Occurrence and Prevention of White Spot Syndrome (WSSV) in Malaysia

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Abstract

Southeast Asia is a significant area for world shrimp culture. However, in recent years, the production of cultured shrimp has markedly decreased as a result of serious viral disease outbreaks. Especially, the increased severity of widespread White Spot Syndrome Virus (WSSV) infection is the most serious threat to stable aquacultural production. In the case of Malaysia, outbreak of this disease was almost the same as Thailand situation that was WSSV disease occurring as serious problem from 1996. A lot of farms have given up shrimp culture due to heavy loss incurred by WSSV until 1997. Field research was performed through 1998 and 1999 on the occurrences of WSSV at farms in Malaysia. Dark-field microscope observation and Japanese PCR methods for PRDV detection were used for this research, because both methods were confirmed to use for detection of Malaysian strain of WSSV. From this research, WSSV outbreaks were found in Penang, Keda and Sarawak State, and information of the occurrence of viral disease was obtained. Almost all prawns in the ponds were dead by this disease. These dead prawns were showing many clear White spots on their carapace. Pathogenic viruses were confirmed by PCR from the samples WSSV. In this etiological study, as for the infection routes, it could be supposed two ways. One was that the prawn fry had been infected with this virus, and another was from supplied seawater containing the virus particles. As one of the preventive countermeasures against viral diseases, inactivation methods for WSSV were studied. This viral inactivation was tested with formalin and halogenous disinfectants and sodium hypochlorite. These chemicals were mixed with the virus and injected to the healthy prawns. As a result of these experiments, no mortality was shown using0.25% effective chloride in sodium hypochlorite and 0.5 ppm formalin. From these results, sodium hypochlorite of halogenouse disinfectants showed the effective inactivation even under lower concentration. It was suggested that these disinfectants were extremely useful for the WSSV inactivation.

Introduction

Southeast Asia is an area of great significance in world shrimp culture. However, in recent years, the production of cultured shrimp has markedly decreased as the result of serious viral diseases. The increased severity of widespread White Spot Syndrome Virus (WSSV) infection is the most serious threat to stable aquacultural production. Features of this WSSV were that the diseased prawns were often showing obviously White spots on their carapace and that the high mortality was occurred from 80% to 100% in only few days after infection (Nakano *et. al.* 1994, Chou *et. al.* 1995).

The first occurrence of WSSV was in Taiwan in 1992 (Chou *et. al.* 1995). In Japan it first appeared in 1993, a new acute viral disease occurred at the kuruma prawn farm (Nakano *et. al.* 1994). These prawns were imported from China as fry. This disease was called Penaeid Acute Viremia (PAV) in Japan and the pathogenic virus was named Penaeid Rod-shaped DNA virus (PRDV) from their particle shape (Inouye *et. al.*1996).

WSSV was also reported in 1993 in Korea (Park *et. al.* 1998) and the disease spread into Thailand in 1994 (Wongteerasupaya *et. al.* 1995). Furthermore, this disease occurred in India in 1994 and 1995 (Karunasagar *et. al.* 1997), and lastly, it was recognized in the Philippines in 1999, which was far away from other east and Southeast Asian countries (Magbanua *et.al.* 2000). Since WSSV occurred at first in 1992, this disease has spread among most Asian countries. Recently, WSSV has spread widely in the world, not only in Asian countries, but also in the USA (Lightner 1996), Central America and South America (O.I.E. 2003). In the case of Malaysia, WSSV was reported in 1994 at almost the same period that Thailand reported the disease (Wang *et. al.* 1999). It was estimated that this disease reached Malaysia through importation of the post larvae from Thailand. WSSV spread rapidly in the peninsular Malaysia, and the disease caused enormous economic losses. The outbreaks of this disease became the most serious problem facing prawn culture farms in Malaysia since 1996. In such conditions, the international collaborative project "Development of Technology for the Diagnosis and Prevention of Shrimp Viral Diseases" was planned by the Japan International Research Center for Agricultural Sciences (JIRCAS). This project was carried out in Malaysia by the Fisheries Research Institute (FRI), Department of Fisheries, Ministry of Agriculture, Malaysia. This project investigated the occurrence of prawn diseases in Malaysian farms and viral inactivation techniques by using disinfectants for disease control.

