Tissue distribution of white spot syndrome virus (WSSV) in shrimp and crabs

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ABSTRACT: White spot syndrome virus (WSSV) has caused mass mortalities of cultured shrimp and crab. In this study, polymerase chain reaction (PCR) was used to determine the pattern of WSSV virus multiplication in tissues of shrimp and crabs. Tested specimens were divided into three groups based on WSSV PCR results. Group I comprised specimens whose tissues were all 2-step WSSV PCR negative; Group II comprised lightly infected specimens which had at least some tissues positive after re-amplification; Group III comprised heavily infected specimens whose tissues tested mostly 1-step WSSV PCR positive. In very lightly infected specimens (Group II), WSSV was particularly prevalent in gills, followed in order of decreasing prevalence by hemolymph, abdominal muscle, stomach, pleopods, heart, midgut, integument, pereiopods, eyestalk and the hepatopancreas. (This order, which is based on a larger sample size, is slightly different to the order reported in our previous paper). Also, because of a problem with false negatives, WSSV is likely to be more prevalent in the eyestalk than its position in this list would imply. WSSV tissue distribution in crabs, *Charybdis feriatus*, *Charybdis natator*, *Portunus pelagicus*, and *Portunus sanguinolentus*, was also investigated. Almost all the tested tissues or organs of the heavily infected crabs (Group III) were one-step PCR positive, while tissues or organs of the lightly infected crabs were positive only after re-amplification. In lightly infected specimens the prevalence of WSSV was particularly high in the gills, pereiopods and hemolymph, followed in order of decreasing prevalence by the stomach, eyestalks, maxillipeds, heart, integument, reproductive organs, midgut, abdominal muscle, nervous tissue and hepatopancreas. We therefore suggest that the best sources for PCR template preparation during non-destructive screening of asymptomatic carrier brooders, would be (a small piece of) the gills or (a small aliquot of) the hemolymph. There is also some evidence to suggest that an ablated eyestalk might be a good alternative, provided that the compound eye is removed before use.

KEY WORDS: WSSV in shrimp and crabs; tissue distribution; PCR

INTRODUCTION

White spot syndrome (WSS) is one of the most important shrimp diseases. It is of global occurrence and it affects most of the commercially cultured shrimp species (Inouye et al. 1994, Cai et al. 1995, Chou et al. 1995, Lightner 1996, Flegal 1997, Lotz 1997, Spann & Lester 1997). The clinical signs of this disease include white spots in the exoskeleton and epidermis, lethargy, a pink to reddish-brown coloration, the gathering of affected shrimp around the edges of ponds throughout the day and a rapid reduction in food consumption. The causative agent of WSS is an enveloped, non-occluded, rod-shaped DNA virus known as white spot syndrome virus (WSSV) (Wang et al. 1995, Lightner 1996). WSSV is also referred to as white spot syndrome baculovirus (WSBV) (Lo et al. 1996a, 1996b, 1997), and it is apparently identical or closely related to penaeid rod-shaped DNA virus (PRDV) (Inouye et al. 1994, 1996), hypodermal and hematopoietic necrosis baculovirus (HHNBV) (Cai et al. 1995), and systemic ectodermal and mesodermal baculovirus (SEMBV) or white spot virus (WSV) (Wongteerasupaya et al. 1995, 1996). Because these viral agents appear to be very similar in morphology, histopathology and genome structure (Lo et al. 1998), they have been regrouped and are now often collectively referred to as WSSV (Lightner 1996).

In cultured shrimp, WSSV infection is characterized by a wide range of target tissues, rapid disease onset and high mortality. During the viremic phase of infection, the virus is present in many organs. We previously conducted a combined study using currently available nucleic acid diagnostic tools and conventional histological observations using light (LM) and transmission electron (TEM) microscopy to examine the sites for virus multiplication in *Penaeus monodon* (black tiger shrimp). Sixteen parts excised from shrimp specimens were examined: pleopods, gills, stomach, abdominal muscle, hemolymph, gut, heart, pereiopods, lymphoid organs, epidermis, nervous tissue, hepatopancreas, testes, ovaries, spermatophores, and eye stalks. All these tissues/organs were found to support WSSV replication (Lo et al. 1997). In lightly infected specimens, WSSV was particularly prevalent in pleopods, followed in order of decreasing prevalence by gills, hemolymph, stomach, abdominal muscle, reproductive organs, midgut, heart, periopods, lymphoid organ, integument, nervous tissue, and the hepatopancreas (Lo et al. 1997). WSSV tissue distribution data from lightly infected specimens helps to suggest the most appropriate tissue source for polymerase chain reaction (PCR) testing. Thus, in the present paper, a larger sample size was used to reconfirm WSSV prevalence in pleopods, gills, hemplymph, stomach, abdominal muscle, heart, periopods and integument.

