JARQ 40 (1), 93 – 97 (2006) http://www.jircas.affrc.go.jp

Examination for Viral Inactivation of WSSV (White Spot Syndrome Virus) Isolated in Malaysia Using Black Tiger Prawn (*Penaeus monodon*)

Norihisa OSEKO[1,3]*, Toh Thye CHUAH[2,4], Yukio MAENO[1,5], Beng Chu KUA[2,6] and Veloo PALANISAMY[2,6]

[1] Fisheries Division, Japan International Research Center for Agricultural Sciences (JIRCAS) (Tsukuba, Ibaraki 305–8686, Japan)
[2] Fish Health Section, Fisheries Research Institute (Batu Maung, Penang 11960, Malaysia)

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 such as white spot syndrome (WSS) outbreaks. In the case of Malaysia, outbreaks of this disease have been a serious problem since 1996. As one of the preventive countermeasures against WSSV, virus inactivation has been carried out against kuruma prawn in Japan. In the recent studies, it became clear that there are differences among local strains of WSSV. Furthermore, kuruma prawn shows resistance against WSSV. For these reasons, methods to inactivate Malaysian isolates of WSSV were studied with black tiger prawn (*Penaeus monodon*). Viral inactivation was tested using the disinfectants formalin and ethanol, the halogenous disinfectants sodium hypochlorite and Isodine^{R}, and also using U.V. irradiation. These chemicals were mixed with the virus and injected into healthy prawns. As a result of these experiments, no mortality was observed at the concentrations of more than 0.25 ppm formalin, 0.5% effective chloride in sodium hypochlorite, and 2.5 ppm effective povidone-iodine in Isodine^{R}. From these results, sodium hypochlorite of halogenous disinfectants showed effective inactivation even at a low concentration (0.5 ppm). On the other hand, this virus was inactivated completely by U.V. irradiation at a dose of 3×10^4 μW·sec/cm^2. These results were similar to the virus inactivation of a Japanese strain with kuruma prawn (*Marsupenaeus japonicus*).

Discipline: Aquaculture
Additional key words: diseases, formalin, halogenous disinfectants, Isodine^{R}, sodium hypochlorite

Introduction

Southeast Asia is an area of great significance for world shrimp culture. Most of the shrimp cultured have been black tiger prawn (*Penaeus monodon*). However, in recent years, the production of cultured shrimp has markedly decreased as a result of several serious viral diseases. Especially, widespread severity of white spot syndrome virus (WSSV) infection is the most serious threat to stable aquacultural production. Features of this white spot syndrome (WSS) are that the diseased prawns often show obvious white spots on their carapace and that a high mortality occurs from 80% to 100% in only a few days after infection[1]. In the case of Malaysia, WSS was reported in 1994 and at almost the same period in Thailand[13,16]. WSS spread rapidly in peninsular Malaysia, and since 1996 this disease has caused enormous economic losses against the prawn culture industry in Malaysia.

In our etiological study, it was clear that the occur-

This paper reports the results obtained in the joint project on "Development of Technology for the Diagnosis and Prevention of Shrimp Viral Diseases" sponsored by Japan International Research Center for Agricultural Sciences (JIRCAS).

Present address:
[3] Aquatic Animal Health Division, National Research Institute of Aquaculture (Watarai, Mie 509–1234, Japan)
[4] National Prawn Fry Production and Research Center (Kota Kuala Muda, Kedah 08500, Malaysia)
[5] Seikai National Fisheries Research Institute (Nagasaki, Nagasaki 851–2213, Japan)
[6] National Fish Health Research Center (Batu Maung, Penang 11960, Malaysia)
*Corresponding author: fax +81–599– 66–1962; e-mail ohseko@fra.affrc.go.jp
Received 4 January 2005 ; accepted 1 June 2005.

rences of WSS were recognized after the rainy season when seawater is mixed with affected sediments. From these results, it could be supposed that supplied seawater inflowing into the ponds contained the virus particles as one of the infection routes. This was important for establishing preventive measures against a WSS epidemic. If the supplied seawater was disinfected, the disease occurrence could be controlled. On the other hand, once WSS breaks out, there is a high risk that this disease will recur in the same pond. WSSV also spreads rapidly and widely to previously non-affected neighboring areas, as it is a highly contagious pathogen. For this reason, it is important that not only the supplied seawater but also affected culture ponds and gears should be sterilized by disinfectants immediately, and then should be dried thoroughly and exposed to sunshine, if the viral disease has occurred. These are the principal preventive measures to protect against intrusion by the pathogens. Therefore, it was important to develop the methods of inactivation for viral pathogens using general disinfectants and ultraviolet (U.V.) irradiation.

