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Long-term presence of white spot syndrome virus (WSSV) in a cultivated shrimp population without disease outbreaks

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ABSTRACT: PCR and *in situ* hybridization analysis were used for detection of white spot syndrome virus (WSSV) in an infected, cultured shrimp population over a long period in the absence of disease outbreaks. The shrimp were derived from a single WSSV-carrier brooder and cultured first in a tank and then in outdoor ponds. Prior to harvest at 13 mo, no 1-step PCR-positive specimens were found, even though most tested specimens were found to be 2-step PCR-positive. At 7 mo, 2-step PCR-positive tissues were found in 5 sampled shrimp. Heart, gill, integument, muscle and stomach tissues best supported viral replication. At 13 mo several shrimp died, and 1-step PCR-positive individuals were found for the first time. Although superficially healthy, 10% of the surviving adults had tiny white spots on their carapace, and *in situ* hybridization analysis revealed WSSV-positive cells in 40% of the specimens examined. As before, most were found in the stomach, integument and gills, and only very few in the lymphoid organ and other organs. These observations contrasted to those for experimentally infected shrimp with gross signs of terminal WSSV infection, where strong positive signals were also observed in the lymphoid organ and in other organs of ectodermal or mesodermal origin. Our results showed clearly that whatever the source, WSSV was carried in the shrimp population at a low intensity (i.e. nested PCR was required for detection) for a very long time in the absence of massive mortality. We hypothesize that disease outbreaks do not occur if shrimp defense mechanisms manage to contain low-intensity viral infections under low-stress culture conditions. Conversely, outbreaks may occur under stressful conditions.

KEY WORDS: WSSV · Carrier brooder · Offspring · Virus multiplication sites · *In situ* hybridization · *Penaeus monodon*

INTRODUCTION

White spot syndrome (WSS) is an important shrimp disease that affects most of the commercially cultured shrimp species globally (Inouye et al. 1994, Cai et al. 1995, Chou et al. 1995, Lightner 1996, Flegel 1997, Lotz 1997, Spann & Lester 1997, Lo et al. 1999). 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,b, 1997), and it is apparently identical or closely related to the 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; also referred to as white spot virus, WSV; Wongteerasupaya et al. 1995, 1996). Because these viral agents appear to be very similar in general characteristics, they have been regrouped and are now often referred to as WSSV (Lightner 1996).

We previously confirmed the presence of virus particles in developing oocytes, follicle cells and connective tissue cells in the ovary (Lo et al. 1997). Investigation of WSSV infection in brooders and their offspring re-

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vealed that 2-step WSSV PCR-positive brooders could produce larval offspring that were either 2-step WSSV PCR-positive or -negative (Lo et al. 1997). This equivocal result led us to consider whether or not 2-step WSSV PCR-positive samples were actually WSSV-infected by vertical transmission from the brooder or merely WSSV-contaminated by the water and/or virus attached to the surface of the egg or larva. Thus, we continued to culture the progeny of 1 of the 2-step WSSV-positive brooders (designated S14 in Lo et al. 1997), and to monitor WSSV in them by PCR and *in situ* hybridization. Here we report the results of these studies and attempt to draw some conclusions concerning the transmission mode of WSSV, its maintenance in nature and its pathogenic mechanism.

MATERIALS AND METHODS

Offspring of a WSSV carrier brooder. The *Penaeus monodon* brooder S14 was captured from natural coastal waters around southern Taiwan on 20 July 1995 and immediately transported to reproduction facilities (a 500 l Fiberglass Reinforced Plastic [FRP] tank kept in darkness and containing 450 l aerated, filtered 33‰ seawater at 30 ± 1°C, pH 8.3) at the Tung Kang Marine Laboratory. Upon arrival, S14 was subjected to 2-step WSSV diagnostic PCR (Lo et al. 1996a,b) using a pereiopod, and it showed positive only in the second step. A few hours after arrival at the laboratory, S14 spawned. Before reaching the zoeal stage, the nauplii were transferred to six 500 l FRP tanks with 35 000 nauplii in each tank. Great care was taken to eliminate possible external sources of WSSV through to postlarval stage 30 (PL_{30}). The larvae were fed cultured algae (*Skeletonema costatum*) once daily and a commercial, artificial, microparticulated diet 3 times daily. After the mysis stage, a continuously flushing system replaced about 40% of the seawater in the culture tanks each day. The survival rate from the naupliar stage to PL_3 was 70%. At PL_3, shrimp were transferred to outdoor 2500 l FRP tanks containing 33‰ seawater (2000 l) at ambient temperature (23 to 26°C), where they were fed a commercial diet 3 times daily. In addition to the commercial diet, the PL_3 to PL_{10} were also fed brine shrimp nauplii. Approximately 7% of the seawater in the 2500 l tanks was replaced by continuous flushing each day. The survival rate from PL_3 to PL_{30} was 50%. The shrimp were subsequently cultured to the adult stage in a small, concrete, outdoor pond (200 m^2) at a density of 24 m^{-2}.

