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NOTE

Safety and protective effect of a disinfectant (STEL water) for white spot syndrome viral infection in shrimp

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ABSTRACT: The efficacy of STEL water for protection against white spot syndrome virus (WSSV) infection was evaluated using shrimp. The LC_{50} of residual chlorine (Cl^-) in STEL water for broodstock and 2-mo-old shrimp were 2.3 and 3.2 ppm, respectively. All 2-month-old shrimp raised in seawater containing more than 40 µl $2\,l^{-1}$ of a WSSV-infected tissue homogenate died within 3 d post-exposure (dpe). Thus, a 10-fold dose of 400 µl $2\,l^{-1}$ was used in the disinfection tests. Low concentrations of STEL water effectively prevented mortality of shrimp at this challenge dose. All 2-month-old shrimp exposed to seawater with 400 µl of viral homogenate disinfected with STEL water at Cl^- concentrations over 0.125 ppm for 1 and 10 min, lived until 5 dpe. With 5-mo-old shrimp, all positive control shrimps died within 3 dpe, whereas most shrimp reared in seawater disinfected with STEL water for 1 h before addition of homogenate lived until 5 dpe. Results suggested that continuous disinfection of seawater with STEL water may be effective for preventing WSSV infection in shrimp.

KEY WORDS: STEL water · WSSV · Shrimp · Disinfection

INTRODUCTION

White spot syndrome virus (WSSV) is a major cause of mortality in cultivated shrimp (Lightner 1996). In Taiwan, the disease was first discovered in 1992 (Chen 1995), but it was later reported elsewhere in Asia and in the southeastern region of the United States (Inouye et al. 1994, Momoyama et al. 1994, Nakano et al. 1994, Takahashi et al. 1994, Cai et al. 1995, Magbanua et al. 2000). Since 1993, massive mortalities due to this virus have also occurred in penaeid shrimp cultured in Korea (Park et al. 1998). Gross signs of infection include abnormal red discoloration of the body and white spots within the cuticle. Several diagnostic methods including PCR, *in situ* hybridization, electron microscopy, and histopathology have been developed for WSSV (Lo et al. 1996, Takahashi et al. 1996, Lu et al. 1997, Nunan et al. 1997, Park et al, 1998, Tapay et al. 1999). However, no good therapeutic treatment has yet been established for its control, and preventive measures are the only recommended methods of protection against it (Flegel et al. 1997). One of these measures is to treat seawater with disinfectants before it is used in shrimp hatcheries and in shrimp rearing ponds.

Before being drawn into shrimp ponds, seawater is sometimes disinfected using sodium or calcium hypochlorite to prevent viral, bacterial, and parasitic infections. However, application of these disinfectants is

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expensive, may be harmful to human health, may contribute to environmental pollution, and may cause direct harm to shrimp. Here we examined the use of electrolyzed water (STEL water) as an alternative to sodium or calcium hypochlorite. This is an inexpensive process that uses salt water to produce hypochlorous acid and we evaluated it for safety and protective effect against WSSV infection in shrimp.

MATERIALS AND METHODS

Generation of STEL water. Hypochlorous acid can be produced by electrolysis of saltwater in the following way:

$$2NaCl + 2H_2O \rightarrow 2NaOH + H_2 + Cl_2$$

$$Cl_2 + H_2O \rightarrow \mathbf{HOCl} + HCl$$

STEL is a commercial machine that produces a stream of modified saltwater (STEL water) using an electrolytic process (Choonwae Humantech Corporation). The water exerts a strong disinfecting effect at low concentration because HOCl can easily penetrate cell walls. At the levels used in this study, it had no harmful effect on human health and only a weakly unpleasant smell. To generate 5 l of STEL water, 2 l of 10% NaCl solution (w/v in distilled water) and 6 l of tap water were electrolyzed by the STEL machine (electrical consumption: 400 W; production capacity: 50 to 80 l h^{-1}; maximum working time: 10 h d^{-1}). The pH of the STEL water produced in this way was 7.5 ± 1.0 and the concentration of free radical chlorine was 400 to 600 ppm.

pH measurement of sodium hypochlorite and calcium hypochlorite solutions. Sodium hypochlorite (Yuhanrox) (Yuhanclorox) and calcium hypochlorite (Star-Chlon) were adjusted to 100, 200, and 400 ppm with distilled water; the pH of each solution was then measured.

