White Spot Syndrome – What We Have Learned about the Virus and the Disease

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ABSTRACT

One of the lessons ultimately learned from the first dramatic outbreaks of white spot syndrome virus (and other shrimp viral diseases) in the early 1990's was that aquaculture management practices needed to be improved. Subsequent research revealed that critical factors included: broodstock sourcing, postlarva sourcing, screening techniques and strategies, diverse transmission pathways, critical infection levels, disease susceptibility, and stressors. In Taiwan, Penaeus monodon brooders are usually captured from the wild. In this wild populations WSSV prevalence runs at about 58-67%, and the infected brooders may pass the virus on to their offspring (via transovum transmission). Nauplii infection status appears to be a key indication in predicting the subsequent outcomes of culturing in grow-out ponds, but it relatively inconvenient and expensive to measure nauplii infection status directly. We have found, however, that only heavily infected brooders are likely to produce heavily infected nauplii, and these brooders can be recognized because they are WSSV positive even before spawning. Conversely, lightly infected brooders (i.e. brooders that only become WSSV positive after spawning; the stress of spawning triggers rapid replication of the virus) and WSSV-free brooders produce nauplii that are, at worse, only lightly infected. These lightly infected nauplii are still able to perform well in grow-out ponds. This paper reviews the work done in the last decade by several research groups, and shows how studies on the key aspects of the biology of WSSV infection have led to improved disease management solutions that are now widely used by the shrimp aquaculture industry.

BACKGROUND: THE VIRUS ITSELF

White spot syndrome virus (WSSV) was initially described as a non-occluded baculovirus, but even while the molecular data were still limited, (the preliminary WSSV-DNA sequence analysis), the morphological characteristics and the general biological properties of the virus had already highlighted its uniqueness (Lo *et al*., 1996a; 1997; Wongteerasupaya *et al*., 1996). Recent data, including the genome sequence and phylogenies based on DNA polymerase and protein kinase, suggest that WSSV is a member of a new virus family tentatively named as Nimaviridae (Liu *et al*., 2001; van Hulten *et al*., 2001a; Vlak *et al*., 2001; Yang *et al*., 2001; Chen *et al*., 2002a). The size of the WSSV genome has been differently reported for different isolates: 305107 bp (GenBank Accession No. AF332093; Yang *et al*., 2001), 292967 bp (GenBank Accession No. AF369029; van Hulten *et al*., 2001a),

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and 307287 bp (GenBank Accession No. AF440570) for viruses isolated from China, Thailand and Taiwan, respectively. Only a few WSSV genes have been studied beyond the sequence analysis (Tsai *et al*., 2000a; 2000b; van Hulten *et al*., 2000; 2001a; 2001b; 2002; Liu *et al*., 2001; Zhang *et al*., 2001; Chen *et al*., 2002a; 2002b; Huang *et al*., 2002a; 2002b; Lin *et al*., 2002; Tzeng *et al*., 2002; Zhang *et al*., 2002). The function of most genes remains unknown and needs to be studied extensively.

White Spot Syndrome

White spot syndrome (WSS) is a shrimp viral disease which has had and continues to have a serious economic impact on cultured shrimp worldwide. Marine shrimp aquaculture production grew rapidly in the 1970s. In 1980, world production of farmed shrimp was 100 thousand metric tons, and in 1993, it reached 710 thousand metric tons. But since then, largely due to the outbreak of WSS, world production has stagnated and, in many countries, it has even gone down over the last few years. In 1996, estimated lost production in Thailand alone peaked at 70,000 metric tons. Cumulative lost production for all Asian countries between 1993 and 1996 probably amounted to several hundred thousand tons. By 2001 the cumulative global loss to WSS was probably of the order of 1 million metric tons or more (Flegel and Fegan, in press).

