

Prevalence of Three Shrimp Viruses in Zhejiang Province in 2008*

Xiao-wei Yu¹, Jian-ping Wang², Wei Zhang³ and Zheng-li Shi^{3**}

(1. Faculty of Life Science and Biotechnology, Ningbo University, Ningbo 315211, China; 2. Ningbo Academy of Ocean and Fishery, Ningbo 315012, China; 3. State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China)

Abstract: White spot syndrome virus (WSSV), Taura syndrome virus (TSV) and Infectious hypodermal and haematopoietic necrosis virus (IHHNV) are three shrimp viruses responsible for major pandemics affecting the shrimp farming industry. Shrimps samples were collected from 12 farms in Zhejiang province, China, in 2008 and analyzed by PCR to determine the prevalence of these viruses. From the 12 sampling locations, 8 farms were positive for WSSV, 8 for IHHNV and 6 for both WSSV and IHHNV. An average percentage of 57.4% of shrimp individuals were infected with WSSV, while 49.2% were infected with IHHNV. A high prevalence of co-infection with WSSV and IHHNV among samples was detected from the following samples: Bingjiang (93.3%), liuao (66.7%), Jianshan (46.7%) and Xianxiang (46.7%). No samples exhibited evidence of infection with TSV in collected samples. This study provides comprehensive information of the prevalence of three shrimp viruses in Zhejiang and may be helpful for disease prevention control in this region.

Key words: Molecular detection; White spot syndrome virus (WSSV); Taura syndrome virus (TSV); Infectious hypodermal and haematopoietic necrosis virus (IHHNV)

The emergence of numerous aquatic viral diseases since the 1990s has significant impact on the shrimp industry worldwide^[3]. White spot syndrome virus (WSSV), taura syndrome virus (TSV) and infectious hypodermal and haematopoietic necrosis virus (IHHNV) are three major viruses which has caused severe diseases in shrimp farming worldwide.

WSSV is the sole species of the new family

Nimaviridae (genus *Whispovirus*)^[9] and a major shrimp pathogen that is highly virulent to all aquatic crustacean^[8]. Since the 1990s, WSSV has spread to almost all countries where shrimp are cultivated and remains a major threat due to its wide host range, genetic variation, and changes in virulence^[6].

IHHNV was first isolated among juvenile blue shrimp *P. stylirostris* in Hawaii in 1981^[7] and has been detected in a number of other penaeid species around the world, including the Americas, Oceania, and Asia^[2]. The virus caused mortalities of up to 90% in shrimp. In China, IHHNV was detected first among

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** Corresponding author.

Phone/Fax: +86-27-87197240, Email: zshi@wh.iov.cn

shrimp and crab samples collected in Hainan provinces and northern China during 2001-2004 [15].

TSV is one of the most economically significant viruses infecting penaeid shrimp in the western hemisphere [4]. It was first found in white shrimp *Penaeus* (or *Litopenaeus*) *vannamei* in Ecuador during 1992, and subsequently, in Taiwan in 1998 [12], China mainland in 2001 [1], and Thailand in 2003 [10].

After *P. vannamei* was introduced into mainland China, many farmers switched to raising this newly introduced species. *P. vannamei* had become the most popular and highest yield cultured shrimp in Zhejiang province, the major shrimp producing region in China. Unfortunately, the importation of *P. vannamei* stocks into China from other geographical regions has resulted in the introduction of TSV and IHHNV to domestic shrimp populations.

In this study, we sampled shrimp from major shrimp cultivating farms in Zhejiang province, China in 2008, and assayed for the presence of WSSV, TSV and IHHNV using PCR methods. The results represent the first systematic survey of WSSV, IHHNV, and TSV infections among cultivated shrimps within the Zhejiang province of China, and can be used in the development of national bio-security plans for the Chinese aquaculture industry.