LC_{50} of STEL for 2-mo-old shrimp and broodstock. Preliminary tests were conducted to determine the approximate LC_{50} of STEL water in broodstock shrimp *Penaeus orientalis*. Based on these results, various concentrations of STEL water were used to determine 3 d LC_{50} for broodstock (59 to 67 g body weight [bw]) in 400 l seawater in round-drum tanks and for 2-mo-old shrimp (5 to 7 g bw) in 10 l seawater in polycarbonate aquaria (26 × 42 × 18 cm) with a wire net. STEL water was added to each tank and aquarium so that the Cl^- concentrations (residual chlorine analyzer, Pangaea21) were 14.2, 7.1, 3.6, 1.8, and 0.9 ppm. Subsequently, 10 2-mo-old shrimp and 8 broodstock shrimp were added. Mortality was measured daily for 3 d, and the LC_{50} was determined using Origin program.

Preparation of WSSV homogenate and PCR amplification of VP28 gene. Healthy shrimp and shrimp infected with WSSV were obtained from a shrimp farm located on the western coast of Korea. Internal organs, including the lymphoid organ and stomach, were collected and homogenized in a ceramic mortar with a 20-fold volume of sterile phosphate buffered saline (pH 7.2, PBS). The homogenate was centrifuged at 3000 rpm (800 × *g*) for 10 min, and the upper layer was collected and stored at –70°C in 1 ml aliquots. Presence of WSSV in the homogenate was verified through PCR amplification using a primer pair (F, 5′-CTC GTC ATG GAT CTT TCT TT-3′; R, 5′-CTC GGT CTC AGT GCC AGA GT-3′) newly designed from the VP28 envelope gene (Fig. 1). Briefly, the homogenate was mixed with SNET lysis buffer [20 mM Tris-HCl, pH 8.0; 5 mM EDTA, pH 8.0; 400 mM NaCl; 1% (w/v) SDS; 1 mg ml^{-1} Proteinase K] and incubated for 3 h at 55°C. DNA was extracted with phenol-chloroform, precipitated in isopropyl alcohol with sodium acetate, washed twice in 70% alcohol, and resuspended in TE buffer (pH 8.0). The PCR mixture (100 µl) contained 2 µg of DNA, 100 pmol of each primer, 1 × PCR buffer containing $MgCl_2$, 200 µM dNTP, and 2.5 units of Taq polymerase (PCR core Kit, Boehringer Mannheim). PCR conditions were as follows: 3 min of denaturation at 95°C followed by 30 cycles denaturation for 30 s at 94°C, annealing for 30 s at 56°C and extension for 1 min at 72°C, followed by final extension for 7 min at 72°C. The PCR product was detected by electrophoresis of 9 µl of the reaction solution in 1.5% agarose gel containing 1 µg ml^{-1} ethidium bromide.

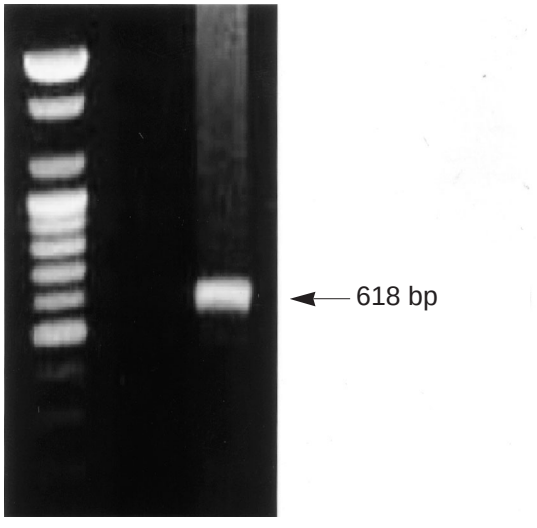

Fig. 1. WSSV infection was confirmed by PCR amplification of VP28 envelope gene. DNA was extracted from each homogenate of uninfected (lane 1) and infected shrimps (lane 2). A 618 bp PCR amplification indicates a positive reaction for the VP28 envelope gene

