6.2.4 White Spot Syndrome of Crustaceans

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A. Name of Disease and Etiological Agent

White Spot Disease is lethal to most commercially-raised penaeid shrimp species and is caused by the highly virulent, OIE-notifiable crustacean pathogen, known as White Spot Syndrome virus (WSSV). It was first reported in *Peneaus japonicus* in northeast Asia during 1992-1993 (Bondad-Reantasco, 2001; OIE, 2003) and was initially called penaeid rod-shaped DNA virus (PRDV). This virus was later referred to as hypodermal & hematopoietic necrosis baculovirus (HHNBV), white spot baculovirus (WSBV) and systemic ectodermal and mesodermal baculovirus (SEMBV), as similar outbreaks in penaeid shrimp species occurred across Asia (Zhan et al., 1998). Ultrastructurally, the rod-shaped virions (80-120 x 250-380 nm) are enveloped and have a unique tail-like appendage (Wongteerasupaya et al., 1995; Durand et al., 1997). Although initially described as a non-occluded baculovirus, this large double-stranded DNA virus has now been reclassified as a Whispovirus in the family Nimaviridae (Van Hulten et al., 2001; Mayo, 2002; Escobelo-Bonilla et al., 2008). To date, little genetic or biologic variation has been demonstrated in different WSSV isolates (OIE manual, 2003; Lightner, 2004; Kiatpathomchai et al., 2005).

B. Known Geographic and Host Ranges of the Diseases

WSSV has a wide host range with outbreaks reported in over 80 aquatic crustacean species worldwide (Zhan et al., 1998; Chapman et al., 2004; Escobedo-Bonilla et al,, 2008). Natural shrimp infections were first reported in farmed *P. monodon, P. chinensis, P, japonicus, P. indicus, P. merguiensis, Litopenaeus vannamei* and *P. setiferus* with experimental infections also induced in *Farfantepenaeus aztecus, L. stylirostris* and *F. duorarum* (Lightner, 1996; Lightner et al., 1998; Bondad-Reantasco, 2001). Since its initial detection, the occurrence of WSSV infections spread rapidly throughout Asia into India, Indonesia, Korea, Malaysia, Thailand and Vietnam with its first detection in the United States in 1995 (Lightner et al., 1998). In 1999, WSSV was responsible for a major pandemic in the Central and South Americas (Jory & Dixon, 1999; Moss, 2002; Rodríguez et al., 2003). The most recent reports of White Spot Disease in U.S. farmed crustacean species have been in farmed *L. vannamei* in Hawaii (CEI, 2004; HDOA, 2008) and in crayfish populations in Louisiana (CEI, 2007).

C. Epizootiology

The occurrence of rapid environmental changes (e.g. water temperature, hardness, salinity) and/or extended periods of low dissolved oxygen levels have been associated with outbreaks of WSSV in shrimp (Zhan et al., 1998; Bondad-Reantasco, 2001, Sanchez-Martinez et al., 2007). Increased mortalities due to WSSV infection have also been reported to be associated with low water temperatures in shrimp (Vidal et al., 2001; Rodriguez et al., 2003) with higher survival rates demonstrated experimentally in WSSV-infected *L. vannamei* maintained at 32˚C (Vidal et al., 2001; Granja et al., 2006). Based on results from a WSSV crayfish infectivity study, it was hypothesized WSSV-infected crayfish could serve as inapparent carriers at low temperatures and later develop clinical white spot disease when exposed to higher temperatures (Jiravanichpaisal et al., 2004).

