DETECTION OF WHITE SPOT SYNDROME VIRUS INFECTION USING NESTED PCR IN *PENAEUS MONODON* CULTURED IN EXTENSIVE FARMS

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Abstract

White Spot Syndrome Virus (WSSV) is a major shrimp disease in South East Asia. It cause high mortality and losses huge economic to the shrimp farming countries. In the present study, shrimps were collected from natural habitats (mangrove forest) and extensive trap and hold farms located in Pyapon area, Ayeyarwady Region from May to September 2019. The samples were examined for WSSV using PCR and Nested PCR methods. Two out of ten samples from natural habitats showed positive in nest PCR in May 2019. The prevalence of infection ranged 20% to 60% in the shrimps collected form trap and hold systems during sampling from May to August 2019. However, all samples collected in September 2019 showed negative in both PCR and nested PCR. Since the nest PCR detects latent or carrier stage, the result in the present study indicated that shrimps collected from natural habitats and traps and hold farms are latent or carrier stage.

Keyword: WSSV, *Penaeus monodon*, PCR, Myanmar, Trap and hold farm

Introduction

Local shrimp farmers in Ayeyarwady Region and Rakhine state in Myanmar have used traditional trap and hold system for shrimp production. The shrimp farms are constructed near the mangrove forest with inlet and outlet channels to introduce and discharge water to the rivers. The system consists of an earthen pond with three to five sluice gates made up of wood or cement. During high tide, the ponds were filled with brackish water containing wild larvae of different species including shrimps larvae. Shrimp are harvested by releasing the water during the low tide by using fishing nets at the sluice to sieve the outgoing water. Depending on the pond size, harvest time takes 3 to 5 days (interview survey with U Phone Myint Naing, shrimp farmer, Kyonkan Village, Pyapon). Using the trap and hold system, shrimps (*Peneaus* spp., *Metapeneaus* spp.), crab and fishes are produced. Among them, *P. monodon* is the valuable commercial species for trading and local demand.

In Ayeyarwady Region, Pyapon Township is the main areas of producing of *P. monodon* using trap and hold farming system. The farm types are defined based on farm size such as small and medium-scale farms (up to 20 ha) and large scale farms (up to 50 ha) and commercial owned by private investors with an area up to 200 ha (Joffre *et al*., 2012). The majority of farms in Pyapon Township are larger than 10 ha.

Local farmers rely on *P. monodon* production rather than other aquatic species because of high market demand and price. However, shrimp production in trap and hold farming system has decreased since 2010 without significant reason. Shrimps have been infected with various viruses, bacteria, fungi, parasites, algal toxins, nutritional deficiency or adverse environment. The most lethal for *P. monodon* are White Spot Syndrome Virus (WSSV) and Yellow head virus (YHV) in Asia (Flegel, 2009). The three main pathogens affecting *P. monodon* in Myanmar are WSSV, Taura syndrome virus (TSV) and YHV (Tun *et al.*, 2020). In 2010, Department of Fisheries conducted scanning of disease for 40 individual of *P. monodon* collected from Ayeyarwady Division. Among

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them, four samples were found positive for TSV. Yellow Head virus (YHV) was also detected in shrimp which were foreseen to export in 2014 (NACA and FAO, 2015).

There is no information for the distribution of shrimp diseases in trap and hold farming systems in Ayeyarwady Region, Myanmar although shrimp farming is one of the most important productions in aquaculture industry of the country. The present study was undertaken to examine the occurrence of WSSV, one of the lethal pathogens for shrimps, in trap and hold system in Pyapon Environs, Ayeyarwady Region.

Materials and Methods

Study area

Two study areas were chosen to collect the sample. Post larvae shrimp were collected from Mangrove forest (natural habitats) before entering the trap and hold farm. In addition, three trap and hold farms located in Pyapon Township, Ayeyarwady Division, 16º4′48″ N - 95º43′16″ E. (Fig.1. A) were selected to collect the shrimp monthly.

Study period

Study period lasted from May, 2019 to November, 2019.

Collection of specimens

A total of ten post larvae of *Penaeus monodon* were collected from mangrove forest in May 2019 to examine the WSSV infection in natural habitat. Collection of post larvae in mangrove forest was conducted only one time before entering the area of trap and hold farms.

To collect the samples from trap and hold farms, three farms near the mangrove area were selected. The areas of farms were 40 acres (16ha). The farms are two meters apart each other. A total of five sample of *Penaeus monodon* were collected from each farm from May to September 2019. The shrimps brought to Aquatic Animal Diseases Laboratory, Fisheries and Aquaculture, University of Yangon alive. In the laboratory, length and weight of the shrimps were measured. The whole tissue of post larvae and pleopods of adult individuals were collected separately and fixed with 70% ethanol in 1.5ml micro centrifuge tube until DNA was extracted (Plate 1).