Determination of WSSV challenge dose. To evaluate the virulence of WSSV, groups of 20 2-mo-old shrimp were placed in 2 l seawater in polycarbonate aquaria (22 × 27 × 13 cm). Subsequently, 1 ml, 0.2 ml, 40 µl, 8 µl, or 1.6 µl of viral homogenate was added. The control aquarium contained no homogenate. Mortality was recorded for 3 d and the viral infection in the dead shrimp was verified by PCR amplification as described above. The optimum challenge dose was considered to be the concentration at which all shrimp were killed within 3 d post exposure to the virus.

Evaluation of protective effect of STEL water on WSSV infection. Three experiments were performed. In Expt 1, 10 2-mo-old shrimp were placed in 2 l seawater in a polycarbonate aquarium (22 × 27 × 13 cm). In preliminary tests, all shrimp exposed to over 40 µl of viral homogenate died (see Table 2). Therefore, a 10-fold quantity of 400 µl was used as the challenge dose for the STEL tests. STEL water at different Cl^- concentrations (2.0, 1.0, 0.5, 0.25, 0.125, and 0.0625 ppm) was mixed with 400 µl viral homogenate for either 1 or 10 min before it was added to the aquaria. For the positive control, 400 µl of untreated viral homogenate was added to the aquarium. No viral homogenate was added to the negative control aquarium. Mortality was measured for 3 d.

In Expt 2, 10 5-mo-old shrimp (27 to 32 g bw) were placed in 14 l seawater in each of 5 polycarbonate aquaria (26 × 42 × 18 cm). In 3 aquaria, 1 ml viral homogenate was mixed with STEL water at different Cl^- concentrations (1.5, 1.0, and 0.5 ppm) for 10 min before addition to the aquaria. The other 2 aquaria served as positive controls (1 ml viral homogenate added) and negative control (no homogenate added).

In Expt 3, 10 5-mo-old shrimp were placed in each of 3 aquaria containing 14 l of seawater, which had been disinfected with STEL water (1.5, 1.0, and 0.5 ppm of Cl^-) for 1 h, before further addition of 1 ml of the viral homogenate to each aquarium. The positive and negative controls were as for Expt 2. Mortality was recorded for 5 d and DNA was extracted for all dead shrimp to verify WSSV infection by PCR.

RESULTS

pH measurement of sodium hypochlorite and calcium hypochlorite solutions

At concentrations of 100, 200, and 400 ppm, the pH of the sodium hypochlorite solutions were 10.3, 10.6, and 10.8, respectively, and those of the calcium hypochlorite solutions were 9.1, 9.8, and 10.4, respectively.

LC_{50} of STEL for broodstock and 2-mo-old shrimp

Based on the results of preliminary tests (data not shown), the concentration range tested in experiments for measuring definitive LC_{50} was 0.9 to 14.2 ppm Cl^-. The 3 d LC_{50} values were 2.3 and 3.2 ppm Cl^- for broodstock and 2-mo-old shrimp, respectively (Table 1).

Determination of WSSV virulence

The virulence of WSSV was evaluated in 2-mo-old shrimps. All shrimps raised in seawater with over 40 µl $2\,l^{-1}$ of viral homogenate died within 3 dpe (Table 2). Twelve out of 20 shrimp died within 3 d in seawater with 8 µl $2\,l^{-1}$ of viral homogenate, whereas no deaths occurred in seawater with 1.6 µl $2\,l^{-1}$ of homogenate or in the control. Therefore, the challenge dose was set at 400 µl $2\,l^{-1}$, 10 times the dose that killed all shrimp.