Samples were taken at different life stages, from egg through to 13 mo adults, and prepared by quick freezing in liquid nitrogen. They were then stored at –20°C until used.

PCR monitoring of WSSV in S14 offspring. During growth from larvae to adults, the shrimp were tested for WSSV infection using WSSV PCR. Samples of eggs, larvae (nauplius, zoea and mysis) and post larvae (PL_1, PL_2 and PL_3) were pooled to weigh about 0.2 g after seawater had been removed by centrifugation at 1000 × *g*, 5 min. Samples of PL_{20} were individual as were pereiopods (0.2 g) from individual shrimp (3 to 13 mo old). These were used for template DNA isolation following the method described by Lo et al. (1996a,b, 1997). Because PCR testing of specimens younger than zoea tended to produce borderline positive results, each pooled egg and larval sample was tested using more PCR replications than for older (PL_1 and up) specimens.

PCR analysis of WSSV tissue distribution in S14 offspring at 7 mo. At 7 mo, tissue from the heart, gills, integument, abdominal muscle, stomach, pereiopods, eyestalks, hepatopancreas and pleopods of 5 shrimp were examined for WSSV tissue distribution by WSSV diagnostic PCR.

WSSV diagnostic PCR. Four primer sets (pms 94 F1/R1, pms 94 F2/R2, pms 146 F1/R1 and pms 146 F2/R2) were utilized for 2-step WSSV diagnostic PCR (Lo et al. 1996a). Before PCR, the quality of extracted DNA was checked with a primer set (143F and 145R) that amplified a highly conserved region of the 18S rRNA sequence of decapods (Kim & Abele 1990, Lo et al. 1996a). The sequences of all the primers used are shown in Table 1. The thermal cycling program and reaction conditions for PCR were the same as those described previously (Lo et al. 1997).

Detection of WSSV in S14 offspring by *in situ* hybridization. At harvest (13 mo post spawning), 20 random specimens of S14 offspring (F1 to F20) were collected.

Table 1. PCR primer sequences used in this study

Primer set	Primer sequence
143/145	143F: 5'-TGCCTTATCAGCTNTCGATTGTAG-3'[a]
	145R: 5'-TTCAGNTTTGCAACCATACTTCCC-3'
pms 94 F1/R1	pms94F1. 5'-CGGTCTCAGTAATTCGTC-3'
	pms94R1: 5'-CCTCCATTTGCTGCAGTG -3'
pms 94 F2/R2	pms94F2: 5'-CGATACTGCCATTGAAAGC-3'
	pms94R2: 5'-GCCCTGGAGAACACTTCC-3'
pms 146 F1/R1	pms146F1: 5'-ACTACTAACTTCAGCCTATCTAG-3'
	pms146R1: 5'-TAATGCGGGTGTAATGTTCTTACGA-3'
pms 146 F2/R2	pms146F2: 5'-GTAACTGCCCCTTCCATCTCCA-3'
	pms146R2: 5'-TACGGCAGCTGCTGCACCTTGT-3'

[a]N represents G, A, T or C

Each specimen was fixed whole in Davidson's fixative (Lightner 1996) after which the cephalothorax was excised and embedded in Paraplast/Plus. Sections (4 µm) were made and mounted onto positively charged Superfrost/Plus™ microscope slides (Fisher Scientific) for *in situ* hybridization with a WSSV-specific digoxigenin-labeled probe using the methods described previously (Chang et al. 1996, Lo et al. 1997). The presence or absence of WSSV-positive cells in tissues of the stomach, the epidermis, gills, the lymphoid organ, the heart, the abdominal muscle, the hepatopancreas and reproductive organs was recorded.

