore, HF membranes were used for dipstick assay. In order to avoid the uneven rewetting problem, each batch of membranes were tested before being used for dipstick assay.

3.4.2 Condition for antibody coating

Microparticles are available with difference sizes ranging from 0.015 to 25,000 µm. They are made from various materials (polymers and minerals) (Bangs, 1999). It has been suggested that the size of microparticles used in dipstick assay be 0.1-0.4 µm depending on membrane porosity. The antibody will be bound to the microparticles for detection in dipstick assay, and the use of microparticles made from polystyrene (PS) has been suggested since proteins adsorb onto PS readily and permanently (Bangs, 1999).

Studies have shown higher protein binding by PS microparticles (PS-MP) which have

been modified by carboxylic acids (Suzawa *et al.*, 1982; Shirahama *et al.*, 1985; Bale *et*

al., 1992). Based on the above considerations, carboxylate-modified polystyrene

microparticle (CM-MP) of 0.279 μm in diameter was chosen for use in the present

dipstick assay.

Affinity purified IgG fraction of polyclonal anti-VP27.5 antibody was coupled to

CM-MPs by adsorption. The CM-MPs' surface capacity for IgG saturation was

calculated according to Bagchi and Birnbaum (1981) using the following parameters and

formula:

CM-MP diameter = 0.279 μm

CM-MP surface area = 2.03×10^{16} nm^2/mg (solid CM-MP)

IgG molecular weight = 150 kDa

IgG molecules/mg = 4.01×10^{12}

IgG dimension = 46 nm^2/molecule IgG (at pH 6 to 7)

$$\frac{\text{amount of IgG (in mg)}}{\text{amount of CM-MP (in mg)}} = \frac{\text{Surface area of 1 mg of CM-MP}}{\text{IgG dimension x Number of IgG molecules in 1 mg}}$$

$$= \frac{2.03 \times 10^{16} \text{ nm}^2/\text{mg (CM-MP)}}{46 \text{ nm}^2/\text{molecule IgG} \times 4.01 \times 10^{12} \text{ molecules IgG/mg}}$$

= 0.11 mg IgG/mg CM-MP at saturation

The optimal condition for adsorption reaction was determined empirically in order

to reach the maximum IgG binding capacity of CM-MPs, which is 110 μg IgG/mg CM-

MP at saturation. Variables such as pH, ionic strength, buffer type and pKa of the buffer affect the adsorption of IgG molecule. The quantity of IgG used for adsorption is optimized to increase binding efficiency and to avoid wasting of reagent. To stabilize IgG-CM-MP complex, bovine serum albumin (BSA) and fish skin gelatin (FSG) (0.1 to 1%) were tested.

Three types of buffer (MES, Tris, and Phosphate) with different pKa value were used for adsorption reaction. The working range of 2-(N-morpholino)-ethanesulfonic acid (MES) buffer is between 20 and 100 mM. MES (pKa 6.1 at 25°C) stabilizes the pH of solutions in the range of 5.5 to 7.0. The working range of Tris-(hydroxymethyl)-aminomethane (Tris) buffer is between 20 to 200 mM. Tris (pKa 8.06 at 25°C) stabilizes the pH of solutions in the range of 7.3 to 8.0. Phosphate buffer (Na_2HPO_4-NaH_2PO_4, pK_2 7.2, pK_3 12.3 at 25°C) stabilizes the pH of solutions in the range of 6 to 13. Affinity purified IgG fraction of polyclonal antibody against recombinant truncated VP27.5 protein was used in the adsorption reaction.

The 25 mM MES buffer (pH 6.1) gave very efficient adsorption. The minimum quantity of IgG used to saturate microparticles was 150 ug IgG/mg CM-MP (Table 3), but IgG-conjugated CM-MP aggregated easily within one day. This is not recommended since aggregated CM-MP in dipstick assay will reduce test sensitivity. Either BSA or FSG (0.1%, 0.5%, and 1%) were added to stabilize the IgG-CM-MP. The IgG conjugated microparticles were stabilized for about one week (4°C) by 1% FSG. The stabilizing effect of BSA was not as good as FSG.

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Table 3. Adsorption of IgG to CM-MP using 25 mM MES buffer (pH 6.1).

IgG added (ug IgG/mg CM-MP)	70	150	225	300	450	600
IgG adsorbed (ug IgG/mg CM-MP)	70	110	107	97	110	85

A 25 mM Tris buffer was tested for adsorption reaction at a higher pH which is close to isoelectric point of IgG (pI 7.8). The Ig-CM-MP was stabilized by 0.1% of FSG and stayed stable for over one month (4°C). However, the adsorption efficiency was very low (Table 4). The addition of 150 mM NaCl in the buffer did not increase the adsorption efficiency which only reached less than 50% of the IgG saturation capacity of CM-MP. If CM-MP is not saturated with IgG, it may bind to other proteins in the test sample. When using unsaturated IgG-CM-MP as detector reagent in dipstick assay, the sensitivity and specificity of the assay will be compromised.

Table 4. Adsorption of IgG to CM-MP using 25 mM Tris buffer (pH 8.0).

