On the vaccination of shrimp against white spot syndrome virus

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On the vaccination of shrimp against white spot syndrome virus

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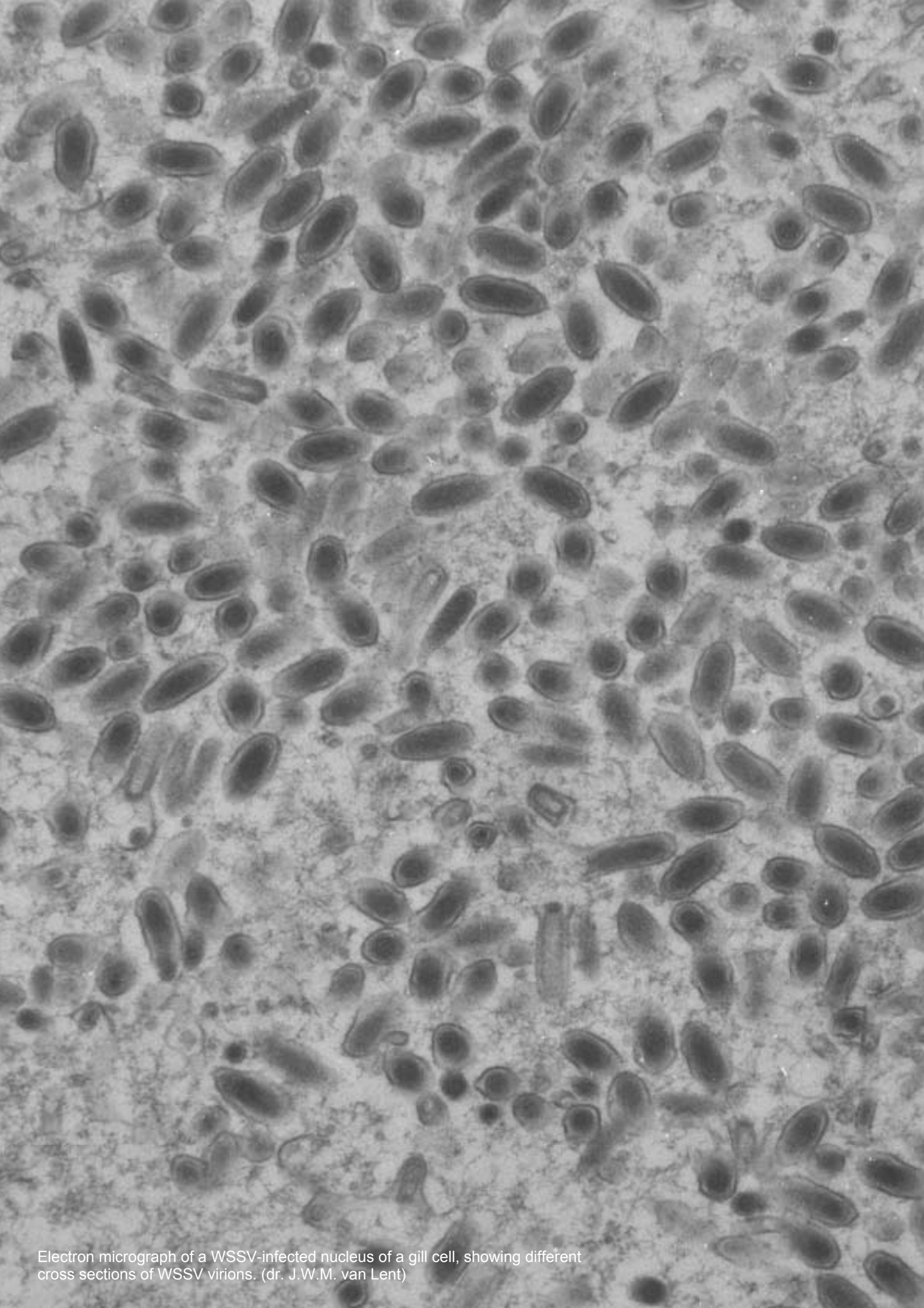
chapter 1

GENERAL INTRODUCTION



Electron micrograph of a WSSV-infected nucleus of a gill cell, showing different cross sections of WSSV virions. (dr. J.W.M. van Lent)

Shrimp culture

Shrimp farming has been practiced at a small scale for centuries in coastal South-east Asia where farmers raised incidental crops of wild shrimp in tidal ponds. The first successful captive spawning and culture through to marketable-sized shrimp was achieved in Japan in the 1930's, where wild female *Penaeus japonicus* shrimp with mature ovaries were captured and spawned in captivity (Hudinaga, 1935, 1942). This technique, called 'sourcing' has since been used worldwide for experimental and commercial culture of numerous other shrimp species. The importance of 'sourcing' is illustrated by its market prices in South-east Asia where a single *P. monodon* female can sell for US$500-$2,000 or more (figure 1.1). However, 'sourcing' limits farmers to the use of indigenous species and the supply of the broodstock animals is dependent on seasonal availability, migratory movements, weather, natural rhythms, and diseases in wild populations, making this source of shrimp far from reliable. Originally, the shrimp species cultured was determined by the availability of local species. However, the development of commercial suppliers of broodstock and consequent international shipping removed this restriction. Currently there are about four important shrimp species used in shrimp culture; *P. merguiensis, P. indicus, P. chinensis*, *P. monodon and Litopenaeus vannamei* of which the latter two species account for the largest part of the annual shrimp yields (app. 75%). Although shrimp culture occurs in all tropical areas of the world, China, Thailand, Vietnam, Indonesia and India are responsible for producing roughly 75% of the total annual shrimp culture production.

Figure 1.1. The most valued of cultured shrimp species, the Giant Tiger shrimp, *Penaeus monodon.*

The current industrial scale and economic importance of shrimp farming has only developed in the last two decades. Since the 1970's farmed shrimp production has seen an almost exponential growth, in contrast to the wild-caught shrimp production growth of a mere 3.8% per year. Shrimp production accounted for a trade value of 7.9 billion US$ in 2001 (FAO, 2003), making it the most valuable marine product in the world trade.

Shrimp farming can be divided in three levels of intensity: extensive, semi-intensive and intensive. The use of either of these levels is highly dependent on region specific conditions. Extensive or traditional farming is predominantly found in poor regions with low costs of land, labor, capital and energy. Broodstock is mostly obtained by netting post-larvae in adjacent mangroves and coastal waters. The World Bank estimates that currently 59% of all shrimp farms are extensive, generating roughly 20% of the total shrimp production. Semi-intensive farms have a higher input of labor and capital and employ technical aids such as pumps, artificial food, fertilizers, hormones, and other aquaculture chemicals to increase productivity. Approximately 10% of the shrimp farms are intensive, using even more advanced technical and chemical aids, increasing yields by 10 times compared to semi-intensive farms (Primavera, 1991).

Besides these economic figures, shrimp farming contributes to the livelihood of people in especially poor regions through direct benefits such as improved food supply, employment and income through the sale of “high-value” products. Indirect benefits of aquaculture are an increase in availability of aquaculture products and subsequent decrease in prices, an increase in employment at peripheral support businesses (e.g.food/seed/repair/manufacturing) and benefits from common pool resources, resulting in an increased overall sustainability (Edwards, 2000).

Though shrimp culture has been, and potentially still is, a success story, it is not without its problems. One of the major concerns is the negative influence of shrimp culture on the environment. Shrimp farming has always been associated with the massive loss of mangroves habitats. Especially extensive farming has contributed to the destruction of natural habitats as this type of farming is often practiced in poor regions with bad enforcement of law and frequent abandonment of farms because of limited funds. Overall estimates are that 1-1,5 million hectares of coastal lowlands have been destructed by shrimp farming (Rosenberry, 1998; FAO, 1999).

Pollution of the natural environment by organic wastes, eutrophication and chemical contaminations by wastewater is also a point of concern. As the water of ponds has to be exchanged regularly or chemically ‘cleaned’, this wastewater is let off in the surrounding environment, causing a negative influence on the natural balance. Though zero-discharge, recirculation and purification systems have been developed, the costs limit its use to a small number of farms (Lin *et al*., 2003).

Due to different factors (e.g. monocultures, increased farm sizes, increased shrimp densities and poor management practices) disease incidences have increased dramatically. This has prompted a massive worldwide transport of shrimp stocks, including exotic species which replace the disease stricken local shrimp species. As escapes are almost impossible to prevent, these new species can threaten the local ecosystem by hybridization and competition with local species and possibly introduce new pests and diseases (Perez *et al*., 2000). The recent introduction of Taura Syndrome Virus infected *L. vannamei* in certain shrimp farming areas has proven this point all too well. Another important point of discussion associated with aquaculture as a whole is the question whether it will ever become a net producer of proteins or will continue to deplete the ocean resources. Shrimp food contains a considerable amount of fishmeal and fish oil (approximately 30%), this combined with a food conversion index greater than one (i.e. it takes more than one kilo of fish to produce one kilo of shrimp) this threatens the global fish supplies and endangers the expansion of shrimp culture (Sargent and Tacon, 1999).

Shrimp culture would not be able to exist without the input of shrimp larvae. Whether the post-larvae are harvested from the wild or originate from wild caught fertilized females, this harvesting has put an enormous pressure on estuarine populations worldwide. With the increased banning of these practices and increased disease incidences, more pressure is put on the development of laboratory strains bred without the input from ocean stocks. In this way, a consistent, reliable source of post-larvae (PL) seed stock can be obtained to support commercial shrimp culture and establish the basis for genetic selection to increase disease resistance and other culture characteristics.

Shrimp diseases

Shrimp are susceptible to a wide variety of pathogens, including parasites, fungi, protozoa, rickettsiae, bacteria and viruses. Like in any production system, these pathogens cause a considerable loss in production and consequently income. In the mid 1990's it was estimated that around 40% of the worldwide shrimp production, representing a value over $3 billion, was lost due to infectious diseases (Lundin, 1996). The main contributors to these losses are viral diseases. Of the about 20 known shrimp viruses today, six are especially important due to their epizootic spread and economic impact; Monodon Baculovirus (MBV), Infectious Hypodermal and Hematopoietic Necrosis Virus (IHNNV), Taura Syndrome Virus (TSV), Yellow Head Disease Virus (YHV), *Monodon* Slow Growth Disease (MSGS) and White Spot Syndrome Virus (WSSV). Of these viruses YHV, TSV and WSSV are notifiable diseases (Office International des Epizooties, Paris, France). However, the latter of the three, WSSV, is considered the most important disease in terms of spread and economic losses. This virus had, and still has the greatest impact on shrimp culture to date. The first reports of the virus and its characteristic high mortalities in shrimp farms came in 1992 from the Fuzhan and Quangzhou provinces in China (Nakano *et al*., 1994). From there the virus spread further into South-east Asia from Japan down to Thailand and Indonesia and later into India and the near East. In 1995 the virus crossed the Pacific Ocean, possibly by transportation of infected post larvae, as the virus was detected in Texas, North America and one year later in south Carolina (Rosenberry, 1996). Not until 1999 did the virus reach the Latin-American pacific shrimp farming countries where it caused major problems in Ecuador, Peru and Mexico.

Fuelled by its sudden appearance and mass mortalities, much research was focused on this at that point unknown virus, resulting in numerous scientific reports describing the virus using several different names such as: "Systemic ectodermal and mesodermal baculovirus" (SEMBV), "Rod-shaped virus of *Penaeus japonicus*" (RV-PJ), "Penaeid rod-shaped DNA virus" (PRDV), "Hypodermal and hematopoietic necrosis baculovirus" (HHNBV), "Chinese baculovirus" (CBV) and "White spot baculovirus" (WSBV). However, the name "White spot syndrome virus" (WSSV) is nowadays the official name approved by the ICTV (Vlak *et al*., 2005) and used by the majority of research groups.

As the name of the virus already suggests, one of the prominent symptoms of WSSV infections are white spots located on the exoskeleton of the shrimp, especially on the carapace and tail. In earlier stages of infection, the shrimp becomes lethargic, stops feeding and sometimes exhibits a reddish to pink discoloration (Chou *et al*., 1995). Under farming conditions, infected shrimp surface and approach the edge of the ponds more often than usual and 7-10 days after the initial detection of the disease up to 100% of the shrimp may be moribund or deceased (Lightner, 1996).

Socio- economic consequences of WSSV

The increase in importance of shrimp farming from an economic perspective has been illustrated in the "shrimp culture" section above. However, the social importance of WSSV can be best described with some figures of the number of people involved in, and dependent on the shrimp industry. A well-described case is Ecuador that was particularly hard-hit by a WSSV epidemic in 1999. During its peak year in 1998 (Figure 1.3), shrimp exports reached

an estimated $900 million worth, accounting for over 3,5% of the total GDP of Ecuador and constituting 15% of the world market, making it the second largest shrimp producing country of the world (Rosenberry, 1999). During this period, direct employment related to the shrimp farming industry was over 200,000 individuals, or just under 2% of the population, this included 76,000 larvae collectors, 2,300 laboratory workers, 103,000 farm employees, 20,000 packaging plant workers and 17,000 involved in other forms of peripheral support (Banco Central de Ecuador, 2002). In 1999 the WSSV crisis had such an impact that a state of emergency was declared, as production plummeted by over 65% in 2 years, resulting in a decrease in export of a half billion US dollars. Moreover, estimates are that over 130,000 jobs were lost in the first year alone that the virus struck, a reduction of nearly one half of those directly employed by the industry. Nearly 100,000 of the total 175,000 hectares of ponds were abandoned by early 2001. Of the 75 processing plants that operated in 1998, only 25 were still open by 2001.

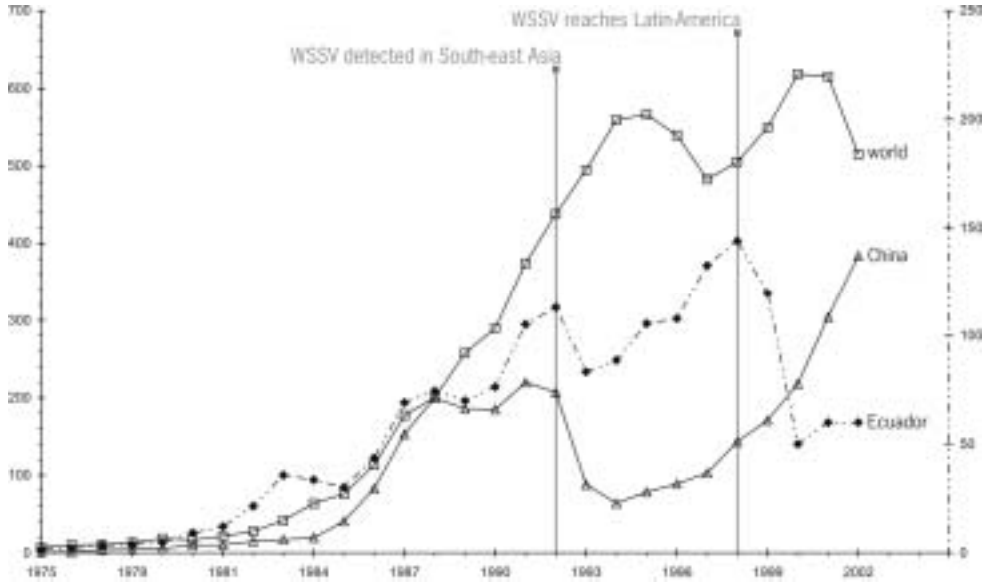


Figure 1.3. Shrimp production in metric tons from 1975 until 2002. Left Y-axis represents world and Chinese production, right Y-axis represents Ecuadorian production. Indicated are the first detection of WSSV and detection in Latin-America (FAO, 2003).

Unfortunately, this has not been an isolated case as shrimp production all over the world has declined or been completely wiped out by the WSSV epizootic. Although difficult to estimate, losses by WSSV alone have been estimated at a staggering $20-30 billion since the start of the epizootic (Lundin, 1996). It is needless to say that the impact on the shrimp industry and people involved, as witnessed in Ecuador, is felt all over the shrimp farming areas.

WSSV host range

WSSV has an exceptionally broad host range including all cultured shrimp species but also a large number of crab, lobster and crayfish species (Lo *et al.*, 1996; Lightner, 1996; Flegel, 1997; Chang *et al.,* 1998; Supamattaya, *et al.,* 1998; Wang *et al.*, 1998; Rajendran *et al.,* 1999; Chen *et al*, 2000; Shi *et al*., 2000; Corbel *et al.,* 2001; Hossain *et al.,* 2001; Jiravanichpaisal *et al.,* 2001; Chakraborty *et al.,* 2002; Hameed *et al.,* 2003; Takahashi *et al*., 2003; Edgerton, 2004). Table 1.1 shows a list of confirmed hosts detected either by natural or experimental infection. The susceptibility to WSSV differs significantly between hosts. In some species, WSSV results in a non-lethal or latent infection, making these species potential virus reservoirs and important sources of infection in shrimp ponds.

Table 1.1. List of confirmed WSSV host species.

Scientific Name	Common Name	Type of Infection[1]	Reference
Shrimp			
Alpheus brevicristatus	Snapping shrimp	N	Takahashi *et al*., 2003
Alpheus lobidens	Apping shrimp	N	Takahashi *et al*., 2003
Aristeus sp.	Red shrimp	N	Chakraborty *et al.,* 2002
Exopalaemon orientalis	Oriental prawn	N, E	Flegel, 1997; Chang *et al.,* 1998; Wang *et al.,* 1998
Farfantepenaeus aztecus	Northern brown shrimp	E	Lightner, 1996
Farfantepenaeus duorarum	Pink shrimp	E	Lightner, 1996
Fenneropenaeus penicillatus	Red tail shrimp	N	Chou *et al*., 1995; Lo *et al*., 1996; Wang *et al.,* 1998
Fenneropenaeus chinensis	Fleshy shrimp	N	Flegel, 1997
Heterocarpus sp.		N	Chakraborty *et al.,* 2002
Litopenaeus vannamei	Whiteleg shrimp	N, E	Lightner, 1996
Litopenaeus setiferus	Northern white shrimp	E	Lightner, 1996
Macrobrachium rosenbergii	Giant freshwater shrimp	N, E	Lo *et al*., 1996; Flegel, 1997; Chang *et al.,* 1998; Wang *et al.,* 1998; Rajendran *et al.,* 1999; Hossain *et al.,* 2001; Chakraborty *et al.,* 2002
Macrobrachium idella	Sunset shrimp	E	Rajendran *et al.,* 1999
MarsuPenaeus japonicus	Kuruma shrimp	N, E	Takahashi *et al*., 1994; Lo *et al*., 1996; Wang *et al.,* 1998
Metapenaeus ensis	Greasyback shrimp	N, E	Lo *et al*., 1996; Chang *et al.,* 1998; Wang *et al.,* 1998
Metapenaeus dobsoni	Kadal shrimp	N, E	Rajendran *et al.,* 1999 ; Hossain *et al.,* 2001; Chakraborty *et al.,* 2002
Metapenaeus monoceros	Speckled shrimp	E	Rajendran *et al.,* 1999
Metapenaeus elegans	Fine shrimp	N	Chakraborty *et al.,* 2002
Palaemon adspersus	Baltic prawn	E	Corbel *et al.,* 2001
Palaemon styliferus	Grass shrimp	N	Lo *et al*., 1996; Flegel, 1997
Parapenaeopsis stylifera	Kiddi shrimp	N	Hossain *et al.,* 2001; Chakraborty *et al.,* 2002
Penaeus monodon	Giant tiger shrimp	N, E	Chou *et al*., 1995; Wongteerasupaya *et al*., 1995 ; Lo *et al*., 1996; Chakraborty *et al.,* 2002
Penaeus indicus	Indian white prawn	N, E	Flegel, 1997; Rajendran *et al.,* 1999
Penaeus merguiensis	Banana prawn	N	Flegel, 1997
Penaeus semiculcatus	Green tiger prawn	N, E	Lo *et al*., 1996; Wang *et al.,* 1998; Rajendran *et al.,* 1999
Penaeus stylirostris	Blue shrimp	E	Lightner, 1996
Solenocera crassicornis	Coastal mud shrimp	N	Hossain *et al.,* 2001

Squilla sp.	Mantis shrimp	N, E	Hossain *et al.,* 2001; Chakraborty *et al.,* 2002
Trachypenaeus curvirostris	Southern rough shrimp	E	Chang *et al.,* 1998; Wang *et al.,* 1998
Crabs			
Cancer pagurus	Edible or rock crab	E	Corbel *et al.,* 2001
Calappa lophos	Box crab	N, E	Flegel, 1997; Chakraborty *et al.,* 2002
Calappa philargius	Box crab	E	Hameed *et al.,* 2003
Charybdis annulata	Swimming crab	N, E	Hossain *et al.,* 2001; Hameed *et al.,* 2003
Charybdis cruciata	Red sea crab	N	Hossain *et al.,* 2001; Chakraborty *et al.,* 2002
Charybdis feriata	Coral crab	N	Lo *et al*., 1996
Charybdis granulata	Swimming crab	E	Chang *et al.,* 1998
Charybdis hoplites	Swimming crab	N	Chakraborty *et al.,* 2002
Charybdis lucifera	Swimming crab	N, E	Chakraborty *et al.,* 2002; Hameed *et al.,* 2003
Charybdis natator	Hairyback crab	N	Flegel, 1997
Doclea hybrida		E	Hameed *et al.,* 2003
Gelasimus marionis nitidus		N	Hossain *et al.,* 2001
Grapsus albolineatus	Rock crab	E	Hameed *et al.,* 2003
Halimede ochtodes	Hairy crab	E	Hameed *et al.,* 2003
Helice tridens	Shore crab	N	Lo *et al*., 1996
Liagore rubromaculata		E	Hameed *et al.,* 2003
Liocarcinus depurator	Harbour crab	E	Corbel *et al.,* 2001
Liocarcinus puber	Velvet swimming crab	E	Corbel *et al.,* 2001
Lithodes maja	Deepsea king crab	E	Hameed *et al.,* 2003
Macrophthalmus sulcatus	Ghost/fiddler crab	N	Hossain *et al.,* 2001
Mantura sp.		N	Flegel, 1997
Matuta miersi	Moon crab	E	Hameed *et al.,* 2003
Metopograpsus messor	Purple climber crab	N, E	Rajendran *et al.,* 1999; Hossain *et al.,* 2001
Paradorippe granulata		E	Hameed *et al.,* 2003
Parthenope prensor	Elbow crab	E	Hameed *et al.,* 2003
Philyra syndactyla	Purse crab	E	Hameed *et al.,* 2003
Podophthalmus vigil	Long-eyed Swimming Crab	E	Hameed *et al.,* 2003
Portunus pelagicus	Sand crab	N, E	Lo *et al*., 1996; Supamattaya, *et al.,* 1998; Chakraborty *et al.,* 2002
Portunus sanguinolentus	(Blood) spot crab	N, E	Lo *et al*., 1996; Chang *et al.,* 1998; Chakraborty *et al.,* 2002; Hameed *et al.,* 2003
Pseudograpsus intermedius	Mosaic crab	N	Chakraborty *et al.,* 2002
Scylla serrata	Mud crab	N, E	Lo *et al*., 1996; Kanchanaphum *et al*., 1998; Supamattaya, *et al.,* 1998; Rajendran *et al.,* 1999; Chen *et al*., 2000; Chakraborty *et al.,* 2002, Hameed *et al.,* 2003,
Scylla tranquebarica	Mangrove crab	E	Rajendran *et al.,* 1999
Sesarma sp.	Marsh crabs	N, E	Flegel, 1997; Kanchanaphum *et al*., 1998; Rajendran *et al.,* 1999
Somannia-thelpusa sp.	Black rice crab	E	Flegel, 1997
Thalamita danae	Swimming crab	N, E	Flegel, 1997; Hameed *et al.,* 2003
Uca pugilator	Calico fiddler crab	E	Kanchanaphum *et al*., 1998
Lobsters			
Acetes sp.	Krill	E	Supamattaya, *et al.,* 1998
Panulirus homarus	Scalloped spiny lobster	E	Rajendran *et al.,* 1999
Panulirus longipes	Longlegged spiny lobster	E	Flegel, 1997
Panulirus ornatus	Ornata spiny lobster	E	Flegel, 1997; Rajendran *et al.,* 1999
Panulirus penicillatus	Pronghorn spiny lobster	E	Chang *et al.,* 1998

Panulirus polyphagus	Mud spiny lobster	E	Rajendran *et al.,* 1999
Panulirus versicolor	Painted spiny lobster	E	Chang *et al.,* 1998
Scyllarus arctus	Small European locust lobster	E	Corbel *et al.,* 2001
Crayfish			
Astacus leptodactylus	Turkish crayfish	E	Corbel *et al.,* 2001
Cherax destructor albidus	Yabby	E	Edgerton, 2004
Cherax quadricarinatus	Australian redclaw	E	Shi *et al*., 2000
Orconectes limosus	Spinycheek crayfish	E	Corbel *et al.,* 2001
Pacifastacus leniusculus	Signal crayfish	E	Jiravanichpaisal *et al.,* 2001
Procambarus clarkii	Red swamp crayfish	E	Chang *et al.,* 1998; Wang *et al.,* 1998
Insects			
Ephydridae sp.	Shore fly	N	Lo *et al*., 1996

[1]N: natural infection; E: experimentally infected

Detection of WSSV

Because early monitoring of any disease is critical for disease management and control, much effort has been put into the detection of WSSV. Besides the traditional observation of gross- and clinical signs and morphological pathology using light- and electron microscopy, histopathology and histochemistry, a whole array of molecular technologies has been developed for the detection of WSSV. WSSV is a large double-stranded DNA containing virus with an ovoid to bacilliform shaped virion containing one rod-shaped nucleocapsid and replicates in the nucleus. In addition to the use of *in situ* hybridization techniques, polymerase chain reactions (PCR) and immunological detection methods have been developed for the detection of WSSV. Besides the published PCR-based methods (Lo *et al*., 1996, 1997; Nunan and Lightner *et al*., 1997; Belcher and Young *et al*., 1998; Hsu *et al*., 2000; Tapay *et al*., 1999; Kiatpathomchai *et al*., 2001; Tsai *et al*., 2002; Vaseeharan *et al*., 2003; Kono *et al*., 2004; Quere *et al*., 2002) and immunodetection methods (Anil *et al.,* 2002; Liu *et al.,* 2002; Nadala and Loh, 2000; Poulos *et al.,* 2001; Zhan *et al*., 2003; Dai *et al*., 2003; Okumura *et al*., 2004; van Hulten *et al*., 2000a; You *et al.,* 2002; Zhang *et al*., 2002a,b), a large number of commercial detection kits, based on *in situ* hybridization, PCR and immunodetection, are also available (EBTL, DiagXotics, IQ2000, BIOTEC).

WSSV genome

Early research using restriction analysis suggested the double-stranded WSSV DNA genome to be about 290 kb in size (Yang *et al*., 1997) and the presence of some genetic variation between WSSV isolates (Wang *et al*., 2000b; Lo *et al*., 1999). In 2001 the complete genome sequence of two WSSV isolates was published (Van Hulten *et al*., 2001c (AF369029); Yang *et al*., 2001 (AF332093)) and in 2002 a third sequence was submitted to GenBank (AF440570). These sequences revealed a considerable difference in genome size, ranging from 292,967 bp (AF369029) to 307,287 bp (AF440570) with the third sequence in-between with 305,107 bp (AF332093).

The genome of the smallest sequenced isolate (van Hulten *et al*., 2001c (AF369029)) encompasses 184 putative ORFs, of which only 11 have homologues in public databases,

mainly representing genes encoding enzymes for nucleotide metabolism, DNA replication and protein modification. Ten gene families, consisting of two to four ORFs with pair wise similarities of 40% or higher, were also identified on the genome. Further unique features of the WSSV genome are the presence of an extremely long ORF of 18,234 nucleotides (ORF167) with unknown function, a collagen-like ORF (ORF30), and nine non-coding regions (hr), dispersed along the genome, each containing a variable number of about 250 bp-long homologous tandem repeats (Van Hulten *et al*., 2001c).

The principle sources of the difference in genome size are located in two major polymorphic loci. The larger of the two contains a single deletion of up to 13 kb (variable region ORF23/24), but intermediate-sized deletions within this region are also found. Although up to thirteen ORFs are located within this deletion, one of which codes for a protein found in the virion of WSSV-isolates lacking the deletion, there is no evidence it has an influence on the virus' pathology. The second origin of variation is a variable region (ORF14/15) which has deletions of up to 8 kb containing approximately six ORFs, one of which belongs to one of the nine identified gene families. Less obvious sources of genetic variation are differences in the number of repeat units in four of the nine *hr*s and a number of single nucleotide polymorphisms. Overall however, there is a very small amount of genetic variation as there is a 99,3% pairwise nucleotide identity between the three sequenced isolates known, suggesting that all known isolates probably evolved recently from a common ancestor, and are closely related. (Marks *et al*., 2004).

Phylogenetic analysis based on the DNA polymerase (van Hulten *et al*., 2001c), ribonucleotide reductase (Van Hulten *et al*., 2000b), two protein kinase genes (Van Hulten *et al*., 2001a) and a non-specific endonuclease (Witteveldt *et al*., 2001) confirmed the unique taxonomic position of WSSV, with no or only distant relationships to other families of large dsDNA viruses. Therefore, WSSV has been classified as the sole species (Whispovirus) of a new monotypic family called *Nimaviridae* (genus *Whispovirus*) (Vlak *et al*., 2005).

WSSV histopathology and tissue tropism

Research on WSSV infected shrimp using histology and *in situ* hybridization reported hypertrophied nuclei with marginated chromatin, especially in epithelial cells of the stomach and gills, but also in the epidermis, haemocytes (granular and semi-granular cells) and connective tissues. In later stages of infection, cytoplasmatic disintegration leading to large voids at these lysed cell sites was observed (Inouye *et al*., 1994; Wongteerasupaya *et al*., 1995; Durand *et al*., 1996; Nunan and Lightner, 1997; Wang *et al.*, 2002).

Later studies investigating the tissue tropism of WSSV detected the virus in many other organs, including the hepatopancreas, lymphoid organ, antennal gland, muscle tissue, pleopods, pereiopods, hemapoietic tissue, heart, midgut, hindgut, nervous tissue, compound eyes and stalks, ovaries, testes and spermatophores (Chang *et al.*, 1996; Lo *et al.*, 1997; Hameed *et al.*, 1998; Kou *et al*., 1998).

WSSV replication and morphogenesis takes place in the nucleus of its host and begins with the *de novo* formation of fibrillar viral envelopes in the nucleoplasm and the formation of long empty tubules build up of cylinder formed stacks of rings. Some studies have suggested that these tubules are broken into nucleocapsid precursors and partially

enveloped, leaving an open extremity through which the nucleoproteins enter. The envelope then closes, leaving a characteristic appendage at the closure site (Durand *et al*., 1997; Wang *et al.*, 1999; Wang *et al.*, 2000a).

WSSV virion morphology

WSSV virions are ovoid to bacilliform shaped with a characteristic tail-like appendage at one end of the virion (Figure 1.4). Within this virion a rod shaped nucleocapsid with a clear striated appearance is located, constructed out of 16 stacked rings of two parallel rows of globular subunits of about 10 nm (figure 1.5) (Durand *et al*., 1997; Nadala *et al*., 1998). Each nucleocapsid contains one copy of the circular double stranded DNA genome of WSSV. Early SDS-PAGE protein analysis of virions of different isolates showed little difference (Wang *et al*., 2000b). More detailed analysis of purified virions initially revealed the presence of five major structural proteins which were consequently isolated and characterized. According to their respective size in SDS-PAGE, they were named VP26, VP28 (Van Hulten *et al*., 2000a), VP24 (Van Hulten *et al*., 2000c), VP15 and VP19 (Van Hulten *et al*., 2002). To determine the location of the major structural proteins, the viral envelope was removed and analysis of the remaining nucleocapsids showed that only VP15, VP24 and VP26 could be detected, suggesting that VP19 and VP28 reside in the envelope (Van Hulten *et al*., 2000a) (figure 1.6). Other authors reported a different location of VP26. In an experiment using gold labeled VP26-specific antibodies a positive signal in intact virions and not in nucleocapsids was found (Zhang *et al*., 2002a), suggesting VP26 may be an integument protein.

At the onset of this thesis only the five major structural proteins as mentioned above were known but further experiments using mass spectrometry on purified virions identified another 34 minor structural proteins (table 1.2) (Huang *et al*., 2002ab; Chen *et al*., 2002; Tsai *et al*., 2004 and Leu *et al*., 2005). Recently, a sixth major virion protein of considerable size (664 kDa) was identified as a major nucleocapsid protein (Leu *et al*., 2005).

Figure 1.4. EM picture WSSV virions

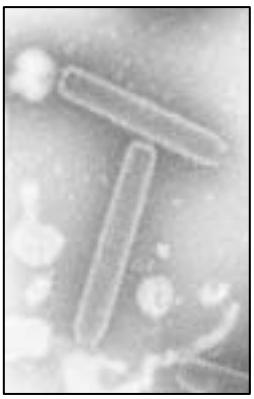


Figure 1.5. EM picture of WSSV nucleocapsids

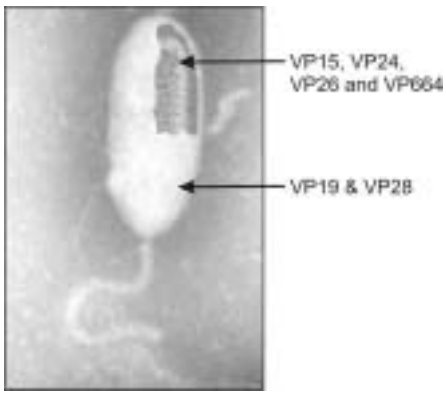


Figure 1.6 Schematic drawing of virion with nucleocapsid and major structural proteins

Table 1.2. WSSV (structural) proteins detected in virions. Shown are the names of the ORF encoding the proteins, number of residues (aa), theoretical mass (M_r), size observed in SDS page, their location in the virion and references. The five major structural proteins are indicated in bold.

WSSV-Th[1]	Gene name	aa	M_r (kDa)	Size on SDS-PAGE (kDa)	Location[3]	Reference
ORF167	vp664	6077	664	664, 186, 161	Nc.	Leu *et al*., 2005
ORF30	vp1684	1684	169	180	n.d.	Huang *et al*., 2002b
ORF134	vp136a	1219	135	136	n.d.	Tsai *et al*., 2004
ORF16	vp136b	1243	138	136	n.d.	Tsai *et al*., 2004
ORF41	vp110	972	108	110	n.d.	Tsai *et al*., 2004
ORF6	vp800, vp95	800	89.4	90	n.d.	Huang *et al*., 2002b; Tsai *et al*., 2004
ORF160	vp75	786	87.6	75	n.d.	Tsai *et al*., 2004
ORF112	vp674, vp73	674	76.2	76	n.d.	Huang *et al*., 2002b, 2005; Tsai *et al*., 2004
ORF158	vp60a	465	51.1	60	n.d.	Tsai *et al*., 2004
ORF183	vp60b, vp544	544	61.8	60	n.d.	Huang *et al*., 2002b, 2004;Tsai *et al*., 2004
ORF29	vp448	448	50.2	55	n.d.	Huang *et al*., 2002b
ORF36	vp53a	1301	144	53	n.d.	Tsai *et al*., 2004
ORF72	vp53b	968	108	53	n.d.	Tsai *et al*., 2004
ORF132	vp53c	489	56.3	53	n.d.	Tsai *et al*., 2004
ORF119	vp51a	486	51.5	51	n.d.	Tsai *et al*., 2004
ORF128	vp51b, vp384	384	43.2	50	n.d.	Tsai *et al*., 2004; Huang *et al*., 2002b
ORF151	vp51c, vp466	466	51.9	50	Env.	Tsai *et al*., 2004; Huang *et al*., 2002b; Wu *et al*., 2005
ORF118	vp41a, vp292	292	33.2	33	n.d.	Tsai *et al*., 2004; Zhang *et al*., 2004
ORF120	vp41b, vp300	300	34.4	34	n.d.	Tsai *et al*., 2004; Huang *et al*., 2002b
ORF150	vp39a	419	47.5	39	n.d.	Tsai *et al*., 2004
ORF162	vp39b	283	32	39	n.d.	Tsai *et al*., 2004
ORF129	vp38a	309	35.5	38	n.d.	Tsai *et al*., 2004
ORF170	vp38b	321	35.8	38	n.d.	Tsai *et al*., 2004
ORF58	vp36a	297	33.1	36	n.d.	Tsai *et al*., 2004
ORF127	vp36b, vp281	281	31.6	32	Env.	Huang *et al*., 2002a,b; Tsai *et al*., 2004; Wu *et al*., 2005; Zhang *et al*., 2004
In deletion	WSSV493[2], vp35	228	26.3		Nc.	Chen *et al*., 2002
ORF102	vp32	278	31.4	32	n.d.	Tsai *et al*., 2004
ORF163	vp31	261	30	31	n.d.	Tsai *et al*., 2004
ORF1	**VP28**, p204	204	22.1	28	Env.	Van Hulten *et al*., 2000c, 2001c; Yang *et al*., 2001; Huang *et al*., 2002b; Zhang *et al*., 2002a, 2004; Tsai *et al*., 2004
ORF153	**VP26**, p22	204	22.2	26	Nc./Env	Van Hulten *et al*., 2000c, 2001c; Yang *et al*., 2001; Huang *et al*., 2002b; Zhang *et al*., 2002b; Tsai *et al*., 2004
ORF31	**VP24**, vp208	208	23.2	24	Nc.	Van Hulten *et al*., 2000a, 2001c; Yang *et al*., 2001; Huang *et al*., 2002b; Tsai *et al*., 2004; Zhang *et al*., 2004
ORF149	vp184	891	100		n.d.	Huang *et al*., 2002b
ORF182	**VP19**, vp121	121	13.2	19	Env.	Van Hulten *et al*., 2002; Yang *et al*., 2001; Huang *et al*., 2002b; Tsai *et al*., 2004
ORF109	**VP15**, p6.8	80	9.2	15	Nc.	Van Hulten *et al*., 2001a, 2002; Yang *et al*., 2001; Zhang *et al*., 2001a; Witteveldt *et al*., 2005
ORF141	vp13a	100	11.1	13	n.d.	Tsai *et al*., 2004
ORF155	vp13b	117	13.1	13	n.d.	Tsai *et al*., 2004
ORF168	vp68, vp12b	68	6.8	7	n.d.	Huang *et al*., 2002b; Tsai *et al*., 2004; Zhang *et al*., 2004
ORF34	vp95	95	11	12	n.d.	Huang *et al*., 2002b
ORF161	vp11	433	48.2	11	n.d.	Tsai *et al*., 2004

[1] numbering according to van Hulten *et al*., 2001
[2] numbering according to Yang *et al.,* 2001
[3] Env: envelope; Nc.: nucleocapsid; n.d.: not determined

Control methods

In view of the impact WSSV has on shrimp farming, combined with the potential threat of WSSV on natural ecosystems, there is a strong demand for the development of control methods and strategies. Several management strategies aimed at minimizing the risk of WSSV outbreaks have been developed. For example, nauplii and PL's could be purchased from reliable sources which maintain good biosecurity measures and test animals before shipping. Other measures include sampling and testing of animals in hatcheries at least two times a week, minimizing stress, the use of non-specific immunostimulants, stocking at appropriate times and lower densities. Another important procedure is the sampling and/or removal of potential carriers of WSSV either by application of chemicals or by avoiding water exchange. Unfortunately, many of these management techniques can only be adopted by solvent farms having enough trained personnel, a combination usually found in intensive farms and not in the more numerous semi-intensive farms. Other than rigorous sanitation and good chain management practices, no adequate measures to control WSSV are available.

What is needed is a cheap and simple vaccine giving sufficient protection against WSSV outbreaks, a strategy that has been so successful in controlling viral and bacterial diseases in man and animals. However, invertebrates seem to lack a true adaptive immune response system and have to rely on various innate immune responses (Kimbrell and Beutler, 2001). Although considered less sophisticated, this innate immune system is able to rapidly and efficiently recognize and destroy non-self material, including pathogens (Lee and Söderhäll, 2002). The innate immune response consists of cellular and humoral responses. Haemocytes are responsible for most of the cellular responses, including encapsulation, phagocytosis, melanization, cytotoxicity, cell-to-cell communication, clotting, and the proPO activating system. Humoral factors, originating from granulocytes, include lectins, defensive enzymes, reactive oxygen intermediates, and the synthesis of a wide array of antimicrobial peptides (Söderhäll, 1999; Lee and Söderhäll, 2002; Kimbrell and Beutler, 2001; Destoumieux, *et al*., 2000). Immunostimulation of shrimp upon contact with products of microbial origin has already been demonstrated (Song *et al*., 1994; Alabi *et al*., 1999). Effective vaccination of *Penaeus monodon* and *Penaeus japonicus* using inactivated Vibrio spp. has been reported by several researchers (Kou *et al*., 1989; Itami *et al*., 1998 and Teunissen *et al*., 1998).

In contrast to the well-studied effect of microbial immunostimulants on the immune system, there is limited information on the immune response upon viral infections. Pan *et al*. (2000) tested tissue extracts from crab, shrimp and crayfish against a variety of viruses for the presence of viral inhibitors. These authors found a 440 kilo Dalton (kDa) molecule, able to non-specifically inhibit infections of six types of both RNA and DNA viruses. Furthermore, an upregulation of the lipopolysaccharide and β-1,3-glucan binding protein gene was observed upon infection with WSSV (Roux *et al*., 2002). This gene is known to be involved in the proPO cascade, which was only known to be upregulated in bacterial and fungal infections. In addition, upregulation of protease inhibitors, apoptotic peptides and tumor-related proteins have been observed (Rojtinnakorn *et al*., 2002). *In vivo* experiments with *Penaeus japonicus* demonstrated the presence of a 'quasi-immune' response in shrimp after re-challenging survivors of both natural and experimental infection with WSSV (Venegas *et*

al., 2000). After this re-challenge the observed mortality of the initial survivors was lower compared to challenged naïve shrimp.

Aim and outline of the thesis

At the onset of the thesis, an increasing knowledge of the WSSV genome led to the functional identification of several genes (Van Hulten *et al*., 2000ab; *Witteveldt et al*., 2001; van Hulten *et al.,* 2001a, van Hulten *et al*., 2002) and ultimately the total sequencing of the genome (van Hulten *et al*., 2001c; Yang *et al*., 2001). This increasing genomic information accelerated and facilitated potential approaches to the development of a WSSV vaccine in shrimp.

As with every vaccine, the first focus had been on the structural proteins of the virus and since all major structural proteins were identified, the first candidate proteins were quickly selected. Being one of the major structural proteins and thus a potential candidate for vaccination, the protein VP15 was investigated (**Chapter 2**). Based on sequence homology to some baculovirus DNA-binding proteins and its location in the nucleocapsid this protein was suspected to be (one of) the basic histone-like proteins of WSSV. DNA and RNA binding experiments were performed to determine whether VP15 is indeed able to bind nucleic acids and if it has sequence and/or structural preferences.