Comparison of WSSV *in situ* hybridization and PCR results for S14 offspring and experimentally infected shrimp. For these tests, the results from *in situ* hybridization analysis were evaluated semi-quantitatively. Tissues were classified based on numbers of hybridization positive cells per 200 high power microscopic fields (magnification ×400) as – for none, + for 1 to 10, ++ for 11 to 100, +++ for 101 to 1000 and +++ for >1000. This analysis was applied to both S14 offspring and experimentally infected, 1-step WSSV PCR-positive *Penaeus monodon*. Three shrimp (10 to 15 g) were experimentally infected using a method modified from Durand et al. (1996). Specifically, virus inoculum was prepared from a homogenate of the gills, stomach and exoskeleton (0.1 g ml^{-1} in 0.9% NaCl) of WSS infected *P. monodon*. After centrifugation at 3000 rpm (800 × *g*) for 10 min, the supernatant was diluted 1:100 with 0.9% NaCl and filtered through a 0.45 µm filter. For experimental infection, 50 µl of the filtrate was injected into each shrimp at the dorsal lateral area of the fourth abdominal segment, between the tergal plates forward into the third abdominal segment. Two days after injection, experimentally infected shrimp were checked by WSSV diagnostic PCR (Lo et al. 1996a,b). All the specimens were 1-step PCR-positive, indicating that they were heavily infected with WSSV. These experimentally infected, 1-step WSSV PCR-positive specimens (E1 to E3) were used as the patently infected control group for comparative tissue tropism analysis.

RESULTS

Gross signs of WSS in S14 offspring

During the entire S14 offspring culture period, mass mortality did not occur, and no obvious WSS was apparent until the 13th month, when 2 or 3 shrimp died per day in the few days before harvest. The shrimp were harvested when their body weight was about 60 to 80 g. Immediately after harvest, the shrimp were carefully examined and they looked healthy, although 10% had tiny, just barely visible spots on their carapace.

PCR monitoring during growth period

Throughout the culture period, most tested specimens were 2-step WSSV PCR positive (Table 2, Fig 1). Only 1 of the 2 egg sample replicates and 1 of the 2 nauplius replicates were 2-step WSSV PCR-positive. On average, about half of the pooled mysis, zoea, PL_1, PL_2, and PL_3 samples were 2-step WSSV PCR-positive, but at PL_{20}, all 6 of the individual specimens were 2-step PCR-positive. After PL_{20}, the percentage of WSSV-positive specimens decreased. Nonetheless, at 13 mo, some 1-step PCR-positive individuals were found.

PCR analysis of WSSV tissue distribution at 7 mo

With DNA templates prepared from pereiopods, only 3 of the 5 tested shrimp were 2-step WSSV PCR-positive with the pms 94 primer set (Table 2). However, all 5 of the specimens were 2-step PCR-positive with the pms 94 primer set in at least 1 of the other tested tissues, with a particularly high proportion of WSSV PCR-positive results being found in the heart, gills, integument, and abdominal muscle (Table 3).

Table 2. Detection of WSSV in S14 offspring by 2-step WSSV diagnostic PCR. Values represent the number of DNA samples (or replications from 1 DNA sample for egg and nauplius) positive in the first and second step PCR per number of DNA samples examined

Shrimp stage	Source of DNA samples	1-step PCR pms 146	1-step PCR pms 94	2-step PCR pms 146	2-step PCR pms 94
Egg	1 pooled sample	0/2	0/2	1/2	1/2
Nauplius	1 pooled sample	0/2	0/2	0/2	1/2
Zoea	6 pooled samples	0/6	0/6	3/6	6/6
Mysis	4 pooled samples	0/4	0/4	1/4	3/4
PL_1	6 pooled samples	0/6	0/6	2/6	6/6
PL_2	6 pooled samples	0/6	0/6	5/6	5/6
PL_3	6 pooled samples	0/6	0/6	2/6	3/6
PL_{20}	6 individuals[a]	0/6	3/6	4/6	6/6
3 mo	6 pereiopods[b]	0/6	0/6	0/6	3/6
4 mo	6 pereiopods	0/6	0/6	2/6	4/6
5 mo	6 pereiopods	0/6	0/6	2/6	2/6
7 mo	5 pereiopods	0/5	0/5	0/5	3/5
13 mo	2 pereiopods	2/2	2/2	2/2	2/2