MATERIALS AND METHODS

Shrimp sampling

The collection of shrimp specimens was conducted during August to September 2008 from 12 independent farms within Zhejiang province of China. The 12 farms are shrimp culture demonstration areas, which represent leading shrimp producers in the Zhejiang province and are located in cities along the

coast of the province including Jiaxing, Hangzhou, Shaoxing, Ningbo, Taizhou and Wenzhou (Fig. 1). Adult shrimp including moribund and healthy shrimp were collected at each pond. The date, clinical syndrome (s) and number of samples collected were documented at the time of collection and are summarized in Table 1. Samples were immediately transported to Ningbo Academy of Ocean and Fishery and stored at -70°C until they could be transferred to the Wuhan Institute of Virology, Chinese Academy of Sciences (CAS) for analysis.

Nucleic acid extraction from tissue samples

For DNA extraction, 0.1mg of frozen gill tissue was homogenized using 300 µL lysis buffer containing 0.4mol/L NaCl, 2 mmol/L EDTA and 10 mmol/L Tris-HCl (pH 7.5) in a 1.5 mL tube and centrifuged at 6000 ×g for 10 min. Supernatant was collected and digested with sodium dodecyl sulphate and proteinase K to a final concentration of 2% and 200 µg/mL,



Fig. 1. Map of Zhejiang province showing the geographical distribution of farms where shrimp samples were collected. Hollow circles indicate the farms; Filled circles indicate the major cities in Zhejiang province.

Table 1. Origin and characteristics of shrimp samples from 12 independent farms in Zhejiang Province.

City	Farm	Clinical syndromes	No. of samples
Jiaxing	Jianshan	Red hue on the shell, White spots on the exoskeleton	32
Hangzhou	Xiaoshan	Red hue on the shell and tail	18
	Binjiang	Red hue on the shell and tail	22
Shaoxing	Lihai	None	17
Ningbo	Xianxiang	Red hue on the shell, White spots on the exoskeleton	30
	Xinqiao	Red hue on the shell and tail, exoskeleton is soften	18
	Yuexi	Red hue on the shell	15
	Fuhai	None	22
Taizhou	Shaliu	Red hue on the shell and tail	14
	Liuao	White spots on the exoskeleton, red hue on the shell	22
Wenzhou	Yongxing	Red hue on the shell	37
	Lingkun	Red hue on the shell	22

respectively. Samples were incubated at 56°C for 3 h and nucleic acid was separated from the proteinaceous components using phenol-chloroform extraction. Purified DNA was precipitated with cold ethanol or isopropanol and re-suspended in 30 µL double-distilled water. The quality and quantity of each sample was determined spectrophotometrically using a Biophotometer (Eppendorf, Germany). All DNA extractions were stored at -20°C until use.

Total RNA extraction from tissue was carried out using Trizol reagent according to the manufacturer's directions (Invitrogen). Briefly, 100 µg tissue was homogenized in 1 mL Trizol reagent and incubated for 5 min at room temperature. Subsequently, 0.2 mL chloroform per 1 mL of Trizol reagent was added to each sample and mixed. Sample was again incubated at room temperature for 5 min and then centrifuged at 12000×g for 10 min at 4°C. Supernatant was transferred to a 1.5 mL tube and 0.5 mL isopropanol per 1 mL Trizol was added to each sample to precipitate the soluble RNA. RNA pellet was rinsed with 700 µL 75% ethanol, and then finally resuspended in 30 µL of DEPC water. The concentration and purity of each sample was measured as before. RNA samples were stored at -80°C until use.