Nakano et al. reported the methods of virus inactivation of PRDV[9], which is considered to be WSSV. The virus that was used in this report was isolated from kuruma prawn (*Marsupenaeus japonicus*) in Japan. Live kuruma prawn instead of culture cells was employed for measurement of virus inactivation, because the shrimp cell line has not been established yet. In the recent studies, it became clear that there are differences among local strains of WSSV that are widely distributed in areas all over the world[5,15]. Kuruma prawn also shows resistance against WSSV[11,12]. Sensitivity between different prawn species against six geographic isolates of WSSV was described by Wang et al.[14]. The aim of this study was the application of these virus inactivation methods for the black tiger culture farmers in Malaysia and other Southeast Asian countries. Hence, the methods following those of Nakano et al.[9] were examined using the virus strain isolated in Malaysia and black tiger prawn, instead of a Japanese strain and kuruma prawn.

Materials and methods

1. Measurement of viral infectivity (LD_{50})

The infectivity (lethal dose 50%: LD_{50}) of WSSV was used as the criterion for the virus inactivation tests. The gills of diseased prawn were homogenized in 9 times its volume of PBS, and this homogenate was filtrated using an HA filter (pore size 0.45 μm). This filtrate was the original viral fluid that was used for the inactivation tests afterwards. To measure the infectivity of this virus fluid, 10-fold serial dilutions of the fluid were made from 10^4 to 10^7. These diluted fluids were injected into each of 10 healthy prawns (average body weight 20.0 g) at the dose of 0.1 mL/prawn. Mortality and clinical signs were observed daily for two weeks.

2. Viral inactivation by disinfectants

Viral inactivation was tested using chemicals, such as formalin, ethanol and halogenous disinfectants including sodium hypochlorite and Isodine^R (povidone-iodine was the effective ingredient). These chemicals were mixed with the virus and then made to react together at 25ºC. After the reaction, the resultant products were injected intramuscularly into 10 healthy prawns at the dose of 0.1 mL/prawn. Mortality was monitored for 2 weeks after the injection. Furthermore, all dead prawns, excluding decomposed ones, and all surviving prawns were applied to PCR test for detection of the virus. PBS instead of the treated virus solution was injected into the prawns using the same conditions to serve as the negative control.

(1) Formalin: Formalin is used for extermination of fish parasites. It was prepared at five concentrations of 0, 0.1, 0.25, 0.5, and 1% (V/V). Four mL of each formalin solution were mixed with 0.1 mL of the virus fluid, and then allowed to react together for 10 min. These reactions were stopped by 100 times dilution, and then these reactants were provided for artificial infections.

(2) Sodium hypochlorite: Sodium hypochlorite is a halogenous disinfectant. It was added into 50 mL of the viral fluid diluted $10^{4.6}$ times, and then allowed to react together for 10 min. The final concentrations of effective chloride were 0, 0.5, 1.0, 2.5, and 5.0 ppm. These reactions were stopped using sodium thiosulfate.

(3) Isodine^R: Isodine^R is a halogenous disinfectant. It was added into 50 mL of the virus fluid diluted $10^{4.6}$ times, and allowed to react together for 10 min. The final concentrations of effective povidone-iodine were 0, 1.5, 2.5, 5.0, and 10.0 ppm. These reactions were stopped using sodium thiosulfate.

(4) Ethanol: Ethanol is a general disinfectant. It was prepared at five concentrations of 0, 10, 20, 30, and 40% (V/V). Four mL of each ethanol dilution were mixed with 0.1 mL of the virus fluid, and then reacted together for 1 min. The reactions were stopped using 100 times dilution, then these reactants were provided for artificial infections.

3. Virus inactivation by U.V. irradiation

U.V. light was set in a clean bench and the U.V. dosage was regulated at 100 μW·sec/cm^2. The original virus fluid was diluted at 1/4,000 ($10^{-3.6}$) by sterile PBS, and 1.5 mL of this diluted viral fluid was spread evenly onto a

glass petri dish of 5 cm in diameter. The petri dish was put on ice and agitated slowly on a shaker set on the clean bench. U.V. light was irradiated against the thin layer of viral fluid for 0, 10, 20, 50, 100, and 300 sec. After irradiation, these inactivated viruses were injected into 10 healthy prawns for each group. PBS was used instead of virus solution for the negative control.

