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NOTE

Comparison of a novel *in situ* polymerase chain reaction (ISPCR) method to other methods for white spot syndrome virus (WSSV) detection in *Penaeus vannamei*

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ABSTRACT: *Penaeus vannamei* were experimentally injected with white spot syndrome virus (WSSV) and tested for WSSV at different times post-injection (p.i.) by 1-step polymerase chain reaction (PCR), 2-step PCR, *in situ* hybridization (ISH) and *in situ* polymerase chain reaction (ISPCR) in order to compare sensitivity of the methods. With 1-step PCR, 4 of 15 shrimp tested positive for WSSV at 12 h p.i., and all tested positive by 24 h p.i. With 2-step PCR, 13 out of 15 samples tested positive at 2 h p.i. and all were positive by 4 h p.i. Using *in situ* hybridization, 1 sample tested positive at 18 h p.i. and all were positive by 36 h p.i. With ISPCR, 1 out of 5 samples was positive at 2 h p.i. and all were positive by 8 h p.i. Two-step PCR showed the highest sensitivity, followed by ISPCR, 1-step PCR and ISH. Although ISPCR revealed WSSV in 9 of 10 *P. vannamei* that tested positive for WSSV using 2-step PCR, none of the shrimp examined showed clinical signs of WSSV infection or detectable WSSV with 1-step PCR. The major infected organs were muscle and the hepatopancreas.

KEY WORDS: *In situ* polymerase chain reaction (ISPCR) · Polymerase chain reaction (PCR) · *In situ* hybridization (ISH) · WSSV · *Penaeus vannamei* · Latent infection

INTRODUCTION

White spot syndrome virus (WSSV) is the causative agent of white spot syndrome (WSS) in cultured penaeid shrimp. Outbreaks of WSS in cultured shrimp occur in many regions of the world, but especially in southeast Asia (Inouye et al. 1994, Chou & Huang 1995, Wang et al. 1995, Wongteerasupaya et al. 1996, Lightner 1996). There are many effective and convenient methods currently available for the detection of WSSV infections. These include PCR (He et al. 1996, Lightner 1996, Lo & Ho 1996, Takahashi et al. 1996,) *in situ* hybridization (ISH) (Lightner 1996, Lo & Ho 1996, Huang et al. 2000, Lu et al. 2000), and real-time quantitative PCR (Durand & Lightner 2002). However, 1-step PCR and *in situ* hybridization can only detect WSSV in shrimp that show gross signs of the disease. Two-step PCR can detect WSSV in lightly infected brood stock, post-larvae and juveniles (Lo & Ho 1996, 1997, Deng et al. 2000). Light infection with WSSV is one of the major causes for WSS outbreaks in shrimp, since environmental stress can change light infections to acute ones and cause incidences of disease in shrimp ponds (Peng et al. 1998, Hsu et al. 1999, He et al. 2000). To understand light WSSV infections and how they lead to disease outbreaks, it is crucial to develop sensitive methods for detecting them in different tissues or cell types. In this paper, we describe an *in situ* polymerase chain reaction (ISPCR) method for the detection of WSSV, and compare its sensitivity with

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that of 1-step PCR, 2-step PCR and *in situ* hybridization. We also use the method to locate WSSV in tissues and cells of lightly infected *Penaeus vannamei*.

MATERIALS AND METHODS

Laboratory infections. *Penaeus vannamei* (aged 65 d) were obtained from a shrimp culture pond in Daya Bay, Guangdong Province, PR China. The average body length was 6 to 7 cm. Before WSSV injection, 10 shrimp were randomly chosen for WSSV detection by 2-step PCR to confirm the absence of WSSV infection in those shrimp. This would confirm with 95% confidence, freedom from WSSV at a prevalence of 26% or more (Cameron 2002).

Moribund *Penaeus vannamei*, with white spots on the cuticle and found to be WSSV positive using 1-step PCR, were used to prepare inoculum for the challenge tests. Muscle (2 g) was homogenized in 20 ml of 1× phosphate buffer solution (PBS) (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g K_2HPO_4, adjust pH to 7.4, add dH_2O to 1 l) and then centrifuged at 4250 × *g* for 15 min at 4°C. The supernatant was passed through a 450 nm filter unit and filtrates (50 µl) were injected intramuscularly at the third abdominal segment into 260 healthy shrimp, while 20 control shrimp were injected with the same volume of PBS.