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Lo et al. (1996) and Lo and Kou (1998) have already used PCR, *in situ* hybridization, and transmission electron microscopy to show that WSSV positive specimens exist in captured populations of wild crabs (*Calappa philarigus*, *Charybdis feriatus*, *Charybdis natator*, *Helice tridens*, *Portunus pelagicus*, *Portunus sanguinolentus*, *Scylla serrata*) collected from the natural environment in coastal waters around southern Taiwan. In the present paper, we more fully document the WSSV prevalence in these same captured crab specimens and also investigate the virus tissue distribution in these crabs.

MATERIALS AND METHODS

Shrimp and crabs

Adult *Penaeus monodon* (black tiger shrimp) were either cultured or captured from their natural environment in the coastal waters around southern Taiwan in 1996-1997. Adult crabs *Calappa philarigus*, *Charybdis feriatus*, *Charybdis natator*, *Helice tridens*, *Portunus pelagicus*, *Portunus sanguinolentus*, *Scylla serrata* were all captured from their natural environment in the coastal waters around southern Taiwan on 18th July 1996. In these crabs, the prevalence of WSSV was first tested by WSSV diagnostic PCR using a piece of the last segment of the 5th pereiopod as the PCR template source.

WSSV tissue distribution in Shrimp and crabs

From the organs to be tested, PCR templates were prepared with proteinase K, N-cetyl N,N,N-trimethylammonium bromide (CTAB) treatments, phenol/chloroform extraction and ethanol precipitation (Lo et al. 1996a, Lo et al. 1997). For each sample, the quality of the extracted DNA was checked by PCR with a decapod 18S rRNA gene specific primer pair, before it was subjected to WSSV diagnostic PCR (Lo et al. 1996a). WSSV diagnostic PCR was performed as described previously (Lo et al. 1996b). Specimens were then divided into three groups based on WSSV PCR results. Specimens whose tissues were all two-step WSSV PCR negative were assigned to Group I; Group II comprised lightly infected specimens which had at least some tissues positive after re-amplification; Group III comprised heavily infected specimens whose tissues tested mostly one-step WSSV PCR positive. WSSV tissue distribution analysis in 5 crab specimens (three *C. feriatus* and two *P. sanguinolentus*) was also examined by *in situ* hybridization analysis (Lo et al. 1997).

RESULTS

WSSV tissue distribution in black tiger shrimp

In terms of clinical observation, all of the tested shrimp appeared to be healthy. However, of the 27 shrimp specimens, only five were 2-step PCR negative (Group I), 14 were lightly infected (Group II) and 8 were heavily infected (Group III). In lightly infected specimens (Group II), WSSV was particularly prevalent in the gills, followed in order of decreasing prevalence by hemolymph, abdominal muscle, stomach, pleopods, heart, integument, pereiopods, eyestalks and the hepatopancreas (Table 1). In Group III, WSSV prevalence in the hepatopancreas was relatively low (50%). Of the 8 specimens in Group III, only one specimen was 1-step WSSV PCR negative in abdominal muscle, integument and pereiopods (Table 2).

WSSV tissue distribution in crabs by PCR

The prevalence in several species as revealed by 2-step WSSV PCR was very high (Table 3). WSSV tissue distribution was investigated in three *Charybdis feriatus*, two *C. natator*, one *Portunus pelagicus*, and eight *P. sanguinolentus* specimens. Four of these crabs (two *P. sanguinolentus* and two *C. natator*) were 2-step WSSV PCR negative. The results for the other 10 crabs are shown in Tables 4 & 5. Almost all the tested tissues or organs of the heavily infected crabs (Group III) were one-step PCR positive (Table 5), while tissues or organs of the lightly infected crabs were positive only after re-amplification (Table 4). In lightly infected specimens, the prevalence of WSSV was particularly high in the gills, pereiopods and hemolymph, followed in order of decreasing prevalence by the stomach, eyestalk, maxillipeds, heart, integument, reproductive organs, midgut, abdominal muscle, nervous tissue and the hepatopancreas.