More insight in the function and involvement in the systemic infection of WSSV of the most abundant envelope protein, VP28, was obtained in a neutralization assay (**Chapter 3**). *In vivo* experiments were performed to determine whether WSSV infection in shrimp could be neutralized by serum from a VP28-immunized rabbit. Further neutralization experiments using another shrimp species performed in collaboration with the Medical University of South-Carolina, USA, demonstrated that much care has to be taken in the interpretation of these neutralization assays (**Chapter 4**).

As primary candidates for vaccination trials, the envelope proteins VP19 and VP28 were chosen. Being envelope proteins, these are the first to come in contact with the host and might therefore be involved in either the entry of the virus or recognition by the host defense system, or both. In the vaccination experiments presented in **Chapter 5,** purified and heterologously expressed VP28 and VP19 fusion proteins were injected into shrimp. The subsequent challenge with WSSV was also applied by injection. As immunological memory is one of the important elements of the defense system, a similar vaccination experiment, but with challenges at two different time points was performed (**Chapter 6**). Although the results were encouraging, especially in the light of the assumed absence of an adaptive immune system in shrimp, the application method is not practical from a shrimp farmers perspective. Furthermore, the challenge method employed in these vaccination experiments ignored other areas of immunological intervention and initial virus-host interactions, leaving room for even better responses *in vivo.*

With this in mind, both a different vaccination and challenge method were adopted (**Chapter 7**). To vaccinate the shrimp, food pellets were coated with inactivated bacteria expressing either VP19 or VP28 and fed for 7 days. The consequent challenges, by immersion in WSSV containing seawater, were performed at three different time points. As oral vaccination with VP28 showed such positive effect in *P. monodon*, experiments were

performed to determine if the same effect could be found in *L. vannamei*, another major commercial shrimp species (**Chapter 8**).

In **Chapter 9** the major findings and implications from the previous chapters together with extra results regarding vaccination, neutralization and competition are presented and discussed.

chapter 2

NUCLEOCAPSID PROTEIN VP15 IS THE BASIC DNA BINDING PROTEIN OF WHITE SPOT SYNDROME VIRUS OF SHRIMP

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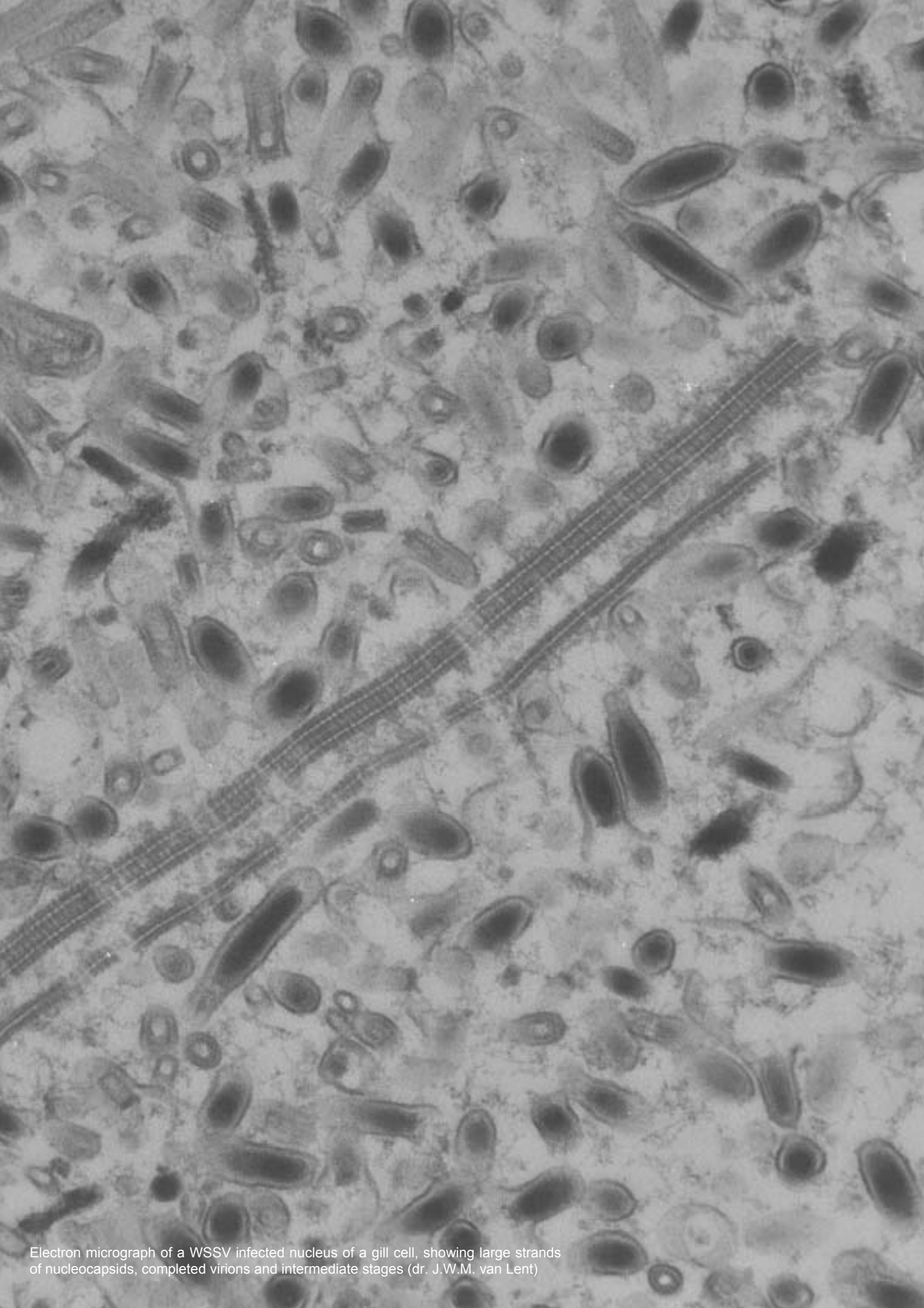

Electron micrograph of a WSSV infected nucleus of a gill cell, showing large strands of nucleocapsids, completed virions and intermediate stages (dr. J.W.M. van Lent)

Abstract

White spot syndrome virus (WSSV) is type species of the genus Whispovirus of the new family Nimaviridae. Despite the elucidation of its genomic sequence, very little is known about the virus as only 6% of its ORFs show homology to known genes. One of the structural virion proteins, VP15, is part of the nucleocapsid of the virus and shows homology to some putative baculovirus DNA binding proteins. These DNA-binding or histone-like proteins are thought to be involved in the condensation and packaging of the genome in the nucleocapsid. Using bacterially expressed VP15 fusion proteins in ELISA and Far-Western experiments showed that VP15 interacts with itself, forming homomultimers, but not with the other major structural proteins of the WSSV virion. Antibodies against phosphorylated proteins revealed that VP15 originating from different sources was not phosphorylated. WSSV VP15 binds non-specifically to double-stranded DNA, but has a clear preference to supercoiled DNA suggesting that VP15 is involved in the packaging of the WSSV genome in the nucleocapsid. This research shed further light on the composition of WSSV virions and the function of one of its nucleocapsid proteins.

Introduction

Since its discovery in Southeast Asia in the beginning of the 1990s, White Spot Syndrome Virus (WSSV) has developed into a devastating epidemic in shrimp. WSSV belongs to the virus family *Nimaviridae,* genus *Whispovirus* (Mayo, 2002) and has a circular dsDNA genome of about 300 kb, coding for approximately 184 open reading frames (ORFs) (van Hulten, *et al*., 2001c; Yang *et al*., 2001). As there is little homology between WSSV ORFs and other known sequences in public databases combined with its unique virion structure (Durand *et al*., 1997), much research has focused on the structural proteins of WSSV. The WSSV virion consists of five major- and 14 minor proteins (Huang *et al.,* 2002a,b; van Hulten *et al.,* 2000a, c, 2002; Zhang *et al.,* 2004). Of the major structural proteins, two are located in the virus envelope (VP19 and VP28) and three are part of the nucleocapsid (VP15, VP24 and VP26). An *in vivo* neutralization assay and vaccination experiments have shown that VP28 is important in the systemic infection of WSSV and protective immunity response of the shrimp (van Hulten *et al.,* 2001b; Witteveldt *et al.,* 2004a,b). For VP19, only vaccination via injection revealed a possible role for this protein in provoking a protective immune response in shrimp (Witteveldt *et al.,* 2004a). Several characteristics of VP15 suggest that it may be the DNA-binding or histone-like protein of WSSV. VP15 is a major structural protein located in the nucleocapsid with a theoretical molecular weight of 6.7 kDa and has a basic pI of 13.2. The amino acid sequence shares some motifs with baculovirus putative basic DNA binding proteins (van Hulten *et al.,* 2001c) and preliminary studies suggested a role of VP15 in DNA binding using a gel mobility shift assay (Zhang *et al.,* 2001a). However, conclusive experimental evidence for the function of VP15 still needs to be obtained. DNA-binding- or histone-like proteins are well known from eukaryotic cells where they assist in the packaging and maintenance of DNA into chromosomes. In this process the histones interact and bind to both DNA and to themselves in multimeric structures. Changes in post-translational

modifications of such proteins, especially phosphorylation, may be involved in the regulation of gene expression (Berger, 2002; Davie and Spencer, 1999; He and Lehming, 2003). Little information is known about the functionality of baculovirus basic DNA-binding proteins. Baculovirus DNA-binding proteins are dephosphorylated prior to assembly of the nucleocapsid (Funk and Consigli, 1993). In viral genomes in general these basic or histone-like proteins are responsible for condensing viral genomes to facilitate packaging into nucleocapsids (Bud and Kelly, 1980; Coca-Prados *et al.,* 1980; Hamatake *et al.,* 1995; Hamatake *et al.,* 1988). In this report, an indepth analysis is performed on the WSSV basic protein VP15 with regard to its phosphorylation status, protein-protein interactions and DNA-binding properties. We conclude that WSSV VP15 is the histone-like DNA binding protein of WSSV.

Materials and Methods

WSSV virus stock and DNA isolation

The virus used in this study originated from infected *Penaeus monodon* shrimp imported from Thailand in 1996 and was obtained as described previously (van Hulten *et al.,* 2000b). In short: hemolymph was collected from WSSV-infected *Orconectes limnosis* crayfish and purified on a continuous sucrose gradient (55%-25% w/w). Centrifugation was performed at 80,000 xg for 1.5 hours at 4˚C. The band containing the virus was removed from the gradient, sedimented by centrifugation at 30,000x g and resuspended in TE. Viral DNA was isolated from purified virions as described in Van Hulten *et al*. (2000c).

Production and purification of recombinant VP15

For bacterial expression, the entire VP15 ORF was cloned in the pMAL-c2x vector (New England Biolabs) resulting in a N-terminal fusion of VP15 with the maltose binding protein (MBP) of approximately 51 kDa. The entire VP15 ORF was also cloned in the pGEX-2T vector resulting in an N-terminal fusion of VP15 with glutathion-S-transferase (GST) of approximately 33 kDa. The DNA fragment encoding the entire VP15 ORF (WSSV ORF 109) was amplified from genomic WSSV DNA by PCR using the forward primer 5'-CGGGATCCATGGTTGCCCGAAGCTCC-3' and reverse primer 5'-TTGCGGCCGCTTAACGCCTTGACTTGC-3'. The amplified PCR product was ligated in the pGEM-T easy vector (Promega) and sequenced. The ORF was removed from the pGEM-T easy plasmid using the restriction enzyme combinations *Bam*HI, *Pst*I and *Bam*HI, *Eco*RI and ligated into the pMAL-c2x and the pGEX-2T vector, respectively. Overexpression of the fusion proteins was performed in *Escherichia coli* DH5α cells for both constructs. After sonication and centrifugation, the fusion proteins were purified by affinity chromatography using amylose resin (New England Biolabs) for the MBP-VP15 protein and Glutathione Sepharose 4B (Amersham Bioscience) for the GST-VP15 protein according to the manufacturers' protocols. The resulting *E. coli* expressing VP15 and the purified proteins were analyzed by SDS-PAGE and Western analysis; the concentration was determined using the Bradford assay (Bio-Rad).

Phosphorylation of VP15

The phosphorylation status of VP15 was investigated using Western-blots of purified WSSV, infected shrimp tissue, MBP-VP15 overexpressed in bacteria (this paper) and VP15 overexpressed in insect cells (van Hulten *et al.,* 2002). Blots were incubated with a mouse monoclonal antibody (diluted 1:200) directed against phosphoserine, phosphothreonine and phosphotyrosine (Spring Bioscience) to detect phosphorylated proteins. Subsequent incubation with horseradish peroxidase-conjugated rabbit anti-mouse antibodies (1:2000) (Dako) for ECL detection (Amersham Biosciences) of the mouse monoclonals was performed according to the manufacturers' protocols.

Protein-Protein interactions: ELISA

An enzyme-linked immunosorbent assay (ELISA) was performed to detect VP15-VP15 interactions. Anti-MBP (New England BioLabs) or anti-GST (Amersham Biosciences) antibodies (100 µl of a 1:5000 dilution in PBS (136 mM NaCl, 2.68 mM KCl, 8 mM Na_2PO_4, 1.76 mM KH_2PO_4 in ddH2O, pH7,4) containing 0.1% Tween 20) were coated on 96-wells plates for one hour at room temperature. After incubation, the plate was washed two times with tap water, once with distilled water and blocked for three hours at room temperature with 100 µl PBS containing 1% Tween-20 and 2% low-fat milk powder. After incubation and washing, the plate was incubated with a serial dilution of proteins or protein combinations as shown in table 2.1 followed by incubation for two hours at room temperature. After washing, the wells were incubated for 1 hour at room temperature with anti-MBP antibodies when the plates were first coated with anti-GST or anti-GST if the wells were first coated with anti-MBP (diluted 1:5000 in PBS containing 1% Tween-20). The plates were washed and subsequently incubated for one hour at room temperature with a secondary antibody (goat anti-rabbit when using anti-MBP and swine anti-goat when using anti-GST) conjugated to horseradish peroxidase (diluted 1:5000 in PBS containing 1% Tween-20). After a final wash, the substrate TMB (Fermentas) was added and incubated for approximately 20 minutes. The absorption was measured at 405 nm after stopping the reaction by adding 100 µl 0.2 M sulphuric acid.

Table 2.1. Set-up of ELISA used for detection of VP15 protein-protein interactions. Indicated are the antibodies used for coating (first column), the protein mixtures (second column), amounts of protein applied (third column) and antibody used for final detection (fourth column).

Antibody coated	Protein mixture	Amount applied per protein (µg)	Detection
α-MBP	MBP-VP15 + GST-VP15	10, 5, 2.5, 1.25, 0.625…..0.001, 0	
α-MBP	MBP + GST-VP15 (control)	10, 5, 2.5, 1.25, 0.625…..0.001, 0	α -GST
α-MBP	GST-VP15 (control)	10, 5, 2.5, 1.25, 0.625…..0.001, 0	
α-GST	GST-VP15 + MBP-VP15	10, 5, 2.5, 1.25, 0.625…..0.001, 0	
α-GST	GST + MBP-VP15 (control)	10, 5, 2.5, 1.25, 0.625…..0.001, 0	α -MBP
α-GST	MBP-VP15 (control)	10, 5, 2.5, 1.25, 0.625…..0.001, 0	

Protein-Protein interactions: Far-Western

Crude bacterial expressions of MBP-VP15, GST-VP15 and GST alone were separated by SDS-PAGE, electrophoretically transferred to Immobilon-P membranes and gradually renatured at 4˚C in HEPES buffer (HEPES buffer: 20 mM HEPES, pH 7; 50 mM NaCl; 5 mM $MgCl_2$; 1 mM EDTA, 1 mM dithiothreitol (DTT); 10% glycerol) containing 5% low-fat milk powder. The blots were washed and incubated with 100 µg of MBP-VP15 or MBP alone in 5 ml incubation buffer (PBS, 1% Tween-20 and 1% low-fat milk powder) for three hours at room temperature, while rocking gently. The blots were subsequently washed three times 10 minutes with incubation buffer and incubated for 1 hour at room temperature with anti-MBP (1:5000 dilution in PBS-T containing 1% low-fat milk powder). The blots were again washed three times 10 minutes with incubation buffer and subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:5000 dilution in PBS-T containing 1% low-fat milk powder) for one hour at room temperature. After washing three times 10 minutes with incubation buffer and two times 10 minutes with PBS, an ECL-detection (Amersham Biosciences) was performed. Using this setup, VP15-VP15 interactions, but also the interaction between the other major WSSV structural proteins (VP28, VP26, VP24 and VP19) was tested.

DNA-binding assay: South-Western

The two VP15 fusion proteins were applied either as a crude bacterial extract or as purified protein and separated in a SDS-PAGE gel and electrophoretically transferred to an immobilon-P membrane. MBP, GST and MBP-VP28 proteins were included as negative controls. After blocking (1.5 hrs at 37˚C in TBS (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl) containing 3% low fat milk powder), the membrane was incubated overnight at 4˚C with 1% blocking reagent (Roche) containing 1.5 µg DIG-labeled DNA per 5 ml. The DNA originated from a PCR product encoding the shrimp phosphoglycerate mutase gene. After incubation, the blot was washed 30 minutes at RT in TBS-T (TBS containing 0.1% Tween-20) containing 3% ELK followed by washing with TBS-T for 10 minutes at RT. The DIG labeled probes were detected using anti-digoxigenin-AP (1:5000 dilution in TBS-T containing 0.3% ELK) (Roche) followed by chemiluminescent detection using CSPD substrate (Roche)

DNA-binding assay: Electrophoretic mobility shift assay (EMSA)

The DNA-binding capacity of VP15 was examined in 1% agarose gels in Boric acid buffer (45mM Boric acid, 45mM Tris-HCl, pH 8.0). Purified plasmid DNA (pET28a) was mixed with purified VP15 at different ratios in a final volume of 20 µl containing 300 mM $MgCl_2$. This mixture was incubated at 37˚C for 30 minutes and mixed with 6X loading buffer (1mM EDTA (pH 8.0), 50% glycerol in water) before loading on gel. Gels were run for at least three hours at 45 V in Boric acid buffer and the DNA was visualized under UV light after staining the gel in running buffer supplemented with ethidium bromide (0.5 µg/ml) for 30 minutes.

DNA-binding assay: Dot spot

Spotting DNA and RNA from different sources followed by incubation with purified MBP-VP15 protein was carried out to investigate the DNA/RNA binding properties of native VP15. Different amounts of DNA and/or RNA from different sources were spotted on a Hybond membrane and bound by exposing the spots to UV-light for two minutes (7 mW/cm^2, Fluo-Link transilluminator, Vilber-Lourmat). The membrane was incubated with 40 µg purified MBP-VP15 or 47 µg of purified MBP as a control in DNA binding buffer (0.01M Tris-HCl, 1 mM DTT, 1mM EDTA, 0.2M NaCl, 10% glycerol, 0.4% NP-40, pH8.0) for three hours, while rocking slowly. Subsequently, the membrane was blocked with blocking buffer (3% BSA in TBS) at 37°C and washed three times 10 min in TBS-T. For detection of the protein, the membrane was incubated with anti-MBP (1:2000 dilution in TBS-T, containing 0,2% ELK) for one hour at room temperature followed by another three washing steps of 10 min and incubation with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:2500 in TBS-T, containing 0,2% ELK) for one hour at room temperature. Finally, the membrane was washed three times followed by ECL detection (Amersham Biosciences).

Results

Production and purification of recombinant VP15

The entire VP15 ORF was successfully overexpressed and purified as an N-terminal fusion with MBP and GST. Bands corresponding to the two fusion proteins were observed at the expected positions of 51 kDa for the MBP and 33 kDa for the GST fusion proteins in a SDS-PAGE gel (Figure 2.1). As control proteins, unfused MBP and GST proteins were overexpressed and purified. Bands corresponding to these proteins were also observed at their expected heights of 42.5 kDa for MBP and 26 kDa for GST.

Phosphorylation of VP15

As phosphorylation of DNA-binding proteins may be involved in the regulation of DNA packaging and gene expression, the phosphorylation status of VP15 from different origins was examined. Western blots of VP15 from WSSV infected tissue, purified virions and of recombinant VP15 (insect cell and bacterial expressed) were assayed using antibodies against phosphoserine, phosphothreonine and phosphotyrosine. Phosphorylated

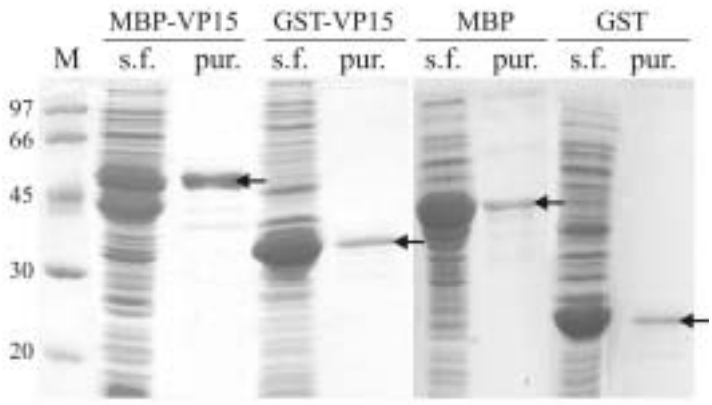

Fig. 2.1. Coomassie stained SDS-PAGE gels of soluble fractions of *E. coli* expressions (s.f.) and purifications (pur.) of MBP-VP15, GST-VP15, MBP and GST proteins. Arrows indicate the overexpressed and purified proteins and the numbers next to the marker (M) indicate molecular weights in kDa.

proteins (Precision Plus Protein standard, Bio-Rad) were included in the experiment as positive control. The Western blots showed no positive signals in the samples containing VP15. The positive controls did show a positive signal at the expected heights (data not shown), suggesting that VP15 is not phosphorylated.

Protein-protein interactions: ELISA

To study the possible interactions between VP15 proteins (homomultimers) ELISA experiments using two differently tagged VP15 fusion proteins (MBP and GST) were performed. In the first experiment wells were coated with anti-MBP followed by incubation with a mixture of GST-VP15 and MBP-VP15 or, as controls, GST-VP15 alone or GST-VP15 plus MBP. Incubation with this mixture of GST-VP15 and MBP-VP15 resulted in a considerably higher absorbance compared to both controls (Figure 2.2a), indicative of a VP15-VP15 interaction. In a second experiment (Figure 2.2b), wells were coated with anti-GST and incubated with the same GST-VP15 and MBP-VP15 mixture and either MBP-VP15 alone or MBP-VP15 plus GST as controls. In this case, the difference between the wells incubated with the mixture of GST-VP15 and MBP-VP15 and the controls was even more profound, confirming a VP15-VP15 interaction. ELISA experiments studying the interaction between VP15 and the other four major structural virion proteins (VP28, VP26, VP24 and VP19 expressed as both MBP and GST fusion proteins) failed to show interactions (data not shown).

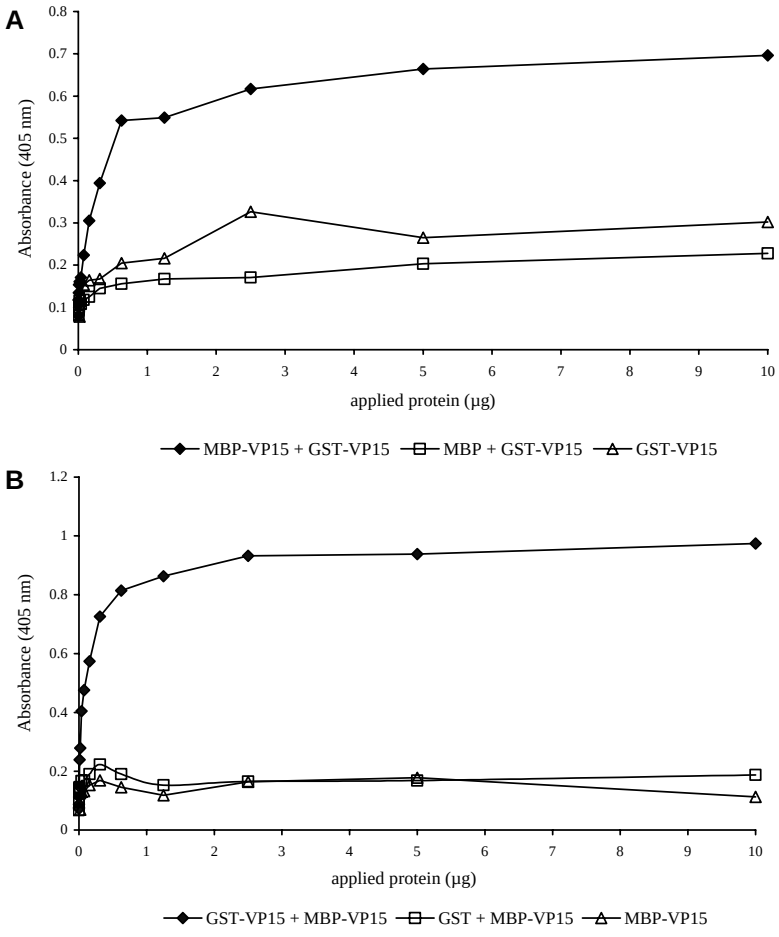

Fig. 2.2. Results of ELISAs using coated wells with anti-MBP (A) and anti-GST (B). Protein mixes were applied as indicated in legend. Detection was performed with anti-GST (A) and anti-MBP (B).

Protein-protein interactions: Far-Western

To independently confirm the VP15-VP15 interaction observed using ELISA, an overlay assay was performed. MBP-VP15, GST-VP15 and GST alone were separated in a SDS-PAGE gel and transferred to Immobilon-P membranes, renatured and subsequently incubated with either purified MBP-VP15 (Figure 2.3a) or purified MBP (Figure 2.3b). MBP(-VP15) was detected using MBP-specific antibodies. Figure 3a shows a clear positive signal where GST-VP15 was applied (lane 2), suggesting that there is an interaction between MBP-VP15 and GST-VP15. No signal was observed between GST and MBP-VP15 (lane 3). To exclude the possibility of an interaction between MBP and VP15, only MBP was used as overlay protein (Figure 2.3b). A positive signal was only detected in the control lane with MBP-VP15 (lane 1) and not in GST-VP15 (lane 2). Separate experiments studying the interaction between VP15 and the other four major structural virion proteins (VP28, VP26, VP24 and VP19 expressed as both MBP and GST fusion proteins) did not show specific interactions (data not shown).

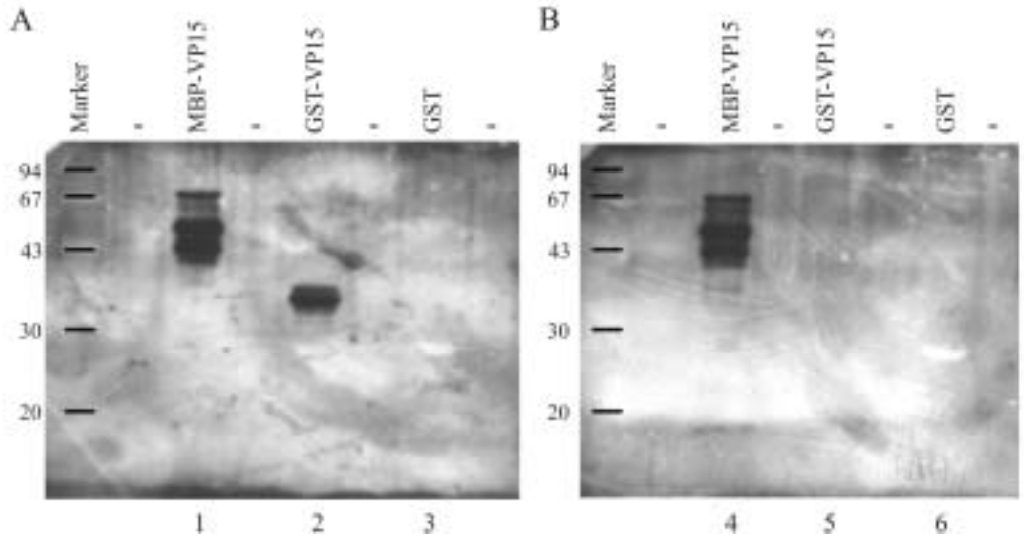

Fig. 2.3. Far-western analysis using SDS-PAGE-separated and blotted proteins indicated above the panel. A: MBP-VP15 as overlay protein and anti-MBP to detect MBP-VP15. B: MBP as overlay protein and anti-MBP to detect binding of MBP. Molecular weights in kDa are indicated next to the marker.

DNA-binding assay: South-Western

A South-Western analysis was performed to verify that the DNA binding properties of VP15 previously observed (Zhang *et al.,* 2001a) are indeed VP15-specific. For this, a membrane containing SDS-PAGE separated extracts and purified proteins of MBP-VP15, MBP, GST-VP15, GST and MBP-VP28 was incubated with DIG-labeled DNA (Figure 2.4). The DNA was consequently detected using CSPD in lanes 1 and 2 containing MBP-VP15, and lanes 4 and 5 containing GST-VP15 fusion proteins at the expected heights. There was no interaction detected in the lanes with MBP, GST and MBP-VP28 (lanes 3, 6 and 7 respectively).

DNA-binding assay: Electrophoretic mobility shift assay (EMSA)

The preference of VP15 for different DNA topologies was investigated using an EMSA experiment. When plasmid DNA is separated in an agarose gel, three major DNA topologies can be distinguished: nicked circle, linear and supercoiled. Different amounts of purified MBP-VP15 were mixed with 500 ng purified plasmid DNA (pET28a), incubated and applied onto an agarose gel. Interactions of DNA with proteins results in a retardation or disappearance of the DNA from the separating gel. In Figure 5 the resulting retardation in mobility is shown. When 0.04-0.12 µg of MBP-VP15 is added, a clear retardation in mobility of only the supercoiled DNA is observed (lanes 2-4). When the amount of MBP-VP15 protein is increased to 0.2 µg, the supercoiled DNA entirely disappears (lane 5). Linearized plasmid DNA disappears at 0.3 µg of MBP-VP15 (lane 6) and ultimately the nicked circle band disappears when 0.4 µg of purified MBP-VP15 is added (lane 7).

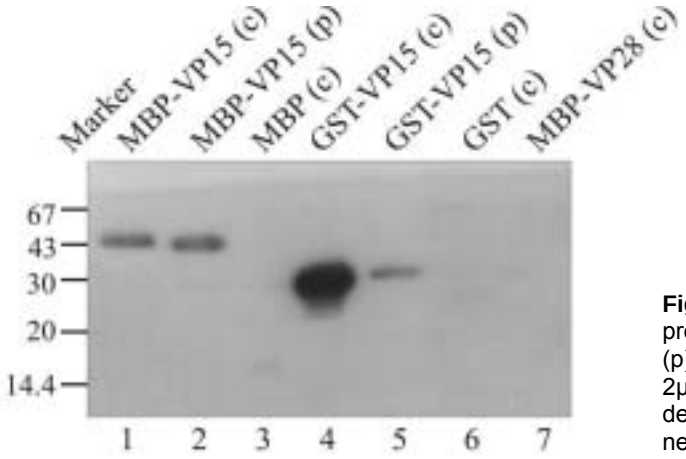

Fig. 2.4. South-Western analysis of different protein samples. (c) crude soluble protein extract, (p) purified proteins. The blot was incubated with 2µg of DIG-labeled DNA followed by CSPD detection. Molecular weights in kDa are indicated next to the marker.

With an increase in VP15 concentration, the DNA is first retarded and with a further increase of VP15 retained in the loading slot, unable to migrate into the gel. This result suggests there is a topological preference of VP15 to supercoiled DNA over both the linearized and nicked circle topology. When 0.3 µg of purified MBP-VP26 is added, no shift is observed, confirming the specific DNA binding properties of VP15 (lane 12).

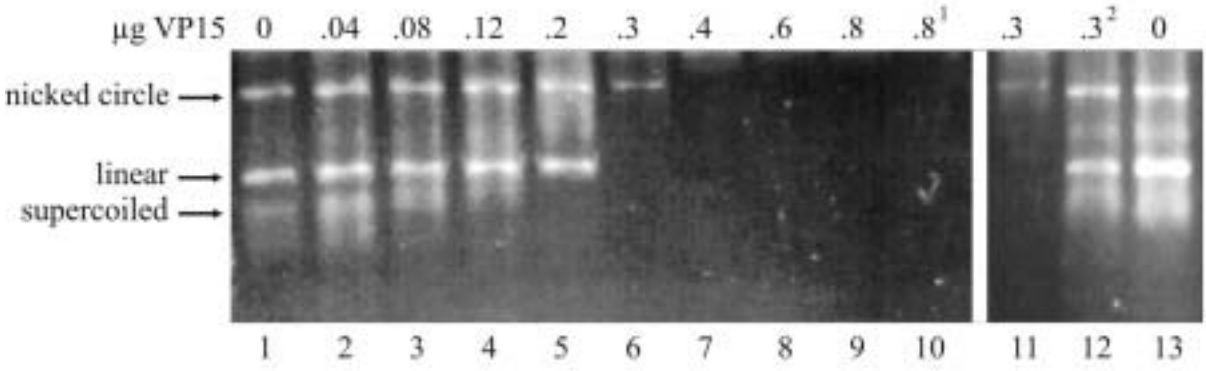

Fig 2.5. Electrophoretic mobility shift assay using 0.5 µg of purified plasmid DNA mixed with purified MBP-VP15 prior to loading. The amount of MBP-VP15 protein used in the different mixtures is indicated. As controls no plasmid [1] and purified MBP-VP26 [2] were included. The different plasmid topologies are indicated with arrows.

DNA-binding assay: Dot spot

For further confirmation of the topological preference of VP15 for supercoiled DNA, and to determine the binding of VP15 to single-stranded DNA, RNA and double-stranded RNA, several Dot-spot assays were performed (Figure 2.6).

First, the binding of VP15 to the double-stranded DNA genomes of both WSSV and the baculovirus AcMNPV in their native (circular) and linearized (*Bam*HI digested) form was evaluated. Figure 2.6a shows that the signal of all circular topologies is similar but considerably higher as compared to the signal observed in their respective linearized forms. When the experiment is repeated with the same DNA, but made single-stranded by heating the DNA (10'at 95°C), the signals are similar to the signals found for the linearized DNA. Finally the binding of VP15 to single-stranded and double-stranded RNA was tested but no positive signal was observed, indicating that there is no binding between VP15 and RNA (Figure 2.6b). When purified MBP instead of the MBP-VP15 was used in these experiments no positive signals were found (data not shown), confirming that the VP15 protein binds specifically to DNA.

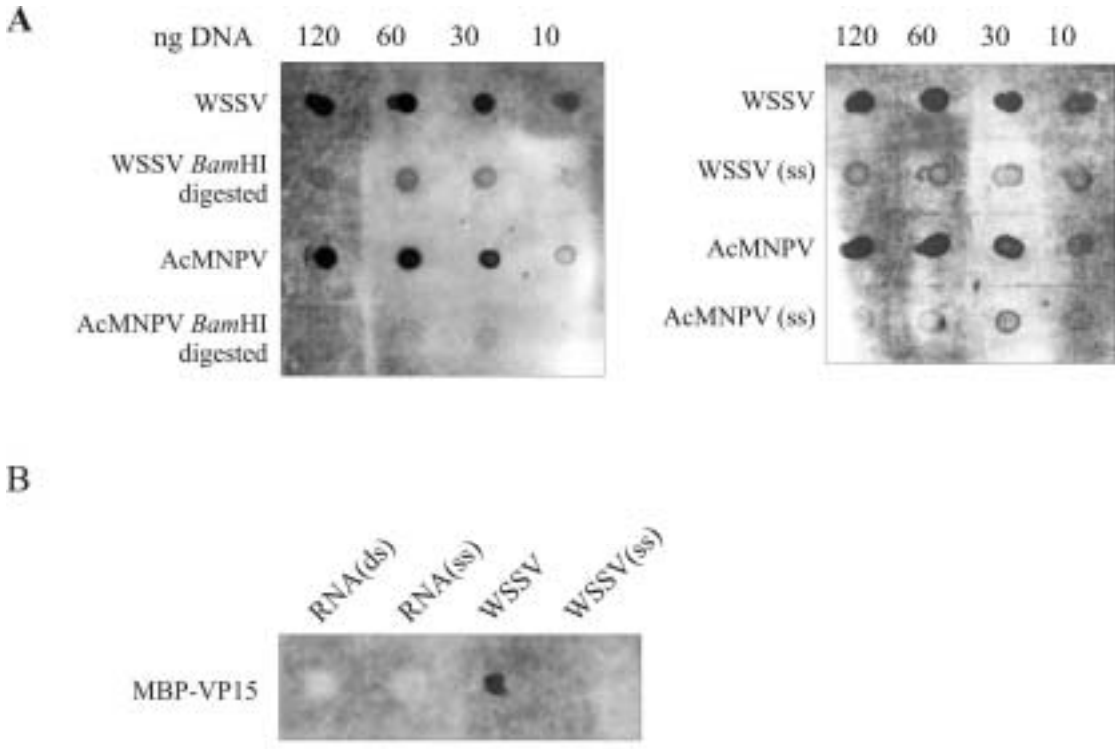

Fig. 2.6. A: Dot-spot of different treatments of circular DNA, overlaid with MBP-VP15 followed by ECL detection. Indicated are the origin of DNA, treatment and the amount applied to the membrane. B: Dot-spot of double-stranded and single-stranded RNA, native WSSV and single-stranded WSSV. Purified MBP-VP15 was used as overlay proteins as indicated and detected by ECL.

Discussion

VP15 is one of the three major structural proteins of the WSSV nucleocapsid (van Hulten *et al.,* 2002). Because of similar motifs found in VP15 and several baculovirus DNA-binding proteins, its location in the virion and its predicted physical characteristics, VP15 was assigned to be a DNA binding protein in the WSSV virion (Maeda *et al.,* 1991; Russell and Rohrman, 1990; van Hulten *et al.,* 2001c; Wilson *et al.,* 1987). Preliminary results using unpurified bacterial expressions of a VP15-GFP fusion protein in an EMSA suggested that WSSV VP15 is a DNA binding protein (Zhang *et al.,* 2001a). However, direct evidence that the observed retardation was due to binding of VP15 to DNA was not shown.

Although the phosphorylation status of nucleocapsid proteins in baculoviruses may be involved in the uncoating process of nucleocapsids (Funk and Consigli, 1993), no phosphorylated VP15 was detected in WSSV purified virions, WSSV infected tissue and recombinant VP15 from *E. coli* and insect cells.

To further study the function of WSSV VP15, its protein and DNA binding properties were investigated. VP15 was expressed N-terminally fused to MBP and GST. Using these tags, VP15 was readily purified and used in protein-protein interaction and DNA-binding studies. Via ELISA (Figure 2.2) and Far-Western experiments (Figure 2.3) VP15-VP15 interactions were demonstrated. The formation of VP15 homomultimers might be involved in the formation of nucleocapsids and although no interaction between VP15 and the four other major structural proteins was observed, it is possible that one or more of the minor WSSV structural proteins (Huang *et al.,* 2002a,b) participates in this process. Despite the fusion of VP15 to the relatively large MBP and GST tags, the protein appeared to remain funtional.

Using the purified VP15 proteins and crude bacterial overexpression lysates, the binding of VP15 to DNA was clearly demonstrated in a South-Western experiment (Figure 2.4). As no DNA was detected in association with MBP, GST and MBP-VP28, the binding of DNA to the VP15 fusion proteins must be due to VP15 and not the fused MBP or GST proteins. Since a PCR product of a shrimp gene was used, the binding of VP15 to DNA seemed sequence aspecific. The latter was confirmed in the EMSA and dot-spot experiments (Figure 2.6).

More insight in the topological preference of VP15 was obtained in an EMSA (Figure 2.5) experiment where a clear preference of VP15 for supercoiled DNA was observed. Supercoiled DNA was the first to show retardation in a serial dilution of VP15. With an increasing amount of VP15 the two other plasmid topologies (nicked circle and linear) also showed retardation, indicating that VP15 has a preference for supercoiled DNA but does not specifically bind to supercoiled DNA. This preference for supercoiled DNA was further substantiated in Dot-spot experiments where the binding of VP15 to circular double-stranded DNA molecules was compared to their linearized counterparts (Figure 2.6a). In these experiments the sequence a-specificity of VP15 was shown, as the signal found in the WSSV and AcMNPV genome was comparably high. Dot-spot experiments using double-stranded RNA, single-stranded RNA and single-stranded DNA all showed very low signals indicating no or a very low affinity to VP15.

A number of proteins have been described to have a preference to bind supercoiled DNA,

which has been referred to as supercoiled-selective (SCS) DNA binding (Paleçek *et al.,* 1997). Because supercoiled DNA contains excess energy which can be released upon binding of proteins, this makes some DNA protein bonds supercoil dependent. Much research has been performed on the human p53 tumor-suppressor protein which plays an important role in the cellular response to DNA damage (Oren and Rotter, 1999). This protein binds to supercoiled DNA even in absence of the target sequence in the DNA molecule (Paleçek *et al.,* 2001) and exhibits protein interactions as it is most abundantly found as tetramers or multiple tetramers (Friedman *et al.,* 1993). From a viral perspective the SCS DNA binding of VP15 might be necessary for condensing the large WSSV genome and packaging into the nucleocapsid.

This research has shed further light on the composition of WSSV virions and the function of one of its structural proteins. Future experiments involving VP15 mutants or specific VP15 domains could reveal the important areas for DNA binding and protein-protein interactions. Finally, pseudotyping experiments using recombinant baculoviruses in which the baculovirus DNA-binding protein has been replaced by WSSV VP15, may give more information on the function of VP15.