Test shrimp were sampled at 2 h intervals from 2 to 20 h and thereafter at 24, 36 and 50 h post injection (p.i.). At each sampling time, 15 shrimp were selected and muscle and pleopods were preserved at −70°C for PCR assays (total sampled = 195). The cephalothoraxes of 5 shrimp were fixed in Davison's AFA (330 ml 95% alcohol, 220 ml 37 to 39% formaldehyde, 115 ml acetic acid and 335 ml dH_2O) for ISH and ISPCR detection. The remaining 65 shrimp were cultured separately, without sampling throughout the test period in order to observe disease development.

PCR amplification. Muscle (approximately 0.1 mg) from the second abdominal segment was homogenized in 10 volumes of TN buffer (50 mM Tris-HCl, pH 7.6, 0.4 M NaCl) on ice and centrifuged at 4250 × *g* for 5 min at 4°C. Digestion solution (50 mM KCl, 10 mM Tris-HCl at pH 8.3, 0.45% Nonidet P-40, 0.45% Tween 20, 80 µg $µl^{-1}$ Proteinase K) was added to the supernatent at a final concentration of 1% followed by boiling for 15 min and placement on ice for 5 min before centrifugation at 8000 × *g* for 5 min at 4°C. DNA template for 1-step PCR comprised 2 µl of the supernate. For 2-step PCR, 2 pairs of primers (PCR: 496F1: 5′-CGT GCC TGA ATC AGT ATG TAC GC-3′, 496R1: 5′-GAC GTT ACA ATA GAC CCA TGT TCG AT-3′, 496F2: 5′-CTC ATG TAC CAA ATC TGG GTT ACG A-3′, 496R2: 5′-CGA TAG ACC ACA AGT TCC GTA GGA-3′) were designed, based on a *Hin*dIII fragment of WSSV DNA sequence from *Penaeus monodon* (Deng et al. 2000). The expected lengths of the amplified fragments were 328 bp (496F1 and 496R1) and 258 bp (496F2 and 496R2). The PCR reaction mixture (25 µl total volume) contained 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 µM of each dNTP, 10 pM of each primer, 1 unit *Taq* DNA polymerase and 2 µl template DNA. The amplification comprised 1 cycle of 94°C for 4 min, 55°C for 45 s, 72°C for 1 min; 34 cycles of 94°C for 45 s, 55°C for 45 s and 72°C for 1 min, followed by a final 8 min extension at 72°C. After completion of the first PCR round, 1 µl of the reaction product was added to 24 µl reaction mixture containing the inner primer pair. This was subjected to a second amplification round using the same PCR conditions. The PBS group provided the negative control template for both 1- and 2-step PCR.

***In situ* hybridization (ISH).** Tissue samples were fixed in Davidson's AFA fixative for 24 h and then processed routinely for tissue sections (Lightner 1996). For histological observation, a series of parallel sections (4 µm thickness) were mounted onto superfrost®/Plus positively charged microscope slides (Fisher Scientific). The sections were dewaxed and rehydrated in a series of graded ethanol before carrying out the ISH protocol described by Lu et al. (2000) and using Digoxygenin (DIG) labeled probe consisting of a 328 bp WSSV 1-step PCR product prepared using a PCR labeling kit (Boehringer Mannheim) according to the manufacturer's instructions. Five shrimp samples from the PBS group were treated in a similar manner as ISH negative controls.

***In situ* polymerase chain reaction (ISPCR).** Tissue sections (6 µm thickness) of *Penaeus vannamei* samples from 0 h to 16 h p.i. were pretreated on slides as in the ISH protocol. After rehydration, sections were incubated in 0.2 M HCl for 10 min, rinsed in 0.1 × PBS, pH 7.4 for 5 min and transferred to a humidified chamber for treatment with 0.25 mg ml^{-1} Proteinase K for 10 min at 37°C. Proteinase K was deactivated at 98°C for 2 min before tissue sections were fixed in 4% paraformaldehyde in PBS for 10 min (Carlos & Muro 1997).

