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Study on the Pathogenesis of the White Spot Syndrome Virus (WSSV) on Juvenile *Penaeus monodon* in Vietnam

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Key words: white spot syndrome virus (WSSV), shrimp, pathogenesis, injection, immunohistochemistry

Abstract

The white spot syndrome virus (WSSV) causes disease and mortality in cultured and wild *Penaeus monodon*. In this study, specific pathogen-free *P. monodon* were injected with WSSV to determine in which primary organs the virus replicates and to analyze viral spread. Shrimps were injected with a low SID_{50} endpoint (shrimp infectious dose resulting in 50% infected shrimp) of $10^{1.5}$ or a high SID_{50} of 10^4 of the virus. Six shrimps per treatment were collected at 0, 3, 6, 9, 12, 15, 18, 21, 24, 30, 36, 42, 48, 54, and 60 h post injection (hpi) for detection of the virus in tissues from 10 organs by immunohistochemistry. In shrimps injected with the low dose, WSSV-infected cells were first detected in the heart and antennal gland 12 hpi, then in the foregut, stomach, and gills at 18 hpi. The integument was infected 24 hpi and the hematopoietic tissue, lymphoid organ, midgut, and connective tissues 36 hpi. In shrimps that received the high dose, the heart, antennal gland, stomach, gill, and connective and hematopoietic tissues were WSSV-positive 12-15 hpi while the foregut and cuticular epithelium were positive 18 hpi and the lymphoid organ and midgut were positive 21 hpi. The present study confirmed the replication of WSSV in *P. monodon* heart, antennal gland, foregut, stomach, gills, cuticular epithelium, hematopoietic tissue, connective tissue, and lymphoid organ.

Introduction

In the 1980s, shrimp diseases were mainly caused by bacteria, fungi, parasites, and viruses such as monodon baculovirus (MBV) and hepatopancreatic parvovirus (HPV). But, since 1993, diseases have been attributed mainly to the yellow head virus (YHV) and white spot syndrome virus (WSSV). The latter virus has a wide host range in crustaceans including crabs, crayfish, and most penaeid shrimp species. Under farming conditions, shrimp mortality reached 100% within 3-10 days of infection (Kasornchandra et al., 1998; Huang et al., 2001).

WSSV was first discovered in Southeast Asia (in Taiwan and China in 1992). Nowadays, it

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has spread to other Asian countries including Japan, Thailand, Indonesia, Vietnam, Korea, Malaysia, and India and to southern Texas and Australia (Lightner, 1996). WSSV is rod shaped (bacilliform), 110-130 nm in width, 250-350 nm in length, and has a tail-like extension, enveloped and containing double-stranded DNA. The entire 305 kb genome sequence was reported by Huang et al. (2001). Two major structural protein genes (*vp*28 and *vp*26) were identified (Van Hulten et al., 2000a), with genes for ribonucleotide reductase, endonuclease, protein kinase, and other structural proteins (Van Hulten et al., 2000b). Analysis indicates it contains 181 open reading frames (ORF), some of which are similar to known viral genes or eukaryotic genes, most of which encode putative proteins without homology to any known protein (Yang et al., 2001). Because of the lack of similarity to any existing virus family, WSSV was allocated to a new family, the Nimaviridae, and to the genus *Whispovirus* (Vlak et al., 2002).

The aim of the present study was to understand the pathogenesis of WSSV on *Penaeus monodon* using a standardized injection inoculation procedure.

Materials and Methods

Sixty-day-old specific pathogen-free (SPF) *Penaeus monodon* shrimp (8.5±2 g) were checked to be free of WSSV, YHV, MBV, HPV, or infectious hypodermal and hematopoietic necrosis virus (IHHNV). The shrimps were acclimatized 4 days prior to infectivity trials. They were fed commercial pellet feeds and kept in water of 28±2°C, 30 g/l salinity, 0.5 mg/l total ammonia, and 0.05-0.15 mg/l nitrite.

The original WSSV isolate came from naturally-infected *P. monodon* in Can Duoc District, Long An Province, Vietnam, and was purified in the Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, Belgium (2004). In a previous study, the dose that resulted in 50% infected shrimp (shrimp infectious dose; SID_{50}) was determined as $10^{5.1}$/ml. A low dose ($10^{1.5}$ SID_{50}) and a high dose (10^{4} SID_{50}) were prepared in phosphate-buffered saline (PBS), pH 7.4.

Six shrimp were kept in each of 17 aquaria containing 126 l sea water. About 0.1 ml WSSV inoculum was injected into the fifth abdominal muscle of each shrimp. Shrimp in eight aquaria were injected with the low dose and shrimp in eight aquaria were injected with the high dose. One aquarium served as a control and shrimp were inoculated with sterile phosphate buffer saline only. In the first experiment, shrimp samples were collected 0, 6, 12, 18, 24, 36, 48, and 60 h post injection (hpi). In the second experiment, shrimp samples were collected 0, 3, 9, 15, 21, 30, 42, and 54 hpi. The six control shrimp were collected at the end of each experiment.