Acknowledgements

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chapter 3

WHITE SPOT SYNDROME VIRUS ENVELOPE PROTEIN VP28 IS INVOLVED IN THE SYSTEMIC INFECTION OF SHRIMP

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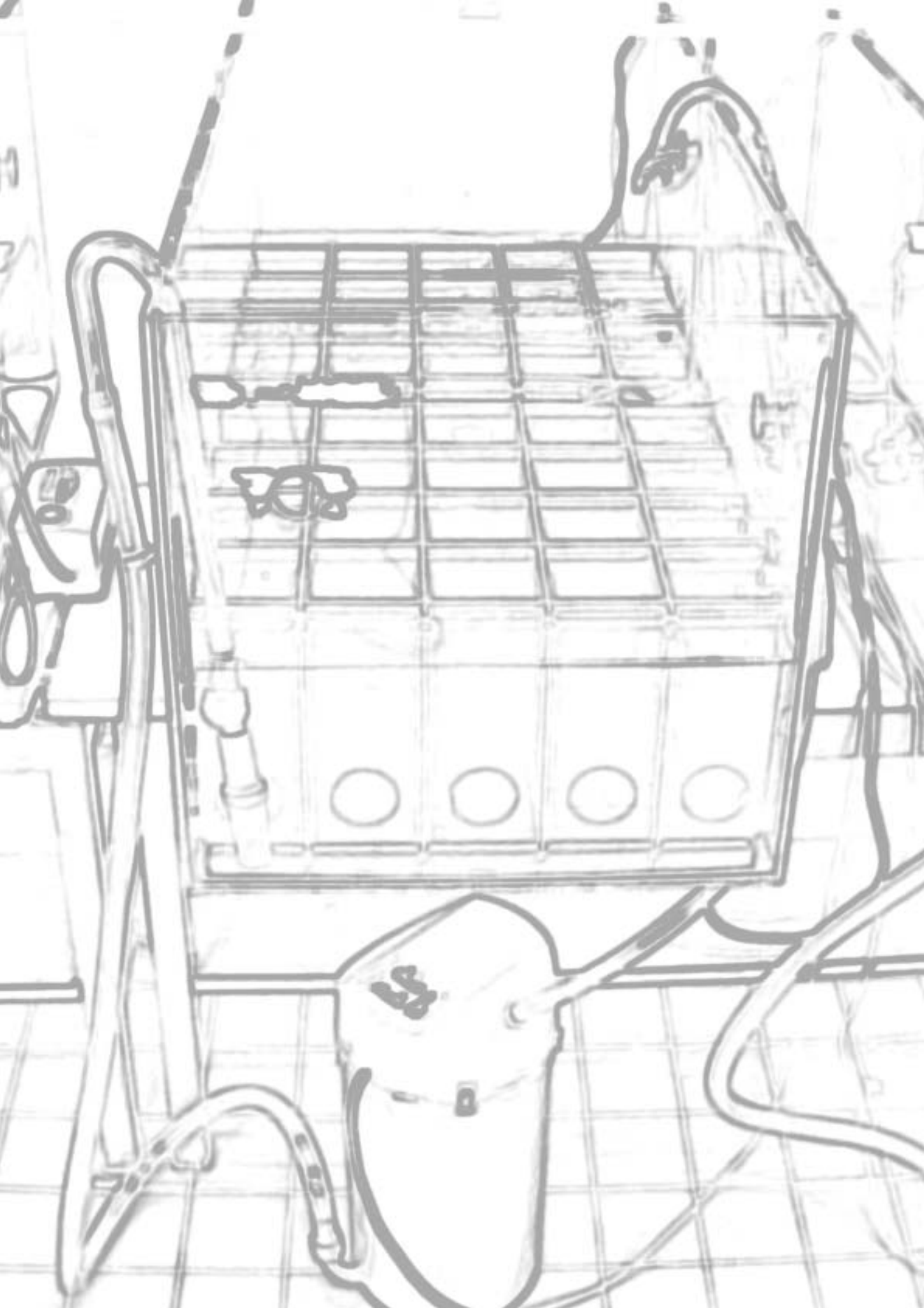

Abstract

White Spot Syndrome Virus (WSSV) is a large DNA virus infecting shrimp and other crustaceans. The virus particles contain at least five major virion proteins, of which three (VP26, VP24 and VP15) are present in the rod-shaped nucleocapsid and two (VP28 and VP19) reside in the envelope. The mode of entry and systemic infection of WSSV in the black tiger shrimp, *Penaeus monodon* and the role of these proteins in these processes are not known. A specific polyclonal antibody was generated against the major envelope protein VP28 using a baculovirus expression vector system. The VP28 antiserum was able to neutralize WSSV infection of *P. monodon* in a concentration-dependent manner upon intramuscular injection. This result suggests that VP28 is located on the surface of the virus particle and is likely to play a key role in the initial steps of the systemic WSSV infection in shrimp.

Introduction

White Spot Syndrome Virus (WSSV) is a major disease agent of penaeid shrimp in Southeast Asia, the Indian continent, and in South and Central America (Rosenberg, 2000). The disease is caused by an ovoid-to-bacilliform virus with a rod-shaped nucleocapsid and a tail-like appendix at one end of the virion (Durand *et al*., 1997; Nadala *et al*., 1998). The virus contains a double-stranded DNA with an estimated size of 290 kbp (Yang *et al*., 1997). Genetic analysis indicates that WSSV is a representative of a new virus group provisionally named whispovirus (Van Hulten *et al*., 2000b; Tsai *et al.*, 2000).

WSSV has a broad host range, infecting several crustacean species, like shrimp, crab, and crayfish (Wang *et al*., 1998). Little is known about WSSV infection and morphogenesis *in vivo*. Upon infection *per os*, infected cells are observed first in the stomach, gill and cuticular epidermis of the shrimp. The infection subsequently spreads systemically in the shrimp to other tissues of mesodermal and ectodermal origin (Chang *et al*., 1996). Research on virus replication and virion morphogenesis shows that DNA replication and *de novo* envelope formation take place in the nucleus (Durand *et al*., 1997; Wang *et al*., 2000a). The mechanism of virus entry into the shrimp and of the spread of the virus in the crustacean body is not known.

The virus particle consists of at least five major proteins with estimated sizes of 28 kDa (VP28), 26 kDa (VP26), 24 kDa (VP24), 19 kDa (VP19) and 15 kDa (VP15). VP28 and VP19 are associated with the virion envelope and VP26, VP24, and VP15 with the nucleocapsid (Van Hulten *et al*., 2000a). Amino acid analysis of VP28, VP26 and VP24 indicated that these proteins have about 40% amino acid identity and that their genes may have evolved from a common ancestral gene (Van Hulten *et al*., 2000c). The role of the envelope and its proteins in the establishment of the systemic infection process has not been determined.

Neutralization experiments have often been performed to study the role of virion proteins or their domains in the infection process. Neutralizing antibodies bind to envelope spikes on the virion and prevent attachment of the virus to the cell surface, cell entry or virus

uncoating (Burton *et al*., 2000). For many vertebrate viruses like poxviruses (Galmiche *et al*., 1999) and hepadnaviruses (Sunyach *et al*., 1999) *in vitro* neutralization experiments involving cell cultures (plaque reduction assays) have been used for this purpose. For invertebrate baculoviruses, *in vitro* neutralization experiments have been exploited to show that *Autographa californica* nucleopolyhedrovirus (*Ac*MNPV) can be neutralized by complexing the budded virions with specific antibodies against the viral envelope protein (GP64) (Volkman and Goldsmith, 1985). These authors showed that the mechanism of neutralization is by inhibition of virus entry and adsorptive endocytosis. However, standardized (primary) shrimp cell cultures are not available and therefore an *in vivo* approach is followed.

In vivo neutralization experiments have been widely used for many vertebrate viruses and have even led to passive immunization strategies. When combined with the use of monoclonal antibodies, this strategy has been used to identify the virion protein epitope(s) involved in the neutralization (e.g. Schofield *et al*., 2000). *In vivo* neutralization assays have also been successfully used in insects, e.g. in inhibiting infection of larvae of the Douglas fir tussock moth *Orgyia pseudotsugata* with its nucleopolyhedrovirus (OpMNPV) using OpMNPV antiserum (Martignoni *et al*., 1980). This strategy has now been applied to shrimp with the added advantage that it is as close as possible to the *in vivo* situation. In this paper we provide evidence that VP28 is directly involved in the systemic infection of the shrimp *P. monodon* by WSSV.

Materials and Methods

Shrimp culture

Cultures of healthy shrimp were performed in a recirculation system at the Laboratory of Fish Culture and Fisheries at Wageningen University. For the experiments shrimp were transferred to an experimental system located at the Laboratory of Virology, Wageningen University, and kept in groups of 10-15 individuals in 60 liter aquariums with an individual filter (Eheim, Germany) and heating (Schego, Germany) at 28°C. *P. monodon* shrimps of approximately 1 gram were used in the titration and neutralization experiments.

White Spot Syndrome Virus stock production

The virus isolate used in this study originates from infected *P. monodon* shrimps imported from Thailand in 1996 and was obtained as described before (Van Hulten *et al*., 2000b). Crayfish *Orconectes limosus* were injected intramuscularly with a lethal dose of WSSV using a 26-gauge needle. After one week the haemolymph was withdrawn from moribund crayfish and mixed with modified Alsever solution (Rodriguez *et al.*, 1995) as an anticoagulant. The virus was purified by centrifugation at 80,000 x g for 1.5 h at 4°C on a 20-45% continuous sucrose gradient in TN (20 mM Tris, 400 mM NaCl, pH 7.4). The visible virus bands were removed and the virus particles were subsequently sedimented by centrifugation at 45,000 x g at 4°C for 1 h. The virus pellet was resuspended in TE (pH 7.5) and the virus integrity was checked by electron microscopy. The virus stock was stored at -80°C until use in the experiments.

VP28 polyclonal antibody

The major WSSV structural envelope protein VP28 was expressed in insect cells using baculovirus *Ac*MNPV-WSSVvp28 (Van Hulten *et al*., 2000a). The protein band containing the VP28 was purified using a Model 491 PrepCell (Biorad) according to the instruction manual. Fractions were collected using a Model 2110 fraction collector (Biorad) and analyzed in a silver-stained SDS PAGE gel. Western blotting using a polyclonal WSSV antibody was employed to determine the VP28-containing fractions. These fractions were pooled and SDS was removed by dialysis against several volumes of 0.1 x TE. The protein was subsequently concentrated by freeze-drying and resuspended in 0.1 x TE (pH 7.5). The purified VP28 protein (100 μg) was injected into a rabbit to produce a polyclonal antibody. The rabbit was boosted with 300 μg of VP28 after six weeks and the antiserum was prepared 2 weeks thereafter.

In vivo injection

P. monodon shrimp of approximately 1 gram were injected intramuscularly with 10 μl of virus solution in 330 mM NaCl in the 4th or 5th tail segment of the shrimp with a 29 gauge needle (Microfine B&D). The shrimps were subsequently cultured for a period of 40 days and the mortality was monitored twice daily. For each group 10 – 15 shrimps were used. Deceased shrimps were monitored for WSSV infection by viewing haemolymph extracts in the electron microscope.

Neutralization assay

Shrimps of 1 gram were injected with WSSV in the presence or absence of VP28 antibody. A negative (330 mM NaCl) and a positive control (virus only) were included (Table 3.1). The total amount of virus administered per shrimp is constant in all groups and is equivalent to 10 μl of the 1 x 10^8 dilution of the virus stock. The pre-immune serum was included as a control for the effect of the serum on shrimp mortality. Several dilutions of the antiserum were incubated with the virus for 1 h at room temperature, prior to injection in shrimp. After injection the shrimps were monitored for 28 days and dead shrimp were examined for the presence of WSSV by electron microscopy.

Table 3.1. Constitution of injection solutions. The first column shows the group numbers, the second the treatment, the third the content of the 10 μl injected volume and the last column the number of shrimps used in each group. The total amount of WSSV injected is the same for group 1 to 5.

Group#	Type	Injection (10 μl total)	# shrimp
1	Positive control	WSSV	10
2	Pre-immune serum control	WSSV + 9 μl pre-immune serum	15
3	10x dilution of VP28 antiserum	WSSV + 1 μl VP28 antiserum	15
4	2x dilution of VP28 antiserum	WSSV + 5 μl VP28 antiserum	15
5	VP28 antiserum	WSSV + 9 μl VP28 antiserum	15
6	Negative control	330 mM NaCl	10

Results

Virus titration

A WSSV virus stock was produced in the crayfish *Orconectes limosus* by intramuscular injection of purified WSSV. In order to determine the dilution resulting in 90-100% mortality in the black tiger shrimp *P. monodon*, an *in vivo* virus titration was performed using animals of approximately 1 gram in weight. The virus stock was diluted in steps from 1 x 10^5 to 5 x 10^{11} times in 330 mM NaCl as indicated (Fig. 3.1) and for each dilution 10 µl was injected intramuscularly into 10 shrimps. Shrimps that were injected with 330 mM NaCl, served as negative control for the infection. All shrimps serving as negative control (not shown) and those having received the 5 x 10^{11} virus dilution survived, whereas mortality due to virus infection occurred in all groups with a lower virus dilution (Fig. 3.1). Administration of virus dilutions of 1 x 10^5 and 1 x 10^7 resulted in almost 100% mortality in a period of 20 days. A delay in mortality was observed when virus dilutions of 1 x 10^8 and 5 x 10^9 were used. The 1 x 10^8 dilution resulted in 90% final mortality, but the time of mortality was delayed and spanned a period of 40 days. The experiment was repeated with the 1x10^7, the 1 x 10^8, and the 5x10^9 dilution yielding essentially the same results. The dilution of 1 x 10^8 was chosen as the virus dose for further experiments as this condition was expected to give the optimal response to the neutralization in terms of mortality reduction.

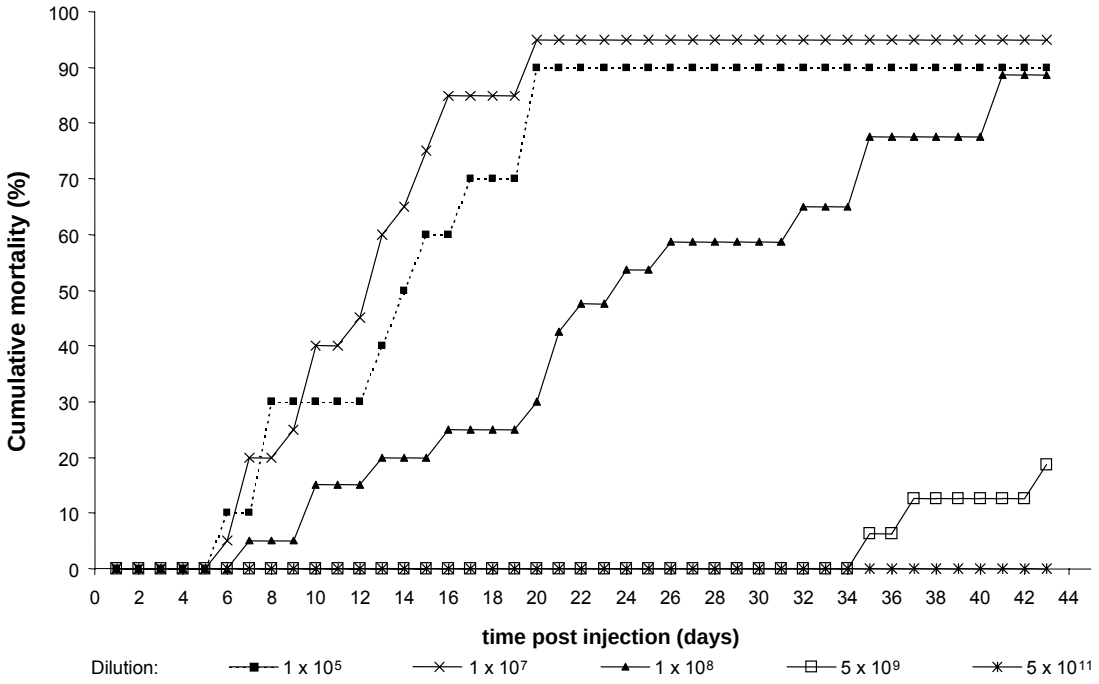

Figure 3.1. Titration of WSSV in *P. monodon* shrimp. The days post injection of the virus are shown on the abcis and the accumulated mortality (in %) on the ordinate. Ten µl of a 1 x 10^5, 1 x 10^7, 1 x 10^8, 5 x 10^9 and 5 x 10^{11} diluted virus stock was injected.

Antibody against recombinant VP28

The major WSSV envelope protein VP28 was expressed under control of the polyhedrin promoter in insect cells using recombinant baculovirus AcMNPV-WSSVvp28 (Van Hulten *et al*., 2000c) and was used after purification to raise specific polyclonal antibodies in rabbits. The VP28 polyclonal antiserum reacted strongly with baculovirus expressed as well as with bacterial expressed VP28 (data not shown).

As VP28 shows a considerable degree of amino acid homology with nucleocapsid proteins VP26 and VP24 (Van Hulten *et al*., 2000c), the specificity of the polyclonal was tested against purified WSSV virions and WSSV nucleocapsids (Fig. 3.2). All major proteins are present in the virion fraction (Fig. 3.2a, lane 2) and only VP26, VP24 and VP15 are present in the nucleocapsid fraction (Fig. 3.2a, lane 3). In the Western analysis (Fig. 3.2b) the VP28 antiserum (1:5000 dilution) shows a clear reaction with the VP28 present in the WSSV virion (Fig. 3.2b, lane 2). A minor reaction was observed with smaller products, most likely VP28 breakdown products. There was no reaction with proteins of the WSSV nucleocapsids (Fig. 3.2b, lane 3). This shows that there is no cross-reactivity of the VP28 polyclonal antiserum with VP26 or VP24, despite the notable degree of amino acid homology.

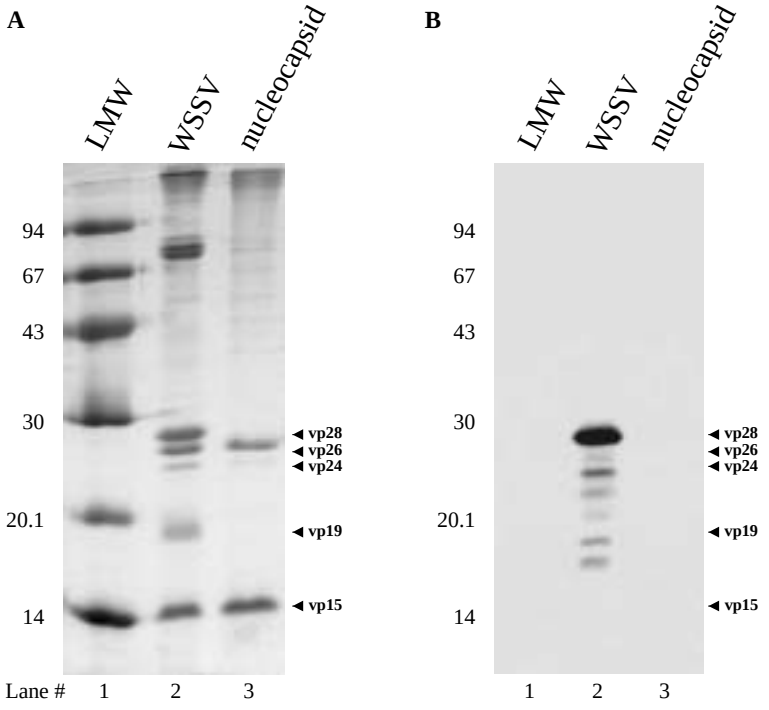

Figure 3.2. (A) 15% Coomassie Brilliant Blue-stained SDS PAGE gel of purified WSSV. Lane 1: Low molecular weight protein marker. Lane 2: purified WSSV virions. Lane 3: purified WSSV nucleocapsids. (B) Western blot of the virions (lane 2), and nucleocapsid (lane 3) of A. VP28 polyclonal antiserum is used and detection is performed with the ECL kit.

WSSV neutralization *in vivo*

The VP28 polyclonal antiserum was used in an *in vivo* neutralization assay in *P. monodon*. A constant amount of WSSV was incubated with various antiserum concentrations (Table 3.1) and injected into shrimps. No shrimp died in the negative control injected only with 330 mM NaCl (group 6). The shrimp in the positive control, which were injected with WSSV only (group 1), showed a 100% mortality at day 23 (Fig. 3.3). Addition of the pre-immune serum (group 2) resulted in a small initial delay in shrimp mortality, which reached 100% at day 25 (Fig. 3.3). When the virus was pre-incubated with a 10-fold dilution of the VP28 antiserum (group 3), shrimp mortality was 100% at day 22. Apparently, the VP28 antiserum at this dilution is not able to neutralize the virus. When the VP28 serum is diluted only 2 times (group 4) or used undiluted (group 5) none of the shrimps died, indicating that WSSV can be neutralized by the VP28 antibodies in a dose dependent manner (Fig. 3.3).

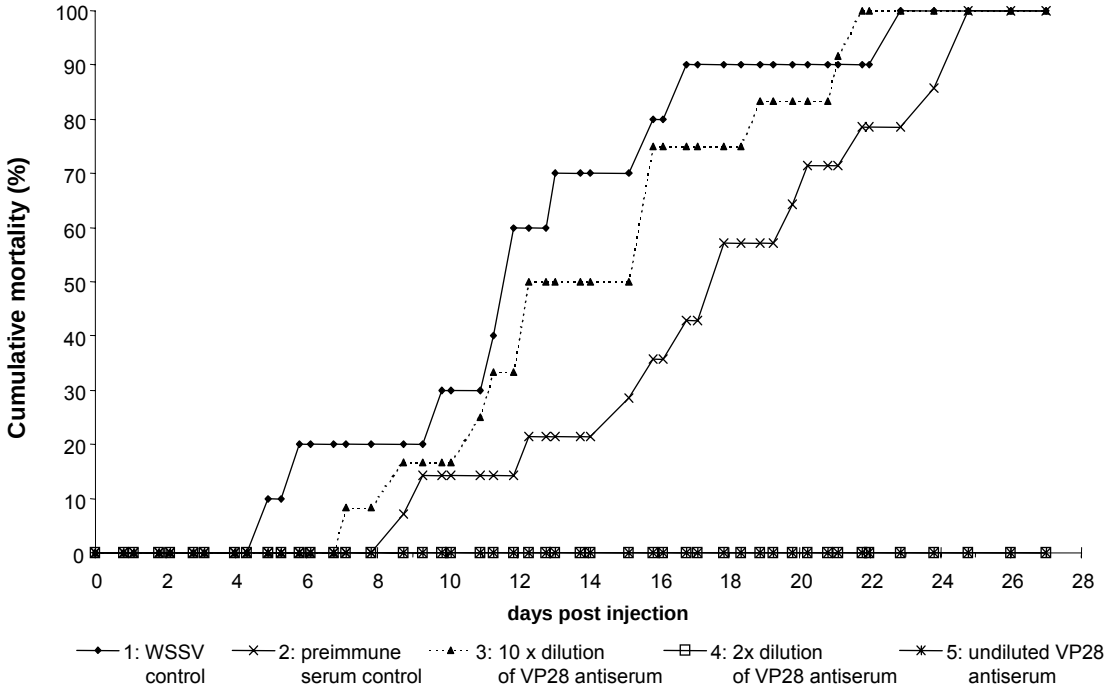

Figure 3.3. Neutralization of WSSV infection in *P. monodon* using VP28 polyclonal antiserum. The days post injection are shown on the abcis and % mortality on the ordinate. The treatments of the five groups used are described in table 3.1.

Discussion

To study the role of the major WSSV envelope protein VP28 in WSSV infection, a specific antiserum was produced against this protein. As there is a significant degree of homology between the envelope protein VP28 and the nucleocapsid proteins VP26 (41% amino acid similarity) and VP24 (46% amino acid similarity) (Van Hulten *et al*., 2000c), a Western analysis (Fig. 2) was performed, confirming the specificity of the VP28 polyclonal antiserum for VP28.

A WSSV virus stock was produced from infected *Orconectes limosus*. Since an absolute measure of virus activity in infectious units cannot be given, we have determined the highest virus dilution of the virus stock that results in almost 100% mortality upon injection of a fixed volume (10 μl) into shrimp. Shrimp were used for the titration of the virus stock, as no reliable cell culture system is available to measure WSSV infection and the effect of neutralization. Since the 1 x 10^8 dilution of the virus stock was the lowest dose still resulting in almost 100% final mortality (Fig. 3.1), this dilution was used for neutralization experiments. In the latter experiments (Fig. 3.3) the dilution of 1 x 10^8 resulted in a somewhat quicker mortality (Fig. 3.3, group 1: WSSV) than in the titration experiment (Fig. 3.1). This difference in response might be the consequence of the use of a different batch of shrimp. However, this does not influence the results of the neutralization experiment, as a control (no antiserum) was included.

To study the role of VP28 in WSSV infection in shrimps, an *in vivo* neutralization test was performed. This test showed that WSSV infection was neutralized by the VP28 polyclonal antiserum (Fig. 3.3) and that VP28 is involved in this process. The pre-immune serum control resulted in a small delay of shrimp mortality. This could be due to compounds present in the serum stimulating the shrimp defense system. WSSV neutralization using the VP28 polyclonal antiserum was concentration dependent; only the two highest antibody concentrations used in this study resulted in neutralization (Fig. 3.3).

VP28 is the major protein in the WSSV envelope, but its location in this structure is not known. The neutralizing activity of the VP28 antiserum shown here might depend on the relative abundance of this protein on the virion envelope. However, mere binding of the antibody to the surface of the virus does not automatically result in virus neutralization. The existence of non-neutralizing antibodies, which bind to virus without diminishing infectivity, has long been recognized (Dimmock, 1984). In other virus systems only anti-envelope antibodies binding to the envelope spike on the virion will be neutralizing or show antiviral activity (Burton *et al*., 2000). Therefore we postulate that VP28 or its neutralization domain is located in the envelope spike of WSSV virions.

Further research is required to reveal the exact role of VP28 in WSSV infection. Neutralization of viral infectivity by antibodies is a complex and, as yet, poorly understood phenomenon. Studies on the functional domains of proteins suggest that neutralization sites and virus attachment sites are often distinct (Ramsey *et al*., 1998). Neutralizing antibodies often inhibit a subsequent stage of infection, which is then responsible for the loss of infectivity. There are only a few examples of residues within neutralization epitopes that are also involved in the attachment of the virus to its cellular receptor (Sunyach *et al*., 1999). For VP28 a similar situation could exist as for the major envelope protein of the budded viruses of AcMNPV, where the virus can be neutralized using antibodies to GP64 (Volkman *et al*., 1984). Further studies with AcMNPV showed that the mechanism of neutralization is not by inhibition of adsorption, but by inhibition of the fusion of the viral envelope with the cell membrane (Volkman and Goldsmith, 1985). However, alternative mechanisms of neutralization are possible. Inhibition can also take place during uncoating of virus or transport of DNA in the nucleus. Furthermore, binding of antibodies can induce conformational changes in virus proteins and these may be relevant for the neutralization process.

The *in vivo* neutralization experiments on WSSV in *P. monodon* with VP28 antibodies suggest that VP28 is located in the 'spikes' of the WSSV envelope and this protein may thus be involved in the systemic infection of WSSV in shrimps. It cannot be excluded that other WSSV envelope proteins, such as VP19, are also involved in this process, either alone or in concert with VP28. Antibodies against VP19 will assist in the elucidation of this point. Future experiments using *in vivo* neutralization will demonstrate which part of VP28 is involved in the neutralization process and what the role of VP28 in WSSV attachment and entry in the systemic infection is.

Acknowledgement

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chapter 4

RE-ASSESSMENT OF THE NEUTRALIZATION OF WSSV INFECTION IN SHRIMP USING MAMMALIAN SERA

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Abstract

White Spot Syndrome Virus (WSSV) is a pathogen of crustaceans, causing severe disease and mass mortalities in shrimp farming operations and potentially threatening wild crustacean populations. A number of proteins present in the envelope of WSSV, including the 22 kDa protein VP28, have been proposed to be involved in the systemic infection of WSSV based on successful neutralization studies using VP28-specific rabbit antisera. In the present study, a series of monoclonal and polyclonal antibodies raised against VP28 were tested for their ability to neutralize WSSV infectivity, with the purpose of identifying epitopes potentially involved in the infection of shrimp. Surprisingly, none of the antibodies tested was capable of inhibiting the infectivity of WSSV when used as protein A-purified immunoglobulin. In contrast, incubation of WSSV with some whole rabbit antisera resulted in strong inactivation of the virus. Serum-mediated inactivation thus can occur independently of anti-VP28 antibodies as pre-immune serum from immunized rabbits and serum form rabbits that had not been immunized with VP28 were capable of neutralizing WSSV. These results underscore the problems of using undefined reagents (e.g. whole rabbit antiserum) in viral neutralization experiments aimed at defining proteins involved in infection by WSSV, and question previous conclusions about the potential of anti-VP28 antibodies to specifically neutralize WSSV.

Introduction

White Spot Syndrome Virus (WSSV) is the causal agent of severe disease in a wide variety of crustacean species, and thus a threat to sustainable aquaculture and potentially to the marine environment. Analysis of the WSSV genome revealed it to be a novel virus and only member of the family *Nimaviridae* (van Hulten *et al.,* 2001c; Yang *et al.,* 2001; Vlak *et al.,* 2005) and offers unique opportunities to study fundamental aspects of viral pathogenicity in invertebrates, as well as the evolution of virus-host interactions. These studies are challenging however, because no crustacean cell line capable of replicating WSSV is available, and because functions and activities cannot be predicted for most of the WSSV genes based on sequence homology. Much of the molecular biology of WSSV has thus focused on structural aspects of the viral particle, leading to the characterization of some of the major protein components of the virion (van Hulten *et al.,* 2000a, van Hulten *et al.,* 2002; Chen *et al.,* 2002; Zhang *et al*., 2002a,b, Leu *et al.*, 2005).

One of the major proteins in the envelope of WSSV is VP28, a protein with a theoretical size of 22 kDa with no significant similarity with known proteins, except for two other structural proteins of WSSV (van Hulten *et al*., 2000c). It dissociates from WSSV nucleocapsids upon treatment with non-ionic detergents (van Hulten *et al*., 2000a), suggesting localization to the lipid envelope. Consistent with this idea, ultrastructural immunolocalisation of VP28 shows association with the outer surface of intact WSSV virions (Zhang *et al*., 2002b). A putative transmembrane domain in the amino-terminal portion of VP28 suggests it may anchor to the envelope as an integral membrane protein. These

observations are compatible with a proposed role for VP28 in the interactions between WSSV and the crustacean host. Such a role is suggested by the report that both intramuscular injection and oral exposure to VP28 can induce immunity to WSSV infection (Witteveldt *et al*., 2004a,b), and the observation that incubation of WSSV preparations with rabbit polyclonal anti-VP28 serum attenuates mortality of live *Penaeus monodon* (van Hulten *et al*., 2001b). In the latter study, pre-immune serum from the rabbit immunized with VP28 showed no significant neutralizing activity, suggesting that anti-VP28 antibodies were responsible for inhibition of infectivity.

The work reported here however, shows that serum from some non-immunized rabbits can inhibit WSSV non-specifically, and that antibodies isolated from whole serum against VP28 lack neutralizing activity. These observations thus question the presence of antibodies recognizing neutralizing epitopes in VP28, and urge caution when interpreting results derived from experiments in which whole serum is used in neutralization studies.

Materials and Methods

Generation of antibodies against recombinant VP28

Two polyclonal antibodies raised in rabbits and two monoclonal antibodies against VP28 were used in this study. One of the polyclonal antibodies has already been described (van Hulten *et al*., 2001b (W-αVP28)), and one was newly raised for this study as described below (C-αVP28).

The coding region of WSSV VP28 was cloned by PCR amplification using the forward primer 5'-CGGGATCCATTGAAGGCCGCGCCATGGATCTTTCTTTCACTCT-3' and reverse primer 5'-CGGAGCTCTTACTCGGTCTCAGTGCCAGA-3'. After sequence confirmation, the product was cloned in a bacterial expression vector (pQE30, Qiagen), and recombinant VP28 (rVP28) bearing an amino-terminal 6XHis tag was purified under denaturing conditions by metal affinity chromatography (Probond, Invitrogen). Briefly, bacterial cells were lysed by freeze/thawing and lysozyme treatment, and insoluble materials were recovered and washed by centrifugation. Solubilisation and chromatography were performed in 6 M Guanidine-HCl, with final elution at pH 4.5. Purified rVP28 was dialyzed twice against 20 mM HEPES, pH 7.4, and the recovered precipitate was re-solubilized in 6M Guanidine-HCl, pH 8.0 and dialyzed again against 20 mM HEPES, pH 7.4. The identity of the purified recombinant product was confirmed by measuring its molecular weight using matrix assisted laser desorption ionisation time of flight (MALDI-TOF). Two rabbits were immunized using 100 μg of purified rVP28, and boosted at 2, 3, and 7 weeks with similar doses (Cocalico Biologicals). Pre-immune serum was collected before immunization and used as control in neutralization experiments.

For monoclonal antibodies, mice were immunized with rVP28, fusion and hybridoma selection was performed using standard methods at the antibody production facility of the Medical University of South Carolina.

To obtain both polyclonal and monoclonal purified immunoglobulins, whole serum or hybridoma supernatant was used in protein-A affinity chromatography (Sigma-Aldrich) using

standard methods, with a final buffer exchange and concentration by ultrafiltration (Vivaspin). The total immunoglobulin was quantified spectrophotometrically, and reactivity towards recombinant VP28 was confirmed by ELISA.

Immunoblotting

For western blots, proteins were resolved by reducing SDS-PAGE and transferred to PVDF (Biorad) using standard methods. After blocking in 4% milk and binding of primary antibodies, membranes were washed extensively and incubated with anti-species specific IgG conjugated to horseradish peroxidase (Biorad). Development was by enhanced chemiluminescence (Amersham) and autoradiography. For dot blots, samples were deposited directly on nylon membranes (Nytran, Schleicher and Schwell) and subsequently treated as for western blots. For detection of VP28 in WSSV-infected material, ca. 3 mL of haemolymph was centrifuged at 76,000 Xg for 40 min. The pelleted virus was re-suspended in 100 µl of phosphate-buffered saline (PBS), and 0.5 µl were used for detection by western blot or dot blot.

Enzyme-linked immunoassays (ELISA)

Purified recombinant VP28 (Witteveldt *et al*., 2004b) or purified WSSV (van Hulten *et al.,* 2001b) were coated onto ELISA plates (diluted in coating buffer: 0.15M Na_2CO_3, 0.35 M $NaHCO_3$, pH 9.6) and incubated O/N at 4°C. After blocking for one hour at RT (100 µl blockbuffer: 2% Tween-20, 1% BSA in PBS), 100 µl of a serum/antibody dilution was added to the wells and incubated for one hour at 37°C. Consequently, the plates were filled with 100 µl HRP-conjugated swine anti-rabbit for the rabbit sera or HRP-conjugated rabbit anti-mouse for the monoclonal antibodies (Dako, 1000x diluted in 0.1% BSA, 0.05% Tween-20 in PBS) and incubated for one hour at 37°C. For the measurement of absorption, 100 µl chromogenic substrate (TMB, Fermentas) was added and read at 630 nm using a photospectrometer.

Neutralization

The WSSV neutralization experiments were performed at two different locations in two different systems. Because the use of WSSV and antibodies in neutralization assays was already shown previously (van Hulten *et al*., 2001b) both the newly raised polyclonal antibody (C-αVP28) as well as the monoclonal antibodies were tested in Wageningen using this setup. As controls, the polyclonal antibody that already showed its neutralizing ability (W-αVP28), the pre-immune serum of C-αVP28 and both a positive (PBS with challenge) and negative control were included (PBS without challenge). The experiments were performed in *P. monodon* with a high serum/antibody ratio (1:9).

In Charleston, the same antibodies were tested in a similar setup using another shrimp species, *L. vannamei,* and a different WSSV isolate (Prior *et al*., 2003). The *L. vannamei* (1-2 g) were kept individually in 260 ml tissue culture flasks with artificial seawater (Marine Environment) and fed approximately half a pellet of commercial feed every day, with

100% water exchange daily. Alternatively, shrimp were kept in groups of 10-17 in 10-15 L tanks connected to a recirculation system with mechanical and biological filtration, as well as UV-sterilization. The haemolymph inoculum was generated by injecting SPF shrimp with head homogenate, and then collecting haemolymph from moribund shrimp (1 part haemolymph with 3 parts of PBS, pH 7.4). The haemolymph was then filtered through 0.45 μm, and its infectivity determined by infection of test animals with serial dilutions (Prior *et al*., 2003). For neutralization, shrimp were acclimated for 2-3 days (34-40 animals per treatment), and injected with 20 μl of WSSV inoculum (positive controls), SPF shrimp extracts (negative controls), or WSSV inoculum mixed with antibodies as indicated. Incubation of WSSV inoculum with antibodies before injection was for 1.5 h at room temperature. Mortality was recorded daily. Commercial rabbit serum with high complement activity was from Sigma-Aldrich (cat# S-7764). Sera from non-immunized rabbits (see figure 3b) were from Sigma-Genosys (serum A) and Cocalico Biologicals (serum B).

Results

Characterization of anti-VP28 antibodies

The specificity of the C-αVP28 antibodies raised for this study was tested by a series of methods including western blot and dot blot (Figure 4.1) which show that the polyclonal and monoclonal antibodies used here specifically recognize both recombinant VP28 and VP28 associated with the virus in tissues of infected shrimp. Similar results were already obtained for W-αVP28 (Van Hulten *et al.,* 2001b).

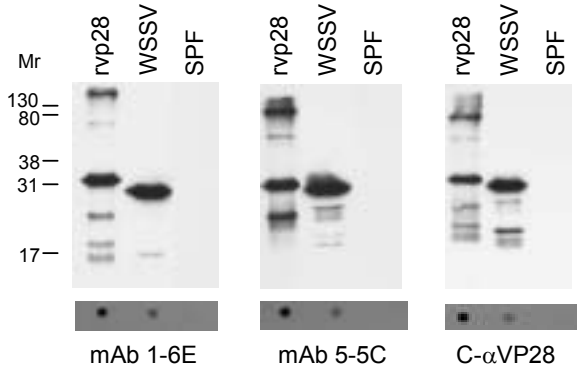

Figure 4.1. Western- and dot blot of purified immunoglobulins from the rabbit polyclonal antiserum C-αVP28 as well as monoclonal antibodies on recombinant VP28 and purified WSSV. The mobility of molecular weight markers (Mr) is indicated to the left.

Using the ELISA technique with dilution series of the different antibodies, the variation in affinity to both purified VP28 and WSSV was determined. When using VP28 as antigen, there is little difference between both polyclonal sera (W-αVP28 and C-αVP28). The pre-immune sera show a low level of background (figure 4.2a). When the same antibodies are used on purified WSSV, the polyclonal antisera again show comparable signals, but the pre-immune sera differ considerably. The signal of the C-pre-immune serum is more than twice as high as the W-pre-immune serum in the lowest two dilutions (figure 4.2b).
In addition, the monoclonal antibodies were used in the ELISA assay and show that both have approximately the same reactivity towards both purified VP28 and WSSV (figure 4.2c).

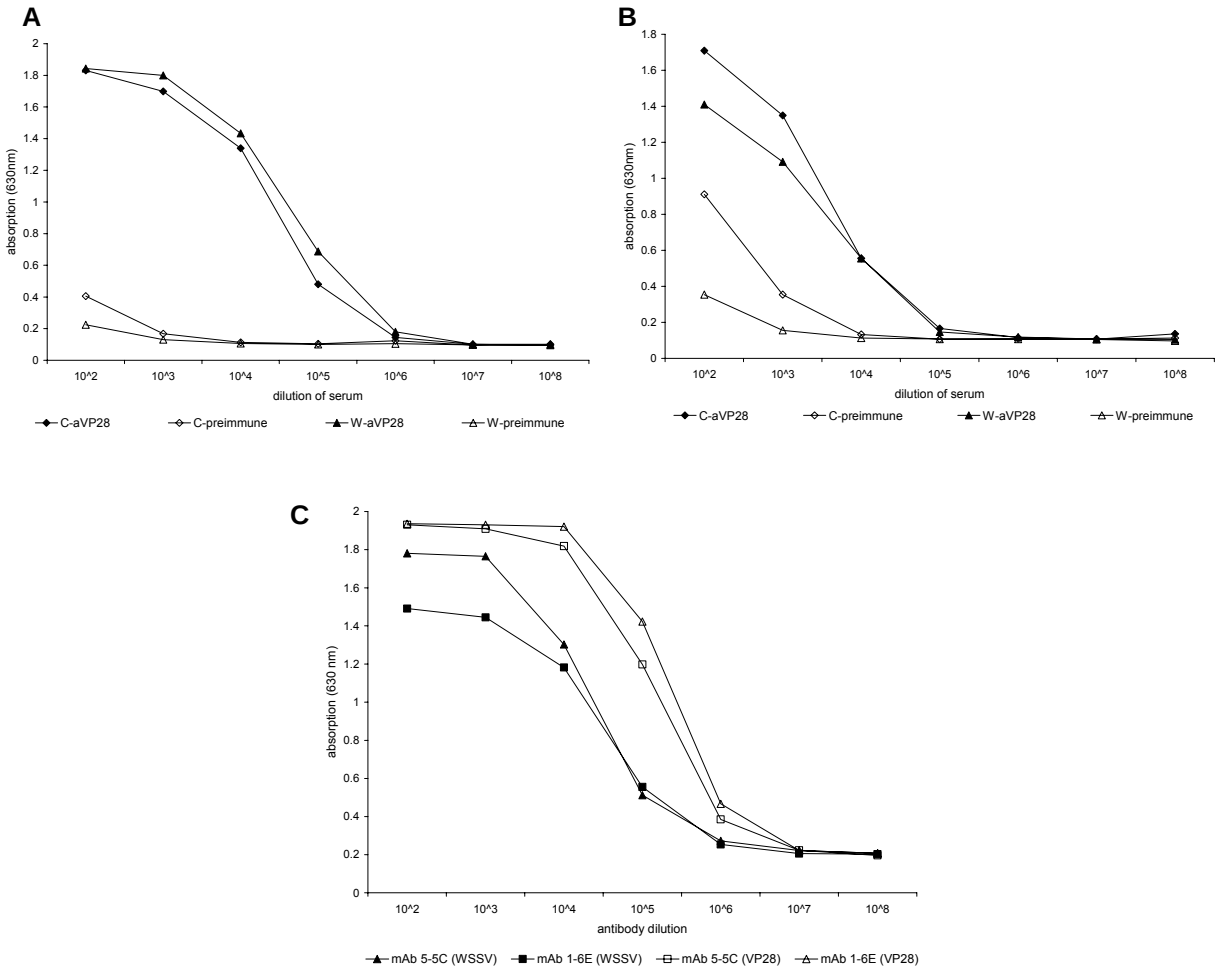

Figure 4.2 ELISA assays using dilution series of different sera and monoclonal antibodies on both purified VP28 and WSSV. A) Shows the signal found using the polyclonal sera W-αVP28, C-αVP28 and both pre-immune sera on purified VP28. B) Shows the signal found using the latter sera of purified WSSV. C) Signals observed using the two monoclonal antibodies (5-5C and 1-6E) on both purified VP28 and WSSV.

Polyclonal and monoclonal antibodies against VP28 lack WSSV neutralizing activity

As the use of WSSV and antibodies in a neutralization assay was already shown previously (van Hulten *et al*., 2001b, Chapter 3) both the newly raised polyclonal antibody (C-αVP28) as well as the monoclonal antibodies were tested using this setup. Conform the previous research, adding W-αVP28 serum to WSSV resulted in a considerable reduction in mortality compared to the positive control, the same was observed for the newly raised serum, C-αVP28. Both monoclonal antisera appeared to confer no level of neutralization at all. More surprisingly however, also the C-pre-immune serum appeared to result in a much lower mortality, even to the same level of neutralization as both α-VP28 sera (figure 4.3a).