ISPCR was performed in a buffer that contained 2.5 mM Mg^{2+}, 200 µm dNTP, 0.5 mM of WSSV primers (PCR: 496F1: 5′-CGT GCC TGA ATC AGT ATG TAC GC-3′, 496R1: 5′-GAC GTT ACA ATA GAC CCA TGT TCG AT-3′), and 3U *Taq* polymerase. The slides were preheated at 70°C to facilitate a hot start, and 65 µl of reaction mix was added to the section within a Gene Frame (Gene Company) capacity 65 µl. The ISPCR was performed in a PCR machine (Gene Company, PTC 100) using the following conditions: 94°C predenaturation of template DNA for 5 min followed by 30 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 90 s and a final 10 min extension at 72°C.

After PCR, slides were post fixed in fresh 4% paraformaldehyde in PBS for 30 min, and then washed in PBS for 5 min. The detection of PCR products in the tissues was performed using a DIG labeled probe as described above.

To avoid false positive results, a control ISPCR was performed in parallel by adding DIG probe-free hybridization solution. In addition, 5 shrimp samples from the PBS group served as ISPCR negative controls.

ISPCR detection of light WSSV infections in shrimp. Fifty shrimp (body length 8 to 9 cm) were selected randomly from a culture pond in Daya Bay where there were no gross signs of a WSS outbreak or WSSV infection. The farm was 10 km from the laboratory and in a region where WSS was prevalent. In all of these shrimp, WSSV was detected by 2-step PCR using DNA extracts from pereopods as the template. Of these shrimp in which WSSV was not detected by 1-step PCR (classed as lightly-infected shrimp), 10 were chosen for ISPCR assays for WSSV.

Table 1. Sensitivity of different detection methods for the WSSV infection in *Penaeus vannamei.* From each phase, tail muscle of 15 shrimp was used to detect WSSV by 1-step or 2-step PCR while 5 samples from each phase were used to perform ISH and ISPCR. Numbers in parentheses indicate the number of animals used per test method. /: no testing for WSSV in the sample using this method. ISH: *in situ* hybridization; ISPCR: *in situ* polymerase chain reaction

Samples	1-step PCR	2-step PCR	ISH	ISPCR
PBS group	0 (15)	0 (15)	0 (5)	0 (5)
2 h	0 (15)	13 (15)	0 (5)	1 (5)
4 h	0 (15)	15 (15)	0 (5)	2 (5)
6 h	0 (15)	15 (15)	0 (5)	3 (5)
8 h	0 (15)	15 (15)	0 (5)	5 (5)
10 h	0 (15)	15 (15)	0 (5)	5 (5)
12 h	4 (15)	10 (10)	0 (5)	5 (5)
14 h	7 (15)	8 (8)	0 (5)	5 (5)
16 h	11 (15)	4 (4)	0 (5)	5 (5)
18 h	13 (15)	2 (2)	1 (5)	/
20 h	14 (15)	1 (1)	3 (5)	/
24 h	15 (15)	/	4 (5)	/
36 h	15 (15)	/	5 (5)	/
50 h	15 (15)	/	5 (5)	/

RESULTS

Artificial infection

Of the 65 shrimp injected with WSSV and monitored without sampling throughout the test period, 100% died within 5 d. No dead shrimp were observed 24 h p.i. Mortality reached 26, 55, 92 and 100% after 2, 3, 4 and 5 d, respectively. All moribund shrimp exhibited typical signs of WSS including red bodies, lack of activity and white spots in the cuticle. None of the shrimp in the PBS-injected control group died.

PCR detection

Using 1-step PCR, the first shrimp was WSSV positive at 12 h p.i. and all were positive at 24 h p.i. With 2-step PCR, the first shrimp was positive at 2 h p.i. and all were positive at 4 h p.i. (Table 1). No positive reactions were obtained in the negative control group.