The major means of transmission of White Spot Disease appears to be via horizontal routes of infection, such as exposure to infected waters or ingestion of dead/moribund cohorts and/or carriers/vectors (Lo et al., 1998; Chang et al., 1996). WSSV virions have been demonstrated to survive in water, decaying tissues and detritus for up to 4 days (Bondad-Reantaso, 2001) with horizontal transmission most likely via various feeding pathways by susceptible crustacean species and aquatic fauna present in the shrimp pond environment (Vijayan et al., 2005). Rotifers, bivalves, polychaete worms and copepods (e.g. artemia) have been demonstrated to serve as possible mechanical vectors (Yan et al., 2004; Vijayan et al., 2005; OIE manual, 2006; Sanchez-Martinez et al., 2007). Trans-ovum vertical transmission is another possibility (Kuo, 1997; Sanchez-Martinez et al., 2007). International commerce of WSSV-infected commodity shrimp has been hypothesized as the most probable route for introduction of WSSV to the Americas (Lightner, 2004), but the actual sources for these outbreaks has not been fully elucidated. Other potential sources for transmission of WSSV include human activities, seabirds, release of ship ballast water and untreated contaminated wastes from shrimp processing plants (Sanchez-Martinez et al., 2007).

Avoidance and implementation of biosecurity production measures, such as using SPF-derived seedstock and pretreatment of water and/or reduced water exchange, have been the major recommended means of control at the farm level to prevent introduction of viruses into shrimp culture systems (Lotz, 1997; Park et al., 2004; Chang et al., 1998b). Following an outbreak, waters from WSSV-infected ponds can be treated with 30 ppm chlorine and/or held a minimum of 4 days before being discharged (Bondad-Reantaso, 2001). Another possible effective preventive measure would be to notify processing plants in advance of the WSSV-infected status of a shipped shrimp lot originating from an emergency harvest (Bondad-Reantaso, 2001).

D. Disease signs

Gross signs. Affected shrimp will initially stop feeding, appear lethargic and swim near the water surface. In the early stage of infection, moribund shrimp can also develop a reddish discoloration to the body due to chromatophore expansion with few to no "white spots" detected (Lightner, 1996; Jory & Dixon, 1999; OIE manual, 2003; Granja et al., 2006). In *P. monodon*, the cuticle becomes loose with characteristic variably-sized, white "spots" (calcium deposits) developing along the inner surface of the carapace as the infection progresses. High mortality rates of up to 100% are reported to occur in shrimp populations within 3 to 10 days following the onset of clinical signs (Sanchez-Martinez et al., 2007). Disease signs are variable in nonpenaeid species, such as crabs, crayfish and lobsters (Chang et al., 1998a; OIE aquatic manual, 2003).

Histologic signs. Development of distinctive hypertrophied, eosinophilic to basophilic, intranuclear inclusions with marginated chromatin is the primary histologic feature detected in infected cells of ectodermal and mesodermal origin (Sanchez-Martinez et al., 2007). These inclusions are observed most commonly in the cuticular epithelial and subcuticular connective tissue cells of the exoskeleton, stomach, appendages, gills and eyestalk and less commonly in the antennal gland, lymphoid organ, hematopoietic tissue and hemocytes. Lesions have also been found in gonadal tissue (Escobelo-Bonilla et al., 2008; Sanchez-Martinez et al., 2007).

Figure 1 WSSV-infected *L. setiferus*. Note reddening of the appendages and lack of white spot cuticular lesions**.**

Table 1. Diagnostic methods available for WSSV testing.
Increasing relative assay sensitivities are categorized (1-4).

Method	Relative sensitivity [1]	Presumptive diagnosis[2]	Confirmatory Diagnosis
Gross signs	1	+	+
Histopathology	3	++	++
Histological immunoassay	3	++	++
ELISA assays	3-4	++	++
PCR assays	4	+++	+++
ISH [3] assays	4	+++	+++

[1] 0-4 increasing sensitivity
[2] applicability: - unsuitable, + limited utility, ++ standard method,+++ recommended method.
[3] in situ hybridization

E. Disease Diagnostic Procedures

All life stages appear to be susceptible to infection with the probability of detection best in late postlarval, juvenile or adult stages exposed to stressful conditions (OIE manual, 2006; Yoganadhan et al., 2003).