[a] PL_{20} with the head removed
[b] A piece of tissue from a pereiopod of each tested individual

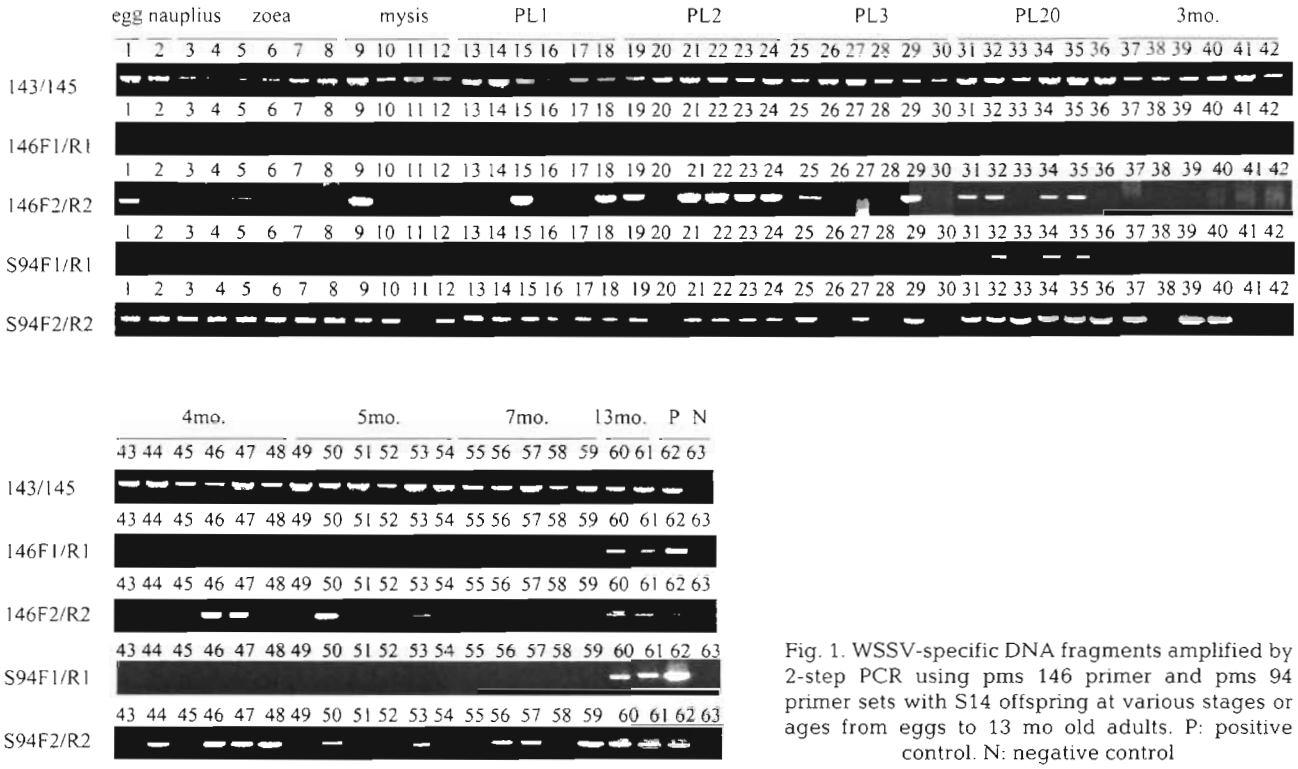

Fig. 1. WSSV-specific DNA fragments amplified by 2-step PCR using pms 146 primer and pms 94 primer sets with S14 offspring at various stages or ages from eggs to 13 mo old adults. P: positive control. N: negative control