DNA extraction

DNA was extracted by PETNAD nucleic acid co-prep kit (GeneReach Biotechnology Corp) (Plate 2). Tissue and pleopods sample (20-25 mg) was ground using disposable plastic grinder with 100 µl PB1 solution. Then PB1 solution (500 µl) was added and vortexed for one minute. A total of 600 µl of PB2 solution, 600 µl of PB3 solution and 600 µl of PB4 solution were added and discharged step by step according to the manual instruction. Finally, 50 µl of PB5 solution was added for elution and stayed at room temperature for one minute and centrifuged. Concentration of extracted DNA were measured NanoDrop 2000 Spectrophotometer and it was stored at -20˚C.

Polymerase chain reaction (PCR) and nested PCR methods for detection of WSSV

Two steps of PCR were used in the present study. First step PCR was to detect the serious infection of WSSV whereas second step PCR was to detect a latent or carrier-state of WSSV (Table1).

Table1 Primers used for PCR analysis of WSSV confirmation (Lo *et. al*., 1996)

PCR	Primers	Sequences 5' to 3'	Product Size
first PCR	146F1	ACT-ACT-AAC-TTC-AGC-CTA-TCTAG	
	146R1	TAA-TGC-GGG-TGT-AAT-GTT-CTT-ACG-A	1447bp
nested PCR	146F2	GTA-ACT-GCC-CCTTCC-ATC-TCC-A	
	146R2	TAC-GGC-AGC-TGC-TGC-ACC-TTG-T	941bp

The volume of the reaction mixture for the first step PCR was 20.0 µl containing 10.0 µl of HS-PCR MasterMix (WizPure™ PCR 2X Master), 0.8 µl of WSSV first PCR Primer Mix, 7.2 µl of ddH_2O 2.0 µl of extracted DNA extract template. In the nested PCR, the amplified PCR products of the first step served as the template DNA for the second step of amplification. After completion of the first step, 20.0 µl of nested PCR containing 10.0 µl of HS-PCR MasterMix ((WizPure™ PCR 2X Master), 0.8 µl of WSSV nested PCR Primer Mix, 7.2 µl of ddH_2O 2.0 µl of first PCR product was prepared. The condition of PCR thermal cycler was described in Plate 3 and Table 2. The positive DNA was provided by the Aquaculture Pathology Laboratory, University of Arizona, United States. The PCR was conducted using thermal cycler (Applied Biosystem 9800) (Plate 3). The condition of PCR thermal cycler was described in Table 2.

Table 2 first and nested PCR amplification for WSSV

Temperature	Time	Cycle	Step
95°C	5 minutes	1	initial denaturing
95°C	30 seconds	25	denaturing
60°C	30 seconds		annealing
72°C	30 seconds		extension
72°C	5 minutes	1	final extension

Gel electrophoresis

PCR products were separated on 1.5% agarose gel containing 30 ml of 1xTAE buffer, 0.15 g of agarose powder and 6 µl of Gel Stain Green (Wizbiosolutions Inc). A 100bp DNA Marker (Wizbiosolutions Inc) was used to compare the base pair PCR results. Gels were visualized under UV Blue light trans-illuminator (Plate 4).

Parameters of soil and water in farms

Water and soil samples from the study sites were collected monthly to analyze water pH, alkalinity, ammonia, temperature, salinity, and soil pH. Water pH, alkalinity, ammonia were checked using the test kits (Advance Pharma Co., Ltd. Bangkok, Thailand). Water temperature was recorded by using a mercury thermometer. The water salinity was measured by using MA 887 Seawater Refractometer. Soil pH was analyzed by using INDEX pH meter ID 1100 USA.