Presumptive

1. Gross observations

a. Lethargy, reddish chromatophore expansion, reduction in feed intake and mortality are the nonspecific clinical signs demonstrated by infected shrimp with the classic subcuticular “white spots” reported primarily in *P. monodon* in the later stages of infection.

Increased bird predation is also seen about affected ponds (see D above).

2. Electron Microscopy

a. Negative staining of preparations, made of suspect infected hemolymph or tissues, allows visualization of the rod-shaped virions with a tail-like appendage **(**Bondad-Reantasco, 2001; Escobedo-Bonilla et al., 2008) at the ultrastructural level**.**

Confirmatory

1. **Histopathology**

a. Davidson-fixed shrimp tissues (Humason, 1979) are processed, sectioned and stained with a modified Mayer's hematoxylin and phloxine/eosin stain (H&E) as described by Bell & Lightner (1988). In WSSV-infected shrimp, intranuclear inclusions with chromatin margination are detected in infected cells of mesodermal and ectodermal origin. (Lightner, 1996; Sanchez-Martinez et al., 2007). The gastric mucosa is a preferred initial site to examine for infection (Escobedo-Bonilla et al., 2007; see Figure 2).

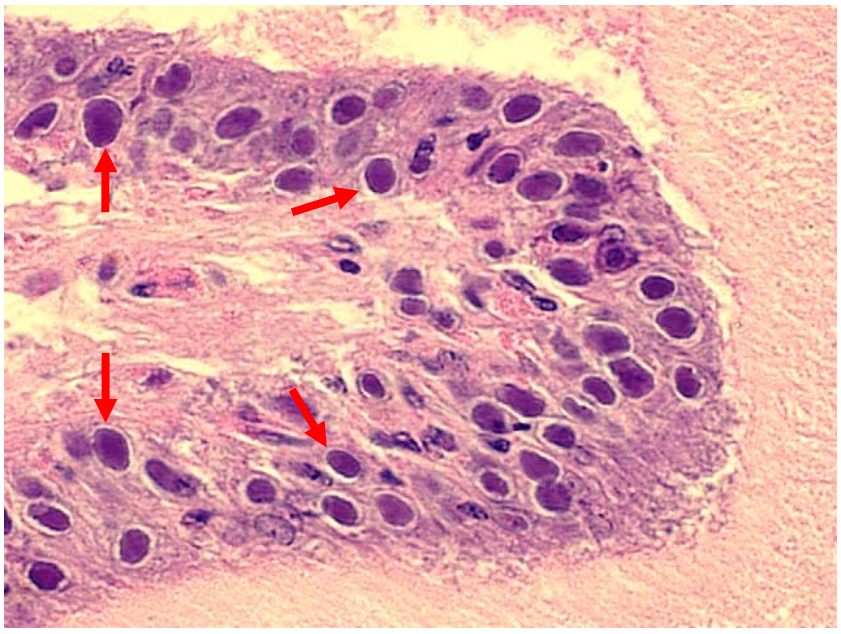

Figure 2. Histologic section of the gastric mucosa demonstrating intranuclear inclusions (arrows) within many WSSV-infected cuticular epithelial cells (H&E stain; 400X).

2. *In situ* hybridization assay

In situ hybridization (ISH), using a digoxigenin-labeled **WSSV**-specific probe, can be performed to confirm suspect histologic findings as outlined in published ISH protocols (Chenggis & Unger, 1993; Nunan et al., 1997; Chang et. al. 1996; Ramis et al., 1998). The WSSV probe can be generated by PCR and randomly-labeled with digoxigenin using the Dig DNA labeling mix (Roche Diagnostics) according to the manufacturer's instructions (see the following PCR reaction).

a. Slide processing

i. De-paraffinize embedded tissue sections mounted on positively-charged slides in a xylene-based solvent.
ii. Rehydrate tissue sections through a series of graded ethanols from 100% to 50% ethanol including one 10 min 1% acid/ethanol soak.