Detection of WSSV by *in situ* hybridization in S14 offspring and experimentally infected shrimp

WSSV positive cells were observed in 40% (8/20) of the tested S14 offspring specimens. In these shrimp, positive signals were observed in the gills, stomach, epidermis, lymphoid organs (Fig. 2), heart, hepatopancreas, and reproductive organs. For these specimens, no WSSV-positive signals were observed in muscle cells. However, the percentage of WSSV-positive cells in the hepatopancreas (1/7; 14%), lymphoid organ (3/7; 43%) and heart (3/6; 50%) was much lower than in the gills (6/8; 75%). It was very high (8/8; 100%) in the epidermis and stomach (Table 4). Tissue tropism for these 8 *in situ* hybridization-positive specimens is shown in Table 4.

Table 3. Detection of WSSV in tissues of S14 offspring at an age of 7 mo by 2-step WSSV diagnostic PCR using pms 94 primers. No DNA samples were positive in the first step of PCR amplification

Shrimp no.	Heart	Gill	Integument	Abdominal muscle	Stomach	Pereiopod	Eyestalk	Hepato-pancreas	Pleopod
F7-1	–	+	+	–	+	–	+	–	–
F7-2	+	–	–	+	+	+	–	–	+
F7-3	+	+	+	+	–	+	–	–	–
F7-4	+	+	+	+	–	–	–	–	–
F7-5	+	+	+	+	+	+	+	+	–
Percentage (%)	80	80	80	80	60	60	40	20	20

In the experimentally WSSV-infected controls (E1, E2 and E3), the relative *in situ* hybridization signal index for gills, stomach, epidermis, lymphoid organ, and heart was mostly at the highest level (+++ to ++++), which suggested that massive virus replication occurred in these organs (Fig. 3). On the other hand, the relative *in situ* hybridization signal index in muscle tissue, the hepatopancreas and reproductive organs was lower. These results contrasted with those for the S14 offspring, which gave lower indices overall and gave low or no hybridization signal in the gills, heart, lymphoid organ and hepatopancreas and gave no signal in muscles.

DISCUSSION

In a previous paper (Lo et al. 1997) we reported on a *Penaeus monodon* brooder (S14) that was captured from the natural environment. It was found to be 2-step WSSV PCR-positive, but became 1-step PCR-positive after spawning, and then died within a few days. Its eggs and nauplii were 2-step WSSV PCR-negative, but the zoeal stage was 2-step WSSV PCR-positive (Lo et al. 1997). In the same paper, surprising and/or equi-

Table 4. Comparison of WSSV tissue tropism in S14 offspring at an age of 13 mo and 3 experimentally infected 1-step WSSV PCR-positive shrimp (E1 to E3) by relative *in situ* hybridization signal index ranging from –: no signal to ++++: high signal (see text for a full definition), nd: not determined

Shrimp no.	Sex	Epidermis	Stomach	Gill	Heart	Lymphoid organ	Reproductive organs	Hepato-pancreas	Muscle cell
E-1	M	+++	++++	++++	++++	+++	+	++	+
E-2	F	++++	++++	+++	++++	+++	nd	++	+
E-3	F	++++	++++	++++	++++	+++	nd	++	+
F13-1	F	–	–	–	–	–	–	–	–
F13-2	F	–	–	–	nd	–	–	nd	nd
F13-3	M	+++	+++	+	nd	–	nd	–	nd
F13-4	F	–	–	–	nd	–	–	–	nd
F13-5	F	–	–	–	–	–	–	–	–
F13-6	F	+++	+++	++	–	–	+	–	–
F13-7	F	++	+++	++	–	nd	+	–	–
F13-8	M	+++	+++	–	+	+	nd	–	–
F13-9	F	++	+++	+	nd	+	+	+	–
F13-10	F	+	++	+	–	–	–	–	–
F13-11	F	nd	–	–	nd	–	–	–	nd
F13-12	M	–	–	–	nd	–	–	–	nd
F13-13	M	–	–	–	–	–	–	–	–
F13-14	M	–	–	–	nd	–	–	–	nd
F13-15	M	–	–	–	nd	nd	–	–	nd
F13-16	F	+++	+++	+	++	+	–	–	–
F13-17	M	++	+++	–	+	–	–	nd	–
F13-18	F	–	–	–	–	–	–	–	–
F13-19	M	–	–	–	–	–	–	–	–
F13-20	F	–	–	–	nd	–	–	–	nd
Overall percentage (%)		13	40	30	27	17	17	6	0
No. of specimens in which this organ was WSSV-positive/no. of tested specimens in which at least 1 organ was WSSV-positive		8/8 (100%)	8/8 (100%)	6/8 (75%)	3/6 (50%)	3/7 (43%)	3/6 (50%)	1/7 (14%)	0/7 (0%)