For further confirmation of these results, the same antibodies, including the W-pre-immune serum, were tested in Charleston using a different WSSV isolate and *L. vannamei* instead of *P. monodon*. For this experiment viral inoculum from homogenized shrimp heads was diluted 3 x 10^{-7} (weight:volume dilutions) and mixed with protein A-purified antibodies

(polyclonal or monoclonal) to a final antibody concentration of 10 µg/mL (to achieve high antibody:VP28 ratios) (van Hulten *et al*., 2001b; Prior *et al*., 2003). The WSSV-antibody mixtures were injected into shrimp after 1.5 h incubation at room temperature. The data in figure 4.3b confirm that the W-αVP28 rabbit antiserum strongly inhibits WSSV-induced mortality of shrimp in this setup, while the corresponding pre-immune serum (W-pre-immune) lacks anti-WSSV activity. However, when using purified IgG instead of the crude serum for any of the sera, no protection is observed at all suggesting that a non-IgG component in the W-αVP28 was involved in the neutralization.

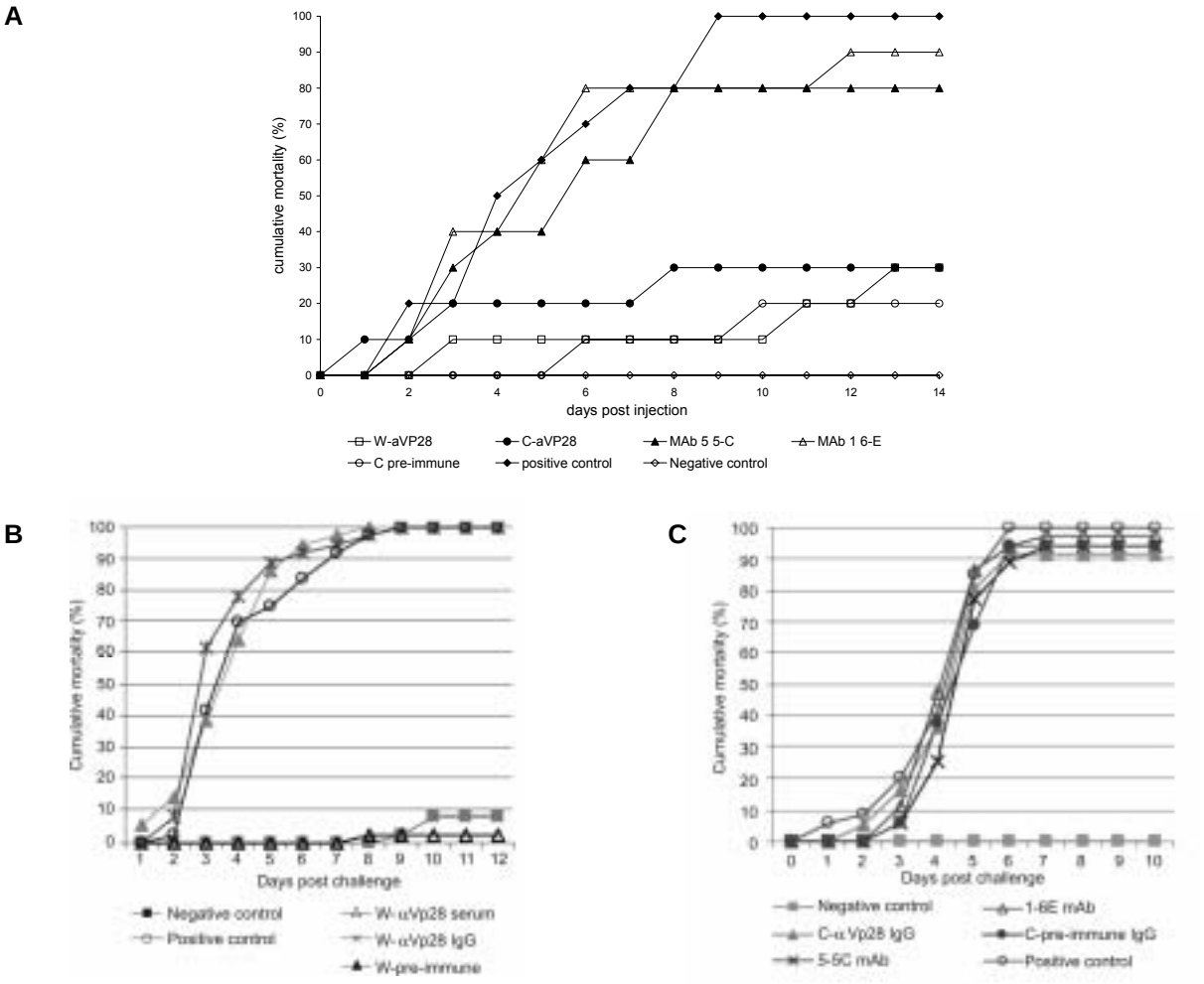

Fig. 4.3 Neutralisation assays using different antibodies. A) Neutralisation assay using *P. Monodon,* two α-VP28 sera, two α-VP28 monoclonal antibodies. As controls a pre-immune serum (C-pre-immune), positive and negative control were included. B) Neutralisation assay using *L. vannamei,* untreated W-αVP28 serum, protein A purified immunoglobulines from the latter serum and its pre-immune serum. A positive and negative control were also included. Neutralisation assay using *L. vannamei,* protein A purified immunoglobulines from C-αVP28 serum its protein-A purified pre-immune serum and two α-VP28 monoclonal antibodies. A positive and negative control were also included.

To verify whether VP28-specific IgG's are indeed unable to neutralize WSSV, protein A-purified antibodies from the newly raised VP28 antibodies (C-αVP28) and two monoclonal

antibodies were tested in a neutralization assay. As shown in figure 4.3c, none of the anti-VP28 specific antibodies raised in this study was able to neutralize the infectivity of WSSV under these conditions. Figure 4.4 shows that the immunoglobulin from both anti-VP28 antisera used in the neutralization experiments retain their reactivity towards recombinant VP28 after purification by protein-A chromatography.

It is possible that the neutralizing activity of anti-VP28 antibodies is dependent upon serum factors, and thus purified immunoglobulins lack neutralizing activity. Taken together, the data presented in figures 4.3 and 4.4 suggest that recognition of VP28 by antibodies is not sufficient for inactivation of WSSV. As the neutralization experiments, performed at different locations and systems, showed similar results, this consistency across experimental systems suggests that the lack of neutralizing capacity of anti-VP28 antibodies is probably not attributable to subtle variations in viral strains, quality of viral preparations, or host species.

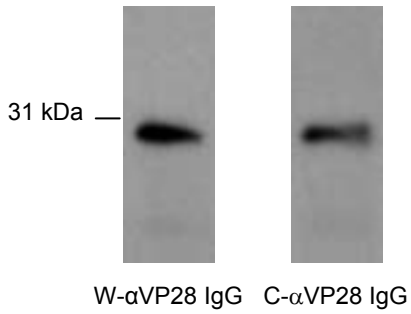

Figure 4.4. Western-blot of rVP28 probed with protein A-purified immunoglobulins from W-αVP28 and the C-αVP28 serum raised in the present study.

Rabbit serum can inhibit the infectivity of WSSV independently of anti-VP28 antibodies

The data presented above indicate that isolated anti-VP28 antibodies lack WSSV neutralizing activity. Thus, specific inactivation of WSSV by anti-VP28 antiserum could be a phenomenon dependent on serum factors as well as other anti-VP28 antibodies (e.g. complement-dependent, antibody-mediated neutralization). To test the hypothesis that anti-VP28 antibodies and serum components are both required for specific WSSV neutralization, neutralization experiments were performed in which the ability of normal rabbit serum to complement the neutralizing potential of isolated anti-VP28 immunoglobulins was tested.

The data in figure 4.5a again shows that whole rabbit serum (C-αVP28 as well as C-pre-immune) can completely abolish WSSV-induced mortality of shrimp. Consistent with the data shown in figure 4.3b, the anti-WSSV activity of C-αVP28 does not co-purify with immunoglobulins, since the isolated antibodies again failed to inactivate WSSV, even when used at a 15-fold higher concentration than before (i.e. 150 $\mu g.ml^{-1}$). Furthermore, commercial rabbit serum alone (of the type used to assay mammalian complement activity) can inactivate WSSV even when used at significantly higher dilutions than the serum from the rabbit used to produce C-αVP28 antibodies (Figure 4.5a, the serum:WSSV ratio (v:v) used for the commercial serum treatment was 0.5, while for C-αVP28 serum the ratio was 8.5).

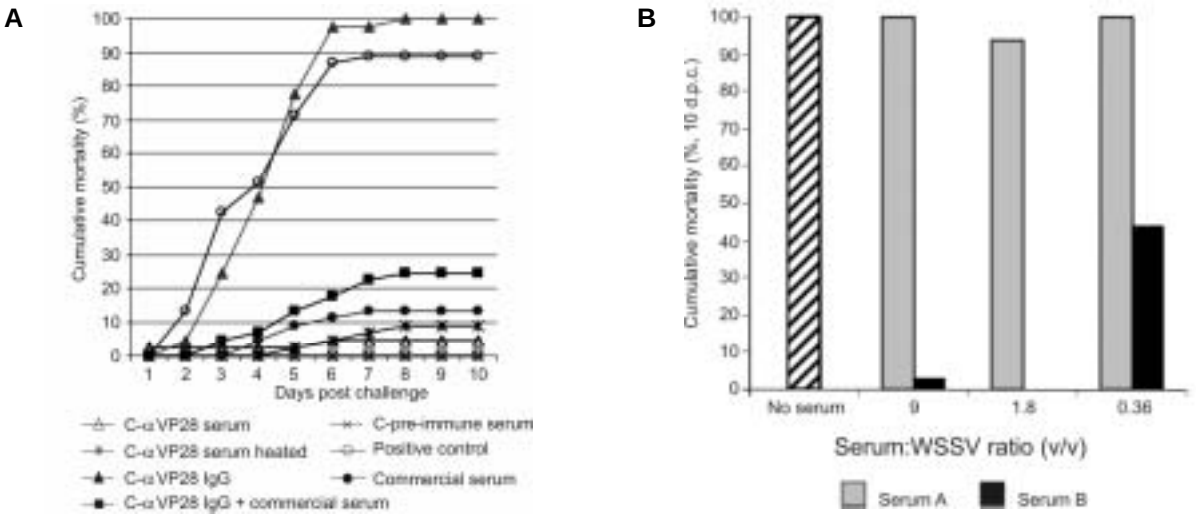

Figure 4.5. Normal rabbit serum can abolish WSSV infectivity: A) 100 µl of WSSV positive haemolymph was mixed with 850 µl of serum as indicated, and 50 µl of PBS. For the C-αVP28 IgG treatment, WSSV-positive haemolymph was mixed instead with 850 µl of PBS and 50 µl of purified antibody (3 mg/ml). For the commercial serum treatment, infective haemolymph was mixed with 850 µl of PBS and 50 µl of complement-rich rabbit serum (Sigma-Aldrich). Heated antiserum was treated at 57 °C for 45 min. After 1.5 h of incubation at room temperature 20 µl of each mixture was injected per animal (n=45). B) 900 µl of undiluted or PBS-diluted normal rabbit serum was mixed with 100 µl of WSSV-positive haemolymph as for panel a). Incubation and injections were also performed as for panel a). Two tanks (34-35 shrimp total) were used per treatment. The cumulative survival 10 days post challenge (d.p.c.) is shown when each serum was used at different concentrations.

Complement activity was probably not responsible for inactivation of WSSV by anti-VP28 serum, since treatment at 57°C for 45 min did not impair the ability of this serum to abolish WSSV infectivity. It is somewhat surprising that some but not all normal rabbit sera can inactivate WSSV (e.g. preimmune serum from W-αVP28 does not inhibit WSSV infectivity, while preimmune serum from the rabbit used to raise the C-αVP28 polyclonal antibody raised for the present study does, figures 4.3b and 4.3a respectively). This suggests a non-specific WSSV inhibitory activity that is subject to individual variability occurs in rabbit serum.

To further explore this issue, sera from two other non-immunized rabbits were used in neutralization experiments, with the results shown in figure 4.5b. One of the two sera tested strongly inactivated WSSV infectivity in a dose dependent manner, while serum from the other rabbit did not significantly inhibit WSSV-induced mortality. Although these experiments do not provide insight into the nature of the anti-WSSV activity present in rabbit serum, or into the tolerance of the virus to this activity, they clearly raise the question of whether or not whole mammalian serum can be mixed with infective WSSV (at 0.36 to 9 parts of serum for 1 part of diluted WSSV inoculum) without strongly affecting WSSV viability.

Discussion

Viral pathogens like WSSV cause severe losses to the aquaculture industry, and likely have an important (yet undetermined) impact on wild crustacean populations. Apart from the commercial and ecological relevance of studying these pathogens, fundamental studies on WSSV-crustacean interactions are likely to provide new insights into the evolution of viral pathogenesis and antiviral immunity. Characterization of some of the major structural components of the WSSV virion provides a basis for addressing some of these questions at the molecular level. Because VP28 is an abundant structural protein in the envelope of WSSV that seems to be exposed to the outer surface of the viral particle, it has been suggested to play a role in the interactions between WSSV and host cells.

A previous study had shown that rabbit serum containing antibodies against VP28 abolished WSSV-induced mortality of *P. monodon* in a dose-dependent manner, while pre-immune serum lacking these antibodies failed to produce comparable effects (van Hulten *et al*., 2001b). Based on these results, it was suggested that available VP28 is required for WSSV to infect and/or cause disease in shrimp. This conclusion is compatible with studies reporting direct binding of VP28 to shrimp cells, and by evidence that VP28 can saturate sites necessary for WSSV attachment *in vitro* (Yi *et al.,* 2004). This latter study also reports that anti-VP28 antiserum can inhibit plaque formation by WSSV in primary cell culture. While these results strongly suggest an involvement of VP28 in mediating essential interactions between the viral particle and host cells, confirmation of such a hypothesis requires that highly specific anti-VP28 reagents be used to inhibit infections by WSSV. The present study was aimed at further testing the specificity of WSSV neutralization by anti-VP28 reagents, by using a panel of monoclonal antibodies and a polyclonal antibody in the form of purified immunoglobulins, free of mammalian serum. It was demonstrated that immunoglobulins, purified from 2 anti-VP28 antisera that inactivate WSSV, lack significant neutralizing activity. These experiments also revealed that normal rabbit serum can strongly inactivate WSSV when used at doses that are comparable to those used by others to assay for specific neutralization. Taken together, these results demonstrate that the anti-VP28 antibodies tested here are not sufficient to neutralize WSSV, and that rabbit serum is capable of inactivating WSSV in a manner independent of anti-WSSV antibodies.

The discrepancies between the conclusions reached by others and those from the present study seem unlikely to be the result of the use of different host species or viral strains/preparations as similar results were obtained in two different virus/host systems at two different experimental facilities.

It remains possible that available VP28 on the surface of WSSV is required for successful infection of host cells. However, the results from the present study suggest that caution should be exercised when interpreting results of virus neutralization using undefined reagents like whole rabbit antiserum. In fact, the specificity of the WSSV neutralization observed by several investigators using anti-VP28 antibodies may prove rabbit-dependent, which is supported by the variable neutralization response to pre-immune sera (Fig. 4.5b). In two other studies, antibodies that potentially target VP28 have been used to successfully neutralize WSSV (Kim *et al.,* 2004; Li *et al*., 2005). These antibodies however were raised against fusion proteins comprising portions of VP28 and vp19, and thus it remains unknown

which (if any) of these envelope proteins may have functioned as the target for neutralization. Additionally, all these experiments were performed using crude sources of antibody (egg yolks from immunized chickens or whole serum from immunized rabbits), and thus non-specific inactivation of WSSV by unknown serum factors remains as a plausible explanation for these results. Another recent article reports on a screen for WSSV envelope proteins involved in infection using an antibody-mediated neutralization strategy (Wu *et al*., 2005). In this study, like in the present report, immunoglobulins purified from rabbit antiserum were used. Interestingly, a modest inhibition of WSSV-induced mortality by purified antibodies against VP281, VP466, and VP68 was observed in these experiments, in stark contrast to the potent inactivation of WSSV observed when whole rabbit serum is used. The same report also refers to positive inhibition of WSSV by purified anti-VP28 polyclonal antibodies. However this statement was supported only by data not shown, and thus interpreting it in the context of the present work is not possible.

Based on the results of the present study, we question the significance of inhibition of WSSV infectivity resulting from mixing large amounts of mammalian serum with infective WSSV. It seems clear that inhibition of WSSV by purified and mono-specific reagents will be necessary to better evaluate the existence of neutralizing epitopes in VP28 or any other structural protein of WSSV.

Acknowledgments

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chapter 5

VACCINATION OF *PENAEUS MONODON* BY INJECTION OF WSSV STRUCTURAL VIRION PROTEINS

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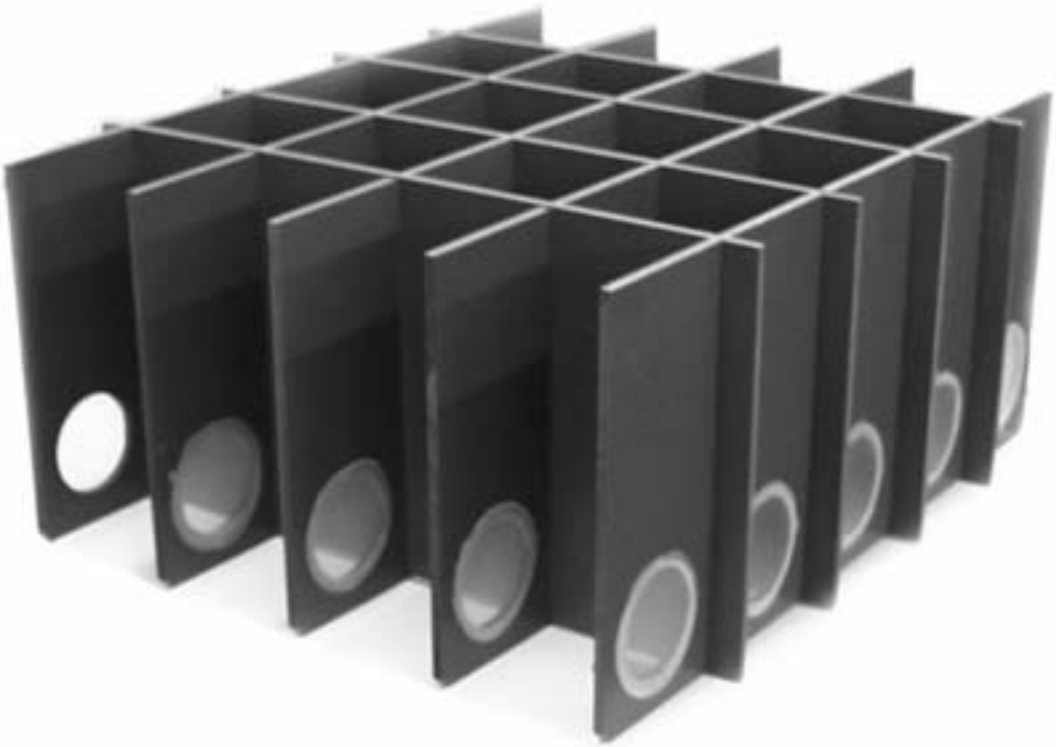

Abstract

White spot syndrome virus is currently responsible for significant health problems in shrimp culture and intervention strategies are being seriously sought. Vaccination of shrimp against this disease could be a viable option. However, as very little is known about the shrimp's immune response to viral infections, the potential of shrimp vaccination is uncertain. In this study we performed vaccinations of shrimp using two major structural WSSV proteins, VP19 and VP28, both present in the virion envelope. Recombinant HIS-VP28 and MBP-VP19 fusion proteins were purified and injected into shrimp that were subsequently challenged with WSSV by injection. Results showed that injection with MBP-VP19 or a mixture of MBP-VP19 and HIS-VP28 significantly slowed or reduced mortality caused by WSSV, suggesting a specific role of VP19 in the systemic shrimp defense response. Furthermore, these results also demonstrate that shrimp can specifically recognize proteins and provoke an immune response, opening the way for vaccination against viruses.

Introduction

Since the discovery of White spot syndrome virus (WSSV) in Asia in 1991/1992, the virus has quickly spread to most shrimp farming areas of the world (Cai *et al*., 1995). Helped by inadequate sanitation and worldwide trade, WSSV has quickly developed into an epizootic disease, causing large economic losses to the shrimp farming industry (Rosenberry, 2002). Besides the economic impact of this disease the natural marine ecology is also threatened, since WSSV is able to infect a large number of crustaceans (Lo *et al*., 1996; Wang *et al*., 1998). WSSV virions are ovoid-to-bacilliform in shape and have a tail-like appendage at one end. The virions can be found throughout the shrimp body, infecting most tissues and circulating ubiquitously in the haemolymph. The enveloped virions contain a single nucleocapsid with a distinctive striated appearance (Loh *et al*. 1997). The virion consists of five major and about thirteen minor proteins (Van Hulten *et al*., 2000a,b, 2002; Huang *et al*., 2002a,b). Two of the major structural proteins, VP28 and VP19, are located in the envelope and three in the nucleocapsid (VP26, VP24 and VP15) (Van Hulten *et al*., 2002). Sequencing of the viral genome revealed a circular sequence of about 300 kb (Van Hulten *et al*., 2001c; Yang *et al*., 2001).

Due to current aquaculture practices and the broad host range of WSSV, intervention strategies including vaccination against this virus would be pivotal to save and protect shrimp farming. However, in contrast to the well-studied effects of microbial immunostimulants on the crustacean immune system (Lee and Söderhall, 2002), information on the immune response to viral infections is limited. In the few studies performed so far, antiviral substances were found to be present in tissue extracts of crustaceans, non-specifically inhibiting different viruses (Pan *et al*., 2000). Also upon infection with WSSV, an upregulation of the lipopolysaccharide and β-1,3-glucan binding protein gene (LGBP), known to be involved in the proPO cascade and upregulation of protease inhibitors, apoptotic peptides and tumour-related proteins, have been observed (Roux *et al*., 2002; Rojtinnakorn

et al., 2002). *In vivo* experiments with *Penaeus japonicus* demonstrated the presence of a quasi-immune response when survivors of both natural and experimental WSSV infections were re-challenged with WSSV (Venegas *et al*., 2000). After re-challenge mortality of the initial survivors was lower than that of challenged naive shrimp. The prospects for shrimp vaccination of against WSSV is best supported by the research performed by Wu *et al*. (2002), who found WSSV neutralizing activity in plasma of infected shrimp from 20 days until over two months after infection.

To better understand the mechanism underlying the observed quasi-immune responses and to answer the question whether this mechanism is WSSV specific, vaccination experiments were performed in *Penaeus monodon* shrimp using specific WSSV proteins. Previous research had shown that one of the WSSV structural proteins, VP28, was involved in the systemic infection of WSSV (Van Hulten *et al*., 2001b). Since both VP28 and VP19 are associated with the virion envelope and involved in initial interaction with the host, these two structural proteins were used in the vaccination experiments. To have maximum control over the amount of proteins the shrimp are exposed to and to be able to use purified proteins, it was decided to vaccinate via injection. Although a number of potential defense and recognition lines are circumvented this way, it still proved a valuable method for testing the vaccination potential of proteins. To be assured of a constant and reproducible challenge pressure, the challenge was also performed by injection, even though this method also circumvents a number of defense or recognition lines.

Materials and Methods

Shrimp culture

Healthy *P. monodon* were imported as post-larvae from Malaysia and maintained in a recirculation system at the aquaculture facility “De Haar” at Wageningen University. Each shipment was tested for the presence of WSSV, monodon baculovirus, yellow head virus, taura syndrome and infectious hypodermal and hematopoietic necrosis virus by PCR. Prior to each experiment, shrimp were transferred to an experimental system located at the Laboratory of Virology at Wageningen University and stocked in 180-liter aquariums. Each aquarium was fitted with an individual filter system (Eheim, Germany) containing pre-conditioned filter material, heating (Schego, Germany) to 28°C and continuous aeration. All experiments were performed in artificial seawater (Instant Ocean, Aquarium Systems) at a salinity of approximately 20 parts per thousand (ppt).

WSSV virus stock

The virus isolate used in this study originated from an infected *P. monodon* shrimp imported from Thailand in 1996 and was obtained as described before (Van Hulten *et al*., 2001b). Crayfish *Orconectes limosus* was injected intramuscularly with a lethal dose of WSSV using a 26-gauge needle (Microfine B&D). After approximately one week, virus was isolated from freshly extracted haemolymph as described by Van Hulten *et al*. (2001b). Virus samples

were examined under the transmission electron microscope for integrity and purity, and stored in aliquots at -80°C until further use.

In vivo titration

Since no crustacean cell lines are available, the WSSV stock was titered by *in vivo* infection experiments as described by Van Hulten *et al*. (2001b). In short, shrimp of approximately one gram were injected intramuscularly with 10 µl of various virus dilutions in 330 mM NaCl (10^3-10^8 times diluted) in the 4th or 5th abdominal segment of the shrimp using a 29 gauge needle (Microfine B&D). After injection, the shrimp were maintained in individual housing to prevent horizontal transmission of WSSV by predation. Mortality was recorded twice a day and dead shrimp were tested for the presence of WSSV by PCR. The obtained time-mortality data were used to determine the desired challenge pressure of 70-90% final mortality for the vaccination experiments.

PCR analysis for WSSV

Muscle tissue retrieved from the tail of dead and surviving shrimp was homogenized and mixed with 200 µl 5% Chelex 100 resin (BioRad) and 16 µl 20 mg/ml proteinase-K. This mixture was incubated overnight at 56°C followed by 10 minutes at 95°C to inactivate the proteinase-K. The samples were tested with two primer pairs. A 16S rRNA primer pair (16S-FW1 5'-GTG CGA AGG TAG CAT AAT C-3'; 16S-RV1 5'-CTG CTG CAA CAT AAG GAT AC-3'), amplifying a 414 bp fragment of ribosomal shrimp DNA was used as a control to verify DNA integrity. A VP26 primer pair (VP26-FW1 5'- ATG GAA TTT GGC AAC CTA ACA AAC CTG-3'; VP26-RV1 5'- GGG CTG TGA CGG TAG AGA TGA C-3') amplifying part of the WSSV vp26 gene (Van Hulten *et al*., 2000a), was used to screen for WSSV positive shrimp.

Expression of recombinant proteins

For bacterial expression of VP19, the entire VP19 ORF was cloned in the pMAL-c2 vector (New England Biolabs) resulting in an N-terminal fusion of VP19 and the maltose binding protein (MBP). The DNA fragment encoding the entire VP19 ORF (WSSV ORF182, Van Hulten *et al*., 2001c) was amplified from genomic WSSV DNA by PCR. Using the forward primer VP19-FW1 (3'-CGG GAT CCA TGG CCA CCA CGA CTA A-5') and reverse primer VP19-RpMAL (3'-GCC TGC AGC CTG ATG TTG TGT TTC TAT A-5') *Bam*HI and *Pst*I restriction sites respectively were introduced. The amplified PCR product was ligated into the pGEM-T easy vector (Promega) and sequenced. The VP19 fragment was removed from the pGEM-T easy plasmid and ligated into the pMAL-c2 vector and electroporated into *E. coli* DH5α cells for protein expression.

The full length ORF encoding the major WSSV envelope protein VP28 (WSSV ORF1, Van Hulten *et al*., 2001c) was first expressed using the pET28a vector which fuses a (HIS)6-tag to VP28 for detection and purification purposes. Expression levels were very low,

probably due to the presence of a strong N-terminal hydrophobic region. Therefore, a new construct without the N-terminal hydrophobic region, was constructed and used for expression. The partial VP28 fragment was amplified from genomic WSSV DNA by PCR using the forward primer VP28PF (3'-AAG GAT CCC ACA ACA CTG TGA CCA AG-5') and reverse primer VP28PR (3'-TAG CGG CCG CAA AAG CAC GAT TTA TTT AC-5') which introduced *Bam*HI and *Not*I restriction sites respectively. This fragment was ligated into the pGEM-T vector and sequenced. After digestion with *Bam*HI and *Not*I, the fragment was ligated at the *Bam*HI and *Not*I site of the pET28a vector (Novagen). The pET28a-VP28 construct was electroporated into BL21 electrocompetent cells for protein production.

Protein production and purification

The MBP-VP19 fusion protein was purified by affinity chromatography using amylose resin (New England Biolab) and the HIS-VP28 fusion protein using TALON metal affinity resin (CLONTECH). The resulting *E. coli* expressions and the purified proteins were analysed by SDS-PAGE and Western-blot. Protein concentration was determined using the Bradford protein assay (Bio-Rad).

Vaccination experiments

For the vaccination experiments, shrimp of approximately one gram were injected intramuscularly in the 4th or 5th abdominal segment with 4 µg of purified protein diluted in 330 mM NaCl in a final volume of 10 µl. The shrimp of the group vaccinated with a mixture of the two proteins received 2 µg of MBP-VP19 and 2 µg of HIS-VP28 proteins. Five days after the initial vaccination, the shrimp were boostered by injecting the same amount of protein. The positive and negative control groups were injected with 10 µl of 330 mM NaCl in the same regime. Two days after the booster the shrimp were challenged by injection of a WSSV dilution aimed at 70-90% mortality in experiment I and 100% mortality in experiment II, except for the negative control shrimp that received 10 µl of 330 mM NaCl. After the challenge, the shrimp were maintained in individual housing to prevent horizontal transmission of WSSV. In experiment I, four experimental groups of 15 individuals each (Table 5.1) were injected with VP19, VP28 or PBS for the positive and negative controls. In experiment II, the same groups as experiment I, but with the addition of a MIX group, injected with both VP19 and VP28 were used. After the challenge of both experiments, the shrimp were placed in individual cages in 180 liter aquaria with heating to 28°C and continuous aeration.

Statistical analysis

Statistical analysis on the mortality curves was performed using the Kaplan-Meier survival analysis (Bull and Spiegelhalter, 1997). After analysis, the significance, hazard ratio and 95% confidence interval were obtained.

Table 5.1. Group names, types of vaccine administered and number of shrimp used in experiments I and II.

	Group name	Vaccine	Number
Experiment I	MBP-VP19	MBP-VP19	15
	HIS-VP28	HIS-VP28	15
	Positive control	-	15
	Negative control	-	15
Experiment II	MBP-VP19	MBP-VP19	15
	HIS-VP28	HIS-VP28	15
	MIX	MBP-VP19+HIS-VP28	15
	Positive control	-	15
	Negative control	-	15

Results

Protein production and purification

WSSV VP19 and VP28 ORFs were overexpressed in E. coli as MBP and (HIS)6-tag fusion proteins, respectively. Bands corresponding to the two fusion proteins were observed at the expected positions (Figure 5.1, lanes 1 and 3). The viral origin of the bands was confirmed by Western blot analysis using an anti-WSSV polyclonal antiserum. After sonication and centrifugation the supernatant of both expressions was used for purification using affinity chromatography. MBP-VP19 was purified using amylose resin and yielded a highly enriched preparation of VP19-MBP fusion protein. Purification of HIS-VP28 using TALON metal affinity resin also resulted in a highly enriched preparation of purified HIS-VP28. (Figure 5.1, lanes 2 and 4). The final concentration of the purified proteins was determined using the Bradford assay.

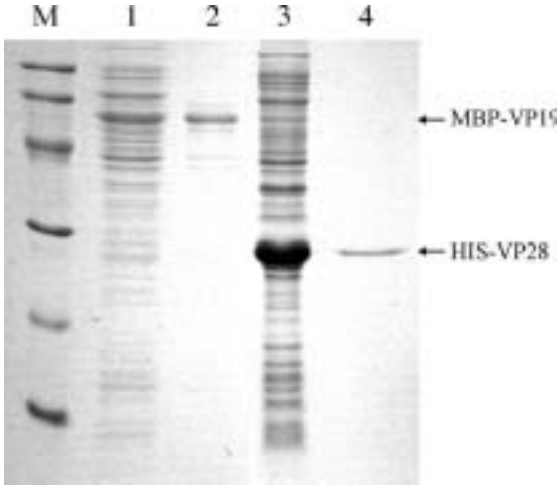

Figure 5.1. Coomassie stained SDS-PAGE gel of MBP-VP19 and HIS-VP28 expressions in *E. coli* cells. M: Low molecular weight marker; Lane 1: total MBP-VP19 expression; Lane 2: purified MBP-VP19; Lane 3: total HIS-VP28 expression; Lane 4: purified HIS-VP28. Numbers on left side indicate the size (in kDa.) of low molecular weight protein markers.

Vaccination with VP19 and VP28 (experiment I)

Shrimp vaccinated with purified MBP-VP19 protein showed a significantly slower mortality and a reduced final mortality when compared to the positive control ($p<0,05$) (Figure 5.2). By contrast, vaccination with HIS-VP28 gave no significant difference in mortality when compared with the positive control. The final mortality in the positive control reached about 60%, which was slightly below the percent anticipated from the dilution used. Random samples of surviving shrimp were tested for WSSV by PCR and found negative.

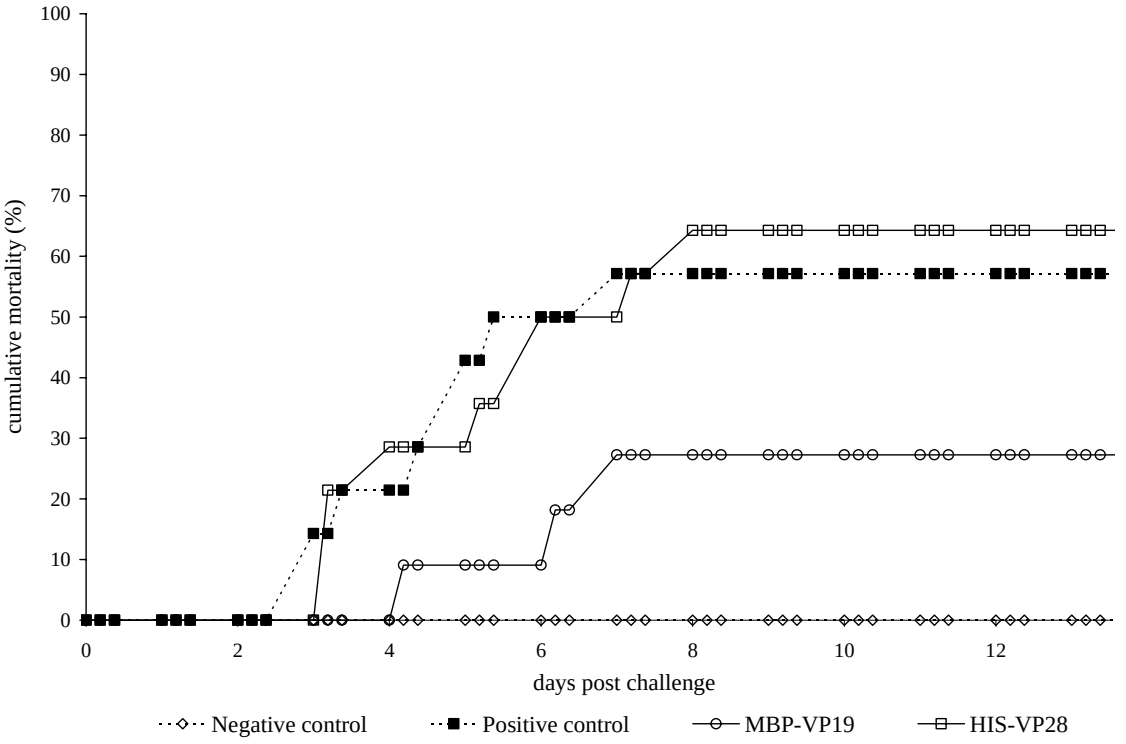

Figure 5.2. Time-mortality relationship of vaccination experiment I. Cumulative mortality rates of shrimp vaccinated with different proteins and challenged by injection of WSSV are plotted against days after challenge. Positive and negative controls are injected with NaCl prior to challenge with WSSV or NaCl, respectively, to exclude injection effects.

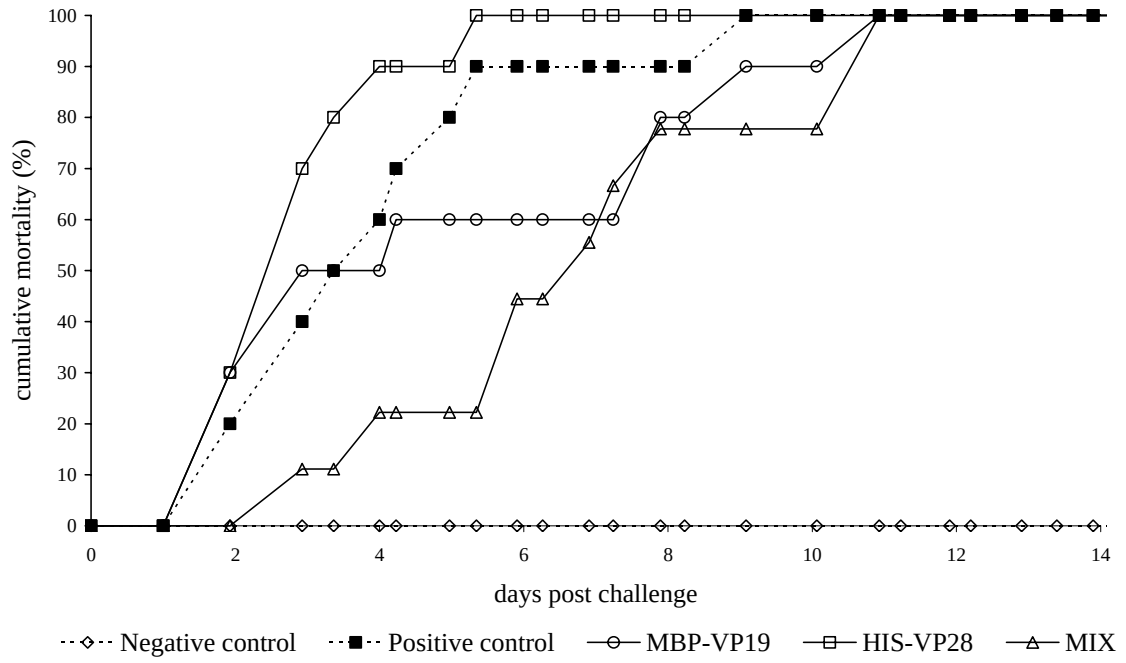

Figure 5.3. Time-mortality relationship of vaccination experiment II. Cumulative mortality rates of shrimp vaccinated with different proteins and challenged by injection of WSSV are plotted against days after challenge. Positive and negative controls are injected with NaCl prior to challenge with WSSV or NaCl, respectively, to exclude injection effects.

Vaccination with VP19, VP28 and a mixture of VP19 and VP28 (experiment II)

The increased challenge pressure in this experiment, gave 100% mortality in 5 days for the positive control group (Figure 5.3). All experimental groups, except the negative control group reached 100% mortality after 10 days. There was no significant difference between the positive control group and the groups injected with either MBP-VP19 ($p=0.16$) or HIS-VP28 alone even though the rate of mortality for MBP-VP19 appeared reduced. However, when both MBP-VP19 and HIS-VP28 are administered, a significant delay in mortality was observed when compared to the positive control ($p<0.02$). There was no significant difference between this group and the one treated with MBP-VP19 alone ($p=0.44$). Random samples of surviving shrimp were tested for WSSV by PCR and found negative.

Discussion

Invertebrates constitute ninety-five percent of all animal species and rely on defense mechanisms primarily based on a broad range of cellular innate immune responses. Because of the lack of a known adaptive immune response, the potential for vaccination against viral pathogens is uncertain. However, a few reports suggested the presence of such a response in crustaceans (Venegas *et al*., 2000; Wu *et al*., 2002) and this has opened up the possibility of vaccination as an intervention strategy to combat viral diseases in shrimp.

Since vaccination with MB-VP19 and in particular a mixture of MBP-VP19 and HIS-VP28 were able to induce a delay or decrease in mortality upon WSSV challenge, . it appears that MBP-VP19 is more important in the shrimp systemic immune response than VP28. It was somewhat surprising that VP28 on its own did not have an effect in view of earlier neutralization experiments (Van Hulten *et al*., 2001b). It is possible that the HIS-VP28 construct is less stable than the MBP-VP19 construct or that MBP on its own may have a protective effect, however, preliminary experiments suggest the latter is not the case (J. Witteveldt, personal communication). Another explanation for its lack of effect may be the removal of its hydrophobic domain during the fusion protein construction. It is interesting that HIS-VP28 did not provoke a protective response on its own but did in consort with MBP-VP19. Possibly, VP19 and VP28 interact, as they do in the WSSV virion, to provoke a more effective response.

It is not clear whether the partial protection we observed is the result of a genuine immune response of the shrimp or the consequence of a coverage of WSSV entry sites by MBP-VP19. As the time between the booster and challenge was only 2 days, the possibility of competition between receptor sites occupied by the injected structural proteins and WSSV virions cannot be excluded.

Venegas *et al*. (2000) and Wu *et al*. (2002) have demonstrated that shrimp, previously exposed to WSSV show antiviral activity in their haemolymph. The trigger needed to obtain this type of protection was, however, unknown. Here we have shown that vaccination with WSSV envelope proteins can induce a similar improved survival. A question still to be answered is whether the observed effect on the WSSV infection is WSSV-specific or whether it is based on a broader antiviral activity. This study further shows which viral

proteins might be involved in this process and demonstrates that the shrimp immune system is able to specifically recognize WSSV structural proteins. Oral vaccination with VP28 and VP19 constructs should indicate whether this strategy is viable and practically feasible. If successful, it would open the way to new control strategies for WSSV and other pathogens.

Acknowledgments

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chapter 6

DURATION AND ONSET OF PROTECTION VIA INJECTION OF A SUBUNIT VACCINE IN *PENAEUS MONODON*

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Abstract

Although invertebrates lack a true adaptive immune response, the potential to vaccinate *Penaeus monodon* shrimp against White Spot Syndrome Virus (WSSV) using the WSSV envelope proteins VP19 and VP28 was evaluated. Both structural WSSV proteins were N-terminally fused to the maltose binding protein (MBP) and purified after expression in bacteria. Shrimp were vaccinated by intramuscular injection of the purified WSSV proteins and challenged two and twenty-five days after vaccination to assess the onset and duration of protection. As controls, purified MBP and mock-vaccinated shrimp were included. VP19-vaccinated shrimp showed a significant better survival ($p<0,05$) as compared to the MBP-vaccinated control shrimp with a relative percent survival (RPS) of 33% and 57% at two and 25 days after vaccination, respectively. Also the groups vaccinated with VP28 and a mixture of VP19 and VP28 showed a significant better survival challenged two days after vaccination (RPS of 44% and 33% respectively), but no longer after twenty-five days. These results show that protection can be generated in shrimp against WSSV using its structural proteins as a subunit vaccine. This suggests that the shrimp immune system is able to specifically recognize and react to proteins. This study further shows that vaccination of shrimp may be possible despite the absence of a true adaptive immune system, opening the way to new strategies to control viral diseases in shrimp and other crustaceans.