b. Permeabilization

i. Incubate sections in Proteinase K for 8 min. at 37°C to permeabilize the tissues to allow probe penetration.
ii. Inactivate the Proteinase K by heating slides up to 95-100°C for 10 min.
iii. Then incubate the slide in 0.4% paraformaldehyde/PBS for 10 min at 4°C to enhance tissue adherence to the slide.

c. ISH hybridization & stringency washes

i. Incubate tissue section(s) in the hybridization solution (containing the WSSV-specific probe) at 100°C for 10 min followed by a 2 hr (or overnight) incubation at 42°C.
ii. Wash slides in decreasing concentrations of SSC (2X to 0.2X with the last soak at 37°C.

d. ISH detection step

i. Incubate slides in an Anti-digoxigenin-alkaline phosphatase conjugated antibody solution for 30 min at 37°C.
ii. Then incubate in a development solution (NBT/BCIP substrate) for 30-60 min. at 37°C.
iii. Counterstain with Bismarck Brown stain and dehydrate slides through graded ethanols (95%-100%) and a xylene-based solvent prior to cover-slipping for histologic evaluation (Figure 3).

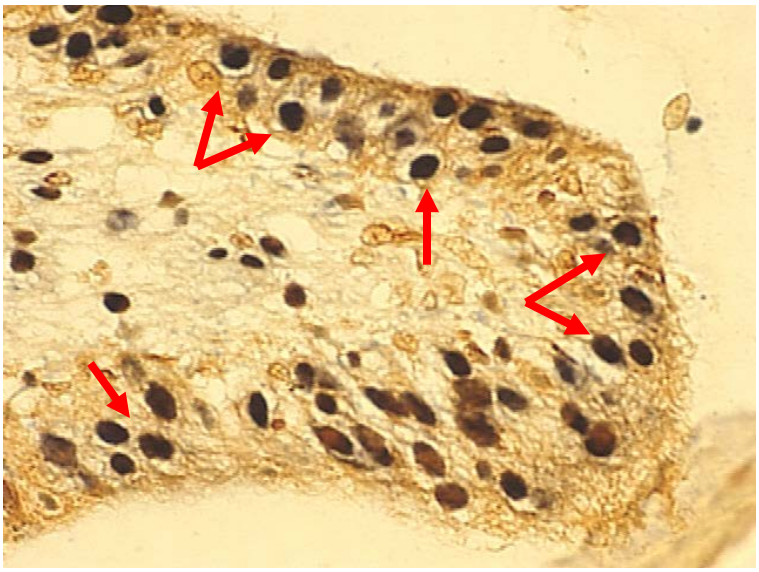

Figure 3. *In situ* hybridization of the gastric cuticular epithelium with intranuclear probe uptake (dark blue-black) by WSSV-infected cells (see arrows). Counterstain with Bismark Brown stain (400X).

3. WSSV PCR assay

Various single-step conventional, nested, and Real-time PCR protocols have been developed for WSSV detection (Lo et al., 1996; Kim et al., 1998; Durand & Lightner, 2002). However, the nested PCR procedure, developed by Lo et al. (1996), is the presently recommended procedure of choice listed in the OIE Manual of Diagnostic Tests for Aquatic Animals (OIE, 2006) for WSSV detection. Samples can be pleopods, hemolymph or whole animals submitted as fresh, frozen or alcohol preserved specimens, dependent on the life stage being tested. Removal of eyestalks is recommended in whole body postlarval samples to prevent PCR inhibition (OIE manual, 2003).