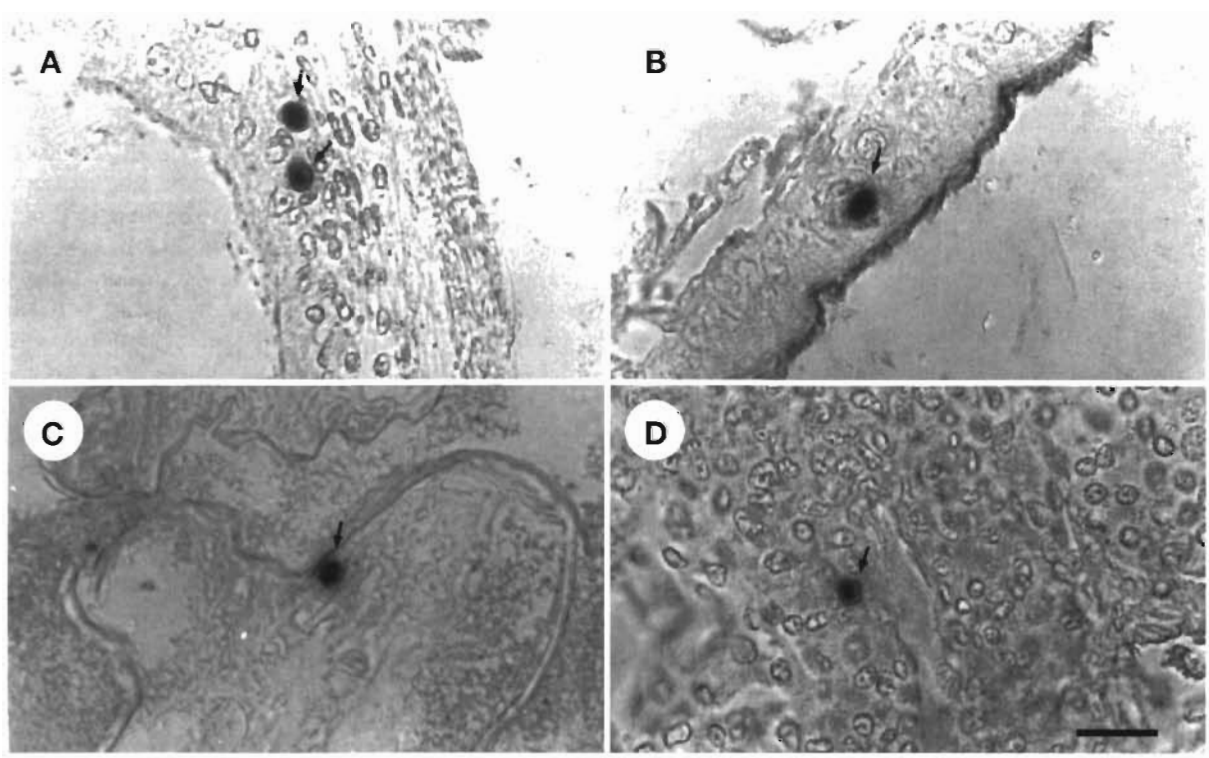

Fig. 2. Detection of WSSV-positive cells (arrows) in (A) stomach, (B) epidermis, (C) gills and (D) lymphoid organ of S14 offspring at the age of 13 mo using *in situ* hybridization. Scale bar = 25 µm

