

Establishment of a Multiplex PCR and an Investigation of Co-Infection Rate of WSSV and IHHNV in *Penaeid vannamei* in Northern of Jiangsu

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Abstract: Based on the conservative regions in genome of WSSV and IHHNV, two specific primer sets, respectively for the two viruses were designed and constructed. A multiplex PCR for co-detection of the two viruses with a specificity of (IHHNV, TSV, YHV, *Vibrio Parahaemolyticus*, etc.) and sensitivity of 1×10^3 template copy numbers was subsequently established after serially optimization. WSSV and IHHNV with different template concentrations could be detected simultaneously. By the multiplex PCR method, 265 diseased shrimp samples and 169 healthy shrimp samples collected from the farms in the Northern area of Jiangsu province were investigated for WSSV and IHHNV co-infection rate in pond-cultured *P. vannamei*. The results show that the infection rate of WSSV was quite high (45~54%) whereas that of IHHNV was very low (6.9~13.7%). The result also indicated that there were 7~10% of diseased shrimp and 0~6.7% of healthy shrimp were co-infected with the two viruses indicated that co-infection of the two viruses was more popular in diseased shrimp than the healthy.

Key words: White spot syndrome virus, infectious hypodermal and hematopoietic necrosis virus, multiplex PCR, co-infection, templates, China

INTRODUCTION

Penaeid vannamei are cultured in the most area of worldwide because of its high production and salt tolerance. In recent years with the breakthrough of freshwater cultivation technology, *P. vannamei* become the dominant species of shrimp cultured in China and the annual production of which is occupied >50% of shrimp cultivated in China and played an important role in the export business. However with the continuous expansion of cultural area and the increasing intensivism, shrimp diseases, especially shrimp viral disease is explosive and epizootic in many aquaculture farms. The aquaculture disease prediction system in some areas shows that White Spot Syndrome (WSS) and Infectious Hypodermal and Hematopoietic Necrosis disease (IHHN) are the most important two viral diseases restraining rapid development of *P. vannamei* aquaculture industry in China.

White Spot Syndrome Virus (WSSV) and Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) were responsible to WSS and IHHN in shrimp, respectively. WSSV has high pathogenic characteristics

and high mortality rate. The mortality rate of shrimp will be up to 100% within 3-10 days post infection (Lightner, 1996). The virus has a broad host tropism and almost all of the crustaceans could be infected with which or become the virus host (Lo *et al.*, 1996). WSSV infection rate of shrimp cultured in China has been maintained at high level (Zhou *et al.*, 2011) and WSS is listed by the World Organization for Animal Health (OIE) as first class disease. IHHNV can infect both wild and cultured shrimp. *P. stylirostri* has high morbidity and mortality (Lightner *et al.*, 1983). Although, *P. vannamei* has the certain resistance of IHHNV, chronic runt-deformity syndrome, RDS caused by the virus will lead to shrimp slow growth and significant economic losses (Kalagayan *et al.*, 1991). Therefore, IHHN is listed by OIE as second class disease. Currently, virus infection was found in shrimp aquaculture in various regions of China (Yang *et al.*, 2007). Previous studies showed that virus can cause arthropod repeated infection characteristics. One kind of the animal can be prevalently infected by two or two more viruses (Chayaburakul *et al.*, 2004; Raia *et al.*, 2009). As both WSSV and IHHNV can infect *P. vannamei* and transmit prevalently (Lo *et al.*, 1996; Yang *et al.*,

2007). The repeated infection of both two viruses will make the significant impact of economic benefit of *P. vannamei* aquaculture certainly. Also there is no obvious disease outbreak symptoms after IHNV-infected and very difficult to make clinical diagnosis. Therefore, finding a rapid and sensitive molecular biology detection method is very necessary to indicate two virus infection, especially co-infection timely. Yangzhou, Taizhou, Nantong, Yancheng, Lianyungang and other regions in Northern of Jiangsu have rich fresh water resource and wide cultured water. *P. vannamei* is the main cultured species in these regions.