a. DNA extraction

Extraction is conducted following the methods of Loy et al. (1996) with minor modifications. Excise a 1-2 mm^3 piece of pleopod or cephalothorax from up to 60 fresh, frozen or ethanol preserved shrimp to obtain a pooled sample not exceeding 300 mg (or pooled samples of 10% sodium citrate-treated hemolymph not exceeding 100 µl). Ethanol-preserved tissues should be soaked in distilled water for 15-20 min to remove alcohol prior to homogenization. Tissues are homogenized in 1000 µl of shrimp lysis buffer (10mM Tris/HCl, 100mM NaCl, 25 mM EDTA, 2% sodium dodecyl sulfate, dd H_2O, pH 8.0) using either a manual tissue grinder or FastPrep tubes (Q-Biogene) according to the manufacturer's instructions (use 600 µl of lysis buffer for 100 µl hemolymph

sample). Centrifuge the homogenate at 650 x *g* for 1 min and incubate 300 µl of the resulting supernatant with 20 µl Proteinase K (20 mg ml^{-1}) for 1 hr in a 65°C water bath. Following incubation, inactivate the Proteinase K by boiling the sample for 10 min followed by centrifugation at 16,000 x *g* for 3 min. to pellet particulate debris. To purify the sample, transfer 100 µl of the supernatant to a ChromaSpin TE-100 column and centrifuge at 700 x *g* for 5 min according to the manufacturer's instructions (Clontech laboratories). The resulting purified lysate can then be stored frozen or analyzed immediately by PCR.

b. PCR amplification

i. Primers (First Step): 146 F1 (5' ACT ACT AAC TTC AGC CTA TCT AG 3')
146 R1 (5' TAA TGC GGG TGT AAT GTT CTT ACG A 3')

ii. First Step PCR (1447 bp amplicon)

Reagent	Vol/RXn	Initial Conc.	Final Conc.
10X Buffer	2.5 µl	10 X (15mM Mg)	1X(1.5mM Mg)
10 X dNTPs	2.5 µl	2.0 mM	0.2 mM
BSA	2.5 µl	10 mg/ml	1 mg/ml
146F1	0.5 µl	50 µM	1.0 µM
146R1	0.5 µl	50 µM	1.0 µM
Taq	0.25 µl	5U/µl	1.25 U/25µl/rxn
H_2O	13.75 µl	-------	-------
DNA	2.5 µl	-------	-------
TOTAL:	25 µl		

iii. Cycling parameters (For both First Step and Nested Procedures)
Initial denaturation of 3 min at 94 °C is followed by
40 cycles of:
1 min at 94 °C
1 min at 55 °C
2 min at 72 °C
Final extension of 5 min at 72 °C, then hold at 4 °C.

iv. Primers (Nested): 146 F2 (5' GTA ACT GCC CCT TCC ATC TCC A 3')
146 R2 (5' TAC GGC AGC TGC TGC ACC TTG T 3')

iv. Nested PCR (941 bp amplicon)

Reagent	Vol/RXn	Initial Conc.	Final Conc.
10X Buffer	2.5 µl	10 X (15mM Mg)	1X(1.5mM Mg)
10 X DNTP's	2.5 µl	2.0 mM	0.2 mM
BSA	2.5 µl	10 mg/ml	1 mg/ml
146F2	0.5 µl	50 µM	1.0 µM
146R2	0.5 µl	50 µM	1.0 µM
Taq	0.25 µl	5 U/µl	1.25 U/25µl rxn
H_2O	13.75 µl	-------	-------
PCR 1 product	2.5 µl	-------	-------
TOTAL:	25.0 µl		

vi. Nested PCR products are electrophoresed on 2% agarose (in 1X TBE) gels, stained with ethidium bromide, and visualized using UV light (see Figure 4.).