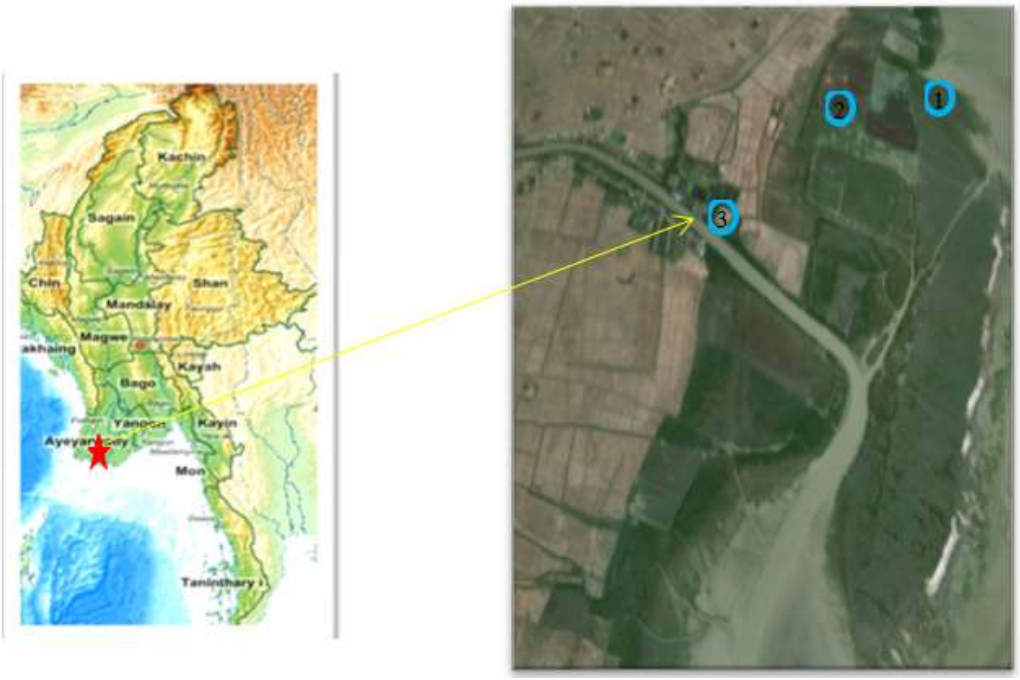


Figure 1 Location of Trap and Hold Shrimp Farms in Pyapon, Ayeyarwady

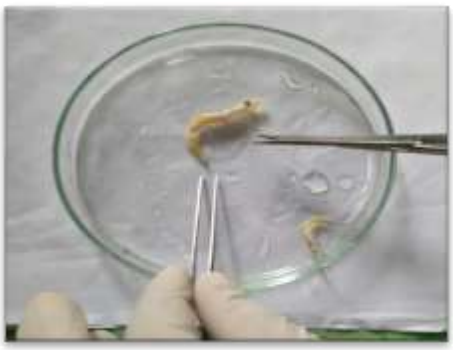


Plate 1 Sampling of *P. monodon*

Plate 2 PETNAD nucleic acid coprep kit (DNA Extraction) (LIR biotech lab-ind resource Co. Ltd, Malaysia)

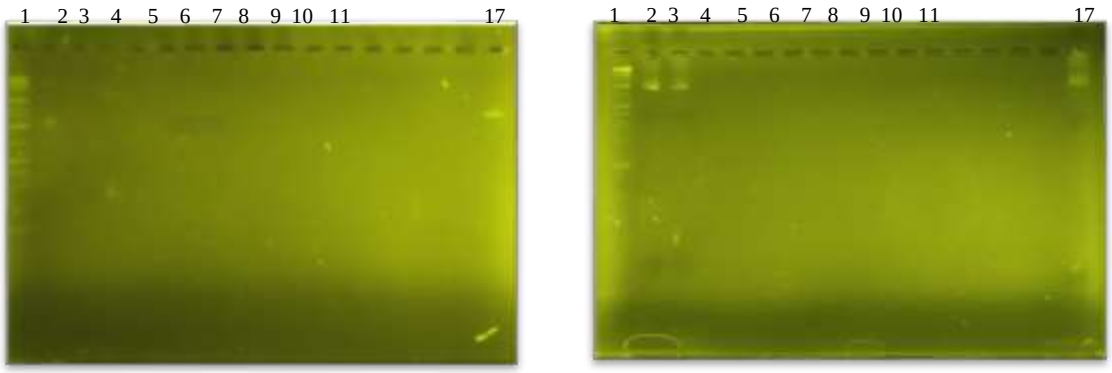
Plate 3 Thermal cycler for PCR assay

Plate 4 Gel electrophoresis

Results

Detection of White Spot Syndrome Virus at Natural Habitats (Magrove forest)

Before entering the cultured farms, 10 individuals of shrimp post-larvae were collected from natural habitats (mangrove forest) in May 2019 to examine the WSSV infection. WSSV infection was negative in the first step PCR in all samples (Plate 5 A). However, two out of ten samples showed positive results in nested PCR (Plate 5 B).