Application for the Existing Diagnostic Method for WSSV

Many diagnostic methods for WSSV were already been reported. In these methods, the dark-field microscopic observation method which were reported by Momoyama *et. al.* (1995) is a quite simple and easy method for detecting this disease. The advantage of this method is that diagnosis could be performed in the ordinary laboratory without expensive and specific machines, and it needed only a biological microscope with wet-type dark-field capacitor. But, this method was adapted only for the case of Kuruma prawn, so that it s needs to be tested for WSSV of black tiger prawn. The PCR method is the most popular diagnosis for detection of WSSV. These methods provide an accurate diagnosis for virus specific genomes. However, new strains sometimes cannot be detected by this method because of minor distinguishable factors between even local strains. For these reasons, it was necessary to confirm whether, local strains of WSSV in Malaysia could be detected by these both diagnostic methods of PAV used in Japan.

(a) Dark-Field Microscopic Observation

This method was tested using hemolymph and stomach samples, collected from prawns artificially infected prawns with WSSV. The stomach samples were provided with raw and 10% formalin fixed tissues. The observation method was followed Momoyama *et. al.* (1995). A lot of small and shiny particulates were observed in the hemolymph samples of diseased prawns. These particulates may be one or more agglutinated particles of WSSV. However, the stomach did not show the same observable

features because the epidermal layer could not be peeled easily from the cuticula. From these results, hemolymph was suitable for simple diagnosis of WSSV using dark-field microscopic observation. On the other hand, this method might led to incorrect diagnostic results because it is difficult to distinguish between the particles of WSSV and the small dust. Therefore, we will need to confirm this viral disease using other accurate diagnostic methods as a counter-check.

(b) PCR Diagnosis Method

PAV detection method of Kimura *et. al.* (1996) was used in this study. This PCR diagnosis method is used in Japan and was developed jointly at Fisheries Research Agency, National Research Institute of Aquaculture (NRIA) and Kumamoto Prefectural Fishery Research Center. Sample DNA was extracted from the muscle of diseased prawns infected with WSSV in Penang Island in August 1998. These DNA were prepared in ten-fold serial dilutions until 10^{-9} and each of the diluted fluids was applied to PCR. Two pairs of PCR (P1-P2, P3-P4) were used in this test, because this was a nested PCR method requiring two-step PCR procedures. The results of PCR, analysis showed that the band was found at the same location in agar gel as the PRDV (about 560 base pair). The detection limit of WSSV was 5-plex (10^{-5}) dilution of the virus sample at the nested step although virus was detected under 3-plex (10^{-3}) diluted samples at the single step alone. These results were showed that both PRDV and this local strain of WSSV were quite similar; at least the regions of primer sequences were almost same. It was very clear that the PAV detection method was useful for screening of the Malaysian local strain of WSSV.

Field Research on WSSV Occurrence in Malaysia

The prawns were mainly produced in the area located on the western coast and southern part of the Peninsular Malaysia. The places on the western coast were Penang, Perak, and Kedah State centering Penang City, and Selangor State centering on Kuala Lumpur City. The southern area was Johor State centered Johor Bahr City. On the eastern coast, prawn production was not as successful. One of the causes must be that prawn ponds were damaged by heavy rain and strong wind in the monsoon seasons. Another cause may be economical. Prawn consumption in this area was lower than west coast with big cities. However, the prawn industry has begun to develop rapidly in the Saba and Sarawaku State on Borneo Island recently. There was a large space for culturing prawns in vast mangroves area there. WSSV became serious problem in the prawn farms starting in 1996, mirroring the same situation of Thailand. Damages were seriously in the Keda State, which was showing a lot of neglected ponds all over. In contrast, YHV disease was not reported in Malaysia although it was a serious problem in Thailand.

Field research occurred in 1998 and 1999 on the occurrences of WSSV at Malaysian fish farm. The diagnoses for WSSV were used with dark-field microscopical observation and PCR, but confirmation of the virus was carried out using PCR of PAV method. Field research sites and periods were shown in **Table 1**.

Area		Main period for investigation	
State	***place***	***1998***	***1999***
Northern part of east coast in Peninsula Malaysia			
Keda State	Sungai Patani	Feb. Jun Aug.	Jan. March*[1]
Penang State	Penang Island	Feb. Jun Aug. *[1]	Jan.
	Butterworth		Jan.
Perak State	Nibong Tebal	Jun Aug.	Jan.
	Taiping		Jan.
Southern Part of Peninsula Malaysia			
Johor State	Gran patha		Aug.*[2]
	Johor Bahru		Jan.
	Batu Pahat		Jan.
Borneo Island			
Sarawak	Kuching		May*[1,2]

*1: WSSV occurrence was confirmed in the field investigation.
*2: Delivery samples