The Early Stages of the WSSV Epidemic in Asia

Thanks to local researchers who have contributed greatly to our understanding of the early stages of WSSV epidemic in China, we now know something of the origins of the WSS epidemic. Outbreaks of WSSV were first found in *Penaeus japonicus* in China in 1992 in Zhangpu, Fujian Province. At the end of 1992 and in 1993, outbreaks of the disease were reported in coastal shrimp farms and in inland ponds (Cai *et al*., 1995; Huang *et al*., 1995a; 1995b; Wang *et al*., 1995). The disease first spread geographically within the species *P. japonicus*, and only later, in 1993, spread to other species including *Penaeus chinensis*, which is the major cultured species in China. Chinese researchers discovered that the disease was associated with a rod-shaped virus called hypodermal and hematopoietic necrosis baculovirus or HHNBV; this is the virus now known as WSSV. They also found that the disease spread inland and along the coast when culture ponds were stocked with *P. japonicus postlarvae* that came from Fujian Province (Fig. 1).

The disease also spread internationally in much the same way (Fig. 1). International transport of live shrimp for aquaculture is attractive economically, but it is also a very rapid and effective means of spreading the virus. Through the import of living broodstock or postlarvae directly to culture facilities, within one year of the first WSSV outbreak in China, the disease had spread to Taiwan, Japan and possibly other countries (Flegel and Fegan, in press).

WSS was first observed in *P. japonicus* in 1992 in farms in the north of Taiwan and soon spread southward. In 1993 *Penaeus monodon* and other shrimp species in farms in the south of Taiwan showed clinical signs of WSS. Although at that time the causative agent was not yet identified, the Taiwanese *P. japonicus* shrimp farming industry was seriously affected by the outbreak of WSS in 1992, with the yield falling by over 90% from 1991 to 1993 (Lo *et al*., 2003).

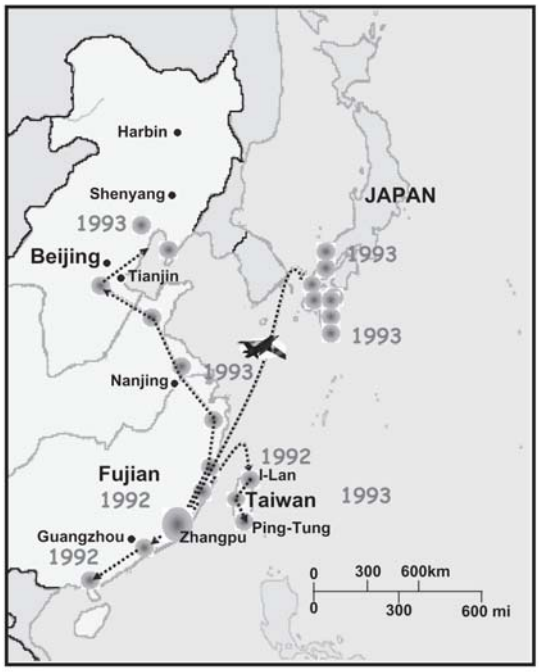

Figure 1. WSSV was spread both within China and internationally via the transport of live shrimp for aquaculture.

WSSV reached Japan in 1993. Japanese scientists very quickly determined that the disease originated with *P. japonicus* postlarvae imported from a Fujian Province hatchery. They also identified the causative agent and published some very good papers immediately, and this led to worldwide attention to the disease (Inouye *et al*., 1994; Momoyama *et al*., 1994; Nakano *et al*., 1994; Takahashi *et al*., 1994)

It is good to be able to report that the Asian Science community reacted very quickly to the disease. For example, within one year of the initial WSS outbreak, the causative agent was identified. Within 2-3 years, molecular diagnostic tools had been developed by several groups working in Japan, Taiwan, and Thailand. Within 3 years, a better understanding of the virus and its transmission modes brought about changes in hatchery and farming practices, which included the use of PCR technology to screen broodstock and larvae (Flegel and Alday-Sanz, 1998).