IgG added (ug IgG/mg CM-MP)	50	75	110	190	250	375
IgG adsorbed(ug IgG/mg CM-MP)	28	33	40	45	53	48

A 25 mM phosphate buffer (pH 7.2) was tested for adsorption reaction. IgG, in approximately twice amount of CM-MP saturation capacity, was needed to saturate the CM-MP (Table 5). IgG-CM-MP was stabilized by 1% of FSG and stayed stable for over three month at 4°C. Even though the IgG adsorption was not as efficient in phosphate buffer as in MES buffer, using phosphate buffer had its advantages. Generally, the biological sample to be assayed is processed in a buffer near physiological pH 7.4 to

maintain its biological activity or characteristics. Since phosphate buffer gave

satisfactory adsorption efficiency and adding 1% FSG provided long term colloidal

stability of IgG-CM-MP complex, CM-MP was coated in phosphate buffer with ~250 ug

IgG/mg CM-MP for dipstick assay.

Table 5. Adsorption of IgG to CM-MP using 25 mM phosphate buffer (pH 7.2).

IgG added(ug IgG/mg CM-MP)	100	150	200	250	300
IgG adsorbed (ug IgG/mg CM-MP)	65	70	90	100	80

Factors affecting the efficient adsorption of IgG to microparticles are pH, ionic

strength, and the charge of the microparticles. Because IgG has a low density of charged

groups (thus low solubility), adding high charged proteins such as FSG enhances the

colloidal stability of the IgG-MP complex. The optimal conditions for adsorption

reaction still need to be determined empirically.

3.4.3 Dipstick assay

1.5 ug of anti-VP27.5 IgG was applied approximated 0.5 cm from the lower end

of a 4 x 0.5 cm strip of Millipore HF membrane. PBS with 0.2% Tween 20 and 1 % non-

fat milk as blocking reagent was used as sample buffer. Compared with other detergents

(e.g. Triton X-100, SDS) and blocking reagents (e.g. casein, BSA), Tween 20 and non-fat

milk efficiently prevented non-specific reaction (false positive). When using purified

VP27.5 protein as antigen, 10 ng of antigen was detected by all the five membranes:

HF75, HF90, HF120, HF135 and HF180 (Figure 37). But it usually took more than 20

min for HF180 membrane to develop a visible signal. HF120, HF135, and HF180

membrane could pick up as little as 1 ng of VP27.5 protein, while HF75 and HF90 membrane failed to develop any signal (Figure 37). Membranes like HF75 and HF90 are also called fast membrane which means they have larger pore size and faster capillary flow rate. Generally, as the membrane capillary flow rate increases, the assay sensitivity decreases (Millipore, 1999). It takes longer time for membrane with low flow rate like HF180 to develop a signal, and sometime it could be clogged by sample and blocking reagent since its pore size is smaller. HF120 and HF135 membranes had good sensitivity and the reaction took about 15 min, they were the choice for this dipstick assay. When WSSV infected shrimp tissue was used, the assay could detect WSSV infection in 1% of gill homogenate (5 ug of total protein) (Figure 38). For now, this wet-format dipstick assay is not as sensitivity as immuno-dot assay in detecting WSSV in shrimp tissue. However, the dipstick assay has great potential for WSSV detection because it is fast and very easy to use.

Though the concept of dipstick, which is a membrane-based lateral-flow immunoassay, is quite simple, developing a dipstick assay is extremely complex and difficult because of the large number of critical components that come together and that interact with each other.

Figure 37. Dipstick assay using Millipore Hi-flow membrane (HF). (A) negative controls. (B) 10 ng of vp27.5 protein as antigen. (C) 1ng of vp27.5 protein as antigen. Lane 1-5, Millipore Hi-flow membranes HF75, HF90, HF120, HF135, HF180.

Figure 38. Dipstick assay of shrimp gill tissue sample. (A) 1% of SFP shrimp gill tissue (5 µg of total protein) (B) 1% of WSSV infection SPF shrimp gill tissue (5 µg of total protein). Lane 1 and 2, Millipore Hi-flow membrane HF120; lane 3 and 4: HF135 membrane.

3.5 SUMMARY

In order to identify genes encoding WSSV structural proteins, a cDNA library was constructed using mRNA isolated from viral infected shrimp gill tissue. A PCR strategy was developed to amplify cDNA without requiring its sequence information. cDNA library was screened for WSSV clones. Twelve WSSC cDNA clones were selected for sequencing and sequence analysis.

A truncated version of the white spot syndrome virus (WSSV) 27.5kDa envelope protein was expressed as a histidine tag fusion protein in *Escherichia coli*. The bacterial expression system allowed the production of up to 10 mg of purified recombinant protein per liter of bacterial culture. Antiserum from a rabbit immunized with the recombinant protein was found to recognize the 27.5kDa viral envelope protein of WSSV isolated from different geographical regions. The antiserum did not recognize any of the other known WSSV structural proteins. A sensitive immuno-dot assay for WSSV was developed using the specific rabbit polyclonal antiserum.

A wet-format dipstick model was developed for WSSV detection. The present study would be of value for the development of a dry-format dipstick with detector reagent (antibody and microparticle complex) dried on a conjugate pad, which would be more desirable for field use.

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