At each sampling, periopods of the six shrimps from one tank were fixed with Davidson's solution for 24-48 h, then transferred to 50% ethanol for at least 24 h before paraffin embedding (Lightner, 1996). Each periopod was cross-sectioned into three parts: from the eye to the mouth, from the mouth to the middle of the heart, and from the middle of the mouth to the distal end. In addition, ten organs were processed and analyzed: the foregut, stomach, midgut, connective tissue, gill, lymphoid organ, heart, antenna gland, hematopoietic tissue, and cuticular epithelium, following the method of Escobedo-Bonilla et al. (2007).

Paraffin-embedded tissue sections were cut at 5 μm, deparaffinized, and rehydrated following the method of Lightner (1996). The endogenous peroxidase was blocked by incubating the slides for 30 min at room temperature in a solution of 1% sodium azide and 0.02% hydrogen peroxidase in Tris buffer (pH 7.4). Sections were incubated for 1 h at 37°C with 2 mg/ml of monoclonal antibody 8B7 raised against the WSSV envelope protein *vp*28 obtained from Poulos and following their method (Poulos et al., 2001). Sections were washed in Tris buffer (pH 7.6) and incubated for 1 h at 37°C with a 1:200 dilution of biotinylated sheep anti-mouse IgG antibodies (RPN1001, Amersham Biosciences). Afterwards, these were washed, incubated for 30 min at room temperature with 1:200 dilution of streptavidine-biotinylated horseradish peroxidase complex (RPN1051 Amersham Biosciences) and washed again. Color development was made with 0.01% of 3, 3'-diaminobenzidine (D8001 Sigma-Aldrich). Sections were counter stained with

hematoxylin [0.5% hematoxylin, 10% $Al_2(SO_4)_3$, 0.25% HgO, 2% glacial acid acetic] and washed in water, dehydrated, and mounted using Baume Canada. The WSSV-infected cells were counted by light microscopy (Olympus CH 40) at magnifications of 40x and 100x.

Results

Clinical signs and pathogenesis of the experimentally-infected shrimp are given in Tables 1-3. Figure 1 shows shrimp tissues infected with WSSV at different hours post injection.

Discussion

In vivo infection experiments with juvenile *P. monodon* confirmed the virulence of WSSV. The WSSV Viet strain used in the current study caused slower mortality than the Thai strains (Lightner, 1996; Escobedo-Bonilla et al., 2006, 2008). In the present study, mortality began at 42 hpi in experiment 2 and 60 hpi in experiment 1 although many organs developed WSSV infection as early as 12 hpi. Working with the same WSSV strain and challenge protocol, mortality