ISH and ISPCR detection

The presence of WSSV with ISH and indirect ISPCR was evident as blue black precipitates (positive signals) in infected cells (Fig. 1). Using ISH, these could be detected in the nuclei of cells in the epithelium, gills and muscle at 18 to 50 h p.i., while no positive signals were observed at any time in the PBS control group (Tables 1 & 2). With ISPCR, WSSV detection increased from 1 shrimp (20%) at 2 h p.i. to 5 shrimp (100%) at 8 h p.i. (Table 1), with positive signals mainly in the muscle and hepatopancreas. However, by 12 h p.i., WSSV was detected in most organs, including the stomach, gills, muscle,

Table 2. Detection of WSSV by ISH and ISPCR in different tissues of *Penaeus vannamei* at various times after injection with WSSV

Specimens	Detection	Gill	Stomach	Muscle	Cuticular epidermis	Hepato-pancreas
PBS group	ISH	0/5	0/5	0/5	0/5	0/5
	ISPCR	0/5	0/5	0/5	0/5	0/5
2 h	ISH	0/5	0/5	0/5	0/5	0/5
	ISPCR	0/5	0/5	1/5	0/5	1/5
4 h	ISH	0/5	0/5	0/5	0/5	0/5
	ISPCR	0/5	0/5	2/5	0/5	2/5
6 h	ISH	0/5	0/5	0/5	0/5	0/5
	ISPCR	3/5	0/5	3/5	0/5	3/5
8 h	ISH	0/5	0/5	0/5	0/5	0/5
	ISPCR	2/5	0/5	5/5	0/5	5/5
10 h	ISH	0/5	0/5	0/5	0/5	0/5
	ISPCR	0/5	0/5	5/5	0/5	5/5
12 h	ISH	0/5	0/5	0/5	0/5	0/5
	ISPCR	3/5	3/5	2/5	2/5	2/5
14 h	ISH	0/5	0/5	0/5	0/5	0/5
	ISPCR	3/5	5/5	2/5	3/5	2/5
16 h	ISH	0/5	0/5	0/5	0/5	0/5
	ISPCR	0/5	5/5	3/5	0/5	2/5
18 h	ISH	1/5	1/5	1/5	0/5	0/5
20 h		0/5	3/5	0/5	0/5	0/5
24 h		3/5	4/5	4/5	3/5	0/5
36 h		0/5	5/5	4/5	0/5	0/5
50 h		0/5	5/5	5/5	5/5	2/5