A. First step PCR result of the shrimp sampled shrimp collected in May 2019

B. Nested PCR result of the in May 2019

Plate 5 First step and nested PCR results of WSSV detection in *P. monodon* from natural habitats

A: Well 1 - Ladder (100bp), Well 2 to11 - Samples from natural habitat, Well 17 - Positive control

B: Well 1 - Ladder (100bp), Well 2 to 11 - Samples from natural habitat,

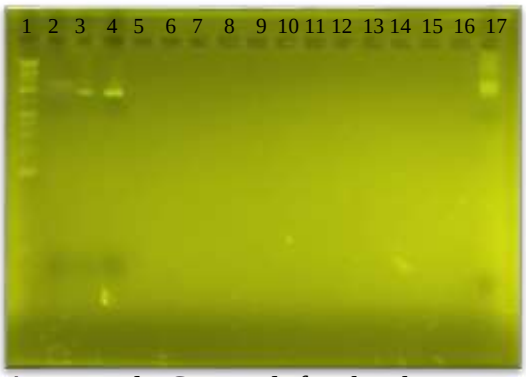
Well 17 - Positive control, Well 2, 3 - WSSV Positive results

Detection of White Spot Syndrome Virus in three trap and hold farms

All samples collected from farm (1), (2) and (3) shows negative results for WSSV infection in first step PCR during the study period.

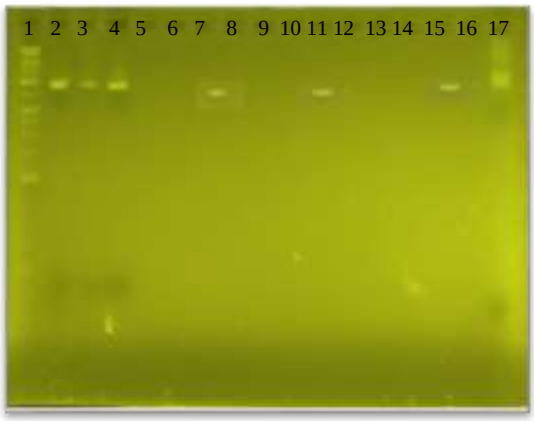
In nested PCR, two samples from the farm (1) were positive in June 2019 (Plate 6 A and Table 3). In July, two samples from the farm (1) and one sample from the farm (2) showed positive in nested PCR (Plate 6 B and Table 3).

All farms showed positive for WSSV in nested PCR in August 2019. The three samples from farm (1), two samples from farm (2), and one sample from farm (3) were positive (Plate 6 C and Table 3). Interestingly, all *P. monodon* samples were negative for WSSV in nested PCR in September 2019 (Plate 6 D and Table 3).

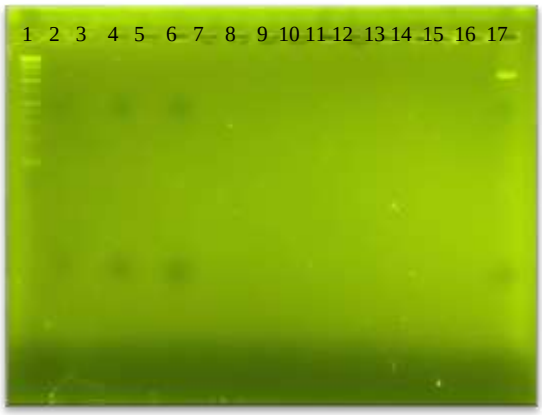


A. Nested PCR result for the shrimp in June 2019

B. Nested PCR result for the shrimp sampled in July 2019



B. Nested PCR result for the shrimp in August 2019



D. Nested PCR result for the shrimp sampled in September 2019

Plate 6 Nested PCR Result for WSSV disease in *P. monodon* (farm 1, 2, and 3) in June – September 2019

Well 1 - Ladder (100bp), Well 2 to 6 - five Samples from farm 1, Well 7 to 11 - five Samples from farm 2, Well 12 to 16 - five Samples from farm 3, Well 17 - Positive control

Table 3 PCR Result for WSSV detection from *P. monodon*

No. of Farm	June				July				August				September			
	+ in 1st PCR	%	+in nested PCR	%	+ in 1st PCR	%	+in nested PCR	%	+ in 1st PR	%	+in nested PCR	%	+ in 1st PCR	%	+in nested PCR	%
Farm (1)	0/5	0	2/5	40	0/5	0	2/5	40	0/5	0	3/5	60	0/5	0	0/5	0
Farm (2)	0/5	0	0/5	0	0/5	0	1/5	20	0/5	0	2/5	40	0/5	0	0/5	0
Farm (3)	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0	1/5	20	0/5	0	0/5	0

Water and Soil Parameters at Extensive Farms

The water temperatures among the three farms were not shown much variation. It was ranged between 27 - 29˚C, 27 - 30˚C and 29 - 31˚C in the farms (1), (2), and (3) respectively during the study period. The range of water pH was 7.2 - 8.3, 7.3 - 8.1, and 7 - 8 in three farms. The range of alkalinity was 100 - 180 ppm in farms (1), (2), and (3). The maximum and minimum

concentrations of ammonia were 0.17 ppm and 0 ppm in the farm (1), 0.3 ppm and 0 ppm in the farm (2), and 0.2 ppm and 0 ppm in the farm (3) from June to September 2019. The range of salinity was 1 - 4 ppt throughout the study period in all farms. The range of soil pH varied 7.06 to 7.23 in the farm (1), 6.95 - 7.36 in the farm (2), and 7.1 - 7.3 in the farm (3) (Table 4).