In order to grasp the transmission and co-infection of both WSSV and IHNV in *P. vannamei* cultures this study establish a multiplex PCR detection method of WSSV and IHNV by adopting molecular biology measures and make investigation of virus co-infection rate to *P. vannamei* cultures in Northern of Jiangsu by adopting this method.

MATERIALS AND METHODS

Experimental materials: During the period of 2009 and 2010, 265 diseased *P. vannamei* samples and 169 non-diseased healthy shrimp samples were collected from Yangzhou, Taizhou, Nantong, Yancheng, Lianyungang and other regions of aquaculture farms. All samples were stored with ice in the incubator and would be taken to the laboratory within 4 h. Samples sources are shown in Table 1.

WSSV positive comparison virus strain was supplied by Jiangsu Institute of Oceanography, Marine Fisheries and Marine Biotechnology Laboratory. IHNV, TSV, YHV and *Vibrio Parahaemolyticus* positive comparison were collected from Ocean University of China.

Primer design and synthesis: Based on the conservative regions in WSSV (AF440570) and IHNV genome (AF218226), a pair of specific primers was designed by adopting the software prime 5 to amplify 1320 and 356 bp products, respectively. WSSV primers were P1 (5'-TAATCAGGTTGCCAAACAA-3'), P2 (5'-CACGGTCAACATGTCTCCTG-3'); IHNV primers were F1 (5'-ATCGGTGCACTACTCGA-3'), F2 (5'-TCGTACTGGCTGTTTCATC-3'). Primers were synthesized by Shanghai Sheng Gong Bioengineering Co., Ltd.

Table 1: *P. vannamei* WSSV and IHNV infection samples composition in Northern of Jiangsu during year, 2009 and 2010

Regions	Yangzhou	Taizhou	Nantong	Yancheng	Lianyungang
Diseased shrimp (Qty)	51	48	60	43	63
Healthy shrimp (Qty)	30	33	39	32	35

Template DNA preparation: A total of 25-50 mg diseased *P. vannamei* cephalothorax were homogenized in the centrifuge tube and mixed equally with 1 mL split solution liquid (100 mmol L⁻¹ NaCl; 10 mmol L⁻¹ Tris-HCl pH value 8.0, 25 mmol L⁻¹ EDTA, pH value 8.0, 2% SDS, 0.5% N-laurylsarcosine; 0.5 mg mL⁻¹ proteinase K) at 65°C water for 1 h, adding 5 M NaCl to make NaCl final concentration to be 0.7 M, slowly adding 10% volume of CTAB/NaCl, mixed equally at 65°C water for 10 min, adding equal volume of 24:1 chloroform/isoamyl alcohol and extracted after mixed equally. Water free ethanol precipitated DNA, 70% ethanol washed. After dried, dissolved in 0.1×TE solution preserved at -20°C.

PCR optimization amplification condition: Optimized under the condition of recommended DNA template concentration by applied the method recommended by Taq DNA polymerase reagents. The cycle times, MgCl₂ concentration, primer concentration, Taq enzyme concentration and other parameters were optimized respectively based on the optimized annealing temperature. Annealing temperatures were 50, 53, 56, 58, 60 and 63°C, respectively. Compared with falling PCR program, falling PCR program was 95°C denatured for 4 min, then denatured 94°C for 30 sec, annealed for 30 sec, 72°C for 1 min each annealing temperature was interval 2°C as 2 cycles. The rest cycles were completed at last 52°C. The total cycle times were set 20, 25, 30, 35 and 40 times, 72°C extended for 10 min. MgCl₂ concentration was set 0.5, 1.0, 1.5, 2.0 and 3.0 μL. Primer concentration was set 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 μL. Taq DNA polymerase concentration were set 0.02, 0.03, 0.04, 0.05, 0.06, 0.08 and 0.10 U μL⁻¹. The total volume was 25 μL which contained 1.0×PCR reaction buffer, 200 μM dNTP, DNA sample 1 μL and deionized water. Amplification product was detected by electrophoresis in 1.5% agarose gel.