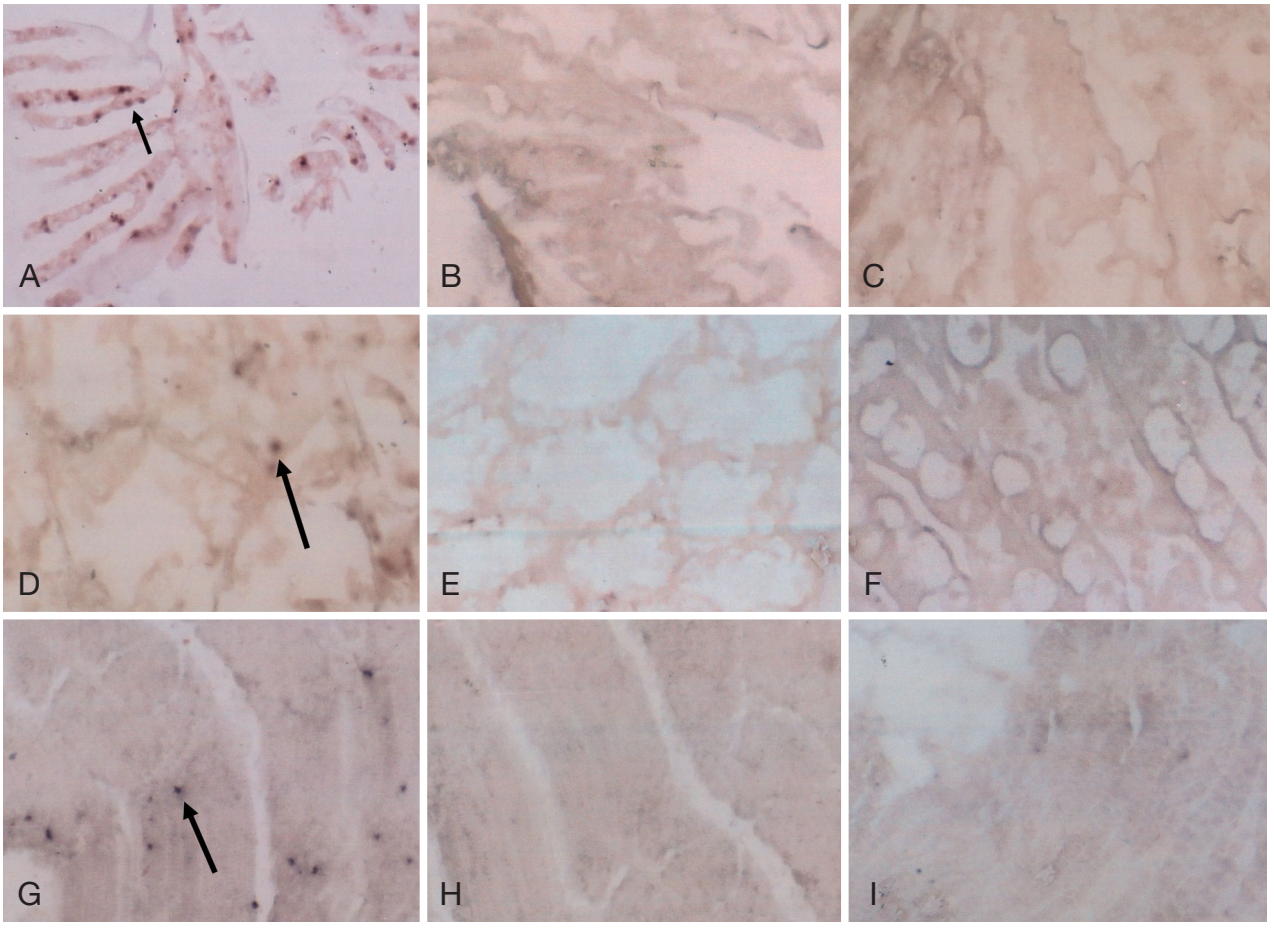

Fig. 1. *Penaeus vannamei*. WSSV detected by ISPCR in shrimp samples 6 h p.i. Positive signals are shown by arrows. (A) Gill, 200×; (B) no signal in the same gill by ISPCR without probe, 126×; (C) no signal in shrimp gill of the PBS group by ISPCR, 126×; (D) hepatopancreas, 200×; (E) no signal in the same hepatopancreas by ISPCR without probe, 126×; (F) no signal in shrimp hepatopancreas of the PBS group by ISPCR, 200×; (G) muscle, 126×; (H) no signal in the same muscle by ISPCR without probe, 126×; (I) no signal in shrimp muscle of the PBS group by ISPCR, 200×

hepatopancreas and epidermis (Table 2). No signals were detected in the probe-blank, control slides or in slides of uninfected control shrimp (Fig. 1).

Detection of light WSSV infections by ISPCR

Of the 10 shrimp that tested negative for WSSV by 1-step PCR but positive using 2-step PCR, none gave positive signals for WSSV by ISH. By contrast, ISPCR gave positive signals for 9 out of 10 shrimp in nuclei of connective tissue cells in the hepatopancreas and in nuclei of muscle cells. In 1 of these 9 shrimp, signals were also found in the sub-cuticular epithelium (Fig. 2). Other tissues, such as gills and the sub-cuticular epithelium of the stomach, gave no WSSV positive signals. No positive signals were detected in 5 healthy shrimp that tested negative for WSSV by 2-step PCR.

DISCUSSION

Our results indicate that ISPCR is at least as effective for WSSV detection in shrimp tissues as PCR, 2-step PCR and ISH. We could detect WSSV by 1-step PCR in the muscle of 4 of 15 shrimp at 12 h p.i. while Xie et al. (2001) reported that they could detect it at 8 h p.i. in *Penaeus monodon*. This discrepancy may be due to the tissue used, the quantity of virus used for inoculum or the different species of shrimp used. Despite this, both studies conclude that WSSV can be detected at 2 h p.i. using 2-step PCR.