Extremely Wide Host Range of WSSV

The most surprising feature of WSSV is its wide range of potential hosts. It infects not only the penaeid shrimps but also a wide range of other decapods including crab, crayfish, and lobster. PCR has also detected the virus in artemia, copepod and insect larvae, but it has not yet been established whether these arthropods are susceptible to actual WSSV infection. In total, there are more than 78 species that have been reported as potential WSSV hosts (Lightner, 1996; Flegel, 1997; Maeda *et al*., 1998, 2000; Peng *et al*., 1998; Wang *et al*., 1998; Otta *et al*., 1999; Sahul Hameed *et al*., 2000; 2001; Corbel *et al*., 2001; Jiravanichpaisal *et al*., 2001; Hossain *et al*., 2001; Huang *et al*., 2001)

Transmission

The virus can be transmitted horizontally either orally by predation on diseased individuals, or by virus particles in the water, presumably through the gills (Chou *et al*., 1995; 1998). The virus is also transmitted vertically, that is from brooder to offspring (Lo *et al*., 1997; Hsu *et al*., 1999; Tsai *et al*., 1999; Peng *et al*., 2001). *In situ* hybridization has detected WSSV-positive cells in reproductive organs, and with TEM, virus particles are readily seen in the nucleus of young oocytes. WSSV-positive cells have been found in the ovary, follicle cells, oogonia, oocytes and connective tissue (Lo *et al*., 1997), but to date positive signals have never been observed in epithelial cells of the spermatophore, sperm cells, perinucleolus oocytes or in yolk stage oocytes. The virus appears to attack only oocytes that are still young, and if a young developing oocyte does become infected, it will die before it reaches maturation. This means that transovarial transmission (that is, through an infected egg) is an unlikely route by which the disease might pass from brooder to offspring. The transovum transmission (egg-mass contamination) pathway, however, is a very real possibility. The susceptibility of the host larvae to this kind of vertical transmission is also critical. For nauplius, zoea and mysis stages, it can be difficult to distinguish between WSSV contamination and WSSV infection, but a preliminary report (Venegas *et al*., 1999) suggests that in these early larval stages, resistance to WSSV may be quite high. If this encouraging finding can be confirmed, it may lead to more practical and effective disease control strategies.

Replication Triggered by Stress

Another notable feature of WSSV is that its replication is easily triggered by stressful conditions. WSSV infection is conveniently classified into 3 stages: the asymptomatic carrier, transition and acutely affected (patent) stages (Lo and Kou, 1998). The carrier stage may persist for months, but in the presence of stress, the disease progresses to the transition and patent stages within a few hours, and once the infection becomes patent, mortality inevitably occurs within a few days. “Stress” includes the stress associated with spawning (Lo *et al*., 1997; Lo and Kou 1998); WSSV loads increase dramatically within several hours of spawning process (Kou and Lo unpublished data; by contrast, IHHNV loads are unaffected).

Environmental conditions can also be a source of stress (Lo *et al*., 1998). One of the most common forms of environmental stress is overcrowding, but temperature, pH and salinity are also important. For intensive culturing, overcrowding is inevitable and it is therefore recommended that only postlarvae known to be WSSV-negative should be cultured intensively. Conversely, if stress can be avoid, then a (non-intensive) pond stocked with WSSV carrier shrimp still has a good chance of yielding a successful harvest. (Withyachumnarnkul, 1999; Peng *et al*., 2001).

WSSV PCR Technology in the Hatcheries

There are two main concerns regarding detection of WSSV. One is the confirmation of disease outbreaks and the other is the certification of broodstock and the post-larvae or fry used to stock rearing ponds. For presumptive diagnosis of outbreaks in suspected ponds, it is sufficient to carry out histological analysis by light microscopy of H&E stained tissues from moribund shrimp (Momoyama *et al*., 1994, 1995; Wongteerasupaya *et al*., 1995;

Lightner, 1996; Flegel *et al*., 1997). Confirmation of WSSV requires more detailed analysis by PCR (Kimura *et al*., 1996; Lo *et al*., 1996b; Takahashi *et al*., 1996; Nunan and Lightner 1997; Kasornchandra *et al*., 1998; Kim *et al*., 1998), Western blot analysis (Nadala *et al*., 1997; Loh *et al*., 1998), *in situ* DNA hybridization (Durand *et al*. 1996; Nunan and Lightner 1997), or transmission electron microscopy (TEM) (Takahashi *et al*., 1994; Wongteerasupaya *et al*., 1995; Durand *et al*., 1997). For definitive diagnosis and certification of WSSV infection status of broodstock and fry, PCR technology is recommended.