Table 1. Field research of the prawn culture farms in Malaysia

In 1998, field research was carried out in February, June and August, and research took place at Penang Island, Nibong Tebal, and Sungai Patani, in Penang, Perak, and Kedah State, respectively (**Fig.1**). In January of 1999, investigations also took place at Taiping in Perak State, Bukit Tambun in Penang State, and Johor Bahru and Batu Pahat in Johor State. Samples from Kuching of Sarawak State were checked on March and May 1999. This Sarawak State was well known to be free of White Spot Syndrome Virus (WSSV) before the study. A lot of farms have given up shrimp culture due to heavy loss incurred by WSSV until 1997. Many shrimp farmers changed their ponds into fishing ponds. In this field research, WSSV was detected at prawn farms of Penang Island in 1998 and killed almost all prawn ponds (**Fig.2**). The dead prawns showed many clear White spots on their carapace (**Fig. 3**). The prawn samples for PCR were collected from Keda, Pera, and Penang State in February, June, and August 1998 and January 1999.Samples from Sungai Patani of Keda state at March and from Sarawak at May in 1999 underwent a PCR analysis. From the result of PCR test showed no virus was detected in the samples collected at Keda, Pera, Penang State in January, 1999. WSSV positive results were found in Penang in August, 1998, Keda in March 1999 and at Sarawak, where WSSV were outbreaks had already been reported. In this field research, viral disease confirmed by WSSV was confirmed for Penang, Sungai Patani and Kuching on August 1998, March and May 1999, respectively. The other research places did not encounter WSSV disease. In this etiological study, it was clear that WSSV came into these farms by two routes. One route was that pathogenic viruses originated from shrimp fry since the disease occurred one month after fry introduction into the pond. WSSV also came via supplied seawater from the ocean because the disease broke out at the rainy season when the seawater was stirred. The culture environments culture also affected the disease outbreak.. The high density of the prawns associated with intensive culture system contributed to the spread of WSSV. The quite low water exchange rate also affected the

disease outbreak.. The low salinity, which was occasionally almost fresh water in rainy season, and affected the disease outbreak.

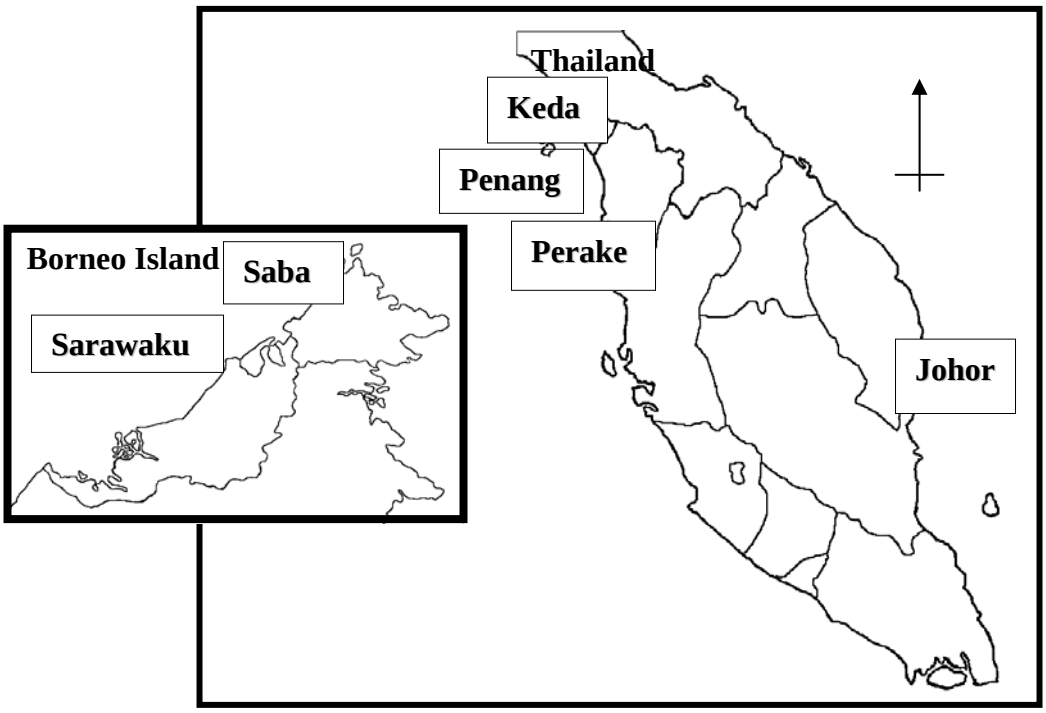

Figure 1. Map of Malaysia

Figure 2. WSSV occurrence in prawn culture pond.

Figure 3. Diseased prawns with White spots on their carapace.