Double PCR specificity, sensitivity and interference detection: Utilizing the total ingested DNA as the template and applying the above WSSV and IHNV primers (P1/P2, F1/F2), PCR was detected on the positive comparison strain by adopting multiplex PCR detection method established in this study.

Positive template was amplified by 10 times serial dilutions (1×10¹⁰ copies μL⁻¹~1×10 copies μL⁻¹) to determine the minimum detection limit. Multiplex PCR detection was carried out on the positive samples, mixed-positive samples and negative comparison samples simultaneously. Repeat detection after 96 h and stored in the template DNA at -20°C to verify stability. Combined WSSV and IHNV standard samples according to the different relative concentrations (1×10⁸, 1×10⁴, 1×10⁵, 1×10⁶, 1×10⁴ and 1×10⁸ copies μL⁻¹), multiplex PCR and

single PCR were detected, respectively and analyzed if the detection between WSSV and IHHNV were existed the mutual interfere phenomena.

WSSV and IHHNV double infection rate survey: The gills of healthy *P. vannamei* and 25-50 mg cephalothorax of diseased *P. vannamei* were taken, respectively. Sample's total DNA were ingested based on the method above and amplify according to the multiplex PCR method. And set up the healthy shrimp group for comparison simultaneously. Amplification products were detected by electrophoresis in 1.5% agarose gel and the results were observed in the gel imaging system and photographed.

RESULTS AND DISCUSSION

WSSV and IHHNV double PCR detection method:

Optimum condition was adopted falling PCR, $MgCl_2$ concentration 1.50 μL , WSSV primer concentration 0.5 μL , IHHNV primer concentration 0.3 μL , Taq DNA polymerase 0.04 μL , 1.0 \times PCR reaction buffer, 200 μM dNTP, DNA samples 1 μL , deionized water to compensate the volume. Falling PCR program was at 95°C denatured for 4 min then denature 94°C for 30 sec, annealing 62-54°C for 30 sec, 72°C for 1 min each annealing temperature is interval 2°C as 2 cycle times. The rest cycles were completed at last 52°C. The total cycle times were set 35 times. About 72°C extended for 10 min. Amplification product was detected by electrophoresis in 1.5% agarose gel and the results were observed in the gel imaging system and photographed. Results are shown in Fig. 1.

Multiplex PCR had good specificity. The obvious bands of PCR reaction to WSSV positive strain, IHHNV positive strain, WSSV and IHHNV mixed strain and healthy shrimp muscle negative comparison strain were clearly found on the electrophoresis figure and conformed to the expected bands size shown in Fig. 1.

Sensitivity detection result showed that two clear bands were appeared when the positive template was diluted to 1×10^3 while there was no purpose amplified band when diluted to 1×10^2 . Details are shown in Fig. 2. Multiplex PCR method had the strong anti-interference characteristics. Different relative concentration virus samples had no obvious anti-interference characteristics to the results. The results are shown in Fig. 2.

WSSV and IHHNV co-infection rate: In early stage of disease outbreak, the body of shrimp is become soft, alone, stay aside or slow swimming in the water, reduction in food consumption or even without eating and is appeared empty stomach. Body, liver, pancreas and even intestine are become red. Liver and pancreas are swelling

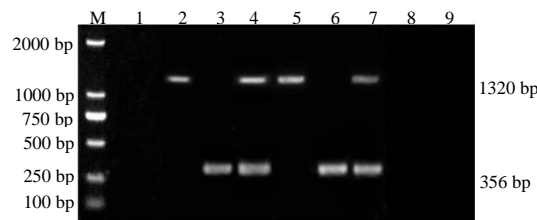


Fig. 1: Results of WSSV and IHHNV Double PCR detection method; 1: Negative comparison; 2: WSSV positive comparison; 3: IHHNV positive comparison; 4: WSSV and IHHNV mixed positive comparison; 5: WSSV positive sample; 6: IHHNV positive sample; 7: WSSV and IHHNV positive sample; 8: Healthy shrimp meat sample; 9: Healthy shrimp gills sample