Table 4 Water and soil quality Parameters in shrimp farms

Parameters	June			July			August			September		
	Farm			Farm			Farm			Farm		
	1	2	3	1	2	3	1	2	3	1	2	3
Temperature (ºC)	27	27	29	27	29	29	29	30	31	29	29	31
pH	8	8.1	8	7.2	7.4	7	7.5	7.3	7.5	8.3	7.7	8
Alkalinity (ppm)	160	140	180	120	110	100	120	150	170	110	100	100
Ammonia (ppm)	0.1	0	0	0	0.1	0.12	0.15	0	0.1	0.17	0.3	0.2
Salinity (ppt)	3	3	2	2	2	1	3	4	2	2	2	2
Soil pH	7.06	6.95	7.13	7.14	7	7.1	7.1	7.21	7.3	7.23	7.36	7.19

Discussion

The present study is the first report for the scanning of White Spot Syndrome Virus in shrimp cultured in extensive trap and hold farms in Pyapon area, Ayeyarwady Region. In the present study, PCR and nested PCR methods were conducted for detection of WSSV in post-larvae lived natural habitats and juvenile to adult shrimps cultured in trap and hold farms. In trap and hold system farms, introducing of shrimp post-larvae are totally depended on natural habitats, mangrove forest (Myanmar Shrimp Association, 2015). To understand the disease transmission of post-larvae from natural habitats to extensive cultured farms, shrimp post-larvae were collected from mangrove and examined for WSSV infection. WSSV infection was negative in the first PCR while it was positive in nested PCR. The result is positive in the first step implies a serious infection with WSSV, when the result is positive only in the second step, a latent or carrier-state infection (OIE, 2019). The present work supports collected shrimps at the natural habitats are carrier-state with the light infection which showed positive with nested PCR only.

WSSV had been introduced to Myanmar by imported post-larvae from Thailand and serious outbreaks of shrimp disease were encountered in 1996-1999 (FAO, 2018). After outbreaks of WSSV in Myanmar, penaeid shrimp from the natural population of Myanmar became carrier state for WSSV in outbreak area (Tun *et. al*., 2017). Therefore, it is assumed that post-larvae from mangroves are latent-state for WSSV. Positive WSSVs were found in nested PCR in farm (1) in June 2019 while farms (2) and (3) were negative. All positive samples from extensive farms were also detected in the nested PCR stage in July and August. Tun *et. al.*, (2017) also reported positive for WSSV infection in nested PCR state and they concluded that WSSV in the Rakhine region was only latent or carrier-state. Yu Wai Hlaing (2018) reported the occurrence of WSSV in intensive shrimp culture farms in Myeik, Tanintharyi Region.

The present work supports collected shrimps at the carrier-state with the light infection which is positive with nested PCR only. Lo and Kou (1998) stated that if the shrimps at the nested state PCR are positive only, they may not display any clinical signs of WSSV. The collected post-larvae of *P. monodon* at natural habitats were not found any clinical signs of WSSV. The collected

samples of *P. monodon* at extensive farms were not displayed in white spots or patches embedded in the exoskeleton.

Water and solid quality in the shrimp farms were examined and the values were acceptable limit for shrimp culture. According to the guideline of FAO (2017), water and soil parameters in the studied area are suitable for the culture of *P. monodon.* To improve the sustainable shrimp production in Pyapone area, *r*egular examination of WSSV in extensive farms is essential for effective health management. Monitoring of shrimp disease in extensive farms is necessary to reduce the possible risk of an outbreak of WSSV.

Conclusion

This is the first report for scanning of WSSV in extensive shrimp farms in the Ayeyarwady Region. The *P. monodon* collected from mangrove area and trap and hold system farms were negative in first stage PCR while it was positive in nested PCR. The present work supports collected shrimps at the asymptomatic carrier-state with the very light infection which is positive with nested PCR only. WSSV can disperse to shrimp farms where water has been shared among the farms. Therefore, a regular scanning for WSSV in farms should be undertaken.

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