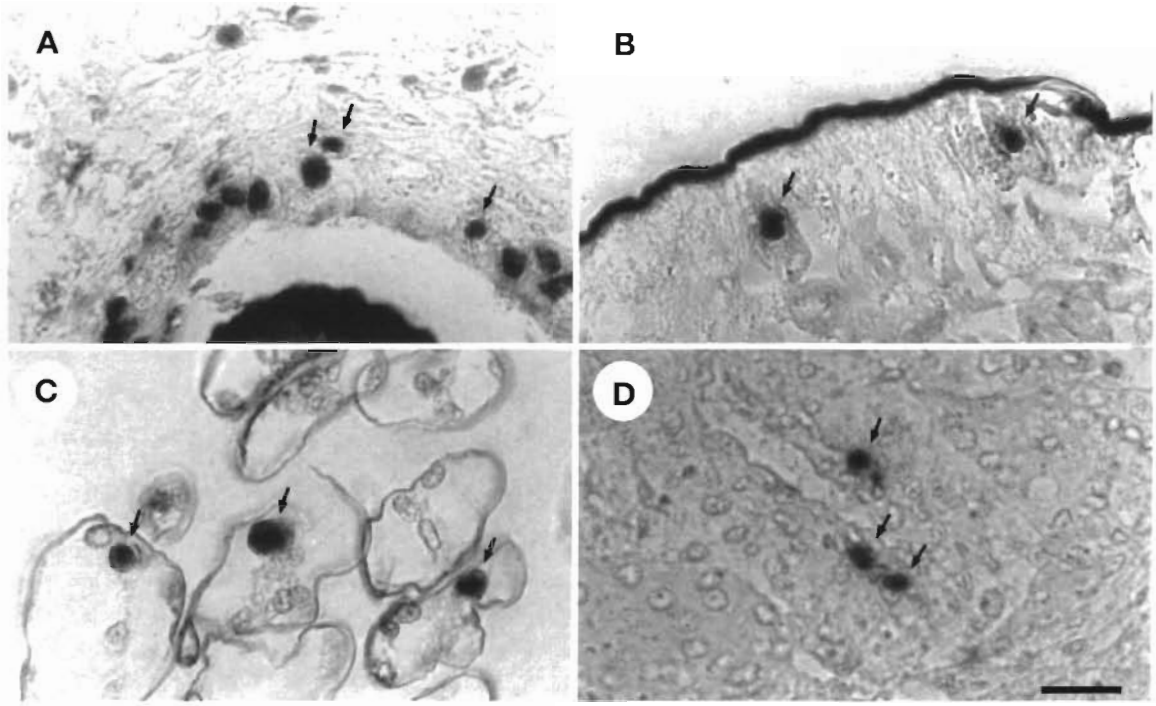

Fig. 3. Detection of WSSV-positive cells (arrows) in (A) stomach, (B) epidermis, (C) gills and (D) lymphoid organ of experimentally infected 1-step WSSV PCR-positive shrimp using *in situ* hybridization. Scale bar = 25 µm

vocal results were also found in 15 other brooders and their offspring. Since it was possible that the amount of WSSV DNA in those tested samples approached the sensitivity limits of the detection method, in the present study more samples were tested and more replications were made. As shown in Table 2, some PCR reactions for eggs and nauplii were 2-step WSSV PCR-positive. The most likely explanation for this is that the WSSV DNA concentration in the tested DNA samples of eggs and nauplii was near the detection limit, so that some samples were positive and some negative. The fact that the percentage of WSSV-positive samples remained fairly low from the egg to the PL_1 stages suggested that the virus might not be able to replicate—or at least not be able to replicate well—in shrimp at these early stages. From PL_2 to PL_{20}, however, the percentage of WSSV-positive samples increased (Table 2). This suggested that the virus was able to replicate at these stages. However, there are other possible explanations that might equally well account for the observed data. For example, one difficulty is that although all the tested individuals at PL_{20} were WSSV-positive, we cannot be certain that all these infections were the direct result of transovarial transmission (i.e. infection via sluffed ovarial tissues). Infections may also have resulted from the spread of the virus horizontally in the shrimp population (i.e. infected by offspring-released virus). Nor can we exclude the possibility of transovum transmission (i.e. infection by virus within eggs). We conclude, however, that under the rearing conditions described here, and without any treatment, offspring of a WSSV-carrier brooder would inevitably be infected with the virus.

