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Disinfectant effect of monopersulfate (MPS) compound to white spot syndrome virus (WSSV) of shrimp

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This study investigated the disinfection effect of monopersulfate (MPS) compound against white spot syndrome virus (WSSV) by bioassay using kuruma shrimp (*Marsupenaeus japonicas*). A WSSV stock was prepared with muscle homogenate from WSSV-infected whiteleg shrimp (*Penaeus vannamei*) and its lethal dose 50% endpoint (LD_{50}) and infectious dose 50% endpoint (SID_{50}) were respectively determined as $10^{-5.63}$ and $10^{-6.79}$ in bioassay using kuruma shrimp, followed by PCR assays. The disinfective effect of MPS compound (1.2 ppm, 2.4 ppm, 4.8 ppm) was performed by bioassay using about 10-fold higher dilution (10^{-4}) of WSSV homogenate. The compound resulted in WSSV inactivation by a concentration-dependent manner. In addition, 4.8 ppm of MPS completely prohibited WSSV infection. To our knowledge, this study is the first report about the usefulness of MPS as a disinfectant to WSSV.

Key words: Shrimp, White spot syndrome virus (WSSV), Monopersulfate (MPS) compound, Disinfectant

INTRODUCTION

White spot syndrome virus (WSSV), which is a rod-shaped, double-stranded DNA virus with an outer lipid bilayer membrane envelope, is a highly contagious pathogen causing massive economic losses worldwide shrimp aquaculture (Bir *et al*., 2017). Since the first report of WSSV infection in Korean shrimp farms (Park *et al*., 1998), the disease has become a major concern for shrimp health management. However, no commercial vaccine and treatment are available for controlling WSSV infection in global shrimp farms including Korea. For this reason, the importance of strict hygiene measures has been suggested for managing WSSV infection on shrimp aquaculture practices, such as the use of pathogen-free shrimp stocks (Alday-Sanz, 2018), and biosecure water and culture systems (Withyachumnarnkul, 1999). There have been some reports about inactivation of WSSV by chemical disinfectants including formalin and sodium hypochlorite (Oseko *et al*., 2006). These disinfectants have been concerned about safeties to handler, environment and shrimp (Park *et al*., 2004).

MPS compounds have been reported to exhibit disinfective effects, such as oxidizing proteins and other parts of the cell protoplasm, inhibiting enzyme systems and degrading cell wall integrity, to a variety of pathogens including *Pseudomonas aeruginosa, Vibrio* sp, avian influenza virus and new castle virus (Masák *et al*., 2014; Min *et al*., 2015; Sonthipet *et al*., 2018). In addition, MPS-containing commercial

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products were confirmed for its safety to animals and the environment (Sonthipet *et al*., 2018). These previous studies showed clear differences in the susceptibilities of pathogens to MPS-based disinfectants. However, there is no information about the disinfection effect of MPS to WSSV. In this study, we examined the disinfective effect of MPS-based product to WSSV by bioassay using kuruma shrimp (*Marsupenaeus japonicas*).

Materials and Methods

WSSV stock preparation

Presently, no cell line is established for culturing WSSV. Therefore, WSSV stock was prepared by filtering homogenates from naturally infected whiteleg shrimp (*Penaeus vannamei*) tissue, according to the method described by Escobedo-Bonilla *et al*., (2005). Briefly, hepatopancreas, gut and shell were removed and then the remaining body was homogenized by glass bead in 10 ml of PBS (pH 7.2). The homogenate was centrifuged at 9,000 × *g* at 4℃ for 30 min. After centrifugation, the supernatant was resuspended with 90 ml of PBS, purified with a 0.45 um syringe filter (Millipore) and stored at -70℃ until used.

Shrimp and rearing condition

Approximately 30-day old kuruma shrimp (*Marsupenaeus japonicas*, 3 cm in length) post-larvae were purchased from a shrimp nursery farm located on the west coast of Korea. They were divided into three glass aquaria containing 50 L filtered seawater and equipping an internal filter and aeration. During acclimation (1 week) and experiment, the water temperature was sustained at 20℃ by air conditioner. The shrimp were fed with *Artemia nauplii* once daily, except one day before and after injection day. Before bioassay, three shrimps within each group were randomly selected for determining WSSV infection by PCR assay.

In vivo titration of WSSV stock

The shrimp infectious dose 50% endpoint (SID_{50}/ml) and the lethal dose 50% endpoint (LD_{50}/ml) of WSSV stock was evaluated by *in vivo* challenging assays using kuruma shrimp. Eighty shrimps were divided into 8 groups. Seven dilutions (10^{-1}~10^{-7}) were prepared by 10-fold serial dilution of WSSV stock in PBS. 100 ul of each dilution was injected intramuscularly (IM) into the shrimps (n=10) per group. The remaining group was injected with only PBS as a mock-injected group. After injection, mortality and clinical symptoms were recorded every day for 5 days. During the experimental period, dead shrimps were stored at 4°C. At the end of the experiment, the survivors were euthanized and stored. Genomic DNA was extracted from all survive and dead shrimps and was used for PCR assays for detecting WSSV DNA.