WSSV tissue distribution in crabs by *in situ* hybridization

Results of PCR testing of the pereiopods of the three *C. feriatus* and two *P. sanguinolentus* specimens are shown in Table 6. One of these crabs was 2-step PCR negative, two were positive only after reamplification and two were positive by 1-step PCR. After *in situ* hybridization, cells showing WSSV positive signals were observed in all of the tested organs. All sections from these crab specimens were also evaluated semiquantitatively. Signal prevalence scores of + (low), ++ (mild), +++ (moderate), ++++ (high) were defined as 1 to 10 positive cells, 11 to 100 positive cells, 101 to 1000 positive cells, and > 1000 positive cells, respectively, per 200 high power microscopic fields (´400 magnification). All the tested tissues of the two 1-step WSSV PCR positive crabs had moderate or high numbers of positive cells, while in the two crabs that were positive only after re-amplification, tissue signal prevalence scores were mostly nil, low or mild. In the infected crabs, the heart, gills and stomach tended to have the highest signal prevalence scores (Table 6).

DISCUSSION

While the results in Tables 2 and 5 are consistent, Tables 1 and 4 show both positive and negative results. In Tables 1 and 4, the shrimp and crabs were in the carrier state, while in Tables 2 and 5, they were in the transition state. It is important to note that they were not in the patent state (i.e., they had no gross signs of WSSV infection). Although the carrier state might persist for months, the transition state usually lasts for only a few hours and once a specimen becomes 1-step PCR positive, it will die within a few days at the most (Lo et al. 1998). So the transition interval is short-lived and during this time the disease/infection can progress rapidly (Lo et al. 1998).

Table 1. WSSV tissue tropism in lightly infected *Penaeus monodon* (Group II) as revealed by 2-step WSSV diagnostic PCR.

	2-step WSSV PCR									
Shrimp no.	Gill	Abdominal muscle	Hemolymph	Stomach	Pleopod	Heart	Integument	Pereiopod	Eyestalk	Hepatopancreas
1	+	-	-	-	+	-	-	-	nd	-
2	-	+	nd	-	-	-	-	+	nd	-
3	+	+	nd	+	+	+	-	-	nd	-
4	+	-	-	+	+	+	-	+	nd	-
5	+	+	+	-	+	-	-	+	nd	-
6	-	+	+	+	+	-	-	+	nd	+
7	+	-	+	+	+	+	+	-	nd	-
8	+	+	+	+	+	+	+	-	nd	-
9	+	+	+	+	+	-	+	-	nd	+
10	+	-	nd	+	-	-	+	-	+	-
11	-	+	nd	+	+	+	-	-	-	-
12	+	+	nd	-	-	+	+	+	-	-
13	+	+	nd	-	-	+	+	-	-	-
14	+	+	nd	+	-	+	+	+	+	+
Prevalence (%)	**78**	**71**	**71**	**64**	**64**	**57**	**50**	**42**	**40**	**21**

In the present study, pms146 F1/R1 and F2/R2 primer sets and the PCR reaction conditions described by Lo et al. (1996a) were utilized for WSSV diagnostic PCR. With this 2-step WSSV diagnostic PCR, it is in fact possible to detect 10-50 copies of target DNA in a PCR reaction, and the sensitivity of the 2-step amplification protocol is about 10^3 to 10^4 times greater than that of 1-step amplification alone. We conclude from the foregoing that to produce the consistent results seen in Table 2 and 5, the virus must have replicated very rapidly, by a factor greater than 10^3 (i.e., to well over the sensitivity threshold of 1-step WSSV diagnostic PCR). So with these pms146 primer sets, a 1-step positive diagnosis with clinically normal animals is a very clear indication of the transition state. It is important to note however, that

Table 2. WSSV tissue tropism in heavily infected *P. monodon* (Group III) as revealed by 1-step WSSV diagnostic PCR.

	1-step WSSV PCR								
Shrimp no.	Gill	Pleopod	Heart	Stomach	Abdominal muscle	Integument	Pereiopod	Hemolymph	Hepatopancreas
1	+	+	+	+	+	+	+	nd	-
2	+	+	+	+	+	+	+	+	-
3	+	+	+	+	+	+	+	+	-
4	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+
8	+	+	+	+	-	-	-	-	-
Prevalence (%)	**100**	**100**	**100**	**100**	**87**	**87**	**87**	**86**	**50**

Table 3. Results of two-step WSSV diagnostic PCR with *Calappa philarigus*, *Charybdis feriatus*, *Charybdis natator*, *Helice tridens*, *Portunus pelagicus*, *Portunu sanguinolentus* and *Scylla serrata* captured from their natural environment in the coastal waters around southern Taiwan.