HE (hematoxylin and eosin stain), ISH and ISPCR can provide information on the cell types infected by WSSV. However, ISPCR provides the highest sensitivity and compares with that of 2-step PCR. The results of ISPCR were consistent with ISH and PCR detection approaches and the traditional methods of pathological identification of WSSV such as HE staining where

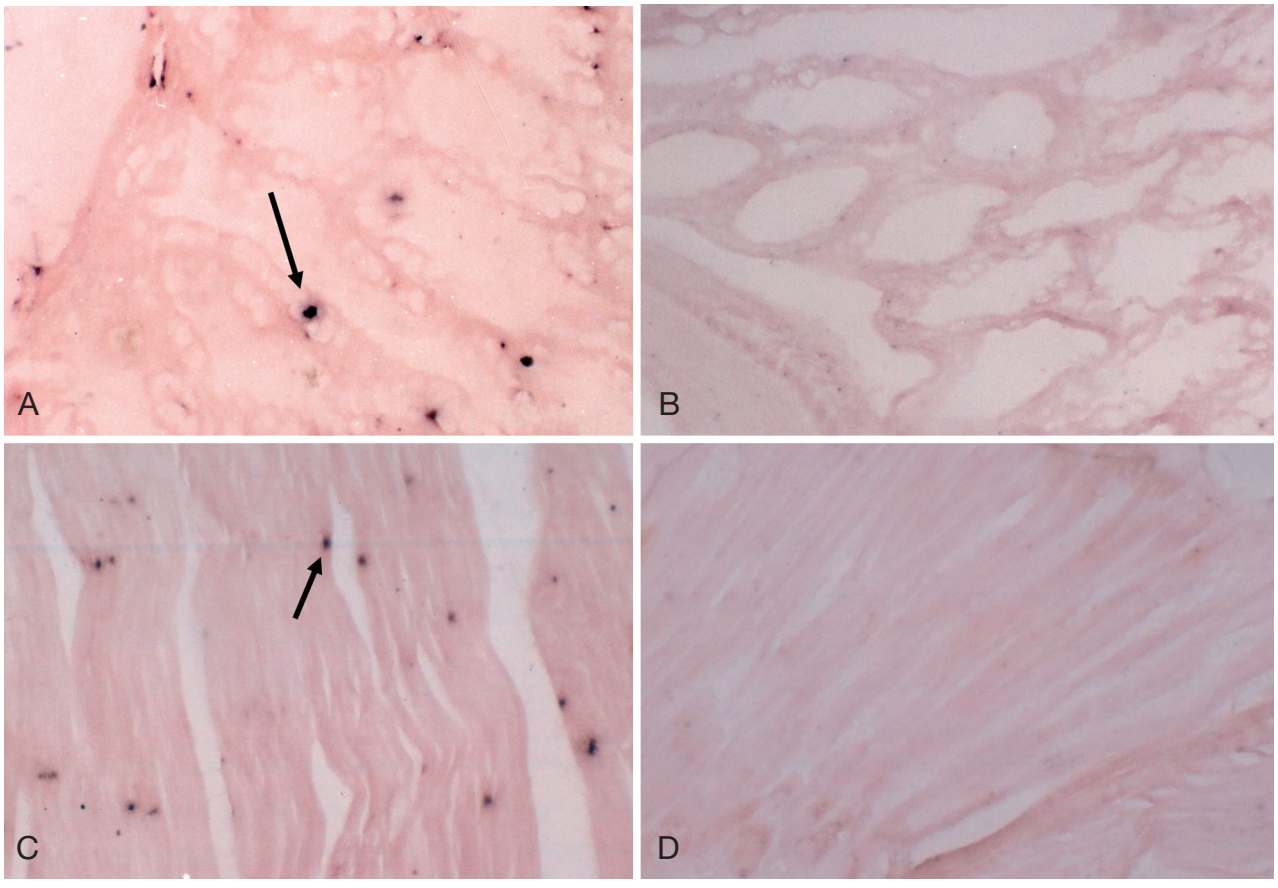

Fig. 2. *Penaeus vannamei*. Results of ISPCR and ISH tests. Positive signals are shown by arrows. (A) WSSV in hepatopancreas detected by ISPCR, 200×; (B) no signals in the hepatopancreas by ISH, 200×; (C) WSSV in muscle detected by ISPCR, 200×; (D) no signals in the muscle by ISH, 200×

mainly mesodermal and ectodermal tissues are positive for infection (Inouye et al. 1994, Lightner 1996, Lo et al. 1997). However, ISPCR has the advantage that it will provide information on the localization of WSSV in tissues of very lightly infected carrier shrimp and shrimp at the early stages of infection.

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