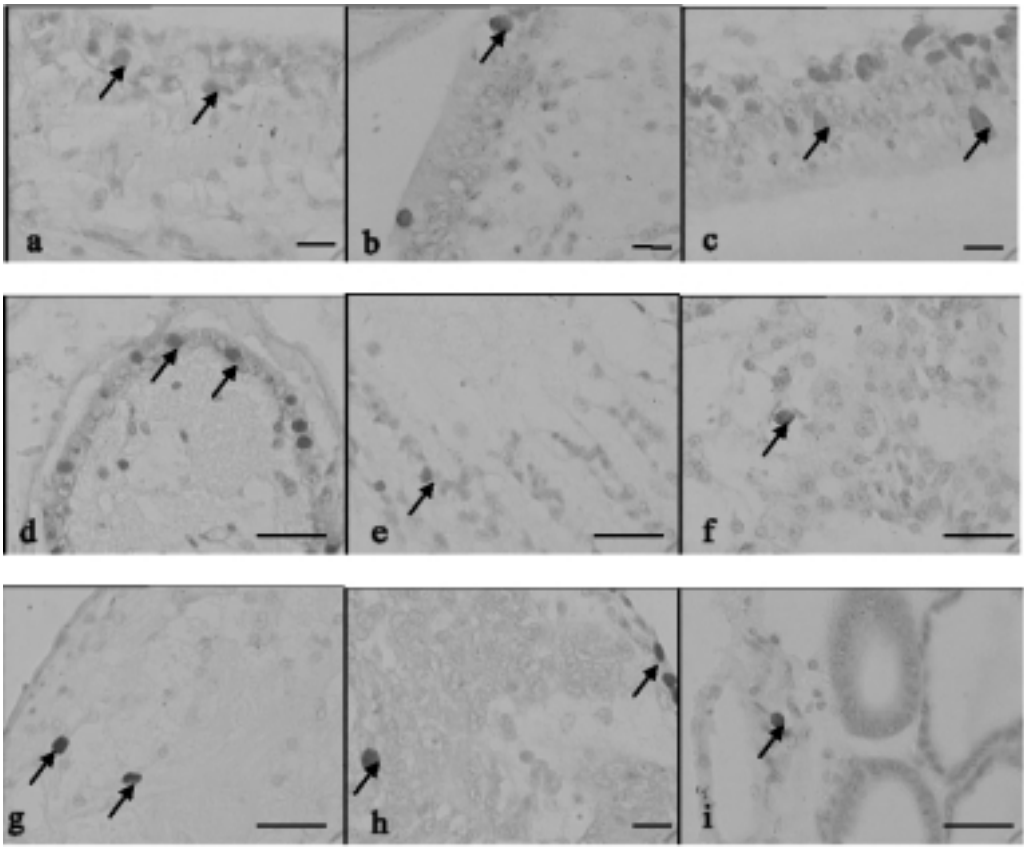

Fig. 1. Penaeus monodon tissues showing nucleus infected with white spot syndrome virus (WSSV) in (a) cuticular epithelium at 48 h post injection (hpi), (b) foregut at 60 hpi, (c) stomach at 60 hpi, (d) midgut at 48 hpi, (e) gill at 60 hpi, (f) antenna gland at 60 hpi, (g) heart at 60 hpi, (h) hematopoietic tissue at 60 hpi, and (i) connective tissue at 48 hpi. Scale bar = 50 µm.

Table 1. Clinical signs of white spot syndrome virus (WSSV) in *Penaeus monodon* injected with a low dose of $10^{1.5}$ SID_{50} (shrimp infectious dose with 50% endpoint) or high dose of 10^4 SID_{50} (no. infected/no. injected).

		Clinical sign					
Dose	*HPI**	*Lethargy*	*Empty gut*	*Reddish*	*White spots*	*Morbidity*	*Dead*
Experiment 1							
$10^{1.5}$ SID_{50}	0	0	0	0	0	0	0
	6	0	0	0	0	0	0
	12	0	0	0	0	0	0
	18	0	0	0	0	0	0
	24	0	0	0	0	0	0
	36	4/18	0	1/18	0	0	0
	48	2/12	2/12	1/12	0	0	0
	60	6/6	2/6	1/6	1/6	0	1/6
Control	60	0	0	0	0	0	0
10^4 SID_{50}	0	0	0	0	0	0	0
	6	0	0	0	0	0	0
	12	0	0	0	0	0	0
	18	0	0	0	0	0	0
	24	2/24	2/24	0	0	0	0
	36	1/18	1/18	0	0	0	0
	48	1/12	3/12	1/12	0	0	0
	60	3/6	3/6	3/6	6/6	1/6	1/6
Control	60	0	0	0	0	0	0
Experiment 2							
$10^{1.5}$ SID_{50}	3	0	0	0	0	0	0
	9	0	0	0	0	0	0
	15	0	0	0	0	0	0
	21	0	0	0	0	0	0
	30	0	0	0	0	0	0
	42	2/12	0	2/12	0	0	0
	54	3/6	2/6	1/6	0	0	0
Control	54	0	0	0	0	0	0
10^4 SID_{50}	0	0	0	0	0	0	0
	3	0	0	0	0	0	0
	9	0	0	0	0	0	0
	15	0	0	2/30	0	0	0
	21	1/24	1/24	1/24	1/24	0	0
	30	1/18	1/18	1/18	0	0	0
	42	1/12	1/12	1/12	1/12	1/12	1/12
	54	1/6	1/6	1/6	1/6	1/6	0
Control	54	0	0	0	0	0	0

* hours post injection

Table 2. Pathogenesis of white spot syndrome virus (WSSV) in *Penaeus monodon* (n = 6 per sample) injected with a low dose of $10^{1.5}$ SID_{50} (no. infected).

Organ	*Hours post injection*							
Experiment 1	*0*	*6*	*12*	*18*	*24*	*36*	*48*	*60*
Heart	0	0	2	0	2	4	5	6
Antennal gland	0	0	1	0	1	4	4	6
Foregut	0	0	0	1	2	4	4	6
Stomach	0	0	0	1	3	4	4	6
Gill	0	0	0	1	1	4	5	6
Cuticular epithelium	0	0	0	0	3	4	5	6
Hematopoietic tissue	0	0	0	0	0	4	5	6
Lymphoid organ	0	0	0	0	0	4	5	6
Midgut	0	0	0	0	0	4	3	6
Connective tissues	0	0	0	0	0	1	0	0
Experiment 2	*0*	*3*	*9*	*15*	*21*	*30*	*42*	*54*
Heart	0	0	0	2	2	6	6	5
Stomach	0	0	0	0	3	6	6	5
Cuticular epithelium	0	0	0	0	3	6	6	5
Gill	0	0	0	0	3	6	6	5
Antennal gland	0	0	0	0	2	6	6	6
Foregut	0	0	0	0	2	6	6	5
Connective tissue	0	0	0	0	2	3	5	5
Midgut	0	0	0	0	0	6	6	5
Lymphoid organ	0	0	0	0	0	4	6	6

began at 36-60 hpi with 100% cumulative mortality at 204-348 hpi in *Litopenaeus vannamei* (Rahman et al., 2008).