	Prevalence of WSSV in crabs detected by PCR													
WSSV	*P. sanguinolentus*		*C. feriatus*		*P. pelagicus*		*S. serrata*		*H. tridens*		*C. natator*		*C. philarigus*	
PCR	No	%	No	%	No	%	No	%	No	%	No	%	No	%
1-step positive	28/48*	58	1/5	20	1/5	20	2/10	20	2/14	14	1/10	10	0/1	0
2-step positive	42/48	87	4/5	80	4/5	80	6/10	60	7/14	50	4/10	40	1/1	100
2-step negative	6/48	13	1/5	20	1/5	20	4/10	40	7/14	50	6/10	60	0/1	0

* Values represent the no. of crabs positive in the first and second step PCR per no. of crabs examined.

Table 4. WSSV tissue tropism in lightly infected crabs (Group II) as revealed by 2-step WSSV diagnostic PCR.

		2-step WSSV PCR														
Crab no.	Species	Pereiopod	Gill	Hemolymph	Stomach	Eyestalk	Maxilliped	Testis	Heart	Integument	Pleopod	Nerve	Midgut	Muscle	Ovary	Hepatopancreas
1	*Portunus sanguinolentus*	+	+	+	-	-	-	-	-	-	-	-	-	-		-
2	*Portunus sanguinolentus*	+	-	-	-	-	-		-	-	-	-	-	-	-	-
3	*Portunus sanguinolentus*	+	+	+	+	+	+	+	+	+	+	+	-	+		-
4	*Portunus sanguinolentus*	+	+	+	+	+	+	-	+	+	-	-	+	-		-
5	*Charybdis feriatus*	+	+	+	+	+	+	+	-	-	+	-	-	-		-
	Prevalence (%)	**100**	**80**	**80**	**60**	**60**	**60**	**50**	**40**	**40**	**40**	**20**	**20**	**20**	**0**	**0**

this is not necessarily true for other primer sets. For example, a 1-step PCR positive result with the more sensitive pms94 primer set (about 10x more sensitive and yielding a shorter amplicon) may sometimes be obtained with specimens that are still only in the carrier state (Lo et al in press, Lo & Kou unpublished data)

In very lightly infected shrimp (Group II), WSSV was particularly prevalent in gills, followed in order of decreasing prevalence by hemolymph, abdominal muscle, stomach, pleopods, heart, integument, periopods, eyestalks and the hepatopancreas (Table 1). This order, which is based on a

Table 5. WSSV tissue tropism in heavily infected crabs (Group III) as revealed by 1-step WSSV diagnostic PCR.

		1-step WSSV PCR														
Crab no.	Species	Heart	Gill	Stomach	Integument	Pleopod	Hemolymph	Nerve	Muscle	Pereiopod	Eyestalk	Maxilliped	Midgut	Hepatopancreas	Testis	Ovary
1	*Portunus sanguinolentus*	+	+	+	+	+	+	+	+	+	+	+	nd	+	+	
2	*Portunus sanguinolentus*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
3	*Charybdis feriatus*	+	+	+	+	+	+	+	+	+	+	+	nd			-
4	*Charybdis feriatus*	+	+	+	+	+	-	-	-	-	-	-	-	-	-	
5	*Portunus pelagicus*	+	+	+	+	+	+	+	+	+	+	+	+			+
	Prevalence (%)	**100**	**100**	**100**	**100**	**100**	**80**	**80**	**80**	**80**	**80**	**80**	**75**	**75**	**66**	**50**

Table 6. WSSV tissue tropism in crabs *Charybdis feriatus* and *Portunus sanguinolentus* as revealed by signal prevalence scores of tissue sections after *in situ* hybridization.