Introduction

Within years after its first discovery in Asia in the early 1990's, White Spot Syndrome Virus (WSSV) has developed into an epizootic disease. Currently, WSSV is found in most shrimp farming areas of the world, where it causes large economic losses to the shrimp farming industry. Besides the economic impact of the disease, the natural marine ecology is also threatened as WSSV is able to infect a large number of crustaceans including crabs and crayfish (Wang *et al.,* 1998; Lo *et al.,* 1996).

WSSV virions are ovoid-to-bacilliform in shape and have a tail-like appendage at one end. The virions can be found throughout the body of infected animals, infecting most tissues and circulating ubiquitously in the hemolymph. The enveloped virions contain a single nucleocapsid with a distinctive striated appearance. The WSSV virion consists of five major and about thirteen minor proteins (van Hulten *et al.,* 2000a,c; van Hulten *et al.,* 2002; Huang *et al.,* 2002a). Sequencing of the WSSV genome revealed a circular sequence of 292,967 basepairs (bp) (van Hulten *et al.,* 2001c), but there is variation in size in geographic isolates of WSSV (Yang *et al.,* 2001).

Due to the current intensity of aquaculture practices and the broad host range of WSSV, novel control strategies including vaccination against this virus would be highly desirable. However, invertebrates lack a true adaptive immune response system and seem to rely on various innate immune responses (Kimbrell and Beutler, 2001). Although considered less sophisticated, this innate immune system is able to rapidly and efficiently

recognize and destroy non-self material, including pathogens (Lee and Söderhäll, 2002). The innate immune response consists of cellular and humoral responses. Hemocytes are responsible for most of the cellular responses, including encapsulation, phagocytosis, melanization, cytotoxicity, cell-to-cell communication, clotting, and the proPO activating system. Humoral response factors originate from granulocytes and include lectins, defensive enzymes, reactive oxygen intermediates and the synthesis of a wide array of antimicrobial peptides (Kimbrell and Beutler, 2001; Lee and Söderhäll, 2002; Söderhäll, 1999; Destoumieux *et al.,* 2000). Immunostimulation of shrimp upon contact with products of microbial origin has already been demonstrated (Song and Hsieh, 1994; Alabi *et al.,* 1999). Even effective vaccination of *P. monodon* and *P. japonicus* using inactivated Vibrio spp. has been reported by several researchers (Kou *et al.,* 1989; Itami *et al.,* 1989; Teunissen *et al., 1998)*.

In contrast to the well-studied effect of microbial immunostimulants on the immune system, there is limited information of the immune response upon viral infections. Pan *et al*. (2000) tested tissue extracts from crab, shrimp and crayfish against a variety of viruses for the presence of viral inhibitors. The authors found a 440 kilo Dalton (kDa) molecule that was able to non-specifically inhibit infection of six types of both RNA and DNA viruses. Furthermore, an upregulation of the lipopolysaccharide and β-1,3-glucan binding protein gene was observed upon infection with WSSV (Roux *et al.,* 2002). This gene is known to be involved in the proPO cascade, which is upregulated in bacterial and fungal infections. Also, upregulation of protease inhibitors, apoptotic peptides and tumor-related proteins have been observed upon WSSV infection (Rotjinnakorn *et al.,* 2002). *In vivo* experiments with *P. japonicus* demonstrated the presence of a quasi-immune response after re-challenging survivors of both natural and experimental infection with WSSV (Venegas *et al.,* 2000). After this re-challenge the observed mortality of the initial survivors was lower compared to challenged naïve shrimp. Wu *et al*. (2002) observed the presence of WSSV-neutralizing activity of plasma of infected shrimp from 20 days up to well over two months after infection. These results suggest the induction of antiviral responses and suggest that vaccination of shrimp against WSSV may be possible.

To test this hypothesis we have used structural WSSV proteins to vaccinate shrimp. The envelope proteins VP19 and VP28 were selected, as both proteins are likely to be the first to come in contact with the host cells and because the envelope protein VP28 was shown to be involved in the systemic infection of shrimp (van Hulten *et al.,* 2001b). VP19 and VP28 were fused to a maltose binding protein (MBP) and used, after purification, to vaccinate shrimp by intramuscular injection. The shrimp were challenged with WSSV by the same technique and showed a higher survival when vaccinated with the WSSV proteins.

Materials and Methods

Shrimp culture

Healthy *P. monodon* shrimp were imported as post-larvae from Malaysia and maintained in a recirculation system at the facility "De Haar" at Wageningen University. Prior to each experiment, shrimp were transferred to an experimental system located at the Laboratory of Virology at Wageningen University and stocked in 180-liter aquariums, each fitted with an individual filter system (Eheim, Germany), heating (Schego, Germany) at 28±1°C and continuous aeration. All experiments were performed in artificial seawater (Instant Ocean, Aquarium Systems) at a salinity of approximately 20 parts per thousand.

WSSV virus stock

The virus isolate used in this study originated from infected *P. monodon* shrimp imported from Thailand in 1996 and was obtained as described before (van Hulten, *et al*., 2001b). Crayfish *Orconectes limosus* were injected intramuscularly with a lethal dose of WSSV using a 26-gauge needle (Microfine B&D). After approximately one week, virus was isolated from freshly extracted hemolymph as described by van Hulten *et al*. (2001b). Virus samples were examined under the transmission electron microscope for purity and stored in aliquots at -80°C until further use.

In vivo titration

Since no crustacean cell lines are available, the WSSV stock was titered by *in vivo* infection experiments as described by van Hulten *et al*. (2001b). In short: shrimp of approximately one gram were injected intramuscularly with 10 µl of different virus dilutions in 330 mM NaCl in the 4th or 5th abdomen segment of the shrimp using a 29 gauge needle. The mortality was recorded twice a day and dead shrimp were tested for the presence of WSSV. The obtained time-mortality relationship was used to determine the desired challenge pressure for the vaccination experiments (≈ 70%).

PCR analysis for WSSV

Muscle tissue from the tails of dead shrimp was homogenized and mixed with 200 µl 5% Chelex 100 resin (BioRad) and 16 µl 20 mg/ml proteinase-K. This mixture was incubated overnight at 56°C followed by 10 minutes at 95°C to inactivate the proteinase-K. The samples were tested with two primer pairs. A 16S rRNA primer pair (16S-FW1 5'-GTG CGA AGG TAG CAT AAT C-3'; 16S-RV1 5'-CTG CTG CAA CAT AAG GAT AC-3'), amplifying a 414 bp fragment of ribosomal shrimp DNA, was used as an isolation control. A VP26 primer pair (VP26-FW1 5'- ATG GAA TTT GGC AAC CTA ACA AAC CTG-3'; VP26-RV1 5'- GGG CTG TGA CGG TAG AGA TGA C-3'), amplifying part of the WSSV VP26 gene (Van Hulten *et al*., 2000a), was used to screen for WSSV-positive animals.

Production of recombinant proteins

For bacterial expression, the VP19 ORF was cloned in the pMAL-c2 vector (New England Biolabs) resulting in an N-terminal fusion of VP19 and the maltose binding protein (MBP). The DNA fragment encoding the entire VP19 ORF (WSSV ORF182) was amplified from genomic WSSV DNA by PCR. Using the forward primer VP19-FW1 (3'-CGG GAT CCA TGG CCA CCA CGA CTA A-5') and reverse primer VP19-R1 (3'-GCC TGC AGC CTG ATG TTG TGT TTC TAT A-5') a *Bam*HI and *Pst*I restriction site respectively, were introduced. The amplified PCR product was ligated in the pGEM-T vector (Promega) and sequenced. The VP19 fragment was removed from the pGEM-T plasmid using the restriction enzymes *Bam*HI and *Pst*I, and after purification from gel (concert nucleic-acid purification system, GIBCOBRL) ligated into the pMAL-c2 vector. For VP28, the complete VP28 ORF was cloned in the pMAL-c2 vector resulting in the N-terminal fusion of VP28 and the maltose binding protein. Using the forward primer VP28-FW1 (3'-cag aat tca tgg atc ttt ctt tca c-5') and reverse primer VP28-RV1 (3'-cag gat cct tac tcg gtc tca gtg c-5') an *Eco*RI and *Bam*HI restriction site, respectively, were introduced. After PCR and cloning into the pGEM-T vector the VP28 insert was removed using the introduced restriction sites and ligated in the pGBKT7 vector (Clontech). The insert was again removed using the restriction enzymes EcoRI and PstI and ligated in the pMAL-c2 vector. Finally, all constructs were electroporated into E. coli DH5α cells. Both the MBP-VP19 and MBP-VP28 proteins and the non-fused MBP protein were overexpressed and purified according to manufacturer's instructions by affinity chromatography using amylose resin (New England Biolabs). The resulting *E. coli* expression samples and the purified proteins were analysed by SDS-PAGE and Western-analysis; the protein concentration was determined using the Bradford assay (Bio-Rad).

Vaccination experiments

For the vaccination experiments, shrimp of approximately one gram were injected with 4 µg of purified protein in 330 mM NaCl at a final volume of 10 µl. The shrimp of the group vaccinated with a mixture of the two proteins received 2 µg of MBP-VP19 and 2 µg of MBP-VP28 proteins (Table 6.1). Five days after the initial vaccination, the shrimp were boostered by injecting the same amount of protein (Figure 6.1). During the vaccination of the groups, the positive and negative control groups were injected with 10 µl of 330 mM NaCl. Two days after the booster half of the shrimp per group was challenged by injection of a specific WSSV dilution, except for the negative control shrimp that received 10 µl of NaCl. The challenged shrimp were maintained in individual cages to prevent horizontal transmission of WSSV. The other half of the shrimp was challenged in the same way 25 days after the last vaccination and also placed in individual cages.

Table 6.1. First column shows the names of the groups used in the experiment. The second column the proteins present in the vaccine administered and the last column the number of shrimp used in the experiments.

Group	Vaccine	# Shrimp
MBP	4 µg MBP	2x12
VP19	4 µg MBP-VP19	2x12
VP28	4 µg MBP-VP28	2x12
MIX	2 µg MBP-VP19+ 2 µg MBP-VP28	2x12
Positive control (C+)	-	2x10
Negative control (C-)	-	2x10

Figure 6.1. Vaccination and challenge scheme of experiment. Shrimp are kept in groups and receive vaccinations on day 0 and 5. Two days after the second vaccination, half of the shrimp per experimental group are challenged, placed in individual cages and observed. The remaining half of the shrimp is kept together for another 25 days after the second vaccination, challenged, placed in individual cages and observed.

Statistical analysis

Statistical analysis on the survival rates between the groups was performed using the $\chi 2$ test at a 5% confidence level. The protection against WSSV after vaccination was calculated as a relative percent survival (RPS) (1 – vaccinated group mortality / control group mortality) *100) (Amend, 1981).

Results

Protein production and purification

The WSSV ORFs encoding VP19 and VP28 were overexpressed as MBP fusion proteins in E. coli. Bands corresponding to the two fusion proteins were observed at the expected height (Figure 6.2, lanes 1 and 3). The WSSV origin of the bands was confirmed by Western analysis using anti-WSSV polyclonal antiserum (data not shown). Non-fused MBP was overexpressed as a control protein according to the same protocol (Figure 6.2, lane 5). The VP19-MBP and VP28-MBP fusion and non-fused MBP proteins were purified by affinity purification using amylose resin (Figure 6.2, lanes 2, 4 and 6). The concentration of the purified proteins was determined using the Bradford assay kit.

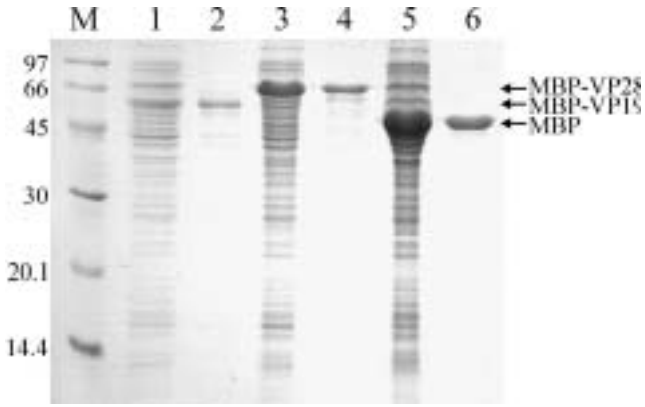

Figure 6.2. Coomassie stained SDS-PAGE gel of MBP-VP19, MBP-VP28 and MBP expressions in *E. coli* cells. M: Protein Molecular mass marker; Lane 1: total MBP-VP19 expression; Lane 2: purified MBP-VP19; Lane 3: total MBP-VP28 expression; Lane 4: purified MBP-VP28; Lane 5: total MBP expression; Lane 6: purified MBP. Numbers on the left indicate molecular mass (kDa.) of the Protein Molecular mass marker.

Vaccination experiment

Vaccination was performed using the WSSV envelope proteins VP28, VP19 or a mix of both proteins. Six experimental groups were set up; four groups of 24 individuals receiving a protein vaccination and two control groups of 20 individuals each receiving a mock vaccination treatment (Table 6.1). The four groups of 24 shrimp received two injections of 4 µg of purified proteins with a five-day interval; the positive and negative control groups received two injections, also with a five-day interval, of 330 mM NaCl. Half of the shrimp of each group was challenged 2 days after the second protein injection. The other half was challenged 25 days after the second protein injection, to test the duration of the WSSV protection. All groups were challenged with a titered WSSV stock, except for the negative control shrimp, which were injected with 330 mM NaCl. After the challenge, the shrimp were kept in individual cages and checked for mortality twice a day.

The resulting time-mortality relationship of the shrimp challenged two days after protein injection is shown in Figure 6.3. The challenge pressure resulted in a final cumulative mortality of 90% for the positive control. The MBP control group showed a final cumulative mortality of 75%, which is significantly lower than the positive control ($p<0.05$). The three groups receiving MBP-VP19, MBP-VP28 or both, all showed a final cumulative mortality of approximately 45%. This is significantly different from both the positive control and MBP group. The RPS values for the MBP-VP19, MBP-VP28 and MIX groups, compared to the positive control and non-fused MBP groups are shown in Table 6.2. Vaccination with MBP-VP28 gives the highest RPS value and also shows a low initial mortality compared to the other groups. Randomly selected survivors from every group were selected and all tested negative for WSSV.

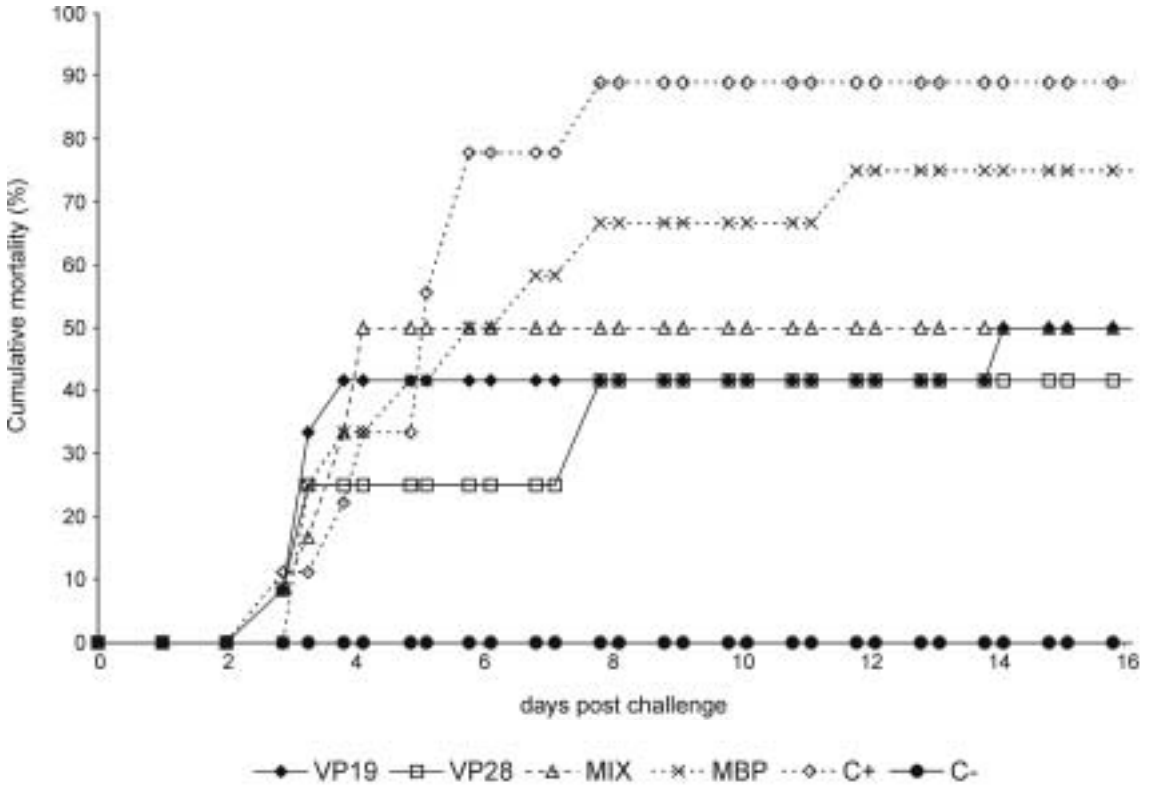

Figure 6.3. Time-mortality relationship of vaccinated shrimp challenged two days after the last vaccination. Cumulative mortality rates of shrimp from the experimental groups as indicated in table 6.1 are plotted against the days after challenge.

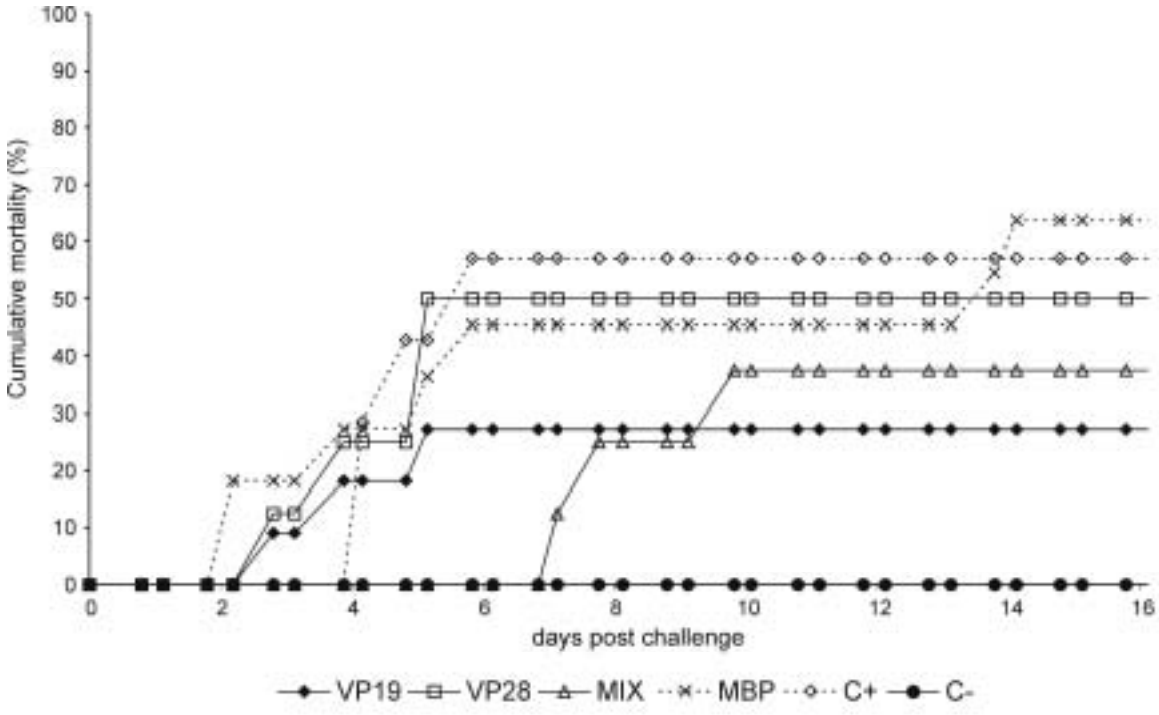

Figure 6.4. Time-mortality relationship of vaccinated shrimp challenged twenty-five days (b) after the last vaccination. Cumulative mortality rates of shrimp from the experimental groups as indicated in table 1 are plotted against the days after challenge.

The challenge at 25 days after the last vaccination was carried out similarly to the first challenge after 2 days. The resulting time-mortality relationships of this experiment can be found in figure 6.4. Although the applied challenge pressure was identical to the first challenge, the positive control showed a final cumulative mortality of 60%. The non-fused MBP group showed a final mortality of 65%, followed by MBP-VP28 with 55%, MIX with 40% and MBP-VP19 with 30%. The $\chi 2$ test only showed a significant difference in survival rates ($P<0.05$) between the MBP-VP19 and the control groups (positive control and non-fused MBP group). The RPS values for the MBP-VP19, MBP-VP28 and MIX groups, compared to the positive control and non-fused MBP groups are shown in Table 6.2. Randomly selected survivors from every group were selected and all tested negative for WSSV.

Table 6.2. Relative percentage survival (RPS) of the vaccination groups compared to the positive control and non-fused MBP groups for the groups vaccinated 2 and 25 days after the last vaccination.

	Positive control		**MBP control**	
Days post vaccination:	2	25	2	25
VP19	44	52	33	57
VP28	53	13	44	21
MIX	44	34	33	41

Discussion

Virus research with WSSV has shown the existence of a quasi immune response as survivors of a WSSV infection showed an increased survival compared to naïve shrimp after a rechallenge (Venegas *et al*., 2000; Wu *et al*., 2002). In this study we have performed experiments to gain more insight in the proteins responsible for this observed quasi immune response. Shrimp were vaccinated using the WSSV envelope proteins VP19 and VP28 fused to MBP and non-fused MBP was included as a control. As the general assumption is that shrimp do not have an adaptive immune response, one challenge was performed shortly after the last vaccination (2 days) to ensure that short-term effects of injection with WSSV proteins would not be missed. As Wu *et al*. (2002) have shown that shrimp become resistant to WSSV between 3 and 4 weeks post initial exposure, a second challenge was performed 25 days after the last vaccination to test the duration of the induced protection. Vaccination was performed via intramuscular injection to ensure the application of a consistent amount of proteins per shrimp. Even though this technique is far from practical under shrimp farming conditions, it is a very suitable in determining the vaccinating potential of proteins. The amount of injected proteins was based on previous unpublished experiments. During the entire period before the challenge the shrimp were kept together. After the challenge the shrimp were placed in individual cages to prevent horizontal transmission by cannibalism, as this increases the applied challenge pressure on the shrimp and reproducibility of the results. Both the challenge 2 and 25 days after vaccination resulted in a significant higher survival when shrimp were vaccinated with the MBP-VP19 fusion protein as compared to shrimp injected with MBP alone (RPS of 33% and 57%, respectively). A significant difference was

also found between the groups vaccinated with either MBP-VP28 or MIX and the MBP group when challenged 2 days after the last vaccination. However, this effect was reduced at 25 days after vaccination and the X^2 test showed that the effect was no longer significant. The experiments also show that injection of shrimp with non-fused MBP results in a small positive effect on shrimp survival when challenged two days after the booster. This suggests that besides the generation of a specific immune response (long term protection provided by injection of virus specific proteins like VP19) a small general immune response can be provoked by injection of a foreign protein like MBP. This immune response may also have long terms effects by enhancing the specific response to WSSV.

These data show that vaccination of shrimp with MBP-VP19 has a positive effect on shrimp survival after challenge with WSSV up to 25 days after vaccination. Most interestingly, the experiments show that the effect is VP19-specific and therefore suggests that the shrimp immune system is capable of specifically recognizing viral protein subunits. Using the developed vaccination and challenge set-up, experiments aimed at locating the VP19 domain(s) responsible for this immune response could be performed. Furthermore the required amount of proteins necessary for eliciting the observed immune response can be optimized using this experimental set-up.

Although the amount of virus injected in both challenges was equal, the second challenge showed generally lower mortality rates compared to the first challenge. This might be due to an increase in mean body weight during the 23 extra days before challenge compared to the first challenge. An interesting question to be answered is whether the observed effect is WSSV-specific or whether it is based on a more broad antiviral activity, possibly showing cross immunity to other shrimp viruses. Because vaccination via injection is neither a viable nor a practical strategy under current shrimp farming conditions, oral vaccination experiments should be performed to see if the same vaccinating potential as observed in this study could be found.

This study is the first to show that the shrimp immune system is able to specifically recognize WSSV structural proteins and that vaccination of shrimp against WSSV might be possible and opens the way to the design of new strategies to control WSSV and other invertebrate pathogens.

Acknowledgements

We thank Angela Vermeesch for her technical assistance, Sietze Leenstra and Aart Hutten of the facility “de Haar vissen” for the shrimp maintenance and Luc Grisez for providing the shrimp. This research was supported by Intervet international BV, Boxmeer, The Netherlands.

chapter 7

ORAL VACCINATION OF *PENAEUS MONODON* FOR PROTECTION AGAINST WSSV

Jeroen Witteveldt, Carolina E. Cifuentes, Just M. Vlak and Mariëlle C.W. van Hulten

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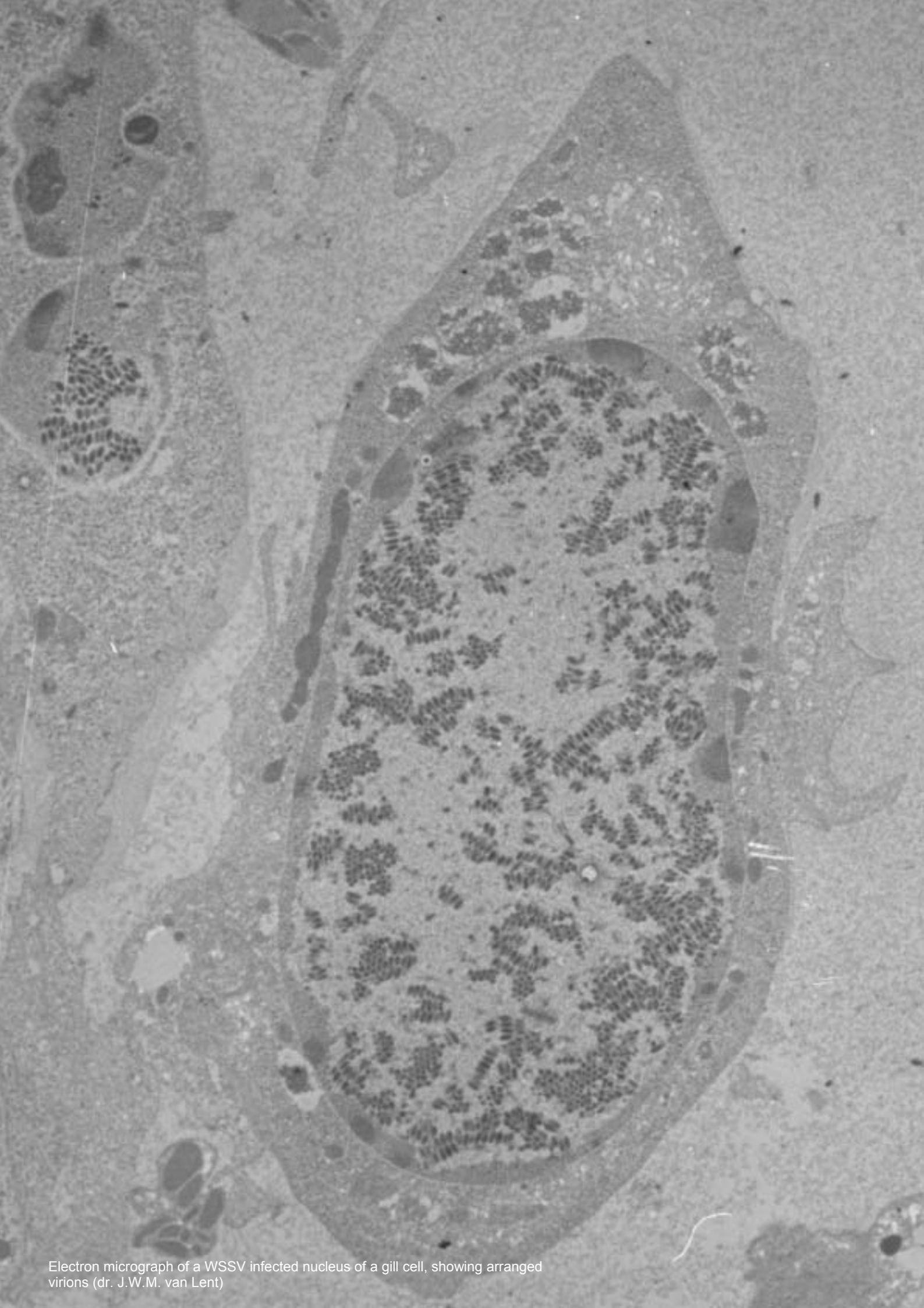

Electron micrograph of a WSSV infected nucleus of a gill cell, showing arranged virions (dr. J.W.M. van Lent)

Abstract

White spot syndrome virus (WSSV) is a worldwide occurring virus that causes high mortalities and considerable economic damages to the shrimp farming industry. No adequate treatments against this virus are available. It is generally accepted that invertebrates such as shrimp do not have an adaptive immune response system such as that present in vertebrates. As it has been demonstrated that shrimp surviving a WSSV infection have higher survival rates upon subsequent re-challenge, we investigated the potential of oral vaccination of shrimp using subunit vaccines consisting of WSSV virion envelope proteins. *Penaeus monodon* shrimp were fed food pellets coated with inactivated bacteria overexpressing two WSSV envelope proteins, VP19 and VP28. Vaccination with VP28 showed a significant lower cumulative mortality compared to shrimp vaccinated with bacteria expressing the empty vectors after challenge via immersion (RPS of 61%), while vaccination with VP19 provided no protection. To determine the onset and duration of protection, challenges were subsequently performed 3, 7 and 21 days after vaccination. A significantly higher survival was observed both 3 and 7 days post vaccination (RPS of 64% and 77%, respectively), but the protection was reduced 21 days after the vaccination (RPS of 29%). This suggests that contrary to current assumptions that invertebrates do not have a true adaptive immune system, a specific immune response and protection can be induced in *P. monodon*. These experiments open up new ways to benefit the WSSV-hampered shrimp farming industry.

Introduction

White Spot Syndrome Virus (WSSV) belongs to a new virus family, the *Nimaviridae* and contains a large circular dsDNA genome of 292,967 base pairs (bp) (van Hulten *et al.,* 2001c), but isolates with larger genomes have also been identified (AF440570; Yang *et al.,* 2001). WSSV virions are ellipsoid-to-bacilliform, enveloped particles with a distinctive tail-like appendage at one end and can be found throughout the body of infected shrimp. The virions contain one nucleocapsid with a typical striated appearance and five major and at least thirteen minor proteins (Huang *et al.,* 2002a; van Hulten *et al.,* 2000a,c; van Hulten *et al.,* 2002).

WSSV, which was first discovered in Southeast Asia around 1992, is currently the most serious viral pathogen of shrimp worldwide. It causes mortalities of up to 100% within 7-10 days in commercial shrimp farms, resulting in large economic losses to the shrimp farming industry (Lightner, 1996). Shrimp culture has been a booming business since the beginning of the nineties and worldwide production was 1 million metric tonnes in 2002 (Rosenberry, 2002). It is one of the few sources for economic development and provides well-paid employment in poor coastal areas (Adger, 1998).

Given the global environmental, economic and sociological importance of shrimp farming and the constraints of high intensity cultivation, the development of vaccines against WSSV would be desirable. The possibility of vaccinating shrimp or invertebrates in general,

seems to be unfeasible since they are assumed to lack an adaptive immune response and rely solely on innate immune responses (Kimbrell and Beutler, 2001). However, a recent study in the copepod *Macrocyclops albidus* showed that the defense system of this invertebrate species reacted more efficiently after a previous encounter to an antigenically similar parasite, implying that a specific memory may exist (Kurtz and Franz, 2003). Furthermore, immunostimulation and vaccination of shrimp using inactivated Vibrio spp. have been reported to provide some protection (Alabi *et al.,* 1999; Itami *et al.,* 1989; Teunissen *et al.,* 1998). Studies on the shrimp immune response to viral infections are limited, although the presence of viral inhibiting proteins and specific upregulation of genes upon viral infection have been demonstrated (Pan *et al.,* 2000; Rojtinnakorn *et al.,* 2002; Roux *et al.,* 2002). *In vivo* experiments with *Penaeus japonicus* demonstrated the presence of a quasi-immune response when WSSV survivors of both natural and experimental infections were re-challenged with WSSV (Venegas *et al.,* 2000). Plasma of the surviving infected shrimp could neutralize WSSV from 20 days up to two months after infection (Wu *et al.,* 2002). These results suggest that some sort of adaptive immune response could exist in shrimp.

To investigate whether protection against WSSV could be induced in shrimp by vaccination, a subunit vaccine was generated using two major structural envelope proteins of WSSV, VP19 and VP28. Neutralization experiments with VP28 have shown it to be involved in the systemic infection of WSSV (van Hulten *et al.,* 2001b). An oral vaccination strategy was adopted since injection vaccinations in shrimp are not practically feasible in shrimp farming. Inactivated bacteria overexpressing the WSSV envelope proteins VP19 and VP28 coated on food pellets were selected for delivery of the WSSV proteins. The onset and duration of the observed protection was further investigated by challenging shrimp on different time points after vaccination.

Materials and Methods

Shrimp culture

Healthy *Penaeus monodon* shrimp were imported as post-larvae from Malaysia and maintained in a recirculation system at the facility “De Haar vissen” at Wageningen University. Each shipment was tested for the presence of WSSV, Monodon baculovirus, yellow head virus, Taura syndrome virus and infectious hypodermal and hematopoietic necrosis virus by PCR. Prior to each experiment, shrimp were transferred to an experimental system located at the Laboratory of Virology at Wageningen University and stocked in 180-liter aquariums, each fitted with an individual filter system (Eheim, Germany), heating (Schego, Germany) to 28±1°C and continuous aeration. All experiments were performed in artificial seawater (Instant Ocean, Aquarium Systems) at a salinity of approximately 20 parts per thousand.

WSSV virus stock

The virus isolate used in this study originated from infected *Penaeus monodon* shrimp imported from Thailand in 1996. Virus stocks were generated in the crayfish *Orconectes limosus* and purified from freshly extracted haemolymph by sucrose gradient centrifugation as described by Van Hulten *et al*. (2001b). Virus samples were stored in aliquots at -80°C.

In vivo titration and WSSV challenge

In order to mimic the natural route of infection and to ensure a constant and reproducible challenge pressure, shrimp were challenged via immersion. To determine the required amount of virus for the desired challenge pressure of approximately 75% mortality, a virus stock was prepared and titrated *in vivo*. Shrimp of approximately one gram were immersed in different dilutions of WSSV for a seven-hour incubation period. The shrimp were removed from the WSSV containing water, rinsed and moved to individual cages to prevent horizontal transmission by cannibalism. Mortality was recorded twice a day and dead shrimp were tested for the presence of WSSV by PCR. WSSV challenge after vaccination was performed identically.

PCR analysis for WSSV

Muscle tissue from the tails of shrimp was homogenized and mixed with 200 µl 5% Chelex 100 resin (BioRad) and 16 µl 20 mg/ml proteinase-K. This mixture was incubated overnight at 56°C followed by 10 minutes at 95°C to inactivate the proteinase-K. The samples were tested with two primer pairs. A 16S rRNA primer pair (16S-FW1 5'-GTG CGA AGG TAG CAT AAT C-3'; 16S-RV1 5'-CTG CTG CAA CAT AAG GAT AC-3'), amplifying a 414 bp fragment of shrimp ribosomal DNA, was used as an isolation control. A VP26 primer pair (VP26-FW1 5'- ATG GAA TTT GGC AAC CTA ACA AAC CTG-3'; VP26-RV1 5'- GGG CTG TGA CGG TAG AGA TGA C-3'), amplifying part of the WSSV VP26 gene, was used to screen for WSSV positive animals (Marks *et al.,* 2003). Twenty-five cycles of amplification were performed at 30 sec. at 94°C, 30 sec. at 52°C and 50 sec. at 72°C for both primer pairs.

Bacterial expression of VP28/19

Expression constructs were generated for VP28 and VP19 in fusion with a (HIS)6 and MBP-tag. The complete VP19 ORF was cloned as a *Bam*HI / *Pst*I fragment into the pMAL-C2 vector (New England Biolabs) after PCR amplification from the WSSV genome using primer VP19-FW1 (5'-CGG GAT CCA TGG CCA CCA CGA CTA A-3') and primer VP19-RV1 (5'-GCC TGC AGC CTG ATG TTG TGT TTC TAT A-3'). Expression of the pMAL-C2-VP19 construct and an empty pMAL-C2 vector (control) was performed in *E. coli* DH5α cells. A partial VP28 fragment (without the N-terminal hydrophobic region (Δ1-29)) was amplified from genomic WSSV DNA by PCR using the primer VP28PF (3'-AAG GAT CCC ACA ACA CTG TGA CCA AG-5') and primer VP28PR (3'-TAG CGG CCG CAA AAG CAC GAT TTA

TTT AC-5') and ligated into the *Bam*HI and *Not*I site of the pET28a vector (Novagen). Expression of the pET28a-VP28 construct and the pET28a vector (control) was performed in BL21 cells.

Protein production and inactivation

The MBP-VP19, (HIS)6-VP28 and control proteins were overexpressed according to manufacturers instructions and analyzed on SDS-PAGE (Laemmli, 1970) and Western blot (Sambrook *et al.,* 1989) using a WSSV polyclonal antiserum (van Hulten *et al.,* 2000a). The bacterial concentration after inactivation was determined using a Beckman DU-7500 photo spectrometer, where an OD_{600} of 1 equaled 10^9 bacteria per ml. The bacteria were inactivated in 0.5% formaldehyde, incubated for 15 minutes at 20°C, checked for inactivation levels and stored at 4°C until further use.

Coating of feed pellets

Commercial pellets of approximately 0.02 grams (Coppens International) were each coated with approximately 108 inactivated bacteria or with PBS for the positive and negative controls. The inactivated bacteria were centrifuged, washed two times in PBS and resuspended in PBS. The bacteria were subsequently mixed with the food pellets and incubated on ice for 15 minutes to allow absorption of the bacterial suspension and coated with cod liver oil to prevent dispersion of the inactivated bacteria in the water.

Vaccination experiments

In the vaccination experiments, groups of 15 shrimp were vaccinated by feeding coated food pellets for seven days as indicated in Table 7.1. For experiment 1 the vaccination was directly followed by a seven-hour immersion challenge in WSSV containing seawater of a pre-determined dilution as described above. For experiment 2 the four groups were subdivided in three even subgroups after the seven days of vaccination, which were subsequently challenged at three, 14 and 21 days post vaccination.

Table 7.1. Setup for the two vaccination experiments. The second column shows the names of the groups used in the experiments, the third column the proteins present in the vaccine administered and the last column the number of shrimp used in the experiments.

	Group	Coating	no. shrimp
Experiment 1	VP19	pMAL-VP19	15
	VP28	pET28a-VP28	15
	VP19 + VP28	pMAL-VP19 + pET28a-VP28	15
	pET + pMAL	pET28a + pMAL-C2	15
	positive control	PBS	15
	negative control	PBS	5
Experiment 2	VP28	pET28a-VP28	3x15
	pET	pET28a	3x15
	Positive control	PBS	3x15
	Negative control	PBS	3x10

Statistical analysis

Statistical analysis of the obtained time-mortality relationships was performed using the Kaplan-Meier survival analysis (Bull *et al.,* 1997) at a 5% confidence level. The protection against WSSV after vaccination was calculated as a relative percent survival (RPS %) calculated as (1 – vaccinated group mortality / control group mortality) *100 (Amend, 1981).

Results

Protein production and purification

The two major WSSV envelope proteins VP19 and VP28 were selected for use as subunit vaccines. Multiple expressions were performed in *E. coli*, using complete and partial ORFs and MBP and (HIS)6-tag fusions. The highest expression of VP28 was obtained in fusion with the (HIS)6-tag when the N-terminal hydrophobic domain was omitted. Expression for VP19 was generally lower and highest in fusion with MBP. Bands corresponding to the fusion proteins were observed at the expected heights (Figure 7.1, lanes 1 and 2). The viral origin of the band was confirmed by Western analysis using anti-WSSV polyclonal antiserum (data not shown). Empty pMAL-C2 and pET28a vectors were overexpressed as control proteins according to the same protocols (Figure 7.1, lanes 3 and 4).

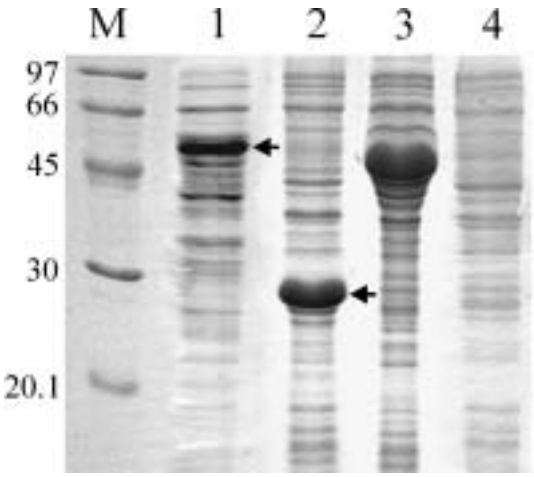

Figure 7.1. Coomassie stained SDS-PAGE gel of *E. coli* overexpressions of VP19 in pMAL-c2, VP28 in pET28a, empty pMAL-C2 and empty pET28a. M: Low molecular weight marker; Lane 1: MBP-VP19 expression; Lane 2: $(HIS)_6$-VP28 expression; Lane 3: pMAL-C2 expression; Lane 4: pET28a expression. Numbers on the left side indicate molecular weight (kDa.) of the marker and arrows indicate the MBP-VP19 and $(HIS)_6$-VP28 overexpression products.

Vaccination experiments

Experiment 1: VP19 and VP28 vaccination

In this experiment, the vaccinating potential of WSSV envelope proteins VP19, VP28 and a mixture of both proteins via oral administration was tested. Four groups of 15 shrimp each were vaccinated as indicated in Table 7.1, directly followed by an immersion challenge. The resulting time-mortality relationship of this experiment is shown in Fig. 7.2. The positive control group and the group vaccinated with a mixture of the empty pMAL-C2 and pET28a vectors showed a cumulative mortality of 67% and 77% respectively. The group vaccinated with VP19 also showed a high cumulative mortality of 83%, indicating that no protection could be obtained using this protein. However, vaccination with either VP28 alone or a mixture of VP28 and VP19 resulted in lower mortalities of 30% and 50% compared to the group vaccinated with the empty vectors (RPS values of 61% and 31% respectively). This mortality was significantly lower for the VP28 vaccinated group compared to the pMAL-C2/pET28a and positive control groups. Randomly selected survivors were checked for the presence of WSSV and all tested negative.