Viral Inactivation Using Disinfectant for Disease Control

Disinfectants are mainly used as a preventive measure to protect against the introduction of pathogens. The methods of inactivation for viral pathogens were developed using general disinfectants in this study. Viral inactivation was tested using chemicals, such as formalin and sodium hypochlorite of halogenous disinfectants. These chemicals were mixed with the virus at 25^{o}C. After the reaction, the resultant products were injected intramuscularly, and the mortality was monitored for 2 weeks after the injection. Formalin, which was used to exterminate fish parasites, was prepared concentrations of 0 to 1% (V/V). Each formalin solution was mixed with virus fluid and reacted together for 10 minutes. After that, the solution was diluted to stop the reaction. Sodium hypochlorite that is used in halogenous disinfectants, was added into the diluted virus fluid and reacted together for 10 minutes. The final concentrations of effective chloride were from 0 to 5.0ppm. These reactions were stopped by sodium thiosulfate. The reactants were then used for artificial infections. No mortality was shown when over 0.25% (V/V) formalin was used. Thus, it became clear that this virus was inactivated by more than a 0.25% formalin concentration. On the other hand no mortality was shown over 0.5 ppm for sodium hypoclorite. Thus, this virus was inactivated by only 0.5ppm of effective chloride concentration in sodium hypochlorite. From these results, sodium hypochlorite of halogenous disinfectants induced an effective inactivation at even lower concentrations. So, it was suggested that this disinfectant was extremely useful for the inactivation of WSSV.

Discussion

The main Malaysian prawn species was the black tiger prawn. There was intensive culture and few water exchanges in the rearing of this species. This culture style was similar between Thailand and Malaysia. These bad environmental conditions caused the prawn diseases. In Japan, PAV was occurred in the cultured kuruma prawns in 1993, and it became a serious problem (Nakano *et. al.* 1993). The PRDV diagnostic method (Momoyama *et. al.* 1995) and PCR (Kimura *et. al.* 1996), preventive techniques by the disinfectants, have been developed WSSV damage became serious starting in 1996 for Malaysia., and one could hypothesize that it had the same pathogenic origin as PRDV. In this study, it was verified that the methods of simple diagnosis and PCR detection, which were developed in Japan, could be adapted to Malaysia. It became clear that these diagnostic methods were effective to use, and these simple or conformed diagnosis could be carried out for WSSV diagnosis in Malaysia as well in Japan. The dark-field microscopic observation method could serve as a simple WSSV diagnosis for heavily infected shrimp. However, it was difficult to confirm the virus with this method and the detection sensitivity was lower. On the other hand, the PCR method has been proven as a useful WSSV diagnostic method. However, this method carries a high cost, and it takes a long time to get the diagnostic result. It also requires special techniques and equipments to run. Therefore, there are advantages and defects in these various methods. Rapidly, low cost, and accurate diagnostic techniques should be developed in the future. Also, Sometimes the PCR method t could not be reacted when the primer setting of the genome sequence was mismatched with different virus strains. However, Malaysian strain was detectable by PCR of the PRDV method. It seemed that there were fewer variations between the PRDV and this local strain of WSSV, at least at the PCR regions . Using this method, WSSV was clearly detected in diseased prawns in Penang 1998, Keda 1999, and Sarawaku State 1999, where the outbreaks of this disease were occurred. The virus could not be detected in other areas. Thus not all prawn farms were necessarily polluted by WSSV, or that all surviving prawns became virus carriers. Almost all prawn farmer gave up managing ponds due to the high WSSV damage. Therefore, the surviving farmers are either virus free or good managers.

In this etiological study, there were two assumed infection routes. One infection route was that the prawn fry had been virus carriers, and another theory is that the virus came from added seawater that contained the virus particles. These results are important for preventing a WSSV epidemic. The disease occurrence must be controlled by screening of the shrimp fry and by disinfecting supplied seawater. Recently, the inspections of prawn fry using PCR have been carried out actively at the fisheries institute of government and by private companies. The occurrence of WSSV has decreased for this reason. However, almost all prawn fry were not tested because of the high cost. Thus, the disease still continues to occur.

Nakano *et. al.* (1998) described that halogenous disinfectants were quite useful for PRDV inactivation. From these results of WSSV, sodium chloride of halogenous disinfectants also induced an effective inactivation for this virus even at lower concentrations. It was suggested that these disinfectants were extremely useful for the inactivation of WSSV. Some farmer began to use the chlorine to sterilize the supplied water in the reserve ponds. But, usage of these disinfectants should be limited to sterilizing of culturing tools and gear, or pond water at lower concentration. This chemical is not only toxic for the virus, but also for the prawns. This study should be

continued, because the WSSV disinfections prevented pathogen intrusion at aquaculture farms and thus has contributed to sustainable aquaculture in Southeast Asia.

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