In the hatcheries, most of the brooders that succeed in spawning are either initially WSSV PCR-negative or are only lightly infected (asympatomatic carrier stage). A tissue distribution analysis of WSSV in these lightly-infected specimens shows the virus to be particularly prevalent in pleopods, followed by gills, and hemolymph (Lo *et al*., 1997; Kou *et al*., 1998). For screening brooders, we therefore recommend testing the pleopods. The best method for sampling is to use red-hot forceps to excise about a small (50 mg) piece of pleopod for PCR template preparation (Fig. 2).

Figure 2. Red hot forceps (A) are used to excise a piece of pleopod (B), which is the recommended tissue source for PCR template preparations for screening brooders.

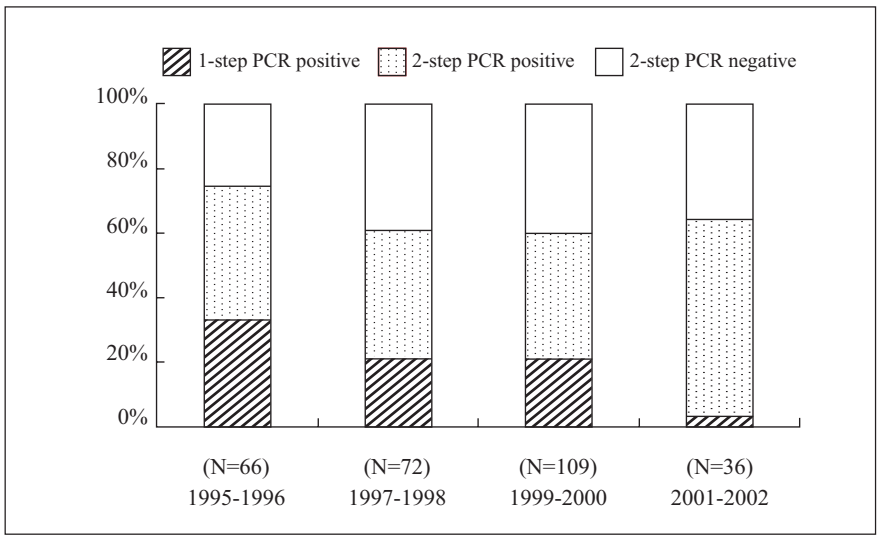

Figure 3. 2-step nested WSSV PCR detection rate in *Penaeus monodon* broodstock immediately after capture.

Figure 3 presents typical screening results for samples of *P. monodon* broodstock collected from Taiwan's coastal waters (near Ping-Tung in South Taiwan) over the last few years, and suggests that WSSV has already been established in the wild population with a prevalence rate of about 58-76%. Of the brooders that are PCR-positive, the majority are in the carrier state, that is they have a very light infection.

Even though the lightly infected carriers (positive only in 2-step WSSV diagnostic PCR) almost always spawn successfully, being WSSV positive nevertheless impacts their spawning behavior in that these lightly infected brooders usually spawn only once or twice rather than repeatedly. Of the heavily infected brooders (positive in 1-step WSSV diagnostic PCR), less than 20% spawned successfully (Fig. 4), while most of the others died within 1 to 4 d after capture without spawning (Hsu *et al*., 1999). Further, even when heavily infected brooders do spawn, their eggs often fail to hatch. One of the reasons for this may be that when healthy shrimp spawn, they swim normally and naturally move their pleopods vigorously to and fro. This movement serves to scatter the eggs and mix them with the sperm released from the spermatophore. By contrast, a heavily WSSV-infected shrimp is lethargic and does not behave in this way, so that its eggs remain clumped together and do not get fertilized.