Disinfective effect of MPS

The MPS [50.69% (w/w)] based product (Virkon Aquatic, Bayer, Korea) was used to investigate the disinfective effect to approximately ten-time higher dilution than LD_{50} of WSSV stock (10^{-4}). The MPS stock of 48 ppm was prepared in the sterilized distilled water. The stock solution was diluted in distilled water for generating three different concentrations of MPS, 1.2 ppm, 2.4 ppm, and 4.8 ppm. 2.5 ml of each MPS concentration was mixed with an equal volume of the diluted WSSV stock. Besides these, control groups included three separate mixtures including WSSV stock alone, 4.8 ppm MPS alone and distilled water alone. The mixtures were incubated at 4℃ for 30 min and vortexed every 10 min. After incubation, the mixtures were supplemented with neutralization buffer (10% fetal bovine serum (FBS) in PBS) stored at 37℃ and separately injected IM immediately into 10 shrimps per group. The mortality and clinical symptoms were recorded every day for 15 days. All dead and survive shrimps were subjected to PCR assays for detecting WSSV.

PCR assay

WSSV infection was examined in homogenates of all shrimps by PCR assay using *vp28* gene-targeting primers designed by Zhu and Quan (2012). The homogenates were generated by glass beat using 50 mg of shrimp muscle. Genomic DNA was extracted from the homogenate using AccuPrep® Genomic DNA extraction kit (Bioneer, Korea). The extracted DNA (200 ng) and primers were added into AccuPower® PCR PreMix (Bioneer, Korea) according to the manufacture's instructions. The amplification was performed in the following thermal conditions: 94℃ at 5 min, the subsequent 35 cycles with 94℃ for 1 min, 55℃ for 1 min and 72℃ for 1 min, and 72℃ for 7 min. The amplicon was analyzed in 1.5% agarose gel and visualized by UV light.

Results and Discussion

WSSV-infected whiteleg shrimp exhibited white spots on the carapace and the extracted DNA was positively reacted with primer in PCR assays. During acclimation, the kuruma shrimps did not show any clinical symptom including reddish color change and white spot. In addition, PCR assays showed a negative reaction in genomic DNA from the randomly selected kuruma shrimps (data not shown).

The virus infection titers (SID_{50}) and mortality (LD_{50}) were calculated by *in vivo* titration using 10-fold serial dilutions of homogenates from WSSV-infected whiteleg shrimp tissue. In the titration, all groups injected until 10^{-5} dilutions exhibited 100% of cumulative mortality during 5 days after injection (Table 1). The 10^{-6} dilution-injected group showed a 20% mortality during the experimental period. However, no mortality was observed in both groups for 10^{-7} dilution injection and mock injection. All dead shrimps showed red color changes in the ventral region and appendages without white spots. Based on these mortalities, the present homogenate has $10^{-5.63}$ LD_{50}.

PCR assays were performed to detect a WSSV-specific gene from carcasses and survivors of all experimental groups (Table 1). No positive PCR reaction was observed in the mock-injected shrimps. The 10^{-7} dilution-injected group had only 2 survivors with PCR positive response. In the case of a 10^{-6} dilution-injected group, it exhibited 60% PCR positivity including 4 survivors and 2 carcasses. In the rest groups showing 100% mortality, all shrimps had a *vp28* gene for WSSV. Therefore, SID_{50} of the homogenate was $10^{-6.79}$.

The disinfective effect of MPS compound was investigated in a bioassay using mixtures of WSSV homogenate with its 3 different concentrations. The results were summarized in Table 2. The homogenate alone injection caused the death of all shrimps during 10 days since first death at 5 day post-injection. All

Table 1. Mortality and number of PCR positive shrimp in groups separately infected with 10-fold serial dilutions of WSSV stock

Dilution	No. of shrimp (n=10)			No. of PCR positive shrimp		
	Alive	Dead	Mortality (%)	Alive	Dead	Total (%)
10^{-1}	0	10	10(100)	-	10/10	10(100)
10^{-2}	0	10	10(100)	-	10/10	10(100)
10^{-3}	0	10	10(100)	-	10/10	10(100)
10^{-4}	0	10	10(100)	-	10/10	10(100)
10^{-5}	0	10	10(100)	-	10/10	10(100)
10^{-6}	8	2	2(20)	4/8	2/2	6(60)
10^{-7}	10	0	0(0)	2/10	-	2(20)
Mock	10	0	0(0)	0/10	-	0(0)