Results and discussion

The results of viral infectivity (LD_{50}) as measured by the mortality of injected prawns showed 100% in all test groups until 10^6 of the serial dilutions, but showed 0% for the 10^7 dilution group. To clarify the infectivity value, prawn injections were carried out again for three viral dilution groups, $10^{6.25}$, $10^{6.5}$, and $10^{6.75}$, between the 10^6 and 10^7 dilutions. The mortality showed 100, 100, and 66% for the $10^{6.25}$, $10^{6.5}$ and $10^{6.75}$ dilution groups, respectively. From these results, the value for the lethal dose of 50% of the population was estimated to be between the viral dilution groups of $10^{6.75}$ and $10^{7.0}$. Therefore, the LD_{50} value of the original viral fluid was estimated to be "$10^{-9.05}$ mL/g < LD_{50} < $10^{-9.30}$ mL/g".

The results of viral inactivation using several disinfectants are shown in Fig. 1. Formalin, sodium hypochlorite, and Isodine^R, resulted in mortalities of 100, 100, 0, 0, and 0% at the formalin concentrations of 0, 0.1, 0.25, 0.5, and 1% (V/V), mortalities of 100, 0, 0, 0, and 0% at the chloride concentrations of 0, 0.5, 1.0, 2.5, and 5.0 ppm, and mortalities of 100, 90, 0, 0, and 0% at the povidone-iodine concentrations of 0, 1.5, 2.5, 5.0, and 10.0 ppm, respectively. It becomes clear that this virus was inactivated by concentrations of more than 0.25% formalin, 0.5 ppm of effective chloride in sodium hypochlorite, and 2.5 ppm effective povidone-iodine in Isodine^R. Furthermore, the mortalities were 100, 100, 100, 0, and 0% for the 0, 10, 20, 30, and 40% (V/V) ethanol concentrations, respectively. It was shown that this virus was inactivated by concentrations of more than 30% ethanol. All dead prawns were virus-positive, but all surviving samples were virus-negative by PCR test. It is therefore suggested that the virus was not able to multiply in the surviving prawns. The results of virus inactivation by U.V. irradiation showed that all prawns could survive in the experimental groups of the U.V. doses of more than 3×10^4 μW·sec/cm^2 (300 sec irradiation; Fig. 2). Almost all dead prawns were virus-positive and surviving samples were virus-negative by PCR viral detection test. This indicates that the virus was inactivated completely by a dose of 3×10^4 μW·sec/cm^2. No mortality and no virus isolation were recognized in all negative control groups.

Inouye et al. reported on the effects of the inactivation against several fish viruses using general disinfectants[2,3]. In these reports, ethanol and halogenous disinfectants, sodium hypochlorite and Isodine^R, could

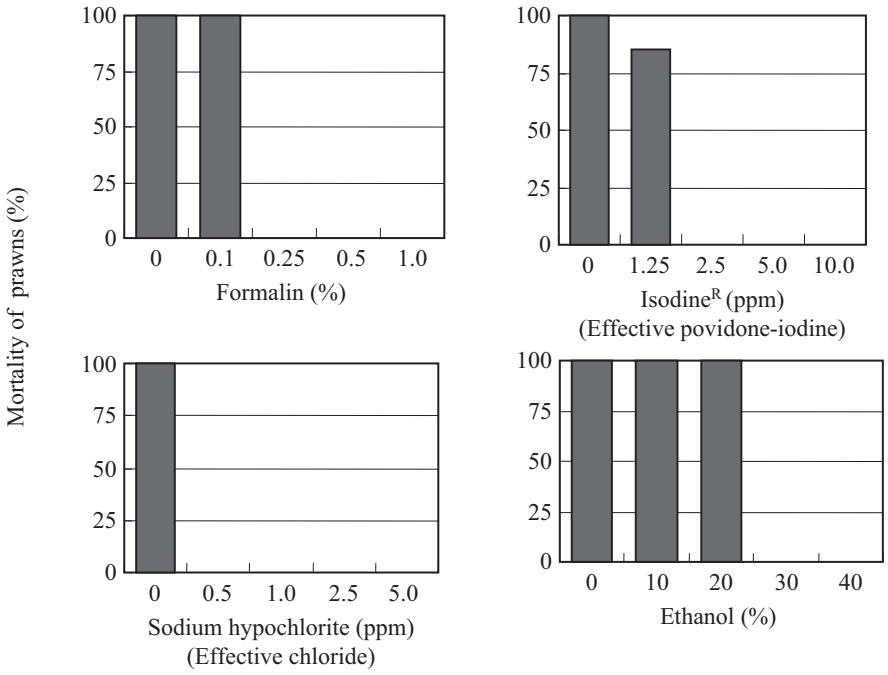

Fig. 1. Virus inactivation effects at several concentrations of the disinfectants formalin, sodium hypochlorite, Isodine^R, and ethanol