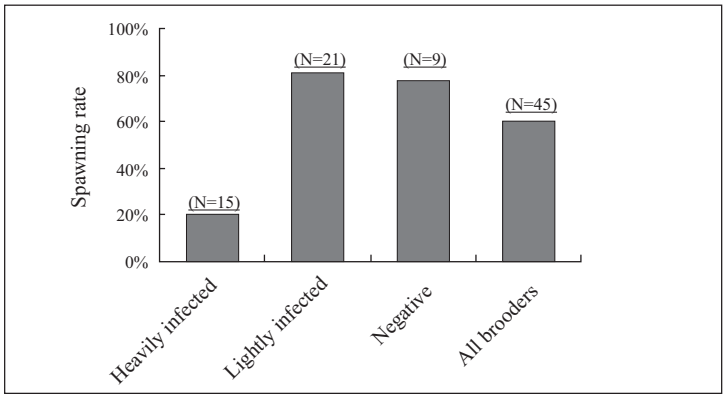

Figure 4. Negative impact of WSSV infection on spawning.

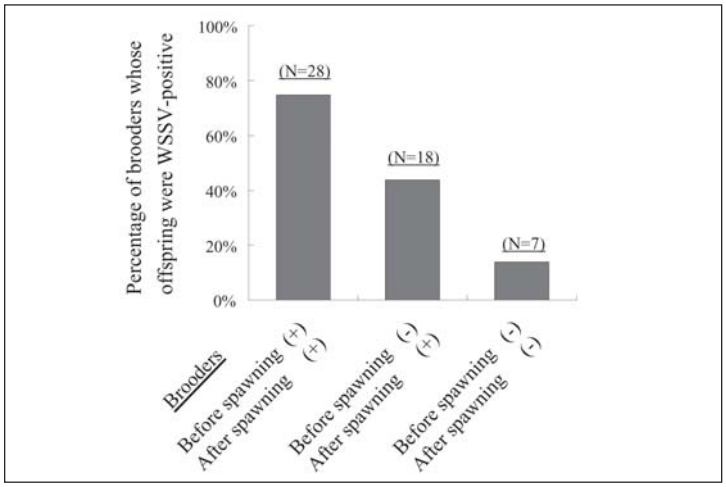

Figure 5. Detection of WSSV by nested WSSV PCR in *Penaeus monodon* brooders and their offspring.

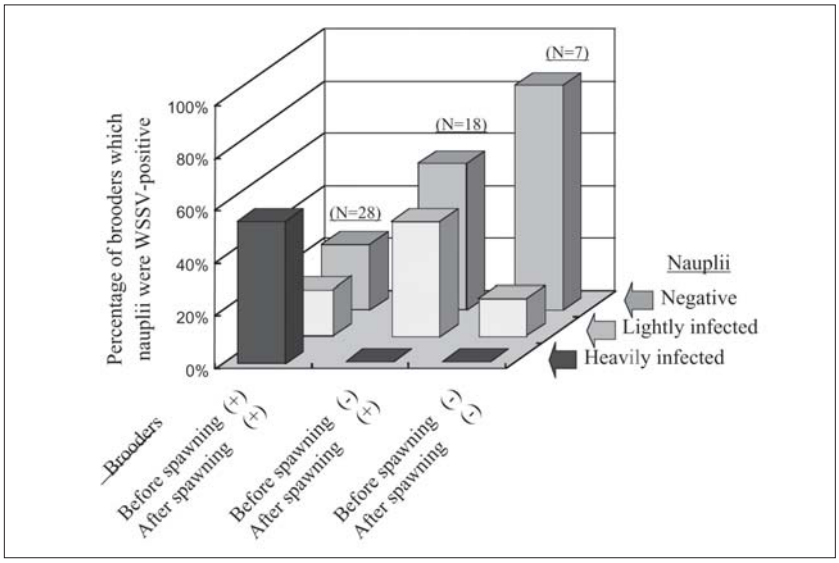

Figure 6. Detection of WSSV infection in *P. monodon* brooders and their nauplii.