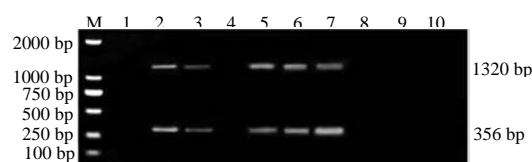


Fig. 2: Results of sensitivity and specificity of WSSV and IHHNV Double PCR detection method; 1: Negative comparison; 2: Template 1×10^4 virus copy concentration sample; 3: Template 1×10^3 virus copy concentration sample; 4: Template 1×10^2 virus copy concentration sample; 5: Template 1×10^6 virus copy of WSSV; Template 1×10^4 virus copy of IHHNV; 6: Template 1×10^5 virus copy of WSSV; Template 1×10^5 virus copy of IHHNV; 7: Template 1×10^4 virus copy of WSSV; Template 1×10^6 virus copy of IHHNV; 8: TSV positive sample; 9: YHV positive sample; 10: Arc germ positive sample

and so on. Head, cuirass and abdominal segment shells are easy to be peeled. There are unscrapped white spots inside some shrimp shells. Table 2 shows that WSSV infection rate of diseased shrimp group was higher. The infection rate in five regions was between 30 and 60%. IHHNV infection rate was lower than WSSV. However, it was attained about 10%. Virus total positive rate was between 45 and 60%. Thereinto, two virus co-infection rates were 9.8, 8.3, 10, 7.0 and 9.5% in Yangzhou, Taizhou, Nantong, Yancheng and Lianyungang cities. However, WSSV virus was detected from the healthy shrimp group in five regions. IHHNV virus was detected in four regions and failed to detect in one region. The total positive rate of both viruses was between 30 and 40%. Thereinto, virus co-infection rates were 6.7, 0, 5.1, 6.3 and 2.9%, failed to detect IHHNV positive infection separately. After

Table 2: *P. vannamei* WSSV and IHHNV infection rate in Northern of Jiangsu during year, 2009 and 2010

Regions	Yangzhou	Taizhou	Nantong	Yancheng	Lanyungang
Diseased shrimp group					
Shrimp sample (Qty)	51.0	48.0	60.0	43.0	63.0
Single WSSV positive shrimp (Qty)	18.0	18.0	25.0	17.0	28.0
Single IHHNV positive shrimp (Qty)	2.0	1.0	2.0	0.0	1.0
Double infection shrimp (Qty)	5.0	4.0	6.0	3.0	6.0
Total virus positive rate (%)	49.0	48.0	55.0	46.5	55.6
Double infection rate (%)	9.8	8.3	10.0	7.0	9.5
Healthy non-diseased shrimp group					
Shrimp sample (Qty)	30.0	33.0	39.0	32.0	35.0
Single WSSV positive shrimp (Qty)	9.0	10.0	12.0	10.0	11.0
Single IHHNV positive shrimp (Qty)	0.0	0.0	0.0	0.0	0.0
Double infection shrimp (Qty)	2.0	0.0	2.0	2.0	1.0
Total virus positive rate (%)	36.7	30.3	35.9	37.5	34.3
Double infection rate (%)	6.7	0.0	5.1	6.3	2.9

undergoing the devastating attack caused by explosive WSSV in the 90s of the last century, the aquaculture enterprises in China have increased the shrimp disease prevention consciousness to the unparalleled extent. Although shrimp immune system disfigurement causes the lack of prevention drug with the continuous improvement of disease diagnosis (Kim *et al.*, 1998; Yang *et al.*, 2005), SPF seed cultivation and variety of immune intensifier and the water quality regulator adopted, shrimp, especially *P. vannamei* aquaculture has been walked out the bottom vale in a short time and achieved the rapid growth of shrimp production in the world since, 2000. With the expansion of farm area and highly focus on disease prevention. It does not seem that the current challenge to the shrimp aquaculture is caused by the declined production because of diseases while is caused by low prices and declined profits because of the fierce market competition. In fact, according to the aquaculture diseases detection prediction, the most important disease which threatens to China's shrimp aquaculture is still the viral disease currently. The economic losses caused by shrimp virus disease are up to 50% and more than the other diseases combination caused. Cultured enterprises should focus on disease prevention and control in order to minimize production costs increase productivity and profitability so that they can stand firmly in the fierce international market competition. Therefore, prevention and control of shrimp viral diseases are still the crux of shrimp aquaculture sustainable development.