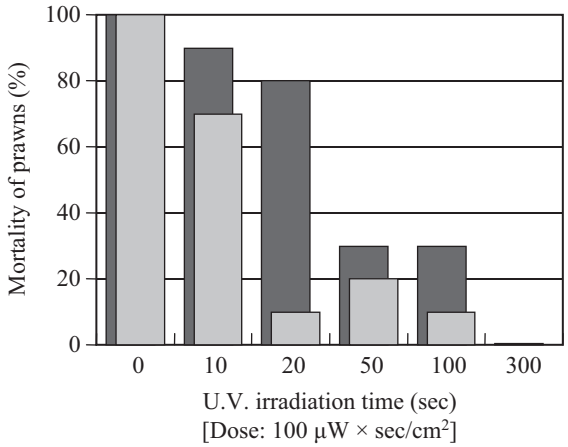

Fig. 2. Inactivation of WSSV by U.V. irradiation
Observational date after injection:
□ : 3 days, ■ : 14 days.

not inactivate free viral particles of IPNV, the salmonid fish pathogen virus belonging to the family Birnaviridae[2]. However, IHNV that belongs to the family Rhabdoviridae and was also one of the pathogenic viruses affecting salmonid fishes, was inactivated by treatment with 40% ethanol for 15 sec and 200 ppm sodium hypochlorite for 2.5 min[3]. In these cases, the viral inactivation effects were quite different between fish pathogen viruses. On the other hand, Momoyama et al. reported that several disinfectants and U.V. irradiation were able to be used for viral inactivation against baculoviral mid-gut gland necrosis (BMN) virus belonging to the family Baculoviridae which is a crustacean viral disease[6]. Inactivation effects of the disinfectants were shown by concentrations of 40% ethanol for 10 min, 5 ppm sodium hypochlorite for 10 min and 5 ppm Isodine^{R} (as active principle-concentrations of chlorine and iodine). In this report, it was found that sodium hypochlorite and Isodine^{R}, which are halogenous disinfectants, were useful in the protection or prevention of WSS viral disease. Additionally, inactivation by U.V. irradiation was also described by Momoyama et al.[7]. BMNV lost its activity by irradiation at doses of more than 4.1×10^5 μW·sec/cm^2.

On the other hand, it was considered that WSSV, the pathogen of black tiger prawn in Malaysia, is almost the same as PRDV that causes PAV in Japanese kuruma prawn[4,8,10]. Nakano et al. reported the viral inactivating effects of several disinfectants against PRDV using kuruma prawns[9]. However recently, differences in geographic isolates of WSSV began to be known and these are widely distributed in areas all over the world[5,15]. It seemed that local strains of WSSV in Japan and Southeast Asia would have different resistance against inactivation. This is because the climate, as well as water temperature, U.V. irradiation, and so on, is quite different in these areas. By contrast, resistance against WSSV was shown in kuruma prawn[11,12]. Sensitivity between different prawn species against six geographic isolates of WSSV was examined by Wang et al.[14]. They described in this report that slight differences in virulence exist among geographic isolates of WSSV, and that susceptibility may vary with species of the host. For these reasons, the inactivation methods described by Nakano et al. need to be compared using the virus strain isolated in Malaysia and black tiger prawn, instead of a Japanese strain and kuruma prawn. The inactivation values of Nakano et al.[9] by disinfectants and U.V. irradiation were 1 ppm sodium hypochlorite for 10 min, 2.5 ppm povidone-iodine for 10 min, 30% ethyl alcohol for 1 min, 5 ppm formalin for 10 min, and U.V. irradiation of 1×10^4 μW·sec/cm^2, respectively[9]. From the comparison of these data, it was clear that the inactivation effect against WSSV in Malaysia using black tiger prawn was almost the same as the case for the Japanese isolate.