Synthesis of cDNA

cDNA synthesis was carried out in 20 µL volumes using the MLV reverse transcriptase enzyme (Promega) according to the manufacturer's protocol with approximately 1.0 µg of RNA and 30 ng of random primers. Total RNA and primers were mixed with 6.5 µL DEPC-treated water and quickly cooled on ice after being heated to 65°C for 5 min. Following, 4 µL 5 × M-MLV Buffer (20 mmol/L Tris-HCl, pH 7.5, 200 mmol/L NaCl, 0.1 mmol/L EDTA, 1.0 mmol/L DTT, 0.01% Nonidet P-40), 1 µL dNTP (10 mmol/L), 25 U RNasin (BioStar) and 200 unit M-MLV reverse transcriptase (Promega) were added to the reaction mixture and incubated at 37°C for 60 min. The resulting cDNA product was either used directly for PCR or stored at -80°C until use.

PCR

Three sets of primers were used to amplify WSSV, IHNV and TSV specific products, respectively. The VP26F/VP26R primer pair, which targets to WSSV VP26 ORF^[14], was used to detect WSSV. While TSV and IHNV were detected using the 9195F/9992R^[11] and 77012F/77035R primer set^[5], respectively. Primer sequences, PCR condition, and the size of the expected PCR products were described in Tan *et al*^[13].

Table 2. Prevalence of WSSV, IHNV and TSV in 12 farms in Zhejiang province, 2008.

Virus	Sample location (Positive/Total)												Total (Prevalence%)
	Jian Shan	Xiao Shan	Bin jiang	Li hai	Xian xiang	Xin qiao	Yue xi	Fu hai	Sha liu	Liu ao	Yong xing	Ling kun	
WSSV	15/15	7/15	15/15	0/15	15/15	15/15	12/15	0/15	4/14	15/15	0/15	0/15	98/179 (57.4%)
IHNV	7/15	0/15	14/15	11/15	7/15	1/15	0/15	6/15	14/14	10/15	10/15	8/15	88/179 (49.2%)
WSSV+IHNV	7/15	0/15	14/15	0/15	7/15	1/15	0/15	0/15	4/14	10/15	0/15	0/15	43/179 (24.0%)

Each PCR reaction was carried out in a 25 μ L volume containing 0.85 μ L of cDNA or DNA, 10 pmol/L of each primer, 0.5 μ L dNTPs (10 mmol/L), 2.5 μ L 10 \times Taq buffer, and 0.5 U of Taq polymerase (BioStar). PCR amplification of WSSV, IHNV and TSV was performed separately in a programmable thermal cycler (Eppendorf Master-cycler). PCR amplicon was analyzed by electrophoresis (110V, 20min) in 1% agarose gel stained with ethidium bromide. To ensure the accuracy of the PCR amplification, PCR product was further purified and sequenced.

RESULTS AND DISCUSSION

Prevalence of WSSV, IHNV and TSV in Zhejiang province

A total of 179 shrimp individuals were collected from 12 independent farms distributed along the coasts of Zhejiang province, China. The results of the PCR assays to determine the presence of WSSV, TSV and IHNV among samples are summarized in Table 2. An average percentage of 57.4% of samples were infected with WSSV while 49.2% of samples were infected with IHNV. A high prevalence of co-infection with WSSV and IHNV among samples was detected from the following samples: Bingjiang (93.3%), liuao (66.7%), Jianshan (46.7%) and Xianxiang (46.7%), and samples infected with WSSV reached 100% in these four farms. No samples exhibited evidence of infection with TSV in collected samples.

The selected PCR amplicon was sequenced and compared with the WSSV and IHNV genome sequence reported in the GenBank database. The sequenced fragments are almost identical to that of the reported WSSV and IHNV (data not shown).

Zhejiang is one of the largest shrimp producer provinces in China, and can export a large number of shrimp products every year, therefore, it is important to conduct regular monitoring of important shrimp pathogens in this region. Our results showed that two major shrimp viruses, WSSV and IHNV, are highly prevalent in the sampled farms. Furthermore, the co-infection by these two major shrimp viruses was detected in 6 sampled farms; some of them reached 93.3%. The results are consistent with the clinical syndromes observed among shrimp specimens at the time of sampling, including the presence of white spots on the exoskeleton, red hue of the shell and tail, lethargy, reduced food intake, and floating at the water's surface.