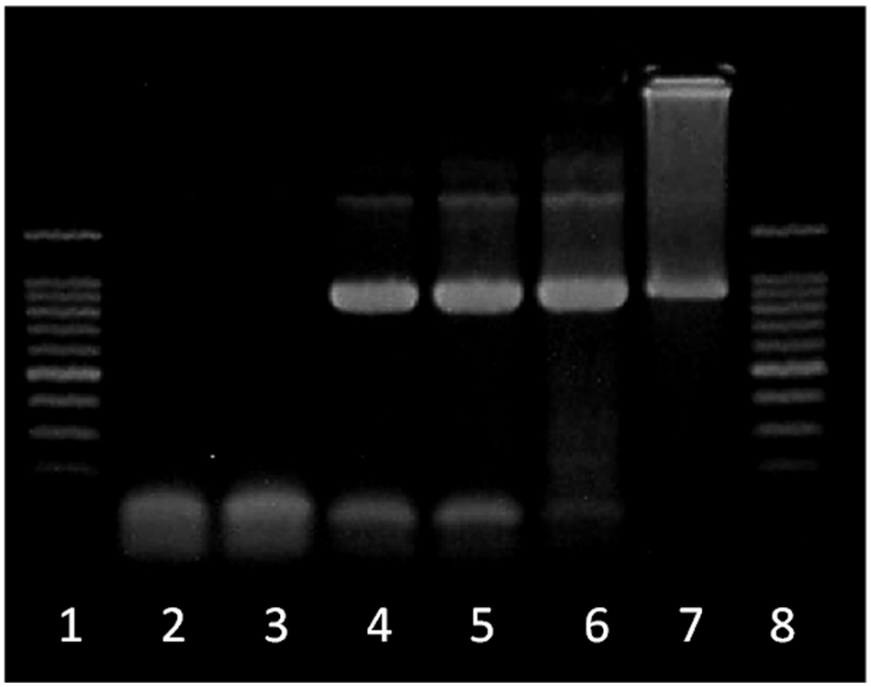

Figure 4. Ethidium bromide-stained PCR gel. Lane 2 is the negative control, Lane 3 is a negative sample, Lanes 4 & 5 are positive samples (941 bp amplicon), Lane 6 is the positive extraction control, Lane 7 is the positive molecular control,

and Lanes 1 & 8 contain the 100 bp DNA standard with the brightest band representing 500 bp.

4. WSSV Bioassay

a. Juvenile SPF shrimp are injected IM with a purified homogenate made from suspect WSSV-infected tissue or exposed *per os* to WSSV-infected tissues.
b. Post-inoculation, histopathology and WSSV-specific PCR are conducted on moribund shrimp, as stated above (OIE Aquatic Manual, 2003).

F. Procedures for Detecting Subclinical Infections

1. Pond-side examinations of statistically significant numbers of shrimp should be conducted during times when environmental conditions are conducive for clinical disease expression. Selected samples should be injected with the appropriate fixative (Bell & Lightner, 1988) and submitted to a lab for diagnostic testing:
    a. Davidson's fixative (Histology /in situ hybridization).
    b. Ethanol (PCR)
2. For confirmation of a new species suspected to be infected with WSSV, suspect positive PCR amplicons can be sequenced (OIE aquatic manual, 2006).
3. Exportation requirements of some importing countries require demonstration of freedom of infection/disease. Testing sample sizes that are statistically significant are important in surveillance testing (Cameron, 2002) and may vary with the country or state of destination.

G. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability or Preservation of the Etiological Agent

1. Histologic/ in situ hybridization analysis
    a. Ship or transport triple-sealed, Davidson-fixed shrimp samples to an aquatic diagnostic laboratory for further diagnostic processing/testing. The method of whole body preservation will vary with the size of the specimen. Contact the diagnostic lab for detailed instructions or follow instructions outlined by Bell & Lightner (1988). Samples should be properly labeled (e.g. pond number, date, species, etc),

2. PCR analysis

a. Ship or transport triple-sealed, ethanol-fixed shrimp samples (injected whole body or pleopods in 70-95% ethanol) or hemolymph to an aquatic diagnostic laboratory for further diagnostic processing/testing. Samples should be properly labeled (e.g. pond number, date, species, etc), and collected in a manner to minimize sample cross contamination, as well as maintained at or below 21 °C.

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