The heart, gills, stomach, cuticular epithelium, hematopoietic tissue, antennal gland, and lymphoid organ of *P. monodon* are major target organs of WSSV replication (Chang et al., 1996). These same organs were selected for enumerating WSSV-infected cells in *L. vannamei* where they are also target organs for WSSV replication (Escobedo-Bonilla et al., 2007; Rahman et al., 2008). The present study confirmed the replication of WSSV in *P. monodon* heart, antennal gland, foregut, stomach, gills, cuticular epithelium, hematopoietic tissue, connective tissue, and lymphoid organ.

Escobedo-Bonilla et al. (2007) developed a standardized WSSV oral inoculation procedure for SPF *L. vannamei* showing that shrimp tissues and hemolymph first become WSSV-positive at 18 hpi when using a low injection dose of $10^{1.5}$ SID_{50} and at 12 hpi when using a high injection dose of 10^4 SID_{50}. At these doses, primary replication was detected in the foregut and gill cells. The antennal gland was also a primary replication site at the high dose (Escobedo-Bonilla et al., 2007). In the present study, the heart and antennal gland were the first organs to develop

Table 3. Pathogenesis of white spot syndrome virus (WSSV) in *Penaeus monodon* (n = 6 per sample) injected with a high dose of 10^4 SID_{50} (no. infected/no. injected).

Organ	*Hours post injection*							
Experiment 1	*0*	*6*	*12*	*18*	*24*	*36*	*48*	*60*
Stomach	0	0	1	6	6	6	6	6
Gill	0	0	1	4	6	6	6	6
Antennal gland	0	0	1	2	6	6	6	6
Heart	0	0	1	1	5	5	5	6
Hematopoietic tissue	0	0	1	0	5	6	6	6
Connective tissue	0	0	1	0	3	1	3	6
Cuticular epithelium	0	0	0	4	6	6	6	6
Foregut	0	0	0	3	6	6	6	6
Lymphoid organ	0	0	0	0	6	6	6	6
Midgut	0	0	0	0	3	6	6	6
Experiment 2	*0*	*3*	*9*	*15*	*21*	*30*	*42*	*54*
Gill	0	0	0	4	6	6	6	6
Stomach	0	0	0	4	6	6	6	6
Cuticular epithelium	0	0	0	4	6	6	6	6
Heart	0	0	0	3	6	6	6	6
Foregut	0	0	0	2	6	6	6	6
Antennal gland	0	0	0	2	6	5	6	6
Hematopoietic tissue	0	0	0	0	6	6	6	6
Midgut	0	0	0	0	6	6	6	6
Lymphoid organ	0	0	0	0	6	6	6	6
Connective tissue	0	0	0	0	6	6	5	6

WSSV-infected cells (at 12 hpi) at the low dose, but all ten organs/tissues were infected by 36 hpi. With the high injection dose, the heart, antennal gland, stomach, gill, connective tissue, and hematopoietic tissue developed WSSV infection by 12 hpi, and all ten organs became infected by 21 hpi. Thus, the high WSSV injection dose increased the rate of WSSV infection development in *P. monodon*. The differences between our results and those of previous studies may be attributed to differences in viral strain and shrimp species.

During disease outbreaks on a shrimp farm, *P. monodon* infected with WSSV were reported to be lethargic, had a reddish body, and died within 7-10 days (Lightner, 1996; Kasornchandra et al., 1998). However, in both the high and low dose treatments in this study, the same disease signs were observed as early as 21 hpi and 36 hpi, respectively. The appearance of white spots was initially noted at 21 hpi and mortality began at 42 hpi in the high dose treatment while neither condition was observed until 60 hpi in the low dose treatment. As such, the 21-36 h incubation period for WSSV infection in *P. monodon* observed in this study was shorter than reported for natural infections on shrimp farms. Thus, this study implies that when the WSSV concen-

tration is high, the onset of disease signs occurs earlier than when the WSSV dose is low. As a result, we recommend that examination of the cephalothorax suffices for early detection of WSSV, since this part of the shrimp contains the organs where WSSV first replicates. For non-destructive sampling, especially of broodstocks, we suggest that only a small piece of the gill filament be checked for the presence of WSSV.

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