Nakano et al. described in their report that halogenous disinfectants were quite effective for PRDV inactivation[9]. Especially sodium hypochlorite of halogenous disinfectants induced an effective inactivation even at low concentrations. Also in this study, halogenous disinfectants were effective for virus inactivation. It was suggested that these disinfectants should be used for sterilization of culture tools and gear, or pond water with a low concentration of disinfectant as is the case in Japan. However, it is well known that the efficiency of chlorine decreases in the presence of organic substances. Therefore, care should be taken in the application of this chemical to gears or water in aquaculture farms. WSSV could be inactivated by a lower U.V. irradiation dose than BMNV. The inactivation value of the U.V. dosage shows that it was possible that the inlet water and affected ponds without water can be sterilized by a general U.V. irradiation system and by sunshine. These procedures are already performed in Japanese farms. Therefore, the same countermeasures should be applicable to the control of this viral disease at black tiger farms in Malaysia and other Southeast Asian countries. This study details WSSV disinfection methods for the prevention of pathogen intrusion into aquaculture farms and will contribute to sustainable aquaculture in Southeast Asia.

Acknowledgments

We appreciate the help of Dato' Hashim Bin Ahmad, Director General of Department of Fisheries Malaysia (DOF), for approving this collaborative project. I also appreciate the assistance of Mr. Ismail Awang

Kechik, Director of Fisheries Research Institute (FRI), for arrangement of my research work. I thank the staff of Fish Health Section in FRI, and also the staff of JIRCAS Penang office.

References

1. Chou, H. Y. et al. (1995) Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan. *Dis. Aquat. Org.,* **23**, 165–173.
2. Inouye, K. et al. (1990) Virucidal activities of various germicides to infectious pancreatic necrosis virus (IPNV). *Fish Pathol.,* **25**(2), 81–86.
3. Inouye, K. et al. (1991) Virucidal activities of various germicides to infectious hematopoietic necrosis virus (IHNV). *Fish Pathol.,* **26**(4), 189–194.
4. Inouye, K. et al. (1996) The penaeid rod-shaped DNA virus (PRDV), which causes penaeid acute viremia (PAV). *Fish Pathol.,* **31**, 39–45.
5. Marks, H. et al. (2004) Genetic variation among isolates of white spot syndrome virus. *Arch. Virol.,* **149**, 673–697.
6. Momoyama, K. (1989a) Virucidal effect of some disinfectants on baculoviral mid-gut gland necrosis (BMN) virus. *Fish Pathol*., **24**(1), 47–49.
7. Momoyama, K. (1989b) Inactivation of baculoviral midgut gland necrosis (BMN) virus by ultraviolet irradiation, sunlight exposure, heating and drying. *Fish Pathol*., **24**(2), 115–118.
8. Nakano, H. et al. (1994) Mass mortalities of cultured kuruma shrimp, *Penaeus japonicus*, in Japan in 1993: Epizootiological survey and infection trials. *Fish Pathol*., **29**, 135–139.
9. Nakano, H. et al. (1998) Inactivation of penaeid rod-shaped DNA virus (PRDV), the causative agent of penaeid acute viremia (PAV), by some chemical and physical treatments. *Fish Pathol*., **33**, 65–71.
10. O. I. E. (2003) White spot disease. *In* Manual of diagnostic tests for aquatic animals, 4th ed., Office International des Epizooties, France, chapter 4.1.2. Available on line at http://www.oie.int/eng/normes/fmanual/A_summry.htm. (Verified 4 Jan. 2005).
11. Pan, D. et al. (2005) Differential gene expression profile in hepatopancreas of WSSV-resistant shrimp (*Penaeus japonicus*) by suppression subtractive hybridization. *Dev. Comp. Immunol.,* **29**, 103–112.
12. Rojtinnakorn, J. et al. (2002) Gene expression in haemocytes of kuruma prawn, *Penaeus japonicus*, in response to infection with WSSV by EST approach. *Fish Shellfish Immunol.,* **13**, 69–83.
13. Wang, Y. G. et al. (1999) Histopathology and cytopathology of white spot syndrome virus (WSSV) in cultured *Penaeus monodon* from peninsular Malaysia with emphasis on pathogenesis and the mechanism of white spot formation. *Dis. Aquat. Org.*, **39**, 1–11.
14. Wang, Q. et al. (1999) Per os challenge of *Litopenaeus vannamei* postlarvae and *Farfantepenaeus duorarum* juveniles with six geographic isolates of white spot syndrome virus. *Aquaculture*, **170**(3–4), 179–194.
15. Wang, Q. et al. (2000) Identification of genomic variations among geographic isolates of white spot syndrome virus using restriction analysis and Southern blot hybridization. *Dis. Aquat. Org*., **43**, 175–181.
16. Wongteerasupaya, C. et al. (1995) A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn *Penaeus monodon*. *Dis. Aquat. Org.*, **21**, 69–77.