WSSV and IHHNV are the common pathogen of *P. vannamei* (Yang *et al.*, 2005; Sankar *et al.*, 2011) which are widespread in Asia and the Pacific region. The survey results show that WSSV and IHHNV single and co-infection rate of healthy *P. vannamei* in Northern of Jiangsu are quite high which is consistent with the related domestic reviews (Yang *et al.*, 2007). In fact, virus co-infection phenomenon in the crustacean host is very widespread (Chayaburakul *et al.*, 2004; Raia *et al.*, 2009). The study shows that (Tang *et al.*, 2003; Melena *et al.*, 2006) when IHHNV infect shrimp potentially, it does not prevent other viruses such as WSSV repeated infection.

But IHHNV can reduce obviously disease risk caused by WSSV through the antagonism function of the viruses so that, the balance can be achieved and latent together through the interaction between two virus and host shrimp. Finally cause multiple virus persistent infection shrimp status. Obviously because virus repeated infection can cause pathogenic factors complicated, clinic symptom and pathological analysis and so on have a big captivation characteristics. It is easy to cause diagnostic error, delay and seriously affect disease prevention timely and accurately. Secondly, *P. vannamei* has quite good tolerance and Disease Symptom (RDS) is not obvious. Its easy to make farmers neglect. Finally because repeated infection is potential and disease is not outbreak (Tang *et al.*, 2003) when the secondary virus WSSV infection volume is quite high caused by the virus mutual restrict. It increases the risk of disease prevention virtually. Because of many species of infected virus and quite big infection volume, once cultured conditions have changed suddenly, diseased will be outbreak quickly. Practicable prevention time and control measures are unavailable and economy losses will be very severe (Zhou *et al.*, 2011; Li *et al.*, 2008). The above shows that at the moment of very popular virus repeated infection, its essential to detect cultured shrimp important virus infection in the body at any time by sensitive and specific molecular biology methods (e.g., PCR). In accordance with the status of shrimp virus repeated infection this study established WSSV and IHHNV multiplex PCR detection method. Samples detection results show that the method has the specificity, sensitive and strong anti-interference characteristics and is suitable to show WSSV and IHHNV single and co-infection in the shrimp body simultaneously and accurately from minor shrimp samples. Therefore, this method can not only save related detection reagent and materials greatly but also win the valued preparation time for disease prediction alarm by shortening greatly double virus diagnostic procedure. This study investigated results indicate that WSSV and IHHNV single and co-infection rate are quite high in the body of cultured *P. vannamei* in Northern of Jiangsu and indicate that WSSV and IHHNV are the main infected virus of cultured

P. vannamei in this region. While the high infection rate of two pathogens in the body of healthy shrimp can warn the potential risk of disease outbreak. Therefore in the progress of cultivation, on one hand, local cultured enterprises should strengthen management and strictly prevent important stimulated factors influence (Zhou *et al.*, 2011; Li *et al.*, 2008) and adopt immunity strengthened reagent to increase shrimp self-immunity power. On the other hand, due to the lack of shrimp particular prevention and treatment drugs, WSSV and IHNV multiplex PCR technique should be adopted to detect virus infection status at any time and carry out preventive measures timely.

CONCLUSION

A multiplex PCR for co-detection of the two viruses with a specificity of (IHNV, TSV, YHV, *Vibrio Parahaemolyticus*, etc.) and sensitivity of 1×10^3 template copy numbers was established after serially optimization to investigate for WSSV and IHNV co-infection rate in pond-cultured *P. vannamei* in this study. This method can not only save related detection reagent and materials greatly but also win the valued preparation time for disease prediction alarm by shortening greatly double virus diagnostic procedure. The investigated results indicate that WSSV and IHNV single and co-infection rate are quite high in the body of cultured *P. vannamei* in Northern of Jiangsu and indicate that WSSV and IHNV are the main infected virus of cultured *P. vannamei* in this region.

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