Another useful way to classify brooders is to assess their WSSV infection states both before and immediately after spawning (Peng *et al*., 2001). Some brooders are WSSV positive both before and after spawning, while others remain WSSV-negative throughout. In other brooders, the virus is initially present but at a level too low to be detectable. In these brooders, the stress of spawning triggers virus replication and converts the post spawning diagnosis to PCR positive. The prevalence of WSSV in the offspring of these three classes of brooders is shown in Figure 5. Figure 5 provides evidence that the virus is transmitted vertically from infected brooders to their offspring: most (75%) of the brooders that were already WSSV positive before spawning produced offspring that were also WSSV positive, whereas less than half of the brooders that become PCR positive after spawning produced infected offspring. One of the PCR negative brooders also produced WSSV positive offspring, possibly because WSSV was present in the brooder at levels that were still too low to be detected even after spawning, or probably because of contamination of the offspring samples. In Figure 6, these data are broken down in terms of the severity of infection in the nauplii. To classify samples of nauplii as heavily, lightly, or uninfected, since quantities of virus were very low, each 50 mg pooled sample of nauplii was tested 5 times: those samples that were WSSV positive in three or more tests were classified as heavily infected; samples with 1 or 2 positive results were lightly infected; and only when there were no positive results in any of the 5 replications was the batch classified as WSSV negative. When broken down in this way, 57% of the heavily infected brooders yielded heavily infected nauplii, whereas nauplii derived from brooders that were WSSV negative at least before spawning were all either lightly infected or WSSV negative (Fig. 6).

A pilot study suggested that the "infection" status of *P. monodon* nauplii might determine their subsequent fate in grow out ponds (Peng *et al*., 2001). In a pond stocked with PL derived from heavily infected nauplii, an outbreak of WSS occurred within 3 weeks and only ~20% of the initial population survived through to harvest, whereas in two other ponds,

which were stocked with PL derived from either lightly infected or WSSV-negative nauplii, respectively, the survival rate (70-80%) and total harvest were both much higher (Peng *et al*., 2001). As shown in Figure 6, those brooders that were WSSV negative before spawning (even if they became WSSV positive afterwards) produced only lightly or negatively infected nauplii. This is very convenient because it means that to select offspring that will likely produce a good harvest, it only needs to be shown that the offspring came from a parent brooder that was PCR negative before spawning.

CONCLUSIONS AND PROSPECTIVES

Increasing knowledge of WSSV and its associated disease has brought about revolutionary changes in hatchery and farming practice. These changes are designed to prevent outbreaks of the disease, and include not only greater attention to environmental conditions but also the use of PCR technology to screen broodstock and larvae (Flegel and Alday-Sanz, 1998). Although this allows for better control of WSSV in a cultivation system, however, if the disease is to be successfully combated, perhaps a WSSV resistant strain of shrimp will eventually need to be developed.

In brooders, spawning usually increases the severity of a WSSV infection. Yet we found that some (< 25 %) brooders caught from wild were able to contain virus and thus prevent it from rapid replication during spawning. It would be very interesting to compare the gene expression profiles of shrimps that perform differently in response to WSSV infection. This may provide insights into the molecular mechanisms that allow the shrimp to contain the virus under stressful conditions. The functional genomics of *P. monodon* thus become increasingly important. A genome-wide analysis of the black tiger shrimp is needed to gain a better understanding of the molecular biology of the shrimp, especially in the fields of reproduction, growth and disease defense. Functional genomic studies will generate useful information by which many biologically and economically significant genes will be identified, and EST analysis and DNA microarray technology may be powerful tools for this study. Furthermore, a genetic analysis of the black tiger shrimp, including development of molecular markers for the use in gene mapping, marker-assisted breeding, stock management, gene function analysis, etc should be performed. All of these approaches will help to develop genetic improvement programs of *P. monodon* to enhance disease resistance, increase productivity, and reduce dependency on wild stocks and thus will be of benefit to shrimp aquaculture industry.

Meanwhile, although many studies demonstrate that the WSSV infection status of the shrimp broodstock and the postlarvae used for stocking plays a key role in successful culturing (Lo *et al*., 1998; Hsu *et al*., 1999; Withyachumnarnkul, 1999; Peng *et al*., 2001), to date full domestication of *P. monodon* (and thus the availability of virus-free brooders) remains elusive, and supplying healthy *P. monodon* broodstock has become the key bottle-neck for shrimp culture industry.

ACKNOWLEDGEMENTS

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