Crab no.	Species	1-step WSSV PCR	2-step WSSV PCR	Heart	Signal prevalence scores: Gill	Stomach	Midgut	Hepatopancreas	Nerve	Integument	Muscle	Pereiopod	Eyestalk	Eyeball	Testis	Vas deferens
1	*Charybdis feriatus*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	*Charybdis feriatus*	-	+	+++	++++	+++	++	+	++	++	++	++	++	nd	+	+++
3	*Portunus sanguinolentus*	-	+	+	++	++	-	-	-	+	-	+	+	-	nd	nd
4	*Portunus sanguinolentus*	+	+	++++	++++	++++	++++	+++	+++	++++	+++	++++	nd	+++	+++	+++
5	*Charybdis feriatus*	+	+	++++	++++	++++	+++	+++	++++	++++	+++	+++	++++	+++	nd	nd

larger sample size, is slightly different to the order reported in our previous paper (Lo et al. 1997). However, pleopods, gills, hemolymph, stomach and abdominal muscle were still the 5 most prevalently infected organs in both studies. Thus, in the carrier state, it is in these organs that the virus most frequently appears and replicates. Also, because of a problem with false negatives (Lo et al. 1997), WSSV is likely to be more prevalent in eyestalks than its position in this list would imply. Therefore, for non-destructive screening of asymptomatic brooder carriers, we recommend that the best sources for PCR template preparation would be (a small piece of) gills or (a small aliquot of) the hemolymph. There is also some evidence to suggest that an ablated eyestalk would be a good alternative, provided that the compound eye is removed before use.

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LITERATURE CITED

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

Chou HY, Huang CY, Wang CH, Chiang HC, Lo CF (1995) Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan. Dis Aquat Org 23:165-173

Flegel TW (1997) Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. In Y. Inui (ed) New approaches to viral diseases of aquatic animals. NRIA International Workshop proceedings. National Research Institute of Aquaculture, Nansei, Watarai, Mie 516-01, Japan. p. 167-187

Flegel TW (1997) Special topic review: Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. World journal of microbiology & Biotechnology 13: 433-442

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

Lightner DV (ed) (1996) A handbook of pathology and diagnostic procedures for diseases of penaeid shrimp. World Aquaculture Soc, Baton Rouge, LA, Section 3.11

Lo CF, Leu JH, Ho CH, Chen CH, Peng SE, Chen YT, Chou CM, Yeh PY, Huang CJ, Chou HY, Wang CH, Kou GH (1996 a) Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction. Dis Aquat Org 25: 133-141

Lo CF, Ho CH, Peng SE, Chen CH, , Hsu HC, Chiu YL, Chang CF, Liu KF Su MS, Wang CH, Kou GH (1996 b) White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods. Dis Aquat Org 27: 215-225

Lo CF, Ho CH, Chen CH, Liu KF, Chiu YL, Yeh PY, Peng SE, Hsu HC, Liu HC, Chang CF, Su MS, Wang CH, Kou GH (1997) Detection and tissue distribution of white spot syndrome baculovirus (WSBV) in captured brooders of *Penaeus monodon* with a special emphasis on reproductive organs. Dis Aquat Org 30: 53-72

Lo CF, Wang CH, Kou GH (1997) White spot syndrome (WSS): pathology, hosts and prevalence in captured shrimp and crabs in Taiwan. In Y. Inui (ed) New approaches to viral diseases of aquatic animals. NRIA International Workshop proceedings. National Research Institute of Aquaculture, Nansei, Watarai, Mie 516-01, Japan. p. 206-217

Lo CF, Hsu HC , Ho CH, Tsai MF , Kou GH, Lightner DV (1998) Specific genomic DNA fragment analysis of different geographical isolates of shrimp white spot syndrome associated viru Dis Aquat Org (in press)

Lotz JM (1997) Special review: Viruses, biosecurity and specific pathogen-free stocks in shrimp aquaculture. World journal of microbiology & Biotechnology 13: 405-413

Spann KM, Lester RJG (1997) Special topic review: Viral diseases of penaeid shrimp with particular reference to four viruses recently found in shrimp from Queensland. World journal of microbiology & Biotechnology 13: 419-426

Wang CH, Lo CF, Leu JH, Chou CM, Yeh PY, Chou HY, Tung MC, Chang CF, Su MS, Kou GH (1995) Purification and genomic analysis of baculovirus associated with white spot syndrome (WSBV) of *Penaeus monodon*. Dis Aquat Org 23: 239-242

Wongteerasupaya C, Vickers JE, Sriurairatana S, Nash GL, Akarajamorn A, Boonsaeng V, Panyim S, Tassanakajon A, Withyachumnarnkul B, Flegel TW (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

Wongteerasupaya C, Wongwisansri, Boonsaeng V, Panyim S, Pratanpipat P, Nash GL, Withyachumnarnkul B, Flegel TW (1996) DNA fragment of *Penaeus monodon* baculovirus PmNOBII gives positive *in situ* hybridization with white-spot viral infections in six penaeid shrimp species. Aquaculture 143: 23-32