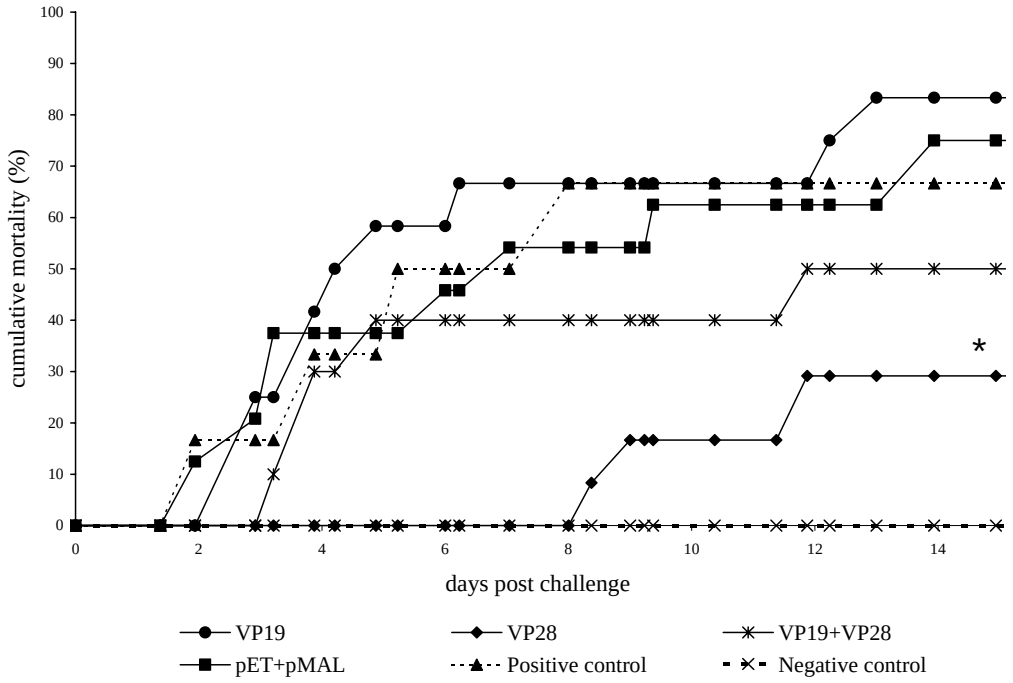

Figure 7.2. Time-mortality relationship of vaccination experiment 1. Cumulative mortality rates of shrimp from the experimental groups as indicated in table 7.1 are plotted against the days after challenge. Lines marked with asterisk are significantly different from the pET+pMAL and positive control groups.

Experiment 2: Onset and duration of vaccination with VP28

As the first experiment demonstrated that vaccination with VP28 resulted in higher survival upon WSSV challenge, the nature of this protection was further analyzed. Shrimp were vaccinated with WSSV envelope protein VP28 and three control groups were included as indicated in Table 7.1. After vaccination, the four groups were subdivided in three even subgroups for subsequent challenge at three different time points.

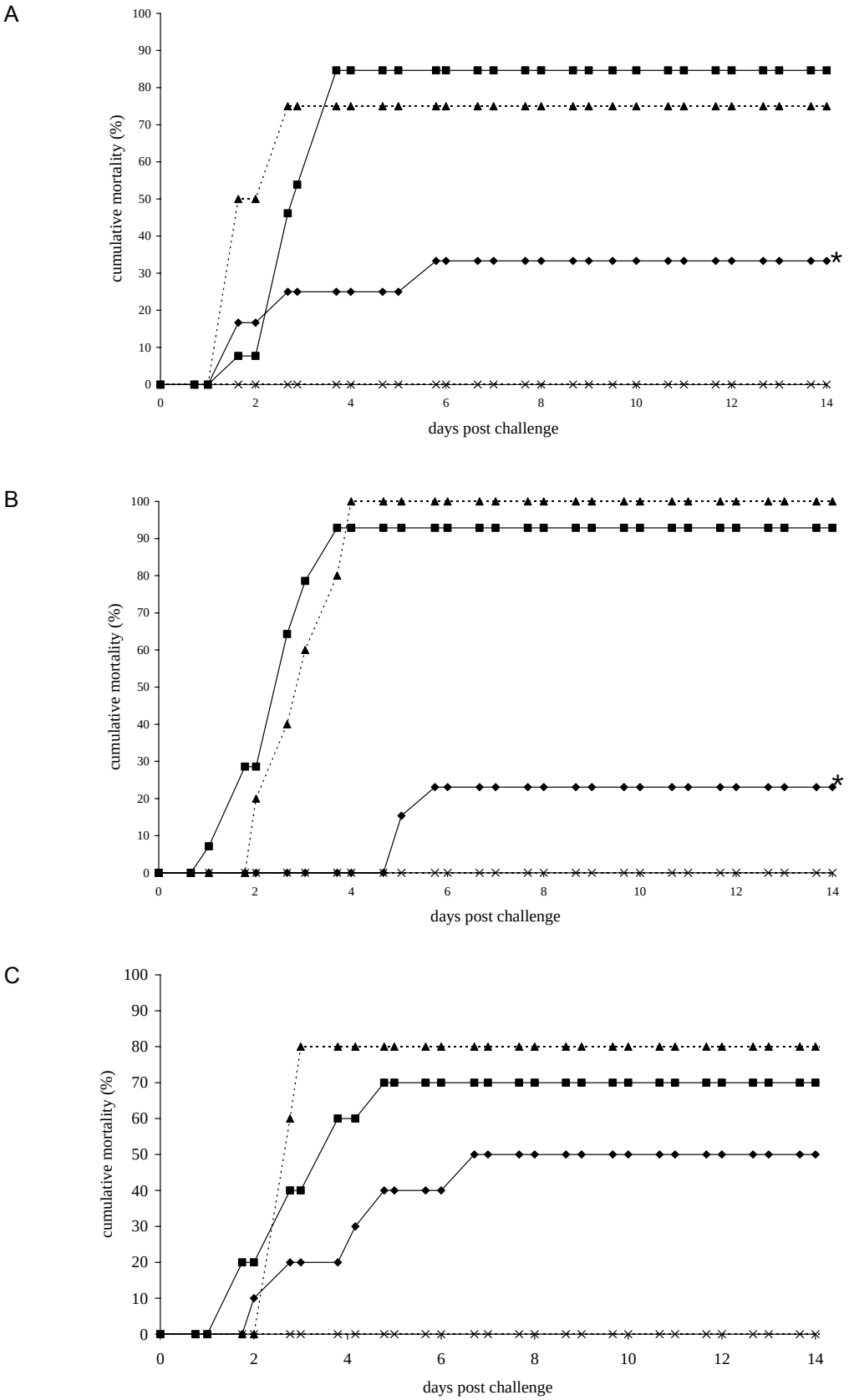

Figure 7.3. Time-mortality relationship of vaccination experiment 2; shrimp are challenged three days (a), seven days (b) and twenty-one days (c) after cessation of feeding coated food pellets. Cumulative mortality rates of shrimp from the experimental groups VP28 (—◆—), pET (—■—), Positive control (- ▲ -) and Negative control (- ✕ -) as indicated in table 7.1 are plotted against the days after challenge. Lines marked with asterisk are significantly different from the pET and positive control groups.

The first challenge was three days after vaccination had stopped, and the resulting time-mortality relationship is shown in Fig. 7.3a. The pET group showed a cumulative mortality of 85% and the positive control group a mortality of 75%. A significantly lower cumulative mortality (30%) was observed in the group fed VP28, resulting in RPS of 64% and 59% when compared to the pET and positive control groups respectively. These results are consistent with an independent experiment (data not shown), where vaccination with VP28 resulted in RPS values of 64% and 62% compared to the pET and positive control group, respectively. For the second challenge, 7 days post vaccination, the group fed pET and the positive control group reached cumulative mortalities of 100% and 93% respectively (Fig. 7.3b). The VP28 vaccination group shows a significantly lower mortality of 23%, resulting in RPS values of 77% and 75% when compared to the pET and positive control groups, respectively. Twenty-one days after the vaccinations, the third challenge was performed. This time, the pET and positive controls groups reached cumulative mortalities of 80 and 70% respectively (Fig 7.3c). The positive effect of feeding VP28 was reduced and this group reached a cumulative mortality of 50% (not significantly different), resulting in RPS values of 29% and 38% compared to the pET and positive control groups, respectively. The negative control groups showed no mortality. Randomly selected survivors from all groups were tested for WSSV and all tested negative.

Discussion

In the study presented here we have analyzed if viral proteins can be used to elicit an immune response in shrimp leading to protection against WSSV. To this end we have used oral vaccination as this is the only practical way to deliver potential vaccines to shrimp. The challenge with WSSV was performed using immersion, as the challenge pressure can be well controlled in contrary to challenge using infected tissue. In a natural situation shrimp become infected through both oral and water-borne routes and the gills are thought to be a major point of viral entry (Chang *et al.,* 1996; Tan *et al.,* 2001). We selected VP28 and VP19 for use in the crude subunit vaccines, as they are the most exposed proteins abundantly present in the WSSV envelope and react strongly with polyclonal antibodies generated against complete virions in rabbits (van Hulten *et al.,* 2000a). As previous studies have shown that the major WSSV proteins are not glycosylated (van Hulten *et al.,* 2002) bacteria were chosen for protein expression and as an antigen delivery vehicle since production for commercial applications is well established and cheap. Vaccination against bacterial diseases in shrimp using inactivated bacteria has been performed earlier (Alabi *et al.,* 1999; Itami *et al.,* 1989; Teunissen *et al.,* 1998) and is commercially available (Norvax ShrimpVib, Intervet International BV). As it is generally believed that the inactivated bacteria induce a general immune stimulation in shrimp, the presence of bacteria in the subunit vaccine might by itself have a positive effect on shrimp survival upon WSSV challenge. However, none of the vaccines lacking VP28 provided protection against WSSV, indicating that protection was VP28 specific. Nonetheless, the presence of the bacterial proteins may still act as an adjuvant in the vaccination.

When a mixture of VP19 and VP28 was used, a lower RPS value was obtained compared to the group vaccinated with VP28 alone. As the concentration of VP28 in the mixture was half of that in the treatment with VP28 alone, this may suggest the existence of a dose-dependent response. Further experiments must elucidate whether the high protection found for up to three weeks after vaccination with VP28 can be extended or further improved by a longer vaccination period, different vaccination schemes using booster feeding or optimization of the amount of vaccine. Challenge experiments using other shrimp infecting viruses such as yellow head virus and Taura syndrome virus may reveal whether the observed effect is virus specific and may give us more insight into the processes involved in the immune response.

Previous experiments have indicated that VP28 plays an important role in the systemic infection of WSSV in shrimp, as it is possible to neutralize WSSV using VP28 antibodies (van Hulten *et al.,* 2001b). As protection against WSSV is maintained up to three weeks after vaccination, it is unlikely that the presence of residual VP28 could block WSSV infection by blocking receptors needed by the virus to enter shrimp cells. The way in which the protection is obtained by the shrimp immune system remains to be resolved. Protection could for example be generated by prevention of entry of WSSV by secreted neutralizing substances or by blocking the virus spread after entry. At the end of the vaccination experiment survivors were checked for the presence of WSSV using one-step PCR and all tested negative for WSSV, indicating that no high levels of WSSV were present in the shrimp. More sensitive nested-PCRs could be used to monitor the entry and possible persistence of WSSV after challenge in vaccinated shrimp.

Altogether these results suggest that a specific memory exists in invertebrates or more specific in crustaceans as the data obtained are in line with the results found for the copepod, which is a minute crustacean (Kurtz and Franz, 2003).
This study is the first to show that oral vaccination of shrimp against WSSV is possible and opens the way for the design of practical strategies to control WSSV and other invertebrate pathogens.

Acknowledgments

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chapter 8

PROTECTION OF PENAEID SHRIMP AGAINST WSSV USING ORAL VACCINATION

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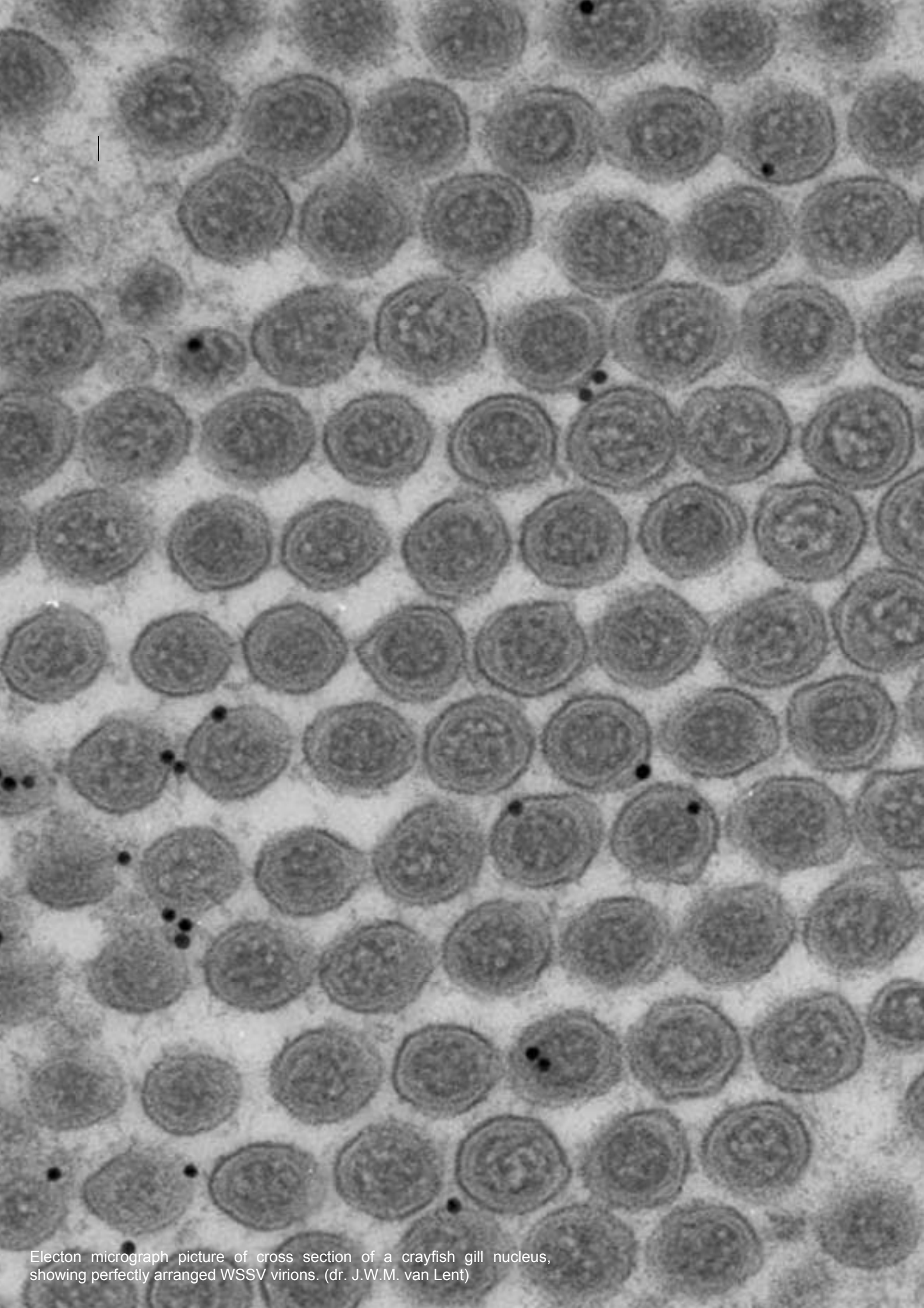

Electon micrograph picture of cross section of a crayfish gill nucleus, showing perfectly arranged WSSV virions. (dr. J.W.M. van Lent)

Abstract

It is generally accepted that invertebrates such as shrimp do not have an adaptive immune response system comparable to that of vertebrates. However, in the last few years several studies have shown or suggested the existence of such a response in invertebrates. In one of these studies, the shrimp *Penaeus monodon* was successfully vaccinated using the recombinant envelope protein VP28 of White Spot Syndrome Virus (WSSV). In an effort to further investigate whether this oral vaccination effect is limited to *P. monodon* or can be extended to other penaeid shrimp, vaccination experiments using an alternative host for WSSV, the Pacific White shrimp *Litopenaeus vannamei*, were performed. In this case, a significantly lower cumulative mortality for VP28 vaccinated shrimp was found compared to the controls. As such, these experiments demonstrate that oral vaccination of the two most important cultured shrimp species, *P. monodon* and *L. vannamei* using a WSSV VP28-based vaccine can be performed successfully. Most likely this increased protection upon vaccination is based on a shared and therefore general mechanism present in all shrimp species, making the design of vaccines against pathogens a viable option for shrimp culture.

Introduction

It has always been assumed that it is not possible to protect invertebrates against pathogens using an induced immune response (e.g. vaccination) or priming (i.e. past experiences with a pathogen). In recent years however, much phenomenological data has been published, suggesting the existence of such adaptive immune mechanisms in invertebrates (Kurtz, 2005). In the last decade viral diseases have caused major problems in the expanding shrimp culture. With the increase in both the social and economical importance of shrimp culture (Lundin, 1996) much research has been aimed at designing intervention strategies. This has resulted in the expansion of knowledge on the immunology of crustaceans and indications that invertebrates may indeed have some sort of adaptive immune system.

In vivo experiments with *Penaeus japonicus* demonstrated the presence of a quasi-immune response when WSSV survivors of both natural and experimental infections were re-challenged with WSSV and showed a higher survival compared to naive shrimp. Moreover, haemolymph of the survivors was able to neutralize virus preparations up to two months after infection (Venegas *et al.,* 2000). Further evidence of a specific immune response in crustaceans was found in a study using *Daphnia magna*. In this case maternal transmission of strain-specific immunity was demonstrated as hosts were less likely to be infected by parasite strains with which their mothers were previously challenged (Little *et al.,* 2003). The presence of immunological memory was demonstrated in the copepod *Macrocyclops albidus* where individuals were less likely to be reinfected with (antigenically) related lines of a natural parasite compared to more unrelated lines (Kurtz & Franz 2003). However, as all of the above examples use whole parasites, this enhanced immunological status might be explained by the recognition of general 'pathogen associated molecular patterns' (e.g. lipopolysaccharide, lipoteichoic acid, mannans, glycans) by 'pattern-recognition receptors' (PRRs) the effect of which has been shown in numerous studies

(Duvik & Söderhäll 1990, Söderhäll *et al.,* 1994, Kim *et al.,* 2000, Romeo-Figueroa *et al.,* 2004). A recent study using the overexpressed White Spot Syndrome Virus (WSSV) envelope protein VP28 as subunit vaccine in the shrimp *Penaeus monodon* was the first study to show that an immune response could be triggered with a protein lacking patterns that can be recognized by known PRRs, suggesting the presence of an unknown type of immune system (Witteveldt *et al.,* 2004a,b).

To further investigate whether this vaccination effect is strictly species-specific or based on a more general mechanism, vaccination experiments were performed using an alternative host for WSSV, the Pacific White shrimp *Litopenaeus vannamei*. This species is of increasing importance in shrimp farming, as it is assumed to have a lower susceptibility to WSSV compared to *P. monodon* (Briggs *et al.,* 2004, Wyban & Sweeney 2001). Nevertheless, the culture of *L. vannamei* is still greatly hampered by massive WSSV outbreaks. In this study, *L. vannamei s*hrimp were orally vaccinated with inactivated bacteria overexpressing VP28 and challenged with WSSV by immersion. The results support the contention that shrimp, i.e. invertebrates, can be vaccinated and made more tolerant to WSSV infection.

Materials and Methods

Unless stated otherwise, the materials and methods were similar to the procedures as previously described in Witteveldt *et al.* (2004b).

Shrimp culture

Specific pathogen-free (SPF) *Litopenaeus vannamei* of the Kona strain (Wyban *et al.,* 1991) were used for the vaccination experiments. Postlarvae (PL 8-12) from Ceatech farms (USA) were maintained at the Laboratory of Aquaculture & Artemia Reference Center (ARC), Ghent University. Each shipment was tested by PCR for the presence of WSSV, *Monodon* baculovirus, yellow head virus, Taura syndrome virus and infectious hypodermal and hematopoietic necrosis virus. Prior to each experiment, shrimp of approximately 5 grams were transferred to an experimental system located at the Laboratory of Virology at Wageningen University and stocked in 180-liter aquariums, each fitted with an individual filter system (Eheim, Germany), heating (Schego, Germany) to 28 $\pm$ 1°C and continuous aeration. All experiments were performed in artificial seawater (Instant Ocean, Aquarium Systems) at a salinity of approximately 20 parts per thousand.

In vivo titration and WSSV challenge

To ensure a constant and reproducible challenge pressure where the virus is introduced via a natural route, shrimp were challenged via immersion (Witteveldt *et al.,* 2004b). To determine the required amount of virus to reach a challenge pressure resulting in about 75% mortality, a WSSV stock was prepared and titrated *in vivo*. Four groups of five shrimp of approximately five grams were immersed in different WSSV dilutions (1, 3, 6 and 9

µl/shrimp) of the WSSV stock for a seven-hour incubation period. After this period, the shrimp were removed from the WSSV-containing seawater, rinsed and moved to individual cages to prevent horizontal transmission by cannibalism. Mortality was recorded twice a day and dead shrimp were tested for the presence of WSSV by PCR. WSSV challenge after vaccination was performed identically.

Bacterial expression of VP28

For the expression of VP28 an expression construct of VP28 fused to a $(HIS)_6$ -tag using the pET28a vector (Novagen) was used (Witteveldt *et al.,* 2004b). An empty pET28a vector was used as a control. Both vectors were transferred into *Escherichia coli* BL21 cells for expression.

Protein production and inactivation

The $(HIS)_6$-VP28 and pET28a control were overexpressed according to manufacturers' instructions and analyzed on SDS-PAGE and Western blot using an anti-WSSV polyclonal serum. The bacterial concentration was determined using a Beckman DU-7500 photo spectrometer, where an OD_{600} of 1 equaled 10^9 bacteria ml^{-1}. The bacteria were inactivated in 0.5% formaldehyde, incubated for 15 minutes at 20°C, checked for inactivation levels and stored at 4°C until further use.

Coating of feed pellets

Commercial pellets of approximately 0.02 grams (Coppens International, The Netherlands) were each coated with approximately 10^8 inactivated bacteria or with PBS for the positive and negative controls as described in Witteveldt *et al.,* (2004b). Briefly, the inactivated bacteria were centrifuged, washed two times in PBS and resuspended in PBS. The bacteria were subsequently mixed with the food pellets, incubated on ice for 15 minutes to allow absorption of the bacterial suspension and coated with cod liver oil to prevent dispersion of the inactivated bacteria in the water. Each shrimp was fed 8 pellets divided into two rations per day.

Vaccination experiments

Groups of 10 shrimp were vaccinated by feeding food pellets coated with either VP28-expressing bacteria, bacteria containing the empty pET28a vector or PBS for the positive and negative controls (table 8.1). The shrimp were vaccinated for seven days, followed by seven days of normal food as this incubation period showed the highest response in previous vaccination studies (Witteveldt *et al.,* 2004b). Subsequently, all shrimp were challenged by immersion, except for 5 shrimp serving as the negative control which were mock infected.

Table 8.1. Vaccination setup showing group name, treatment, challenge material and number of shrimp in each group.

Groups	Treatment	Challenge	# shrimp
VP28	pET28a-VP28 bacteria	WSSV	10
pET28a	pET28a bacteria	WSSV	10
Positive control	PBS	WSSV	5
Negative control	PBS	PBS	5

Statistical analysis

Statistical analysis of the obtained time-mortality relationships was performed using Chi-square test at a 5% confidence level. The protection against WSSV after vaccination was calculated as a relative percent survival (RPS %) calculated as (1 – vaccinated group mortality / control group mortality) *100 (Amend, 1981).

Results and Discussion

Protein production and purification

The major WSSV envelope protein VP28 was selected for use as subunit vaccine as this showed an increase in shrimp survival after oral vaccination in *P. monodon* (Witteveldt *et al.,* 2004b). VP28 was expressed fused to a $(HIS)_6$-tag and without its N-terminal hydrophobic domain. After expression, a band corresponding to the fusion protein was observed at the expected height (Fig. 8.1) and confirmed by Western analysis using anti-WSSV polyclonal antiserum to be of viral origin. An empty pET28a vector transformed in the same bacterial strain was overexpressed as control according to the same protocol (Fig. 8.1).

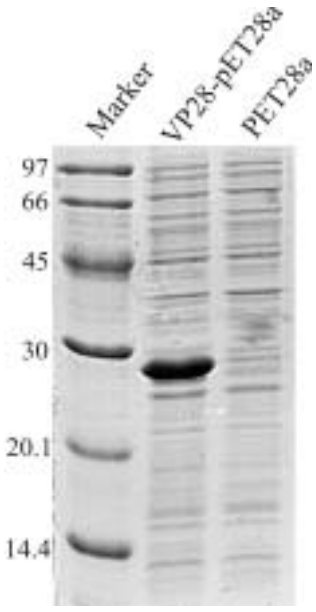

Fig. 8.1. SDS-PAGE gel of *E. coli* expressing VP28 using the pET28a vector and the empty pET28 vector. Numbers on left side of figure indicate size of the marker bands in kDa.

Vaccination experiments

The resulting time-mortality relationship of the titration of WSSV in *L. vannamei* is shown in figure 8.2. A final cumulative mortality of a hundred percent was observed when the shrimp were incubated with 9 µl WSSV stock per shrimp. Incubation with 3 and 6 µl stock per shrimp resulted in a final cumulative mortality of 75%, with the remark that 3 µl, as expected showed a slower mortality development (Van Hulten *et al.,* 2001b). Incubation with 1 µl resulted in an even lower mortality of 67%. Based on this titration, a challenge pressure of 6 µl of WSSV stock per shrimp was selected for the vaccination experiment.

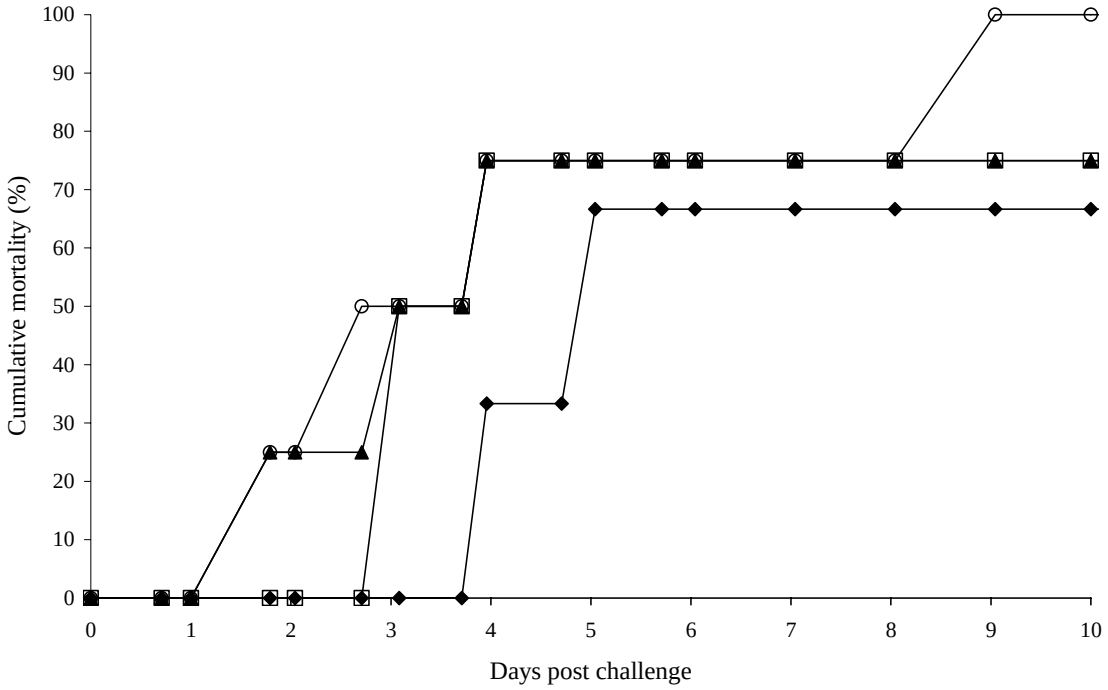

Figure 8.2. Time-mortality relationship of *L. vannamei* challenged with four different WSSV dilutions. Cumulative mortality rates of shrimp challenged with 1 (–◆–), 3 (–⊟–), 6 (–△–) and 9 (–○–) µl of WSSV stock per shrimp are plotted against the days after challenge.

Two groups of *L. vannamei* shrimp were vaccinated with bacteria (VP28 and pET28a) coated food pellets for a period of seven days. Two other groups of shrimp received PBS coated food pellets (table 8.1). After vaccination, all shrimp received normal food for another period of seven days. Subsequently the VP28, pET28a and positive control groups were challenged by immersion using 6 µl WSSV stock per shrimp. At the same time the negative control was mock challenged. The resulting time-mortality relationship is shown in figure 8.3. The shrimp vaccinated with VP28 show a significant lower cumulative mortality (RPS value of 50%) compared to both the pET28a fed shrimp and the positive control. All shrimp that died during the experiment tested positive and all surviving shrimp tested negative for WSSV by one-step PCR.

The seven day interval period between the last vaccination and challenge was chosen as this resulted in the highest level of protection when using *P. monodon* (Witteveldt *et al.,* 2004b). The level of protection in *L. vannamei* is lower when compared to *P. monodon*

(RPS values of 50% in *L. vannamei* and 77% in *P. monodon* when comparing the VP28 to the pET28a groups (Witteveldt *et al.,* 2004b)), but is comparable to other vaccination trials in *P. monodon* using a similar, high, challenge pressure (unpublished results).
This study has shown that the enhanced tolerance of *P. monodon* for WSSV upon vaccination can also be demonstrated in *L. vannamei*. Although these experiments do not explain the mechanism of the observed protection, they do suggest that the protection is based on a general mechanism, conserved among shrimp species and possibly based on a novel type of immunological response.

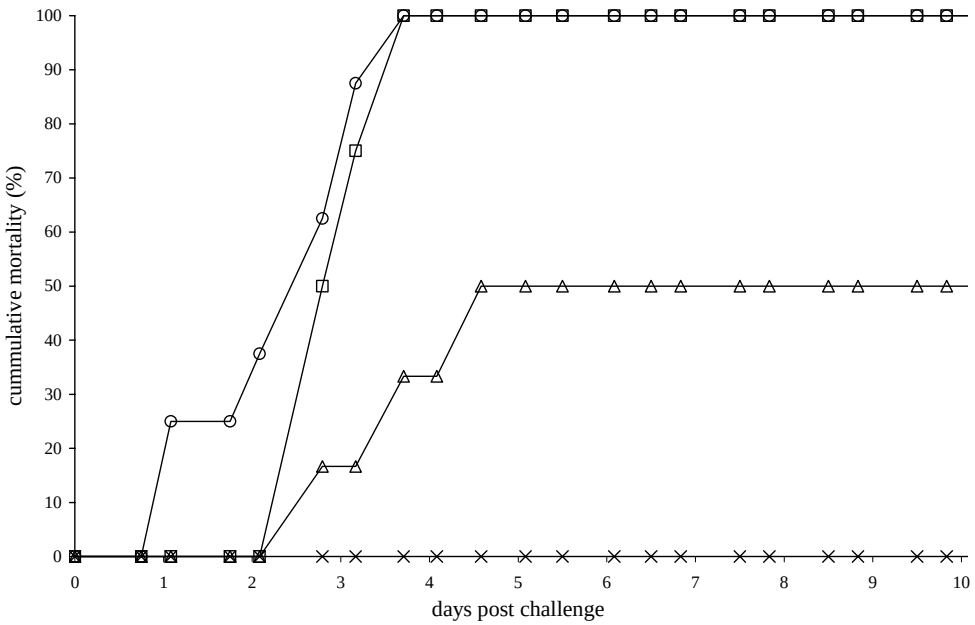

Figure 8.3. Time-mortality relationship after vaccination and challenge of *L. vannamei*. Cumulative mortality rates of shrimp from the experimental groups VP28 (—△—), pET28a (—⊟—), positive control (—○—) and negative control (—×—) are plotted against the days after challenge. The VP28-vaccinated group shows a significantly lower ($p<0.05$) cumulative mortality compared to both the pET28a and positive control groups.

Analysis of the host response upon VP28 vaccination in molecular terms is under way. Effective oral vaccination using the VP28 subunit vaccine has now been demonstrated for the two most important shrimp species in aquaculture, *P. monodon* and *L. vannamei*. Most likely similar results could be obtained for other shrimp species, underlining the value of vaccination strategies to assist the shrimp farming industry in its battle against viral and bacterial diseases.

Acknowledgements

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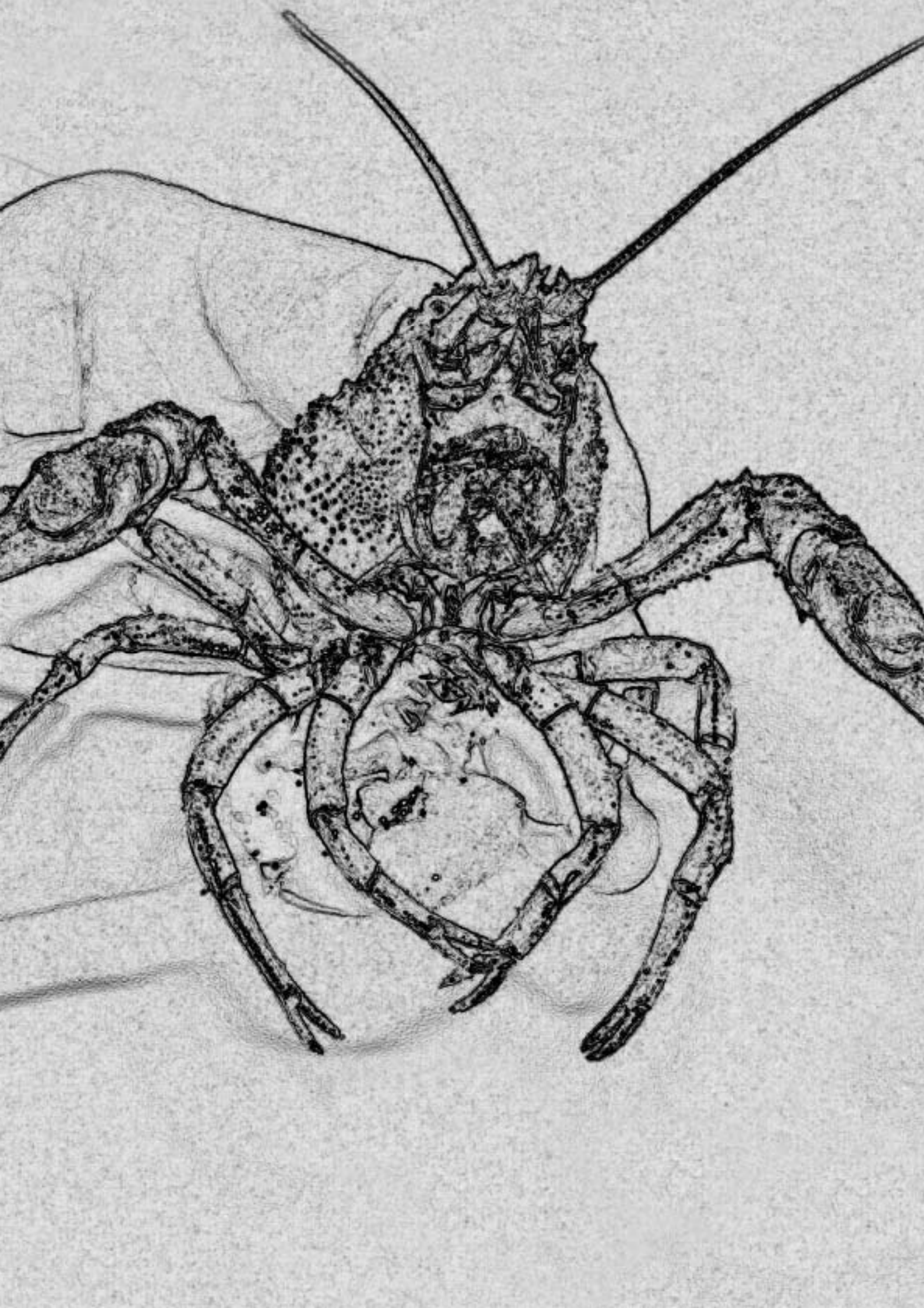
chapter 9

GENERAL DISCUSSION



General discussion

Since its sudden emergence at the start of the nineties, WSSV has had an enormous economical and social impact on the global shrimp production. Unfortunately, measures to control this disease were desperately needed, but not available at the time. In this thesis, the characteristics of several WSSV structural proteins were studied together with their potential as vaccines. In order to assess the potential of the structural proteins to be used as a vaccine, two application systems were evaluated; intramuscular injection and oral application. The structural envelope proteins VP19 and VP28 both induced protection when injected. However, for oral application, which in practice is the more convenient and attractive method, only VP28 showed the potential to be developed into a commercial product. In this general discussion, a short description of the various WSSV structural proteins is given in the context of published data and the experimental results presented in the previous chapters of this thesis, followed by a discussion of the vaccination studies performed with the most promising protein products.

WSSV Structural proteins

The five major structural proteins of WSSV were already identified at the start of the project (Van Hulten *et al*, 2000a,c; van Hulten *et al*., 2002; Nadala *et al*, 1998; Hameed *et al*., 1998; Wang *et al*., 2000; Shih *et al*., 2001; Huang *et al.,* 2002a,b). Coomassie staining and immunodetection revealed that VP26, VP24 and VP15 were located in the nucleocapsid of the virion (Van Hulten *et al*., 2000a). For VP26 there is still some discussion on its position within the virion. In 2002 a report claimed it to be located in the envelope rather than the nucleocapsid. However, because of inaccuracies in the (re-)use of the presented figures, the results are not conclusive. (Zhang *et al*., 2002b). Another report concluded that VP26 resides in between the envelope and nucleocapsid as the authors observed VP26 to be present in both the envelope and nucleocapsid fraction after removal of the envelope (Xie and Yang, 2005). However, this latter report uses a purification process which is considerably different from the one reported by van Hulten (2000a) and might have led to incomplete separation of both fractions. There is no discussion as to the location of VP24 in the nucleocapsid. This protein shares a significant degree of sequence similarity with the strutural proteins VP26 and VP28 suggesting that these proteins evolved by gene duplication (van Hulten *et al*., 2000c). One of the major components of the envelope, VP19 was identified at the same time as the nucleocapsid protein VP15 and both appeared to be not glycosylated, just as all other structural proteins (van Hulten *et al*., 2002). Besides these major structural proteins also a large number of minor (structural) proteins have been identified in WSSV virions (Huang *et al*., 2002ab; Chen *et al*., 2002; Tsai *et al*., 2004; Leu *et al*., 2005), but their structural role remains largely enigmatic.

Its location, basic nature and sequence homology to a number of baculovirus DNA-binding proteins suggested VP15 to be a DNA-binding protein. Using crude bacterial protein samples containing overexpressed VP15, the presence of DNA binding proteins in the

extract, possibly VP15, was suggested (Zhang *et al.*, 2001a). In chapter 2 electrophoretic mobility shift assays, South-Western blots and Dot-spotting experiments convincingly showed that VP15 was indeed able to bind DNA. Moreover, these experiments showed that VP15 has a strong preference for supercoiled DNA over linear single- and double stranded DNA or RNA. This characteristic, also known as supercoiled-selective (SCS) DNA binding (Paleçek *et al*., 1997) makes use of the excess energy contained in supercoiled DNA which is released upon binding of DNA binding proteins such as VP15. These SCS DNA binding properties may be of importance in the packaging of the viral genome during replication as supercoiled DNA is one of the most efficient ways to package DNA and necessary to accommodate large genomes such as WSSV DNA into nucleocapsids.

Subsequent protein-protein interaction studies using ELISA and Far-Western assays showed that VP15 formed homomultimers, but did not interact with any of the other four major structural proteins. The same techniques to study protein-protein interaction also showed that there was no formation of hetero- and homomultimers of the other four major structural proteins. This finding confirms a previous experiment using the yeast two-hybrid system, which also showed the absence of protein-protein interactions (Reijns and van Hulten, unpublished report). To retain the integrity of the virion there should be protein-protein interaction present. Possibly the methods used are not suitable to detect these interactions or the minor proteins of which many are present in the virion are the interacting proteins. Future work using these minor proteins might shed some more light in this issue.

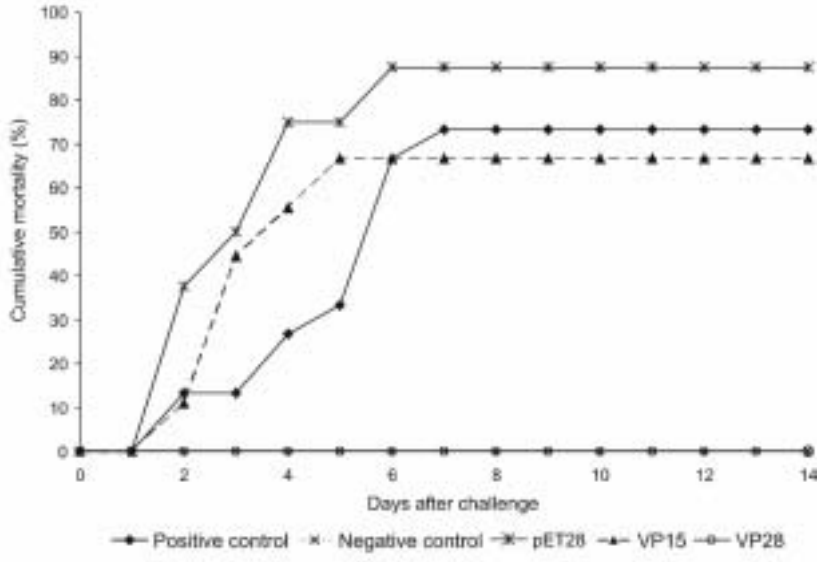


Figure 9.1. Oral vaccination experiment using VP15, pET28 and VP28. Each group consisted of 15 shrimp. The shrimp were challenged via immersion.