In pilot studies on the effects of farm culture conditions on growth and survival of WSSV-infected shrimp, our preliminary data showed that ponds intensively stocked with post-larvae having WSSV infections equal to most of the specimens in Fig. 1 gave predictable mass mortality within a few weeks. Mass mortality sometimes occurred even at semi-intensive density (50 to 100 shrimp m^{-2}) (Lo et al. 1998). By contrast, the offspring of S14 were successfully cultured through to harvest even though many were 2-step PCR-positive (Fig 1). The culture density in the outdoor ponds used was intentionally kept very low (24 shrimp m^{-2}), precisely because we hoped to culture these shrimps for as long as possible. Under such favorable (low-stress, or at least low-density-stress) conditions, we hypothesize that the shrimp defense mechanisms were able to contain the virus. Thus, it was only at 13 mo that 1-step PCR-positive individuals were found for the first time, and some shrimp even died. At this time, the remaining shrimp were harvested and appeared healthy, although close inspection revealed that 10% had tiny white spots on their carapace, and WSSV-positive cells were found by *in situ* hybridization (Table 4). All this

confirmed that the offspring of S14 were indeed infected.

Table 2 also reveals another unexpected result. Although all 6 of the tested PL_{20} specimens were 2-step PCR-positive, only 2 of the 6 tested adolescent shrimp were 2-step PCR-positive at 5 mo (Table 2). This can be explained by sampling methods. For PL_{20}, the entire body was used to prepare the PCR template, but for later stages the PCR template was prepared using only 1 pereiopod. This would decrease the chance of detecting the virus and could lead to an apparent decrease in prevalence. This raises questions about the reliability of pereiopods as a source of DNA template. Indeed, our 7 mo samples for tissue distribution showed that heart, gill, epidermis, muscle and stomach were the tissues that best supported viral replication. Tissues from these organs are therefore recommended as the source of PCR template for monitoring WSSV in shrimp during the growth period. This supplements recommendations made in a previous paper (Lo et al. 1997), in which we suggested excision of a small piece of a pereiopod or pleopod for screening brooders, since this is relatively non-destructive and causes little damage. By contrast, survival of the tested shrimp from culture ponds is far less important and internal organs could be used to do destructive PCR tests if the results more closely reflected the real situation.

Primer sets pms 146 F1/R1 and F2/R2 and the PCR conditions described by Lo et al. (1996a) are routinely utilized for WSSV diagnostic PCR. With this 2-step WSSV diagnostic PCR, it is possible to detect 10 to 50 copies of target DNA in PCR, 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 (Lo et al. 1998). In a previous paper, we classified WSSV infections into 3 states, as (1) the asymptomatic carrier state which was WSSV 2-step PCR-positive only, (2) the transition and (3) patent states, both of which were WSSV 1-step PCR-positive. Under certain triggering conditions (e.g. stress), the carrier state may progress to the transition and patent states. Although the carrier state may 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 most. Thus the transition state is short-lived and is characterized by rapid disease/infection progression (Lo & Kou 1998). We also concluded from our study on WSSV tissue distribution in shrimp that the virus must replicate very rapidly from the carrier state to transition state, i.e. by a factor greater than 10^3 or well over the sensitivity threshold of 1-step WSSV diagnostic PCR (Kou et al. 1998). So with these pms 146 primer sets, a 1-step positive diagnosis is a very clear indication of the transition state. It is important to note, however, that this is not necessarily true for other primer sets, such as pms 94, which has a shorter amplicon and is about 10× more sensitive. The primer set pms 146 F4/R3 yields an amplicon of only a few hundred base pairs and is even more sensitive (Lo et al. 1996a). Using such primers, a 1-step PCR-positive result may sometimes be obtained with specimens that are still in the carrier state only. Paradoxically, for some purposes particular primer sets may actually be too sensitive. While excellent for detecting the presence of WSSV, their increased sensitivity does not necessarily mean that they are more useful for prognosis in the field. An example of this can be seen in Fig. 1: the 3 PL_{20} specimens that were 2-step PCR-positive with the pms 146 primer set yielded 1-step PCR-positive results with the pms 94 primer set. Mortality, however, did not occur in the shrimp population at this stage.

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