Fortunately, no samples exhibit evidence of infection with TSV among all of the sampled farms. This result showed that TSV incidence rate has fallen to very low levels in Zhejiang Province through years of efforts of prevention and control. The sampling locations are Healthy Breeding Base of *P. vannamei*, PCR is used commonly for WSSV detection in hatchery broodstock and in post-larvae to eliminate WSSV infection before entry into the farms.

Therefore, we suspect that the viruses were transmitted to the shrimp during cultivation from potentially contaminated sources such as water, food and/or aquaculture container. In addition to horizontal transmission, environmental factors such as deterioration of ecological environment, intensive culturing practices, abrupt change of temperature, and inappropriate breeding management would further favor the transmission of viral pathogens. These data indicate that in order to prevent introduction of viral pathogens, farmers not only need to choose high-quality postlarvae but also to conduct strict quarantine measures during the culturing process.

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References

1. **Chen A P.** 2003. Review on culture of *Penaeus vannamei* and viral diseases in China during 2002. **Scientific Fish Farming**, 11: 41-42.
2. **Flegel T W.** 1997. Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. **World J Microb Biot**, 13: 433-442.
3. **Flegel T W.** 2002. Emerging shrimp diseases and innovations to prevent their spread. **Diseases in Asian Aquaculture IV Fish Health Section, Asian Fisheries Society, Quezon City Philippines**: 137-149.
4. **Hasson K W L D, Poulos B T, Redman R M, et al.** 1995. Taura syndrome in *Penaeus vannamei*: Demonstration of a viral etiology. **Diseases of Aquatic Organisms**, 23: 115-126.
5. **Krabetsve K, Cullen B R, Owens L.** 2004. Rediscovery of the Australian strain of infectious hypodermal and haematopoietic necrosis virus. **Dis Aquat Organ**, 61: 153-158.
6. **Leu J H, Yang F, Zhang X, et al.** 2009. Whispovirus. **Curr Top Microbiol Immunol**, 328: 197-227.
7. **Lightner D V.** 1996. Epizootiology, distribution and the impact on international trade of two penaeid shrimp viruses in the Americas. **Rev Sci Tech**, 15: 579-601.
8. **Lo C F, Ho C H, Peng S E, et al.** 1996. White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods. **Dis Aquat Organ**, 27: 215-225.
9. **Mayo M A.** 2002. A summary of taxonomic changes recently approved by ICTV. **Arch Virol**, 147: 1655-1663.
10. **Nielsen L, Sang-oum W, Cheevadhanarak S, et al.** 2005. Taura syndrome virus (TSV) in Thailand and its relationship to TSV in China and the Americas. **Dis Aquat Organ**, 63: 101-106.
11. **Nunan L M, Poulos B T, Lightner D V.** 1998. Reverse transcription polymerase chain reaction (RT-PCR) used for the detection of Taura syndrome virus (TSV) in experimentally infected shrimp. **Dis Aquat Organ**, 34: 87-91.
12. **Tu C, Huang H T, Chuang S H, et al.** 1999. Taura syndrome in Pacific white shrimp *Penaeus vannamei* cultured in Taiwan. **Dis Aquat Org**, 38: 159-161.
13. **Tan Y, Xing Y, Zhang H, et al.** 2009. Molecular detection of three shrimp viruses and genetic variation of white spot syndrome virus in Hainan Province, China, in 2007. **J Fish Dis**, 32, 777-784.
14. **van Hulten M C, Westenberg M, Goodall S D, et al.** 2000. Identification of two major virion protein genes of white spot syndrome virus of shrimp. **Virology**, 266: 227-236.
15. **Yang B, Song X L, Huang J, et al.** 2007. Evidence of existence of infectious hypodermal and hematopoietic necrosis virus in penaeid shrimp cultured in China. **Vet Microbiol**, 120: 63-70.