Table 2. Mortality and number of PCR positive shrimp in groups infected with three different concentrations of MPS compound and WSSV alone, MPS compound alone and distilled water alone

Experimental groups	No. of shrimp (n=10)			No. of PCR positive shrimp		
	Alive	Dead	Mortality (%)	Alive	Dead	Total (%)
WSSV	0	10	100	-	10/10	100
VA 1.2 ppm+WSSV	4	6	60	0/4	6/6	60
VA 2.4 ppm+WSSV	8	2	20	0/8	2/2	20
VA 4.8 ppm+WSSV	0	0	0	0/10	-	0
VA 4.8 ppm	0	0	0	0/10	-	0
Mock	0	0	0	0/10	-	0

VA: Virkon Aquatic
WSSV: White Spot Syndrome Virus

tissue DNA from the dead shrimps were positively reacted with specific primers in PCR assays. Contrarily, a 100% survivor rate showed in 4.8 ppm MPS mixture and mock injection groups without postive PCR reaction. Groups injected with mixtures of homogenate and 1.2 ppm, 2.4 ppm MPS exihibited 60% and 20% mortalities, respectively. In addition, the first death of shrimp was shown at 4 day post-injection in the group injected with 1.2 ppm mixture but at 13 day in the group with 2.4 ppm mixture (Fig. 1). In both groups, only dead shrimps were positive for PCR assays. On the other hand, no death and positive PCR reaction were observed in the group injected with the mixture containing only 4.8 ppm MPS.

MPS-based product has been used to keep the water quality in swimming pools (Shukur and Adnan, 2016). In addition, Virkon®-S, which contains 21.41% of MPS, has been used at the range of 5,000 to 20,000 ppm for various purposes as virucidal disinfection. This study showed that MPS compound exhibited a disinfection effect against WSSV. 4.8 ppm of MPS-based disinfectant completely inactivated WSSV in bioassay using a 10-fold higher concentration of WSSV homogenate with $10^{-5.63}$ LD_{50}. It was a first study to determine a disinfective concentration of MPS compound for controlling undesirable con-

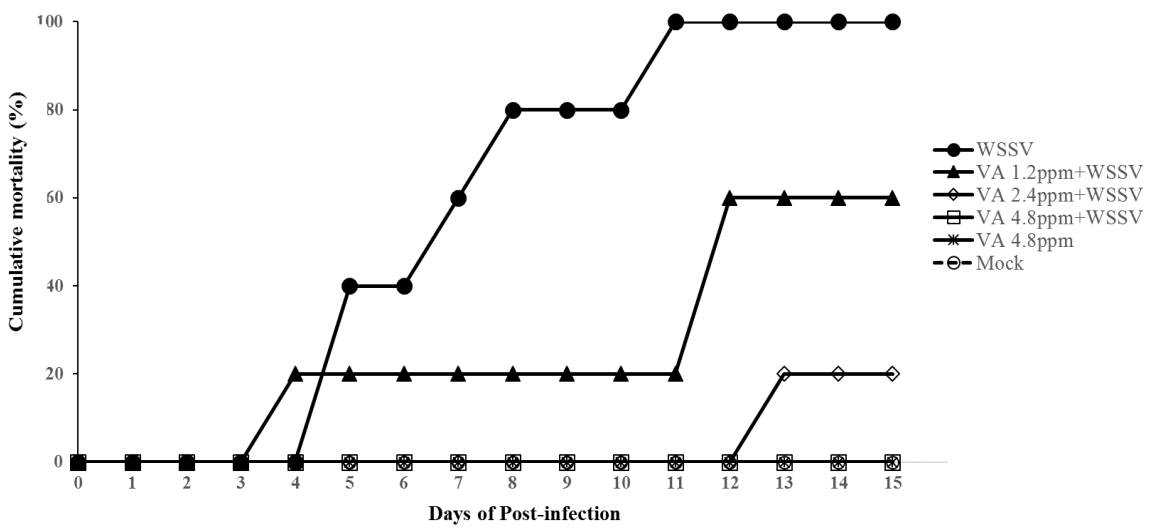

Fig. 1. Cumulative mortality in *in vivo* challenge assay for investigating the disinfectant effect of MPS compounds.

taminations of WSSV. A previous study showed that 2.4 ppm of the present MPS-based product completely inhibited growth of *V. harveyi*, which is a causative agent for shrimp vibriosis (Min *et al*., 2015). Based on the previous and present studies, we suggested 4.8 ppm of the present MPS-based product as disinfectant concentration to control the spread and contamination of shrimp pathogens including WSSV and *V. harveyi*.

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