Although assumed not to be involved in the initial virus-host interactions, nucleocapsid protein-based vaccines are known from a number of different viruses (Cherpillod *et al.*, 2000; Fooks *et al*., 2000; Zhang *et al*., 2001b). Therefore, VP15 was tested as a vaccine in an oral vaccination experiment in which VP15, VP28, a positive control and a negative control were tested. Vaccination with VP28 resulted in a 100% protection, whereas VP15 did not show any positive effect on the shrimp survival after challenge with WSSV (figure 9.1). Most likely VP15 is not involved in the initial infection process or just not very

immunogenic because of its basic nature. The latter has been confirmed by unsuccessful immunization trials in rats, chickens and rabbits (unpublished results).

Whereas the phosphorylation status of DNA binding or histon-like proteins may be involved in the regulation of gene expression in other viruses (Berger, 2002; Davie and Spencer, 1999; He and Lehming, 2003), VP15 in the virion, infected tissue, eukaryotic and prokaryotic expression systems appeared to be non-phosphorylated. This suggests that the phosphorylation status of VP15 does not have a regulatory role in the gene expression of WSSV. Furthermore, pseudotyping experiments using recombinant p6.9 null baculoviruses showed that VP15 was not able to replace the function of the baculovirus' DNA-binding protein p6.9 (Westenberg *et al*., in preparation) suggesting VP15 employs a significantly different mode of action from that of baculoviral p6.9.

VP28 the most abundant and immunogenic (in rabbits) protein in the envelope of the WSSV virion and therefore of particular interest for vaccination purposes. Neutralization assays showed that the protein is involved in the systemic infection of shrimp as anti-VP28 antiserum could neutralize the virus in a dose-dependent manner (Chapter 3). These findings were partly confirmed by neutralization assays using polyclonal antibodies from egg yolk and rabbit serum against a fusion protein of VP19 and VP28 (Kim *et al.,* 2004 and Li *et al.,* 2005). However, as these studies used fusions of VP19 and VP28 it is impossible to ascertain which fraction of the antibodies (α-VP19 or α-VP28) is responsible for the observed neutralization. Studies using primary cells from the shrimp's lymphoid organ confirmed that VP28 is involved in the systemic infection of shrimp as VP28-GFP fusion proteins were able to bind and even enter shrimp cells and compete with WSSV for cell attachment (Yi *et al*., 2004). The observation of VP28 inside the host cells suggests that the entry of WSSV is not based on fusion of the viral envelope to the host cells, but on a process such as endocytosis. Plaque reduction neutralization assays with these shrimp cells using a polyclonal antibody against VP28 showed that WSSV could be neutralized in this setup, but unfortunately a pre-immune serum was not included (Yi *et al*., 2004).

Later research in Wageningen and Charleston revealed that the use of serum (rabbit) in these types of experiments is not without its drawbacks as it appeared that in some cases pre-immune serum is also capable of neutralizing WSSV (Chapter 4). Moreover, protein-A purified serum lost its neutralization potential, suggesting that the observed inhibition by whole serum is not antibody (IgG) mediated. Until the mechanism behind this observation is known one should take care in interpreting neutralization experiments in general. The concept that VP28 plays an important role in the infectivity of WSSV, supported by published data, may in some cases be open to other interpretations.

Vaccinating shrimp

Before actual vaccination experiments can be performed, several important conditions have to be decided upon. One of the most important decisions is the selection of the antigen that will be used. Another is the method of application e.g. how the vaccine will be administered: injection, immersion or oral application in bioencapsulated form (e.g. via *Artemia*). Further considerations are the method of antigen production and preparation (formulation) and the

method of challenge. For WSSV there are several different challenge options: injection with a virus stock, feeding of infected tissue, or immersion in a WSSV dilution. In this regard also the challenge pressure to be administered is of extreme importance. Finally, there is the importance of shrimp maintenance; at what temperature, salinity and physical environment should the different parts of the experiment be done. For each of the two different vaccination methods (injection and oral) some of the above-mentioned consideration will be discussed.

Injection vaccination

The most interesting vaccination candidates are the proteins which are most likely the first to interact with the hosts' immune system: the virion envelope proteins. In the search for the most interesting candidate for vaccination, several vaccination trials using intramuscular injection of antigens were performed. Although not useful in a practical setup (except for broodstock animals), this method was chosen as it guarantees a controlled and reproducible application of a highly purified antigen. For the same reason intramuscular injection with a titrated virus stock as mode of challenge was selected.

A high challenge pressure might result in the masking of minor differences, in other words, immunological reactions that might be present will not result in changes in the rate of- or final cumulative mortality. On the other hand, a (too) low challenge pressure might obscure minor differences as the control infections already result in low mortalities, leaving a small window for the experimental groups. As the first objective was to find a ‘proof of principle’, a final mortality level of 75% was selected as challenge pressure.

During initial titration experiments, shrimp were maintained in groups within one enclosure after the challenge. However, soon it became apparent that *P. monodon* exhibits high levels of cannibalism as dead or moribund animals were quickly consumed by the remaining animals, resulting in unwanted horizontal transmission of the virus. An experiment comparing individually kept shrimp with shrimp kept in cohorts after challenge clearly showed that by maintaining shrimp in groups after challenge the applied challenge pressure is greatly overestimated (figure 9.2). Based on these observations it was decided to keep all shrimp individually after challenge.

Because there is little known on the vaccination of invertebrates, the first injection vaccination protocols were set up analogous to vertebrate vaccination strategies. Hence, the shrimp were injected twice with approximately 4 µg of purified antigen with a five-day interval, mimicking the ‘booster’ effect as used in vertebrate systems. Two days after this ‘booster’ the shrimp were challenged with WSSV. In a number of preliminary experiments, injection with VP19 resulted in a significant lower mortality or delay in mortality when using a low challenge pressure. When applying a high challenge pressure after vaccination, only injection of a mix of VP19 and VP28 results in a significant improvement in survival (Chapter 5). Possibly when injected alone, only VP19 is able to illicit a detectable immune response in contrast to VP28. When both proteins are injected together the combined effect is greater than the single injections, indicating either that a protein-protein interaction of VP19 and VP28 increases the immune response or that the combined immunological stimulation of the separate proteins greatly enhances the response. In Chapter 6 the focus of the injection

vaccination experiments was directed towards confirmation of the results of chapter 5 and determining the onset and duration of the vaccinations. The experiments confirmed that when challenged two days after the booster injection VP19 and a mixture of VP19 and VP28 results in lower mortalities, but this time also VP28 alone resulted in lower mortalities. For these latter experiments VP28 was expressed fused to the same MBP-tag as used for expression of VP19. This tag might in some way positively influence the effect of the fused protein either by an enhanced presentation of the protein or increased stability. An influence of MBP on the immune response is unlikely as injection of purified MBP did not show a detectable effect. When the challenge period was delayed until 25 days after the booster only injection with VP19 and a mixture of VP19 and VP28 showed a significant positive effect. Overall, injection with VP19 appears to result in a higher level of protection compared to injection with VP28, but is exceeded when a mixture of VP19 and VP28 is injected. For completeness, the other major structural proteins VP15, VP24 and VP26 were tested in the same setups, but none of these proteins showed a similar protection level as observed with VP19 and VP28.

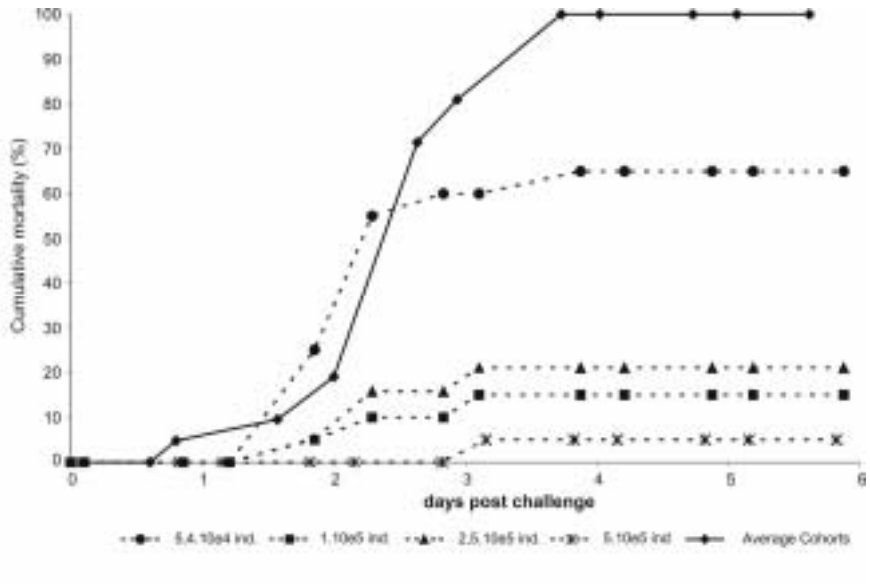


Figure 9.2. Maintenance of shrimp in cohorts vs individually after challenge with WSSV. Four different dilutions were used for the challenge, which are individually plotted for the individually kept shrimp, but plotted as an average for the pooled shrimp (they all reached 100% mortality in approximately the same time.

Based on these experiments a number of conclusions can be drawn. Despite the assumption that invertebrates can only recognize a limited number of conserved products of microbial metabolism (PAMPs), a specific reaction to VP19 and VP28 but not the control proteins was observed. This suggests that invertebrates are capable of specifically recognizing foreign proteins which do not contain patterns recognized by known pattern recognition receptors (PRRs). Since a lower mortality could be observed until at least 25 days post vaccination, this suggest that shrimp also harbor some sort of immunological memory system which was hitherto unknown.

These studies have been a valuable tool for the selection of potential vaccine candidates and proof of principle, however there are inherent drawbacks to this approach. Injection of vaccines, and maybe more importantly WSSV, bypasses a large number of possible (immunological) barriers and sites of immunological interaction. As such, vaccines

applied in this way might not function in an efficient manner or not at all. Moreover, if an immunological reaction is elicited, injection of WSSV could circumvent the areas were these mechanisms are directed to and are not subjected to the full array of the shrimps' immunological responses. Also from a practical, farmers, point of view, the prospect of injecting the entire shrimp population in ponds is not very appealing.

Oral vaccination

Knowing the limitations and drawbacks of vaccination via injection, oral vaccination was tested. This vaccination method does not bypass areas of immunological interaction and is also practically more attainable. For the challenge however, there are a limited number of alternatives to injection. A natural route of infection would be preferable to assure the virus is subjected to all places of immunological interaction in the shrimp. These viral delivery routes could be via infected food through the intestinal tract or via uptake of virions suspended in water through either the gills or again the intestinal tract when ingesting water. Although challenging shrimp using infected material is probably the easiest method and closest to reality, the major constraint is the production of material with a consistent level of virus, ensuring a constant and reproducible challenge pressure. Recently, an oral inoculation protocol, i.e. injecting WSSV straight into the stomach of a shrimp by injection into the mouth cavity, has been developed (Escobedo-Bonilla *et al*, in prep). However, this method can only be used in relatively large animals and results into high levels of stress during the challenge. As an alternative, a challenge protocol via immersion of shrimp in WSSV-containing seawater was tested. This method ensures a constant and reproducible challenge pressure when using the same virus stock. With this challenge method, titration of the virus stock prior to use in the actual vaccination experiments is essential, but easily attainable.

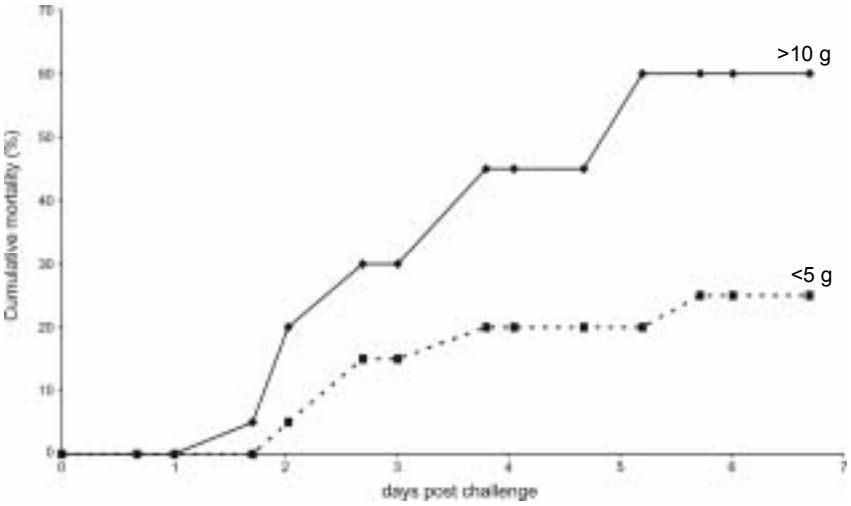


Figure 9.3 The influence of size on susceptibility of *P. monodon* to WSSV after immersion challenge. Two groups of twenty shrimp each were divided in two groups depending on their size and challenged with the same WSSV dosage.

This was confirmed during the first challenge trials when it became obvious that the size of the shrimp is strongly correlated to the observed mortality (Figure 9.3). Apparently, larger shrimp can take up WSSV more efficiently or in larger amounts compared to small shrimp.

Although size dependent differences in the immune status cannot be ruled out, this difference in susceptibility was not observed during injection challenges. The variations may be due to physical differences such as the total gill surface which increases with the shrimp size, increasing the chance of gill-mediated infection. Whatever the underlying mechanism may be, the size of the shrimp has to be included as an extra variable in the correct titration and thus challenge pressure for this challenge method.

Because the vaccine was to be given orally an oral vaccine formulation was developed. To prevent quick degradation and digestion of the presented vaccine it requires a type of bio-encapsulation. Other studies using bio-encapsulated vaccines made use of formaldehyde-inactivated bacteria (Teunissen *et al*., 1998). This approach was also applied on the bacteria overexpressing the WSSV structural proteins (Chapter 7). As bacteria are too small and not very appetizing, they were coated on commercial food pellets, which were subsequently coated with cod-liver oil to prevent quick resuspension of the bacteria when adding it to the water.

Further issues that had to be decided were the amount of vaccine (antigen) that had to be administered and the duration of vaccination. As no information was available on any of these questions, it was arbitrarily decided to vaccinate the shrimp with approximately $4 \cdot 10^8$ - 10^9 bacteria per day for a period of seven days.

Based on experiences in the injection-vaccination and neutralization experiments (chapters 5 and 6), the first candidates for oral vaccination were VP19 and VP28. The results in chapter 7 showed that contrary to the injection vaccination experiments, oral vaccination of VP19 does not improve survival after WSSV challenge. An explanation for this discrepancy could lie in the differences in administration of the vaccine and WSSV challenge used in both vaccination methods. VP19 might not elicit an immune response in shrimp when administered orally, due to rapid degradation of VP19 despite the bio-encapsulation or due to the absence of VP19 receptors (used for either further transportation of VP19 or directly eliciting an immune response) in the intestinal tract. These receptors may be present beyond the intestinal tract and become activated only when VP19 is injected (or infected with WSSV). On the other hand, oral vaccination with VP28 results in a reduction in mortality to a level unlike that observed in the injection vaccinations. Possibly the reverse situation for VP28 exists; it elicits a high reaction in the intestinal tract, but a much lower reaction when injected. Another explanation could lie within the challenge methods: with the immersion challenge method WSSV is confronted with a different set of immunological defenses of the shrimp and may be neutralized by a process which is circumvented by using injection challenge. In that respect, the response on VP28 vaccination via injection could be the same as via oral vaccination, but is obscured because of the challenge method. This leads to an interesting future experiment in which vaccination by injection is followed by challenge via immersion and vice versa. Overall, these oral vaccination experiments have shown that shrimp can specifically recognize and react on WSSV structural proteins.

Besides specific recognition of proteins, the second foundation of an adaptive immune system is memory and therefore also the duration of protection was evaluated (Chapter 7). Although lower than in earlier time points, a reduction in mortality was observed until at least 21 days post vaccination, suggesting the presence of some sort of immunological memory. The mechanism underlying the observed protection is unknown. It

could be that the excess VP28 occupies receptors thereby outcompeting the virus. However, it is hard to perceive a prolonged interaction between VP28 and receptors in the gut/gill to explain the observed long-term protection against the virus. Alternatively, a host defense response could be involved, including the synthesis of antiviral substances. Although in its infancy, there are some reports on the up- and down regulation of several shrimp genes upon infection with WSSV (Bangrak *et al*., 2002, 2004; Rojtinnakorn *et al*., 2002; He *et al*., 2005; Dhar *et al*., 2003).

In an effort to confirm the general principle of oral vaccination of shrimp, another cultured shrimp species of increasing importance, *L. vannamei*, was tested (Chapter 8). Using the setup and parameters that resulted in the highest response in *P. monodon* (seven days vaccination, followed by seven days of normal food and consequent challenge), *L. vannamei* also showed a significantly lower final cumulative mortality compared to the control groups. This shows that a second cultured shrimp species can also be vaccinated and that the underlying mechanism is probably general and conserved between both shrimp species, probably extending to an unknown range of species.

Before the experiments both shrimp species were tested for a number of diseases but only in the case of *L. vannamei* experiments SPF shrimp were used as SPF *P. monodon* shrimp are not (yet) available. Although using infected shrimp for experiments in theory can highly influence the outcome, no indication of this were found in the experiments presented in this thesis, as no symptoms of diseases or PCR positive mortalities in the control shrimp were recorded. This shows that the use of SPF shrimp is not required for these types of experiments, as long as sufficient controls are included and the shrimp are tested before and after the experiments.

Even thought the results are in agreement with an immunological response rather than receptor blocking or interference, the actual mechanism still needs to be elucidated. An experiment was performed to determine if continuous vaccination would further increase protection, which could point towards a mechanism based on receptor blocking/interference. In this experiment shrimp were fed coated food for seven days, challenged three days later and compared to shrimp which continued to receive coated food even after the challenge.

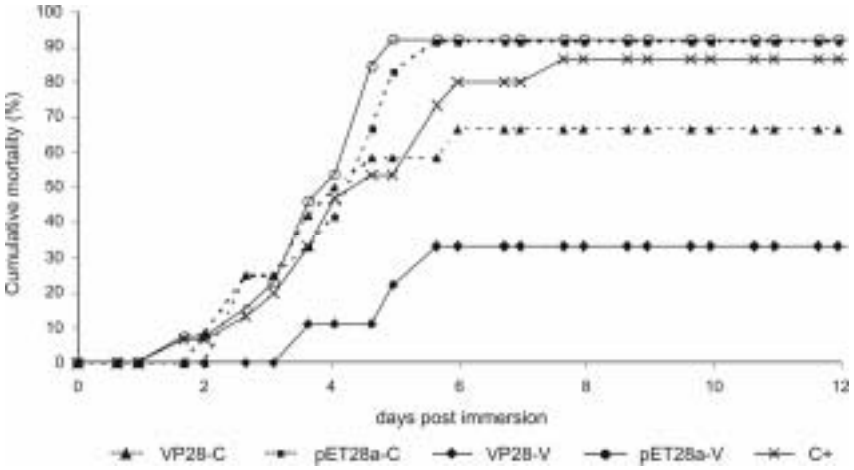


Figure 9.4. Short vaccination vs. continuous vaccination. VP28-V and pET28-V received vaccine for a period of 7 days, whereas the groups VP28-C and pET28-C continued to receive vaccine even after the challenge.

Continuous feeding even has a negative effect on shrimp survival as this group showed a decreased protection compared to the non-continuous vaccinated group (figure 9.4). Although it does not completely answers the question as the negative effect of continuous vaccination could be due to the continuous exposure to bacterial proteins, it does show that continuous vaccination is not advantageous and hence, the observed protection not simply based on competition effects. Future experiments in which the non-continuous vaccinated group receives pET28a control bacteria after the seven days vaccination might shed more light on this matter.

Shrimp immunology: is it vaccination?

There are many different definitions for vaccination, but all include the administration of a product (vaccine) to prevent a specific disease. With vaccination as the main subject of the thesis, this did not seem very promising at the beginning of this study as invertebrates were supposed to lack a true adaptive immune response system and must rely on their innate immune system (Kimbrell and Beutler, 2001). Although considered less sophisticated, this innate immune system is still able to recognize and destroy non-self material, including pathogens (Lee and Söderhäll, 2002). Furthermore, innate immune systems have been evolving for a much longer time compared to the adaptive immune response and must have its (still unknown) benefits as it is used by the majority of organisms (>90%). Despite this lack of an adaptive immune system, immunostimulation of shrimp upon contact with products of microbial origin has already been demonstrated (Song and Hsieh, 1994; Alabi *et al*., 1999). For viral pathogens there is limited information on the immune response. Tests of tissue extracts from crab, shrimp and crayfish against a variety of viruses identified a virus inhibitory molecule in a 440 kDa fraction (Pan *et al*., 2000). Furthermore, an upregulation of lipopolysaccharide, β-1,3-glucan binding protein, protease inhibitors, apoptotic peptides and tumor-related proteins were observed after infection with WSSV (Destoumieux *et al.*, 2000; Rojtinnakorn *et al*., 2002).

In vivo experiments with *P. japonicus* demonstrated the presence of a quasi-immune response when WSSV survivors of both natural and experimental infections were re-challenged with WSSV and exhibited a higher survival compared to naive shrimp. Moreover, haemolymph of the survivors was able to neutralize virus preparations up to two months after infection (Venegas *et al*., 2000). More recent evidence of a specific immune response in crustaceans was found in a study using *Daphnia magna* which demonstrated the maternal transmission of strain-specific immunity as hosts were less likely to be infected by parasites strains with which their mothers were previously challenged with (Little *et al*., 2003). An indication of immunological memory was found in the copepod *Macrocyclops albidus* where individuals were less likely to be reinfected with (antigenically) related lines of a natural parasite compared to more unrelated lines (Kurtz and Franz., 2003). However, as all of the above examples use whole parasites, this increased immunological status might be explained by the recognition of general ‘pathogen associated molecular patterns’ (PAMP’s, e.g. lipopolysaccharide, lipoteichoic acid, mannans, and glycans) by ‘pattern-recognition

receptors' (PRRs). One of the novelties presented in this thesis is the observation that proteins (VP19 and VP28) lacking these PAMPs can also be specifically recognized and acted upon (increased protection) after administration. This suggests an immune system that is able to recognize pathogens via its PAMPs, but also contains a (self-)non-self recognition system, the presence of which has already been suggested by allorecognition experiments (Grosberg, 1988).

However, the question remains whether this observed mechanism is evidence of (self-)non-self recognition and memory or based on a non-immunological mechanism such as receptor binding. One experiment that might shed some light on this issue is challenging VP28-vaccinated shrimp with a different pathogen such as TSV. The result from such an experiment might elucidate if the mechanism really consists of 'specific immunity' (memory) in case vaccination has no effect on another pathogen's success or 'induced immunity' (induced immunity without specificity) in case there is also an increased protection against another pathogen.

If specific memory exists in invertebrates, which underlying mechanism could then be involved? The presence of T-cell receptors and the recombination mechanism such as present in the vertebrate system is unlikely as the number of cells necessary for a homologous system in invertebrates would exceed the available number (Klein, 1989). Differential and long lasting upregulation of the well-known *Toll* and *Imd* pathways might already contain a certain degree of specificity (Boutros *et al*., 2002). Moreover, the recently detected pathway of innate antiviral response (jak-STAT) is specifically activated upon viral infections but further research must elucidate the exact mode of action (Dostert *et al*., 2005). Also receptor molecules such as peptidoglycan recognition molecules and lectins might be differentially upregulated and expressed after infection resulting in specific memory, hard to differentiate from vertebrate adaptive immunity (Steiner, 2004; Kurtz, 2005). Besides these simple and comparatively short-term regulatory mechanisms, more complex types of memory, based on diversity-generating mechanisms might exist. One candidate for this, although much simpler compared to its vertebrate homolog, are fibrogen-related proteins (FREPs) containing one or two Ig superfamily domains and a fibrinogen domain (Zhang *et al*., 2004). Driven by somatic recombinatorial diversification unlike the mechanism found in Ig diversification in vertebrates, a high number of FREPs diversified at the genomic level per individual were identified. Furthermore, even between different vertebrate taxa there are different mechanisms for the somatic diversification of immune receptors (Pancer *et al*., 2004; Bell *et al*., 2003). More evidence of diversification of receptors was found in *Drosophila* immune-competent cells, which were found to be able to express more than 18,000 isoforms of the immunoglobulin (Ig)-superfamily receptor Down syndrome cell adhesion molecule (Dscam). These isoforms were detected in the hemolymph, and hemocyte-specific loss of Dscam impaired the efficiency of phagocytic uptake of bacteria. This molecular diversity of Dscam transcripts is generated through a mechanism of alternative splicing which is highly conserved and points towards an unsuspected molecular complexity of the innate immune system of insects (Watson *et al*., 2005).

All this knowledge suggests that instead of looking for systems homologous to the vertebrate acquired immune system, we have to look for different mechanistic routes by which memory can be achieved (Kurtz, 2005). Considering the current knowledge, it is

plausible to assume that shrimp and even invertebrates in general are equipped with an immune system capable of specific memory. Besides being exciting from a fundamental point of view, the vaccination results described in this thesis imply that vaccinating shrimp against WSSV and other devastating diseases is possible and therefore of high importance for the future of shrimp farming.

Adger, W. N. (1998). Sustainability and social resilience in coastal resource use. CSERGE Working Paper GEC 97-23. Center for Social and Economic Research on the Global Environment. University of East Anglia and University College, London.

Alabi, A. O., Jones, D. A. and Latchford, J. W. (1999). The efficacy of immersion as opposed to oral vaccination of *Penaeus indicus* larvae against *Vibrio harveyi*. *Aquaculture* 178, 1-11.

Amend, D. F. (1981). Potency testing of fish vaccines, p. 447-454. *In* D. P. Anderson and W. Hennessen (ed.), Fish Biologics: Serodiagnostics and Vaccines. S. Karger, Basel, Switzerland.

Anil, T. M., Shankar, K. M. and Mohan, C. V. (2002). Monoclonal antibodies developed for sensitive detection and comparison of white spot syndrome virus isolates in India. *Dis Aquat Organ* 51, 67-75.

Bangrak, P., Graidist, P., Chotigeat, W., Supamattaya, K. and Phongdara, A. (2002) A syntenin-like protein with postsynaptic density protein (PDZ) domains produced by black tiger shrimp *Penaeus monodon* in response to white spot syndrome virus infection. *Dis. Aquat. Org.* 49, 19–25

Bangrak, P., Graidist, P., Chotigeat, W. and Phongdara, A. (2004) Molecular cloning and expression of a mammalian homologue of a translationally controlled tumor protein (TCTP) gene from *Penaeus monodon* shrimp. *J Biotechnol*. 18, 219-26

Banco Central de Ecuador (2002). Infomacion Estadistica Mensual No. 1.803. May 2002

Belcher, C. R. and Young, P. R. (1998). Colourimetric PCR-based detection of monodon baculovirus in whole *Penaeus monodon* postlarvae. *J Virol Methods* 74, 21-9.

Bell, J. K., Mullen, G. E., Leifer, C. A., Mazzoni, A., Davies, D. R. and Segal, D. M. (2003). Leucine-rich repeats and pathogen recognition in Toll-like receptors. *Trends Immunol* 24, 528-33.

Berger S. L. (2002). Histone modifications in transcriptional regulation. *Curr Opin Genet Dev* 12, 142-8

Boutros, M., Agaisse, H. and Perrimon, N. (2002). Sequential activation of signaling pathways during innate immune responses in *Drosophila*. *Dev Cell* 3, 711-22.

Briggs M, Funge-Smith S, Subasinghe R, Philips, M (2004). Introduction and movement of *Penaeus vannamei* and *Penaeus stylirostris* in Asia and the Pacific. *FAO* 2004/10

Bud H. M. and Kelly, D. C. (1980). An electron microscope study of partially lysed baculovirus nucleocapsids: the intranucleocapsid packaging of viral DNA. *J Ultrastruct Res* 73, 361-8

Bull, K., and Spiegelhalter, D. J. (1997). Tutorial in biostatistics: survival analysis in observational studies. *Stat Med* 16,1041-1074.

Burton, D. R., Williamson, R. A., and Parren, P. W. H. I. (2000). Antibody and virus: binding and neutralization. *Virology* 270, 1-3.

Cai, S. L., J. Huang, C. M. Wang, X. L. Song, X. Sun, J. Yu, Y. Zhang and Yang, C.H. (1995). Epidemiological studies on the explosive epidemic disease of prawn in 1993-1994. *J. China Fish* 19, 112-117

Chakraborty, A., Otta, S. K., Joseph, B., Kumar, S., Hossain, M. S., Karunasagar, I., Venugopal, M. N. and Karunasagar, I. (2002). Prevalence of white spot syndrome virus in wild crustaceans along the coast of India Anirban. *Curr Science*. 82, 1392-1397

Chang, P. S., Lo, C. F., Wang, Y. C. and Kou, G. H. (1996). Identification of white spot syndrome associated baculovirus (WSBV) target organs in the shrimp *Penaeus monodon* by in situ hybridization *Dis. Aquat. Org*. 27, 131-139.

Chang, P. S., Chen, H. C. and Wang, Y. C (1998). Detection of white spot syndrome associated baculovirus in experimentally infected wild shrimp, crab and lobsters by in situ hybridization. *Aquaculture*. 164, 233-231

Chen, L. L., Lo, C. F., Chiu, Y. L., Chang, C. F. and Kou, G. H. (2000). Natural and experimental infection of white spot syndrome virus (WSSV) in benthic larvae of mud crab Scylla serrata. *Dis Aquat Organ* 40, 157-61.

Chen, L. L., Leu, J. H., Huang, C. J., Chou, C. M., Chen, S. M., Wang, C. H., Lo, C. F. and Kou, G. H. (2002). Identification of a nucleocapsid protein (VP35) gene of shrimp white spot syndrome virus and characterization of the motif important for targeting VP35 to the nuclei of transfected insect cells. *Virology* 293, 44-53.

Cherpillod, P., Tipold, A., Griot-Wenk, M., Cardozo, C., Schmid, I., Fatzer, R., Schobesberger, M., Zurbriggen, R., Bruckner, L., Roch, F., Vandevelde, M., Wittek, R. and Zurbriggen, A. (2000) DNA vaccine encoding nucleocapsid and surface proteins of wild type canine distemper virus protects its natural host against distemper. *Vaccine* 18, 2927-2936

Chou, H. Y., Huang, C. Y., Wang, C. H., Chiang, H. C. and Lo, C. F. (1995). Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan. *Diseases of Aquatic Organisms* 23, 165-173.

Coca-Prados, M., Vidali, G. and Hsu, M. T. (1980) Intracellular forms of simian virus 40 nucleoprotein complexes. III. Study of histone modifications. *J Virol* 36, 353-60

Corbel, V., Zuprizal, Shi, Z., Huang, C., Sumartono, Arcier, J. M. and Bonami, J. R. (2001). Experimental infection of European crustaceans with white spot syndrome virus (WSSV). *Journal of Fish Diseases.* 24, 377-382.

Dai, H., Gao, H., Zhao, X., Dai, L., Zhang, X., Xiao, N., Zhao, R. and Hemmingsen, S. M. (2003). Construction and characterization of a novel recombinant single-chain variable fragment antibody against White Spot Syndrome Virus from shrimp. *J Immunol Methods* 279, 267-75.

Davie J. R., and Spencer, V. A. (1999). Control of histone modifications. *J Cell Biochem* Suppl 32-33: 141-8

Destoumieux, D., Munoz, M., Cosseau, C., Rodriguez, J., Bulet, P., Comps, M. and Bachere, E. (2000). Penaeidins, antimicrobial peptides with chitin-binding activity, are produced and stored in shrimp granulocytes and released after microbial challenge. *Journal of Cell Science* 113, 461-469.

Dhar, A.K., Dettori, A., Roux, M.M., Klimpel, K.R. and Read, B. (2003) Identification of differentially expressed genes in shrimp (*Penaeus stylirostris*) infected with White spot syndrome virus by cDNA microarrays. *Arch Virol.* 148, 2381-96

Dimmock, N. J. (1984). Mechanisms of neutralization of animal viruses. *J. Gen. Virol.* 65, 1015-1022.

Dostert, C., Jouanguy, E., Irving, P., Troxler, L., Galiana-Arnoux, D., Hetru, C., Hoffmann, J. A. and Imler, J. L. (2005). The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of drosophila. *Nat Immunol* 6, 946-53

Durand, S., Lightner, D. V., Nunan, L., Redman, R. M., Mari, J. and Bonami, J. R. (1996). Application of gene probes as diagnostic tools for White Spot Baculovirus (WSBV) of penaeid shrimp. *Diseases of Aquatic Organisms* 27, 59-66.

Durand, S., Lightner, D. V., Redman, R. M. and Bonami, J. R. (1997). Ultrastructure and morphogenesis of white spot syndrome baculovirus (WSSV). *Dis Aquat Org* 29, 205-211

Duvic, B. and Söderhäll, K. (1990). Purification and characterization of β-1,3-glucan-binding protein from the plasma of the crayfish Pacifastacus leniusculus. *J Biol Chem* 265, 9332–9337.

Edgerton, B. F. (2004). Susceptibility of the Australian freshwater crayfish Cherax destructor albidus to white spot syndrome virus (WSSV). *Dis Aquat Organ* 59, 187-93.

Edwards, P. (2000). Aquaculture, poverty and livelihoods. *Natural Resource Perspectives*, Overseas development institute. 56

Flegel, T. W. (1997). Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. *World Journal of Microbiology and Biotechnology* 13, 433-442.

Food and Agriculture Organization. (1999). Aquaculture pro- duction statistics 1988–1997. Fisheries Circular no. 815 Rev.11. FAO, Rome, 203 pp.

Food and Agriculture Organization. (2003). FishStat Plus: Universal software for fishery statistical time series. http://www.fao.org/fi/statist/FISOFT/FISHPLUS.asp

Fooks, A.R,, Sharpe, S.A., Shallcross, J.A., Clegg, J.C. and Cranage, M.P. (2000) Induction of immunity using oral DNA vaccines expressing the measles virus nucleocapsid protein. *Dev Biol (Basel)*. 104, 65-71

Friedman, P. N., Chen, X., Bargonetti, J., Prives, C. (1993). The p53 protein is an unusually shaped tetramer that binds directly to DNA. *Proc Natl Acad Sci USA* 90, 3319-23

Funk, C. J. and Consigli, R. A. (1993). Phosphate cycling on the basic protein of *Plodia interpunctella* granulosis virus. *Virology* 193, 396-402

Galmich, M. C., Goenaga, J., Wittek, R., and Rindisbacher, L. (1999). Neutralizing and protective antibodies directed against vaccinia virus envelope antigens. *Virology* 254, 71-80.

Grosberg, R. K. (1988). The evolution of allorecognition specificity in clonal invertebrates. *Quartely review of biology* 63, 377-412

Hamatake, R. K., Aoyama, A. and Hayashi. M. (1985). The J gene of bacteriophage phi X174: *in vitro* analysis of J protein function. *J Virol* 54, 345-50

Hamatake, R. K., Buckley, K. J. and Hayashi, M. (1988). The J gene of phi X174: isolation and characterization of a J gene mutant. *Mol Gen Genet* 211, 72-7

Hameed, A. S., Anilkumar, M., Raj, M. L. S. and Jayaraman, K. (1998). Studies on the pathogenicity of systemic ectodermal and mesodermal baculovirus and its detection in shrimp by immunological methods. *Aquaculture* 160, 31-45.

Hameed, A. S., Balasubramanian, G., Musthaq, S. S. and Yoganandhan, K. (2003). Experimental infection of twenty species of Indian marine crabs with white spot syndrome virus (WSSV). *Dis Aquat Organ* 57, 157-61.

He, H. and Lehming, N. (2003). Global effects of histone modifications. *Brief Funct Genomic Proteomic* 2, 234-43

He, N., Qin, Q. and Xu, X. (2005). Differential profile of genes expressed in hemocytes of White Spot Syndrome Virus-resistant shrimp (*Penaeus japonicus*) by combining suppression subtractive hybridization and differential hybridization. *Antiviral Res.* 66, 39-45.

Hossain, M. S., Chakraborty, A., Joseph, B., Otta, S. K., Karunasagar, I. (2001). Detection of new hosts for white spot syndrome virus of shrimp using nested polymerase chain reaction. *Aquaculture* 198,1-11

Hsu, Y. L., Wang, K. H., Yang, Y. H., Tung, M. C., Hu, C. H., Lo, C. F., Wang, C. H. and Hsu, T. (2000). Diagnosis of *Penaeus monodon*-type baculovirus by PCR and by ELISA of occlusion bodies. *Dis Aquat Organ* 40, 93-9.

Huang, C., Zhang, X., Lin, Q., Xu, X., Hu, Z. H. and Hew, C. L. (2002a). Proteomic analysis of shrimp white spot syndrome viral proteins and characterization of a novel envelope protein VP466. *Mol and Cell Prot*. 1, 223-231.

Huang, C., Zhang, X., Lin, Q., Xu, X. and Hew, C. L. (2002b). Characterization of a novel envelope protein (VP281) of shrimp white spot syndrome virus by mass spectrometry. *J Gen Virol* 83, 2385-92.

Hudinaga, M. (1935). Studies on the development of *Penaeus japonicus* (Bate). *Report. Hayatomo Fishery Institute*. 1.

Hudinaga, M. (1942). Reproduction, development and rearing of *P. japonicus*. *Jap. J. Zool.* 10, 305-393.

Inouye K., Miwa, S., Oseko, N., Nakano, K., and Kimura, T. (1994). Mass mortalities of cultured kuruma shrimp, *Penaeus japonicus*, in Japan in 1993: electron microscopic evidence of the causative virus. *Fish Pathology* 29, 149-158.

Itami, T., Yan, Y., and Takahashi, Y. (1989). Efficacy of vaccination against vibriosis in cultured kuruma prawns *Penaeus japonicus*. *J. Aquat. Anim. Health* 1, 234-242.

Itami, T., Asano, M., Tokushige, K., Kubono, K., Nakagawa, A., Takeno, N., Nishimura, H., Maeda, M., Kondo, M. and Takahashi, Y. (1998). Enhancement of disease resistance of kuruma shrimp, *Penaeus japonicus*, after oral administration of peptidoglycan derived from Bifidobacterium thermophilum. *Aquaculture* 164, 277-288.

Jiravanichpaisal, P., Bangyeekhun, E., Söderhäll, K. and Söderhäll, I. (2001). Experimental infection of white spot syndrome virus in freshwater crayfish Pacifastacus leniusculus. *Dis Aquat Organ* 47, 151-7.

Kiatpathomchai, W., Boonsaeng, V., Tassanakajon, A., Wongteerasupaya, C., Jitrapakdee, S. and Panyim, S. (2001). A non-stop, single-tube, semi-nested PCR technique for grading the severity of white spot syndrome virus infections in *Penaeus monodon*. *Dis Aquat Organ* 47, 235-9.

Kim, Y. S., Ryu, J. H., Han, S. J., Choi, K. H., Nam, K. B., Jang, I. H., Lemaitre, B., Brey, P. T. and Lee, W. J. (2000). Gram-negative bacteria-binding protein, a pattern recognition receptor for lipopolysaccharide and β-1,3-glucan protein that mediates the signaling for the induction of innate immune gene in *Drosophila melanogaster* cells, *J Biol Chem* 275, 32721–27

Kim, D. K., Jang, I. K., Seo, H. C., Shin, S. O., Yang, S. Y. and Kim, J.W. (2004). Shrimp protected from WSSV disease by treatment with egg yolk antibodies (IgY) against a truncated fusion protein derived from WSSV. *Aquaculture* 237,21-30

Kimbrell, D. A. and Beutler, B. (2001). The evolution and genetics of innate immunity. *Nat Rev Genet* 2, 256-67.

Kono, T., Savan, R., Sakai, M. and Itami, T. (2004). Detection of white spot syndrome virus in shrimp by loop-mediated isothermal amplification. *J Virol Methods* 115, 59-65.

Kou, G. H., Chen, S. N. and Huang, S. L., (1989). Studies on bacterial infection and vaccination trials for culture *Penaeus monodon* in Taiwan. *Diseases of fish and shellfish*, abstract of the 4th EAFP International conference, 24-28 September 1989, Santiago de Compostella, Spain, p. 94

Kou, G. H., Peng, S. E., Chui, Y. L. and Lo, C. F. (1998). Tissue distribution of white spot syndrome virus in shrimp and crabs. In *Advances in shrimp biotechnology*, pp. 267-271. Edited by T. W. Flegel. Bangkok: National Center for Genetic Engineering and Biotechnology.

Kurtz, J. and Franz, K. (2003). Innate defence: evidence for memory in invertebrate immunity. *Nature* 425, 37-38.

Kurtz. J. (2005). Specific memory within innate immune systems. *Trends Immunol* 26,186-192

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685

Lee, S. Y. and Söderhäll, K. (2002). Early events in crustacean innate immunity. *Fish and Shellfish Immunology* 12, 421-437.

Leu, J. H., Tsai, J. M., Wang, H. C., Wang, A. H., Wang, C. H., Kou, G. H. and Lo, C. F. (2005). The unique stacked rings in the nucleocapsid of the white spot syndrome virus virion are formed by the major structural protein VP664, the largest viral structural protein ever found. *J Virol* 79, 140-9.

Li, H. X., Meng, X. L., Xu, J. P., Lu, W. and Wang, J. (2005). Protection of crayfish, *Cambarus clarkii*, from white spot syndrome virus by polyclonal antibodies against a viral envelope fusion protein. *J Fish Dis* 28, 285-91

Lightner, D. V. (1996). A handbook of pathology and diagnostic procedures for diseases of penaeid shrimp. In *Special publication of the World Aquaculture Society.* LA: Baton Rouge.

Lin, Y. F., Jing, S. R. and Lee, D. Y. (2003). The potential use of constructed wetlands in a recirculating aquaculture system for shrimp culture. *Environ Pollut* 123, 107-13.

Little T. J., O'Connor, B., Colegrave, N., Watt., K. and Read, A. F. (2003). Maternal transfer of strain-specific immunity in an invertebrate. *Curr Biol* 13, 489-492

Liu, W., Wang, Y. T., Tian, D. S., Yin, Z. C. and Kwang, J. (2002). Detection of white spot syndrome virus (WSSV) of shrimp by means of monoclonal antibodies (MAbs) specific to an envelope protein (28 kDa). *Dis Aquat Organ* 49, 11-8.

Lo, C. F., Ho, C. H., Peng, S. E., Chen, C. H., Hsu, H. C., Chiu, Y. L., Chang, C. F., Liu, K. F., Su, M. S., Wang, C. H. and Kou, G. H. (1996). White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods. *Diseases of Aquatic Organisms* 27, 215-225.

Lo, C. F., Ho, C. H., Chen, C. H., Liu, K. F., Chiu, Y. L., Yeh, P. Y., Peng, S. E., Hsu, H. C., Liu, H. C., Chang, C. F., Su, M. S., Wang, C. H. and Kou, G. H. (1997). Detection and tissue tropism of white spot syndrome baculovirus (WSBV) in captured brooders of *Penaeus monodon* with a special emphasis on reproductive organs. *Dis Aqua Org* 30, 53-72.

Lo, C. F., Hsu, H. C., Tsai, M. F., Ho, C. H., Peng, S. E., Kou, G. H. and Lightner, D. V. (1999). Specific genomic DNA fragment analysis of different geographical clinical samples of shrimp white spot syndrome virus. *Dis Aqua Org* 35, 175-185.

Loh, P. C., L. M. Tapay, Y. Lu and Nadala, E. C. (1997). Viral pathogens of the penaeid shrimp. *Adv Virus Res* 48, 263-312.

Lundin, G. G. (1996). Global attempts to address shrimp disease. Marine/Environmental paper No. 4 Lard, water and Natural habitats Division. Environment Department. The World Bank Report, pp. 45.

Maeda S., Kamita, S. G., Kataoka, H. (1991). The basic DNA-binding protein of *Bombyx mori* nuclear polyhedrosis virus: the existence of an additional arginine repeat. *Virology* 180: 807-10

Marks, H., Mennens, M., Vlak, J. M. and van Hulten, M. C. W. (2003). Transcriptional analysis of the white spot syndrome major virion protein genes. *J Gen Virol* 84:1517-1523

Marks, H., Goldbach, R. W., Vlak, J. M. and van Hulten, M. C. (2004). Genetic variation among isolates of White spot syndrome virus. *Arch Virol* 149, 673-97.

Martignoni, M. E., Iwai, P. J., and Rohrmann, G. F. (1980). Serum neutralization of nucleopolyhedrosis viruses (baculovirus subgroup A) pathogenic for *Orgyia pseudotsugata*. *J Invert Path* 36, 12-20.

Mayo, M. A. (2002). A summary of taxonomic changes recently approved by ICTV. *Arch Virol* 147, 1655-63.

Nadala, E. C. B., Jr., Tapay, L. M. and Loh, P. C. (1998). Characterization of a non-occluded baculovirus-like agent pathogenic to penaeid shrimp. *Diseases of Aquatic Organisms* 33, 221-229.

Nadala, E. C., Jr. and Loh, P. C. (2000). Dot-blot nitrocellulose enzyme immunoassays for the detection of white-spot virus and yellow-head virus of penaeid shrimp. *J Virol Methods* 84, 175-9.

Nakano, H., Koube, H., Umezaea, S., Momoyama, K., Hiraoka, M., Inouye, K. and Oseko, N. (1994). Mass Mortalities Of Cultured Kuruma Shrimp *Penaeus Japonicus*, In Japan In 1993: Epizootiological Survey And Infection Trials. *Fish Pathology* 29,135-139.

Nunan, L. M. and Lightner, D. V. (1997). Development of a non radioactive gene probe by PCR for detection of white spot syndrome virus (WSSV). *Journal of Virological Methods* 63, 193-201.

Okumura, T., Nagai, F., Yamamoto, S., Yamano, K., Oseko, N., Inouye, K., Oomura, H. and Sawada, H. (2004). Detection of white spot syndrome virus from stomach tissue homogenate of the kuruma shrimp (*Penaeus japonicus*) by reverse passive latex agglutination. *J Virol Methods* 119, 11-6.

Oren, M. and Rotter, V. (1999). Introduction: p53--the first twenty years. *Cell Mol Life Sci* 55, 9-11

Paleçek, E., Vlk, D., Stankova, V., Brazda. V., Vojtesek, B., Hupp, T. R., Schaper, A. and Jovin, T. M. (1997). Tumor suppressor protein p53 binds preferentially to supercoiled DNA. *Oncogene* 15: 2201-9

Paleçek., E., Brazdova, M., Brazda, V., Paleçek, J., Billova, S., Subramaniam, V. and Jovin, T. M. (2001). Binding of p53 and its core domain to supercoiled DNA. *Eur J Biochem* 268, 573-81

Pan, J., Kurosky, A., Xu, B., Chopra, A. K., Coppenhaver, D. H., Sigh, I. P. and Baron, S. (2000). Broad antiviral activity in tissue of crustaceans. *Antiviral Res* 48, 39-47

Pancer, Z., Amemiya, C. T., Ehrhardt, G. R., Ceitlin, J., Gartland, G. L. and Cooper, M. D. (2004). Somatic diversification of variable lymphocyte receptors in the agnathan sea lamprey. *Nature* 430, 174-80

Perez, J. E., Nirchio, M. and Gomez, J. A. (2000). Aquaculture: part of the problem, not a solution. *Nature* 408(6812): 514.

Poulos, B. T., Pantoja, C. R., Bradley-Dunlop, D., Aguilar, J. and Lightner, D. V. (2001). Development and application of monoclonal antibodies for the detection of white spot syndrome virus of penaeid shrimp. *Dis Aquat Organ* 47, 13-23.

Primavera, J.H. (1991) Intensive prawn farming in the Philippines: ecological, social, and economic implications. *Ambio* 20, 28-33

Prior, S., Browdy, C. L., Shepard, E. F., Laramore, R. and Parnell, P. G. (2003). Controlled bioassay systems for determination of lethal infective doses of tissue homogenates containing Taura syndrome or white spot syndrome virus. *Dis Aquat Organ* 54, 89-96

Quere, R., Commes, T., Marti, J., Bonami, J. R. and Piquemal, D. (2002). White spot syndrome virus and infectious hypodermal and hematopoietic necrosis virus simultaneous diagnosis by miniarray system with colorimetry detection. *J Virol Methods* 105, 189-96.

Rajendran, K. V., Vijayan, K. K., Santiago, T. C. and Krol, R. M. (1999). Experimental host range and histopathology of white spot syndrome virus (WSSV) infection in shrimp, prawns, crabs and lobsters from India. *J. Fish Dis*. 22, 183-191

Ramsey, I. K., Spibey, N., and Jarrett, O. (1998). The receptor binding site of feline leukemia virus surface glycoprotein is distinct from the site involved in virus neutralization. *J. Virol.* 72, 3268-77.

Rodriguez, J., Boulo, V., Mialhe, E., and Bachère, E. (1995). Characterisation of shrimp haemocytes and plasma components by monoclonal antibodies. *J. Cell Sci.* 108, 1043-1050.

Rojtinnakorn, J., Hirono, I., Itami, T., Takahashi, Y. and Aoki, T. (2002). Gene expression in haemocytes of kuruma prawn, *Penaeus japonicus*, in response to infection with WSSV by EST approach. *Fish and Shellfish Immunology* 13, 69-83

Romeo-Figueroa, M. G., Vargas-Requena, C., Sotelo-Mundo, R. R., Vargas-Albores, F., Higuera-Ciapara, I., Söderhäll, K. and Yepiz-Plascencia, G. (2004). Molecular cloning of a β-glucan pattern-recognition lipoprotein from the white shrimp *Penaeus (Litopenaeus) vannamei*: correlations between the deduced amino acid sequence and the native protein structure. *Dev Comp Immunol* 28, 713–726.

Rosenberry, B. (1996). World shrimp farming 1996. *Shrimp News International,* San Diego, California, USA

Rosenberry, B. (1998). World shrimp farming 1998. *Shrimp News International,* San Diego, California, USA

Rosenberry, B. (1999). World shrimp farming 1999. *Shrimp News International,* San Diego, California, USA

Rosenberry, B. (2000). World shrimp farming 2000. *Shrimp News International,* San Diego, California, USA

Rosenberry, B. (2002). World shrimp farming 2002. *Shrimp News International,* San Diego, California, USA

Roux, M. M., Pain, A., Klimpel, K. R. and Dhar, A. K. (2002). The lipopolysaccharide and β-1,3-glucan binding protein gene is upregulated in white spot syndrome virus-infected shrimp (*Penaeus stylirostris*). *J Virology* 76, 7140-7149.

Russell, R. L. and Rohrmann, G. F. (1990). The p6.5 gene region of a nuclear polyhedrosis virus of Orgyia pseudotsugata: DNA sequence and transcriptional analysis of four late genes. *J Gen Virol* 71: 551-60

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Sargent, J. R. and Tacon, A. G. (1999). Development of farmed fish: a nutritionally necessary alternative to meat. *Proc Nutr Soc* 58:377-83

Schofield, D. J., Glamann, J., Emerson, U., and Purchell, R. H. (2000). Identification by phage display and characterization of two neutralizing chimpanzee monoclonal antibodies to the hepatitis E virus capsid protein. *J. Virol.* 74, 5548-5555.

Shi, Z., Huang, C., Zhang, J., Chen, D. and Bonami, J. R. (2000) White spot syndrome virus (WSSV) experimental infection of the freshwater crayfish, Cherax quadricarinatus. *J Fish Diseases* 23, 285-288

Shih, H. H., Wang, C. S., Tan, L. F. and Chen, S. N. (2001). Characterization and application of monoclonal antibodies against white spot syndrome virus. *J Fish Dis* 24, 143-150

Söderhäll, K., Cerenius, L. and Johansson, M. W. (1994). The prophenoloxidase activating system and its role in invertebrate defence. *Ann N Y Acad Sci*. 15, 155-61.

Söderhäll, K. (1999). Invertebrate immunity. *Dev Comp Immun* 23, 263-266.

Song, Y. L. and Hsieh, Y. T. (1994). Immunostimulation of tiger shrimp (*Penaeus monodon*) hemocytes for generation of microbicidal substances: Analysis of reactive oxygen species. *Dev Comp Immun* 18, 201-209.

Steiner, H. (2004). Peptidoglycan recognition proteins: on and off switches for innate immunity. *Immunol Rev* 198,83-96.

Sunyach, C., Rollier, C., Robaczewska, M., Borel, C., Barraud, L., Kay, A., Trepo, C., Will, H., and Cova, L. (1999). Residues critical for duck hepatitis B virus neutralization are involved in host cell interaction. *J. Virol.* 73, 2569-2575.

Supamattaya, K., Hoffmann, R.W., Boonyaratpalin, S. and Kanchanaphum, P. (1998). Experimental transmission of white spot syndrome virus (WSSV) from black tiger shrimp *Penaeus monodon* to the sand crab Portunus pelagicus, mud crab Scylla serrata and krill *Acetes* sp. *Dis Aqua Org* 32:79-85

Takahashi, Y., Fukoda, K., Kondo, M., Chongthaleong, A., Nishi, K., Nishimura, M., Ogata, K., Shinya, I., Takise, K., Fujishima, K. and Matsumura, M. (2003). Detection and prevention of WSSV infection in cultured shrimp. Asian Aquaculture Magazine, November/December, 25-27.

Tan, L. T., Soon, S., Lee, K. L., Shariff, M., Hassan, M. D. and Omar, A. R. (2001). Quantitative analysis of an experimental white spot syndrome virus (WSSV) infection in *Penaeus monodon* Fabricius using competitive polymerase chain reaction. *J Fish Dis* 24, 315-323.

Tapay, L. M., Nadala, E. C. B. and Loh, P. C. (1999). A polymerase chain reaction protocol for the detection of various geographical isolates of white spot virus. *J Vir Meth* 82, 39-43.

Teunissen, O. S. P., Faber, R., Booms, G. H. R., Latscha, T. and Boon, J. H. (1998). Influence of vaccination on vibriosis resistance of the giant black tiger shrimp *Penaeus monodon* (Fabricius). *Aquaculture* 164, 359-366.

Tsai, M. F., Yu, H. T., Tzeng, H. F, Leu, J. H., Chou, C. M., Huang, C. J., Wang, C. H., Lin, J. Y., Kou, G. H. and Lo, C. F. (2000). Identification and characterization of a shrimp white spot syndrome virus (WSSV) gene that encodes a novel chimeric polypeptide of cellular-type thymidine kinase and thymidylate kinase. *Virology* 277, 100-110

Tsai, J. M., Shiau, L. J., Lee, H. H., Chan, P. W. and Lin, C. Y. (2002). Simultaneous detection of white spot syndrome virus (WSSV) and Taura syndrome virus (TSV) by multiplex reverse transcription-polymerase chain reaction (RT-PCR) in pacific white shrimp *Penaeus vannamei*. *Dis Aquat Organ* 50, 9-12.

Tsai, J. M., Wang, H. C., Leu, J. H., Hsiao, H. H., Wang, A. H., Kou, G. H. and Lo, C. F. (2004). Genomic and proteomic analysis of thirty-nine structural proteins of shrimp white spot syndrome virus. *J Virol* 78, 11360-70.

van Hulten, M. C. W., Westenberg, M., Goodall, S. D. and Vlak, J. M. (2000a). Identification of two major virion protein genes of White Spot Syndrome Virus of shrimp. *Virology* 266, 227-236.

van Hulten, M. C. W., Tsai, M. F., Schipper, C. A., Lo, C. F., Kou, G. H. and Vlak, J. M. (2000b). Analysis of a genomic segment of white spot syndrome virus of shrimp containing ribonucleotide reductase genes and repeat regions. *J Gen Virol* 81, 307-316.

van Hulten, M. C. W., Goldbach, R. W. and Vlak, J. M. (2000c). Three functionally diverged major structural proteins of white spot syndrome virus evolved by gene duplication. *J Gen Virol* 81, 2525-2529

van Hulten, M., C. W. and Vlak, J. M. (2001a). Identification and phylogeny of a protein kinase gene of white spot syndrome virus. *Virus Genes.* 22, 201-207.

van Hulten, M. C. W., Witteveldt, J., Snippe, M. and Vlak, J. M. (2001b). White spot syndrome virus envelope protein VP28 is involved in the systemic infection of shrimp. *Virology* 285, 228-233.

van Hulten, M. C. W., Witteveldt, J., Peters, S., Kloosterboer, N., Tarchini, R., Fiers, M., Sandbrink, H., Lankhorst, R. K. and Vlak J. M. (2001c). The white spot syndrome virus DNA genome sequence. *Virology* 286*,* 7-22.

van Hulten, M. C. W., Reijns, M., Vermeesch, A. M., Zandbergen, F. and Vlak, J. M. (2002). Identification of VP19 and VP15 of white spot syndrome virus (WSSV) and glycosylation status of the WSSV major structural proteins. *J Gen Virol* 83: 257-65.

Vaseeharan B, Jayakumar R and Ramasamy P. (2003). PCR-based detection of white spot syndrome virus in cultured and captured crustaceans in India. *Lett Appl Microbiol.* 37, 443-447

Venegas, C. A., Nonaka, L., Mushiake, K., Nishizawa, T. and Muroga, K. (2000). Quasi-immune response of *Penaeus japonicus* to penaeid rod-shaped DNA virus (PRDV). *Dis Aquat Org* 42,83-89.

Vlak, J.M., J.R. Bonami, T.W. Flegel, G.H. Kou, D.V. Lightner, C.F. Loh, P.C. Loh and Walker, P.W. (2005). Nimaviridae. In: *Virus Taxonomy*, VIIIth Report of the International Committee on Taxonomy of Viruses (C.M. Fauquet, M.A. Mayo, J. Maniloff, U. Desselberger and L.A. Ball, Eds.), 187-192. Elsevier/Academic Press, London

Volkman, L. E., and Goldsmith, P. A. (1985). Mechanism of neutralization of budded *Autographa californica* nuclear polyhedrosis virus by a monoclonal antibody: inhibition of entry by adsorptive endocytosis. *Virology* 143, 185-1951.

Volkman, L. E., Goldsmith, P. A., Hess, R. T., and Faulkner, P. (1984). Neutralization of budded *Autographa californica* NPV by a monoclonal antibody: identification of the target antigen. *Virology* 133, 354-362.

Wang, Y. C., Lo, C. F., Chang, P. S. and Kou, G. H. (1998). Experimental infection of white spot baculovirus in some cultured and wild decapods in Taiwan. *Aquaculture* 164, 221-231.

Wang, Y. G., Hassan, M. D., Shariff, M., Zamri, S. M. and Chen, X. (1999). Histopathology and cytopathology of white spot syndrome virus (WSSV) in cultured *Penaeus monodon* from peninsular Malaysia with emphasis on pathogenesis and the mechanism of white spot formation. *Dis Aquat Org* 39, 1-11.

Wang, C. H., Yang, H. N., Tang, C. Y., Lu, C. H., Kou, G. H. and Lo, C. F. (2000a). Ultrastructure of white spot syndrome virus development in primary lymphoid organ cell cultures. *Dis Aquat Org* 41, 91-104.

Wang, Q., Poulos, B. T. and Lightner, D. V. (2000b) Protein analysis of geographic isolates of shrimp white spot syndrome virus. *Archives of Virology* 145, 263-274

Wang, Y. T., Liu, W., Seah, J. N., Lam, C. S., Xiang, J. H., Korzh, V., and Kwang, J. (2002). White spot syndrome virus (WSSV) infects specific hemocytes of the shrimp *Penaeus merguiensis*. *Dis Aquat Organ* 52, 249-59

Watson, F.L., Püttmann-Holgado, R., Thomas, F., Lamar, D.L., Hughes, M., Kondo, M., Rebel, V.I. and Schmucker, D. (2005). Extensive Diversity of Ig-Superfamily Proteins in the Immune System of Insects *Science*, 309, 1874-1878

Wilson, M. E., Mainprize, T. H., Friesen, P. D. and Miller, L. K. (1987). Location, transcription, and sequence of a baculovirus gene encoding a small arginine-rich polypeptide. *J Virol* 61: 661-6

Witteveldt, J., van Hulten, M. C. W. and Vlak, J. M. (2001). Identification and phylogeny of a non-specific endonuclease gene of White Spot Syndrome Virus of shrimp. *Virus Genes* 23, 331-337.

Witteveldt, J., Vlak, J. M., van Hulten, M. C. W. (2004a). Protection of *Penaeus monodon* against white spot syndrome virus using a WSSV subunit vaccine. *Fish Shellfish Immunol* 16: 571-9

Witteveldt, J., Cifuentes, C. C., Vlak, J. M., van Hulten, M. C. W. (2004b). Protection of *Penaeus monodon* against white spot syndrome virus by oral vaccination. *J Virol* 78: 2057-61

Wongteerasupaya, C., Vickers, J. E., Sriurairatana, S., Nash, G. L., Akarajamorn, A., Boonsaeng, V., Panyim, S., Tassanakajon, A., Withyachumnarnkul, B. and Flegel, T. W. (1995). A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn *Penaeus monodon*. *Dis Aquat Org* 21, 69-77.

Wu, J. L., Nishioka, T., Mori, K., Nishizawa, T. & Muroga, K. (2002). A time-course study on the resistance of *Penaeus japonicus* induced by artificial infection with white spot syndrome virus. *Fish Shellfish Immunol* 13, 391-403.

Wu, W., Wang, L. and Zhang, X. (2005). Identification of white spot syndrome virus (WSSV) envelope proteins involved in shrimp infection. *Virology* 332, 578-83

Wyban, J. A. and Sweeney, J. N. (1991). Intensive shrimp production technology. *High Health Aquaculture Inc.*, Hawaii. 158 pp.

Xie, X. and Yang, F. (2005). Interaction of white spot syndrome virus VP26 protein with actin. *Virology* 336, 93-99

Yang, F., Wang, W., Chen, R. Z. and Xu, X. (1997). A simple and efficient method for purification of prawn baculovirus DNA. *J Virol Meth* 67, 1-4.

Yang, F., He, J., Lin, X., Li, Q., Pan, D., Zhang, X. and Xu, X. (2001). Complete genome sequence of the shrimp white spot bacilliform virus. *J Virol* 75:11811-20.

You, Z., Nadala, E. C., Jr., Yang, J., van Hulten, M. C. and Loh, P. C. (2002). Production of polyclonal antiserum specific to the 27.5 kDa envelope protein of white spot syndrome virus. *Dis Aquat Org* 51, 77-80.

Yi, G., Wang, Z., Qi, Y., Yao, L., Qian, J. and Hu, L. (2004) Vp28 of shrimp white spot syndrome virus is involved in the attachment and penetration into shrimp cells. *J Biochem Mol Biol* 37, 726-34.

Zhan, W. B., Chen, J., Zhang, Z. D., Zhou, L. and Fukuda, H. (2003). Elimination of shrimp endogenous peroxidase background in immunodot blot assays to detect white spot syndrome virus (WSSV). *Dis Aquat Org* 53, 263-5.

Zhang, X., Xu, X. and Hew, C. L. (2001a). The structure and function of a gene encoding a basic peptide from prawn white spot syndrome virus. *Virus Res* 79, 137-44

Zhang, M., Emerson, S.U., Nguyen, H., Engle, R.E., Govindarajan, S., Gerin, J.L., and Purcell, R.H. (2001b) Immunogenicity and protective efficacy of a vaccine prepared from 53 kDa truncated hepatitis E virus capsid protein expressed in insect cells. *Vaccine*. 12, 853-7.

Zhang, X., Huang, C., Xu, X. and Hew, C. L. (2002a). Identification and localization of a prawn white spot syndrome virus gene that encodes an envelope protein. *J Gen Virol* 83, 1069-74.

Zhang, X., Huang, C., Xu, X. and Hew, C. L. (2002b). Transcription and identification of an envelope protein gene (p22) from shrimp white spot syndrome virus. *J Gen Virol* 83, 471-7.

Zhang, X., Huang, C., Tang, X., Zhuang, Y. and Hew, C. L. (2004). Identification of structural proteins from shrimp white spot syndrome virus (WSSV) by 2DE-MS. *Proteins* 55, 229-35

More than a decade after its discovery in South-East Asia, White Spot Syndrome Virus (WSSV) is still the most important (viral) pathogen in the shrimp culture industry. Despite the shift from culturing *Penaeus monodon* towards the presumed less susceptible *Litopenaeus vannamei*, the use of specific pathogen free shrimp and the development of more advanced shrimp culturing techniques, WSSV continues to scourge shrimp farms. Therefore there is an urgent need for effective intervention strategies. Vaccination is the generally used method to prevent viral infections in vertebrates. The success of this method depends on the immunological memory generated by the adaptive immune system. Unfortunately, shrimps, as any other arthropod, do not have such an adaptive immune system implying that vaccination would never work. However, some phenomenological observations have been made, indicating that there might be an analogous defense system present in shrimp. With this in mind experiments in this thesis are presented to determine if and how shrimp can be protected against WSSV via vaccination.

At the start of this research project several studies were available describing various major structural proteins present in the WSSV virion including the major virion envelope proteins VP28 and VP19 and virion structural proteins VP26, VP24 and VP15 (see thesis van Hulten, 2001). In this research a number of these proteins were investigated in more detail as potential vaccine candidates. For one of these, the major nucleocapsid protein VP15, it was determined that it was probably (one of) the DNA-binding protein(s) of WSSV (Chapter 2). Experiments revealed that VP15 binds non-specifically to double-stranded DNA, but has a strong preference to supercoiled DNA, suggesting a possible role in the packaging of the WSSV genome. Furthermore, VP15 forms homomultimers but does not interact with any of the other major WSSV structural proteins and unlike other basic DNA-binding proteins VP15 was not phosphorylated.

The next structural protein studied in more detail was VP28 which, because of its abundance and location in the envelope of the WSSV virion, was another potential candidate for use as a WSSV vaccine. The involvement of the VP28 protein in the infection process of WSSV was studied in virus neutralization experiments using polyclonal antibodies generated against the VP28 protein in rabbit (Chapter 3). The antiserum neutralized WSSV infection of *P. monodon* in a dose-dependent manner, whereas the pre-immune rabbit serum did not. These results suggested that VP28 is located on the surface of the virus particle and is likely to play a key role in the initial steps of the systemic WSSV infection in shrimp. Although the results from the neutralization experiments seemed conclusive, further research revealed that the observed neutralization is probably not IgG-based. Experiments showed that some rabbit pre-immune sera are already able to neutralize WSSV and furthermore, purified IgG from sera that neutralized WSSV was not able to neutralize the virus (Chapter 4). Therefore, it could be concluded that in most cases the neutralization is not antibody based, but caused by unidentified serum components.

VP28 and the other major envelope protein VP19 were tested in vaccination and challenge experiments. The first experiments were performed via injection of the antigens and virus as this guaranteed a controlled and reproducible application. Injection with recombinant MBP-VP19 or a mixture of MBP-VP19 and His_6-VP28 significantly reduced and delayed mortality upon WSSV challenge, suggesting a specific role of VP19 in the systemic defense response of shrimp (Chapter 5). To study the onset and duration of the vaccination,

groups of shrimp were challenged two or twenty-five days after vaccination. After the challenge, VP19-vaccinated shrimp showed a significant better survival compared to the controls with a Relative Percent Survival (RPS) of 33% and 57% at two and 25 days after vaccination, respectively. Also the groups vaccinated with VP28 and a mixture of VP19 and VP28 showed a significantly better survival challenged two days after vaccination (RPS of 44% and 33% respectively), but no longer after twenty-five days (Chapter 6).

Although these injection experiments clearly showed that shrimp are indeed capable of specifically recognizing foreign proteins and exhibit a kind of adaptive memory, the injection vaccination technique is far from suited for use under shrimp farming conditions. Therefore the potential of oral vaccination of shrimp using the same viral envelope proteins was investigated (Chapter 7). In this setup *P. monodon* shrimp were fed commercial food pellets coated with inactivated bacteria that overexpressed both envelope proteins VP19 and VP28. In order to approach the natural route of WSSV infection and subject the virus to the full array of immunological responses of the shrimp, the challenge was performed via immersion of the shrimp in WSSV containing seawater. When the challenge was performed three days after a seven-day vaccination period, VP28 vaccinated shrimp showed a significant lower cumulative mortality compared to shrimp vaccinated with bacteria containing empty vectors (RPS of 61%), while vaccination with VP19 provided no protection. To determine the onset and duration of protection of VP28, challenges were performed three, seven and twenty-one days after the seven-day vaccination period. A significantly higher survival was observed both three and seven days post vaccination (RPS of 64% and 77%, respectively), but the protection was reduced twenty-one days after the vaccination (RPS of 29%). These results strongly suggest that a specific immune response and ultimately protection can be generated in an invertebrate species like shrimp.

In an effort to investigate whether the oral vaccination effects were limited to *P. monodon* or based on more universal mechanisms, the vaccination experiments were applied to an alternative host for WSSV, the Pacific White shrimp *Litopenaeus vannamei* (Chapter 8). Also this species showed a significantly lower cumulative mortality upon VP28 vaccination compared to the control groups. This outcome points to a shared and therefore universal adaptive response mechanism present in crustaceans. It is still not clear whether this response is WSSV specific or more generally directed against viruses.

In recent years more evidence has become available suggesting the presence of a specific immune response and adaptive memory in invertebrates. The results presented in this thesis support this view by showing that the shrimp's immune system is able to specifically recognize and react upon WSSV structural proteins or more in general, proteins lacking known pathogen associated molecular patterns. Furthermore, the studies described in this thesis have shown that vaccination of shrimp against WSSV can be successful, which opens the way to the design of new strategies to control WSSV and other invertebrate pathogens.

Een decennium na de ontdekking van het “Witte-vlekken-syndroom-virus” (WSSV) in Zuid-oost Azië is dit virus nog steeds de belangrijkste veroorzaker van ziekte in de wereldwijde garnalenkweek. Ondanks de grootschalige overstap van de tijgergarnaal *Penaeus monodon* naar de veronderstelde resistentere *Litopenaeus vannamei*, het gebruik van ziektevrij uitgangsmateriaal en de ontwikkeling van geavanceerde teeltmethoden blijft WSSV de industrie teisteren. Er is daarom een grote vraag naar de ontwikkeling van een werkzame interventiestrategie.

Voor virusziektes in vertebraten is de meest gebruikte en meest succesvolle interventiestrategie vaccinatie. Het succes van vaccinaties valt of staat echter met het opwekken van een immuunreactie en immunologisch geheugen, gereguleerd door het adaptieve immuunsysteem. Helaas hebben garnalen, net als alle andere ongewervelde dieren, ogenschijnlijk geen adaptief immuunsysteem, met als gevolg dat vaccineren in garnalen wellicht niet zal werken.

Ondanks dat zijn er de laatste jaren verschillende fenomenologische aanwijzingen naar voren gekomen die op de aanwezigheid van afweersystemen in ongewervelden wijzen, analoog aan die bij gewervelde dieren. Met dit in het achterhoofd zijn tijdens dit onderzoek experimenten uitgevoerd om vast te stellen of garnalen via vaccinatie toch tegen WSSV beschermd kunnen worden.

Bij het begin van het onderzoek waren verschillende structurele eiwitten van het WSSV virion, waaronder de envelop-eiwitten VP28 en VP19 en de nucleocapside eiwitten VP15, VP24 en VP26 reeds beschreven. Voor het in dit proefschrift gepresenteerde onderzoek is een aantal van deze structurele eiwitten verder onderzocht op hun mogelijke werking in een vaccin, dat beschrming biedt tegen WSSV infectie.

Voor een van de nucleocapside-eiwitten, VP15, werd ontdekt dat het een DNA-bindend eiwit van WSSV is (Hoofdstuk 2). Bovendien werd aangetoond dat dit eiwit niet-specifiek bindt aan dubbel-strengs DNA, maar wel een sterke voorkeur heeft voor ‘supercoiled’ DNA, wat kan wijzen op een mogelijke rol van VP15 bij het inpakken van het WSSV-genoom. Ook werd vastgesteld dat VP15 homomultimeren vormt, maar geen interacties aangaat met de andere structurele eiwitten van WSSV en, in tegenstelling tot andere DNA-bindende eiwitten, het niet gefosforyleerd is.

Het grote aandeel in het virion van WSSV en de locatie in de envelop maakt het WSSV-envelop-eiwit VP28 tot een potentiële vaccinkandidaat en werd daarom nader onderzocht. Informatie over de betrokkenheid van dit eiwit bij het infectieproces van WSSV werd verkregen door neutralisatie-experimenten met polyklonale antilichamen, opgewekt tegen VP28 in konijnen (Hoofdstuk 3). In *P. monodon* bleek serum van deze geïmmuniseerde konijnen een WSSV-infectie op een concentratie-afhankelijke wijze te neutraliseren, terwijl pre-immuun serum dit niet deed. Deze resultaten suggereren dat VP28 is gelocaliseerd aan de buitenkant van het virion en mogelijk een belangrijke rol speelt in de beginfase van de WSSV-infectie.

Hoewel de resultaten overtuigend waren, bleek uit vervolgonderzoek dat de eerder gevonden neutralisaties waarschijnlijk niet op immunoglobuline (Ig) G’s gebaseerd zijn. Ook bleken sommige pre-immuun-sera van konijnen in staat WSSV te neutraliseren en dat IgG’s, opgezuiverd uit sera die eerder in staat waren WSSV te neutraliseren (Hoofdstuk 3), dit nu niet konden (Hoofdstuk 4). De conclusie die getrokken kon worden is dat in de meeste

gevallen neutralisatie van WSSV niet op antilichamen, maar op voor nu nog onbekende serumcomponenten lijkt te zijn gebasseerd.

VP28 en het andere envelop-eiwit VP19 werden geselecteerd als belangrijkste kandidaten voor vaccinatie- en challenge-experimenten. Om een gecontroleerde en reproduceerbare toediening van antigeen en virus te waarborgen werd voor de eerste vaccinatie en challenge experimenten voor injectie van garnaal gekozen. Injectie met recombinant VP19 gefuseerd met het maltose-binding-protein (MBP) of een combinatie van MBP-VP19 en His_6-VP28 (VP28 met een histidinestaart) resulteerde in een significante daling en vertraging van mortaliteit na een 'challenge' met WSSV. Dit duidt op een mogelijke rol van VP19 in de systemische afweerreactie van de garnaal (Hoofdstuk 5). Om meer over het begin en de duur van de bescherming te weten te komen, werden groepen garnalen op twee en vijfentwintig dagen na vaccinatie met WSSV 'gechallenged'. Wederom lieten met VP19 gevaccineerde garnalen een significant hogere overleving zien ($p<0.05$) in vergelijking met de controles, resulterend in relatieve overlevingspercentages (RPS) van 33% en 57%, respectievelijk twee en vijfentwintig dagen na vaccinatie. Ook de groepen gevaccineerd met MBP-VP28 en een mengsel van MBP-VP19 en MBP-VP28 lieten een hoger overlevingspercentage zien twee dagen na vaccinatie (RPS van respectievelijk 44% en 33%). Na vijfentwintig dagen was het verschil verdwenen (Hoofdstuk 6). De injectie-vaccinatie-experimenten tonen duidelijk aan dat garnalen in staat zijn lichaamsvreemde eiwitten te herkennen en mogelijk in het bezit zijn van een soort adaptief geheugen.

Echter, de gebruikte injectie-techniek is in een kweeksituatie onpractisch en dus onbruikbaar. Vandaar dat de mogelijkheid om via orale vaccinatie van dezelfde eiwitten garnalen te beschermen is getest (Hoofdstuk 7). *P. monodon* garnalen werden gevaccineerd door het aanbieden van voerkorrels bedekt met geïnactiveerde bacteriën, die VP19 of VP28 tot overexpressie brengen. Om een natuurlijke infectieroute na te bootsen en het virus bloot te stellen aan het gehele immunologische repertoire van de gastheer werden de garnalen via immersie in zeewater met WSSV 'gechallenged'. Wanneer de 'challenge' drie dagen na de zevendaagse vaccinatieperiode werd uitgevoerd, resulteerde vaccinatie met VP28 in een significant lagere mortaliteit vergeleken met garnalen die bacteriën kregen zonder VP28 (RPS van 61%), terwijl vaccinatie met VP19 geen significant verschil met de controles liet zien. Om ook bij deze vaccinatiemethode het begin en duur van het vaccinatiegeheugen vast te stellen werden garnalen drie, zeven en eenentwintig dagen na vaccinatie 'gechallenged' met WSSV. Een significant hogere overleving werd gevonden wanneer de 'challenge' drie en zeven dagen na vaccinatie plaatsvond (RPS van respectievelijk 64% en 77%); bij een 'challenge' eenentwintig dagen na vaccinatie was het effect gereduceerd tot een RPS van 29%. Deze resultaten duiden wederom op de aanwezigheid van een specifieke immuunrespons en de mogelijkheid tot het verhogen van de overleving van garnalen via vaccinatie na infectie met WSSV.

Om vast te stellen of deze succesvolle orale vaccinatie beperkt is tot de garnaal *P. monodon*, of dat de relatieve bescherming tegen WSSV gebaseerd is op een meer algemeen voorkomend mechanisme, werden er vaccinaties uitgevoerd van een andere gastheer van WSSV, de Pacifische witte garnaal, *Litopenaeus vannamei* (Hoofdstuk 8). Ook deze soort vertoonde een significant lagere mortaliteit na vaccinatie met VP28 in vergelijking met controles. Daarmee lijkt aangetoond dat orale vaccinatie van de twee commercieel

belangrijkste garnalensoorten mogelijk is en dat de verhoogde bescherming gebaseerd is op een algemeen voorkomend, op een geheugen gebaseerd mechanisme. Het is echter nog niet duidelijk of dit mechanisme WSSV-specifiek of in het algemeen tegen virussen is gericht.

De laatste jaren is er een toenemend aantal aanwijzingen gekomen, die duiden op de aanwezigheid van een specifiek, op geheugen gebaseerd immuunsysteem in ongewervelde dieren. De resultaten van de experimenten gepresenteerd in dit proefschrift onderschrijven deze aanwijzingen door aan te tonen dat het immuunsysteem van garnalen in staat is structurele eiwitten van WSSV, of algemener gesteld, eiwitten zonder bekende pathogeen-geassocieerde moleculaire patronen te herkennen en daarop te reageren. Bovendien bestaat nu de mogelijkheid garnalen tegen WSSV te vaccineren, waarmee een nieuwe richting in de strijd tegen dit virus en andere ziekteverwekkers van ongewervelde dieren kan worden ingeslagen.

Het nawoord, als laatste geschreven maar als eerste gelezen. Een hoofdstuk dat ik het liefst zo kort mogelijk had gehouden opdat niemand vergeten zou worden (iedereen bedankt!), maar waarvoor ik toch wat meer woorden nodig heb.

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Op 15 april 1975 werd ik, Jeroen Witteveldt, geboren in Purmerend. Op mijn 18e levensjaar behaalde ik mijn VWO-diploma aan het St. Ignatius college te Purmerend en vertrok kort daarna naar Wageningen om aan de studie ‘Plantenveredeling en Gewasbescherming’ te beginnen aan de toenmalige Landbouw Universiteit Wageningen. Na een zeer gevarieerde stage in Costa Rica in samenwerking met de Universiteit van Amsterdam begon mijn interesse meer richting de kleinere organismen te verschuiven en begon mij tijdens mijn doctoraalfase derhalve steeds meer te verdiepen in de entomologie en virologie. Na een afstudeervak bij entomologie en een stage aan de Universiteit van Californië, Riverside, USA, rondde ik mijn studie af met een afstudeervak bij het Laboratorium voor Virologie. Na het behalen van mijn doctoraaldiploma kon ik onder leiding van dr. ir. M.C.W. van Hulten en Prof. Dr. J.M. Vlak op dezelfde plek als gastmedewerker komen werken, een functie die begin 2001 in een AIO positie werd omgezet en waarvan de resultaten in dit proefschrift beschreven zijn. Begin 2006 zal ik als Postdoc beginnen bij de MRC virology unit in Glasgow, Schotland.

Witteveldt, J., van Hulten, M. C. W. and Vlak, J. M. (2001). Identification and phylogeny of a non-specific endonuclease gene of white spot syndrome virus of shrimp. *Virus Genes* **23**, 331-7.

Witteveldt, J., Jolink, M., Cifuentes, C. E., Vlak, J. M. and van Hulten, M. C. W. (2002). Vaccination of *Penaeus monodon* against White Spot Syndrome Virus using structural virion proteins. Proceedings of the 5th Symposium on Diseases in Asian Aquaculture (P.J. Walker *et al*. , eds), Brisbane, Australia, Nov. 24-28, 2002

Witteveldt, J., Vlak, J. M. and van Hulten, M. C. W. (2004). Protection of *Penaeus monodon* against white spot syndrome virus using a WSSV subunit vaccine. *Fish and Shellfish Immunology* **16**, 571-9.

Witteveldt, J., Cifuentes, C. E., Vlak, J. M. and van Hulten, M. C. (2004). Protection of *Penaeus monodon* against white spot syndrome virus by oral vaccination. *Journal of Virology* **78**, 2057-61.

Witteveldt, J., Vermeesch, A. M. G., Langenhof, M., de Lang, A., Vlak, J. M. and van Hulten, M.C.W. (2005). Nucleocapsid protein VP15 is the basic DNA binding protein of white spot syndrome virus of shrimp. *Archives of Virology* **150**,1121-1133

Witteveldt, J., Vlak, J. M. and van Hulten, M. C. W. Increased tolerance of *Litopenaeus vannamei* to white spot syndrome virus infection after oral application of the viral envelope protein VP28. *Diseases of Aquatic Organisms* (accepted)

van Hulten, M. C., **Witteveldt, J**., Snippe, M. and Vlak, J. M. (2001). White spot syndrome virus envelope protein VP28 is involved in the systemic infection of shrimp. *Virology* **285**, 228-33.

van Hulten, M. C. W., **Witteveldt, J**., Peters, S., Kloosterboer, N., Tarchini, R., Fiers, M., Sandbrink, H., Lankhorst, R. K. and Vlak, J. M. (2001). The white spot syndrome virus DNA genome sequence. *Virology* **286**, 7-22.

Robalino, J., Payne, C., Parnell, P., Shepard, E., Grimes, A. C., Metz, A., Prior, S., **Witteveldt, J**., Vlak, J. M., Gross, P. S., Warr, G. and Browdy, C. L. Non-specific inactivation of White Spot Syndrome Virus (WSSV) by normal rabbit serum: Re-assessing the role of the envelope protein vp28 in WSSV infection of shrimp. *Virus Research* (accepted)

Marks, H., Ren, X., **Witteveldt, J**., Sandbrink, H., Vlak, J. M. and van Hulten, M. C. W. (2002). Transcription regulation and genomics of White Spot Syndrome Virus. Proceedings of the 5th Symposium on Diseases in Asian Aquaculture (P.J. Walker *et al*. , eds), Brisbane, Australia, Nov. 24-28, 2002

Zuidema, D., van Hulten, M. C. W., Marks, H., **Witteveldt, J**. and Vlak, J. M. (2004). Virus-Host Interactions of White Spot Syndrome Virus. In: Current Trends in the Study of Bacterial and Viral Fish and Shrimp Diseases. pp. 237-255. Edited by K. Y. Leung. Singapore: World Scientific Publishing Cp. Pte. Ltd.

van Meer, M. M. M., **Witteveldt, J**. and Stouthamer, R. (1999). Phylogeny of the arthropod endosymbiont *Wolbachia* based on the *wsp* gene. *Insect Molecular Biology* **8**, 399-408

van Meer, M. M. M., **Witteveldt, J**. and Stouthamer, R. (1999). Development of a micro injection protocol for the parasitoid *Nasonia vitripennis*. *Proceedings of the section Experimental and Applied Entomology of the Netherlands Entomological Society* **93**, 325-329.

PE&RC PhD Education Statement Form

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 22 credits (= 32 ECTS = 22 weeks of activities)

Review of Literature (3 credits)

- Development of White Spot Syndrome Virus (WSSV) vaccination strategies in the black tiger shrimp (*Penaeus monodon*) (2001-2005)

Writing of Project Proposal (3 credits)

- Development of White Spot Syndrome Virus (WSSV) vaccination strategies in the black tiger shrimp (*Penaeus monodon*) (2001)

Post-Graduate Courses (3 credits)

- Fish Vaccination (2002)
- Fish Immunology (2003)
- Meetings of the "NWO-CW study group nucleic acids" (2001-2004)

Deficiency, Refresh, Brush-up and General Courses (2 credits)

- Veilig werken met radioactieve stoffen en stralingsbronnen (2001)
- Time Planning and Project Management (2004)

PhD Discussion Groups (3 credits)

- Weekly work discussion meetings and monthly literature survey meetings attended by PhD students, undergraduate students and staff of the Laboratory of Virology (2001-2005)

PE&RC Annual Meetings, Seminars and Introduction Days (1.5 credits)

- PE&RC annual meeting: 'Food Insecurity'(2001)
- PE&RC annual meeting: 'Ethics in science' (2002)
- PE&RC annual meeting: 'Global climate change and biodiversity' (2003)
- PE&RC weekend (2003)
- PE&RC annual meeting: 'Biological disasters' (2004)

International Symposia, Workshops and Conferences (6 credits)

- Dutch Annual Virology Symposia, University of Utrecht (2002-2005)
- 34th Annual Meeting of the Society for Invertebrate Pathology (S.I.P.) (2001)
- IUMS the World of Microbes, Paris, France (2002)
- 5th Symposium on Diseases in Asian Aquaculture (2002)
- Innate Immunity Workshop, Plön, Germany (2004)
- World Aquaculture 2005, Bali, Indonesia (2005)

Laboratory Training and Working Visits (2.5 credits)

- Vaccination studies in several hosts. Intervet/Norbio R&D Singapore, Lab of Dr. L. Grisez (2003)

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