Table 1. LC_{50} determination for STEL water with broodstock and 2-mo-old shrimp

Shrimp Conc (ppm[a])	Experimental period (d) 0	1	2	3	Total[b]	LC_{50} (ppm[a])
Broodstock						
14.2	8	–	–	–	8/8	2.3
7.1	8	–	–	–	8/8	
3.6	2	5	0	0	7/8	
1.8	0	1	1	0	2/8	
0.9	0	0	0	0	0/8	
0	0	0	0	0	0/8	
Two-mo-old						
14.2	10	–	–	–	10/10	3.2
7.1	5	5	–	–	10/10	
3.6	2	5	2	0	9/10	
1.8	0	0	0	0	0/10	
0.9	0	0	0	0	0/10	
0	0	0	0	0	0/10	

[a]Concentration of residual chlorine (Cl^-)
[b]Number of dead shrimps during the experimental period

Table 2. Mortality of 2-mo-old shrimp after exposure to WSSV homogenate

Dose	Days post-inoculation 0	1	2	3	Total mortality
1 ml $2\,l^{-1}$	0	0	20	–	20/20
0.2 ml $2\,l^{-1}$	0	0	18	2	20/20
40 µl $2\,l^{-1}$	0	0	14	6	20/20
8 µl $2\,l^{-1}$	0	0	4	8	12/20
1.6 µl $2\,l^{-1}$	0	0	0	0	0/20
Control	0	0	0	0	0/20

Table 3. Mortality of 5-mo-old shrimp after exposure to WSSV homogenate previously treated with STEL water (Expt 2)

Cl^- concentration	Days post-exposure					
	0	1	2	3	4	5
1.5 ppm	0	0	0	0	1*	0
1.0 ppm	0	1*	0	0	0	0
0.5 ppm	0	0	0	0	0	0
Positive control	0	0	1*	0	3	6
Negative control	0	0	0	0	0	0

*Vp28 envelope gene was not amplified by PCR assay

Table 4. Mortality of 5-month-old shrimp held in STEL-treated water and then challenged with WSSV homogenate (Expt 3)

Cl^- concentration	Days post-exposure					
	0	1	2	3	4	5
1.5 ppm	0	0	1*	0	0	1*
1.0 ppm	0	0	0	0	0	0
0.5 ppm	0	0	0	0	0	0

*Vp28 envelope gene was not amplified by PCR assay. The positive and negative controls were those for Expt 2

Evaluation of protective effect of STEL water on WSSV infection

In Expt 1, all 2-mo-old shrimp survived to 5 dpe in seawater containing 400 µl of viral homogenate that had been pre-treated for 1 or 10 min with STEL water containing more than 0.125 ppm Cl^-. On the other hand, all shrimp died by 2 dpe in seawater containing the untreated viral homogenate (positive control) or homogenate treated with 0.0625 ppm STEL water for 1 min (10/10 dead by 2 dpe) or 10 min (10/10 dead by 3 dpe). There was no mortality in the unchallenged negative control group.

In Expts 2 and 3 (Tables 3 & 4), all positive control shrimp died within 5 dpe. Most shrimp survived in seawater containing 1 ml of the STEL-treated viral homogenate or in STEL-treated seawater containing 1 ml of viral homogenate. In the tests, some shrimp died due to environmental conditions rather than WSSV infection, since they did not yield positive PCR results for VP28 gene (data not shown). In contrast, WSSV infection was verified by PCR amplification in dead shrimp from the positive control group.

DISCUSSION

The types of chlorine residuals depend on solution pH. For example, the ratio of HOCl to OCl^- is inversely proportional to pH and a 1:1 ratio is achieved at pH 7.53 (Baker 1959). At the time of production, STEL water (400 ppm) had pH 7.5 ± 1.0 meaning that about 50% of its chlorine residuals were present in HOCl form. Over pH 9.0, little HOCl exists, and OCl^- predominates (Baker 1959). When sodium or calcium hypochlorite solutions were prepared, we obtained pH values over 9.0 at concentrations over 100 ppm. This means that there would be little HOCl in such sodium/calcium hypochlorite solutions. Thus, the results suggested that STEL water would have stronger disinfecting ability than sodium/calcium hypochlorite solutions. Moreover, it is inexpensive to produce STEL water because only salt, water, and a low amount of electricity (400 W) are needed for its production (see www.exkor.co.kr/electrochemical/electro.htm).

Several studies have shown that disinfectants including chlorine dioxide, potassium peroxymonosulfate, and sodium hypochlorite have potent virucidal or bactericidal effects (Puente et al. 1992, Romanelli et al. 2000, Wutzler et al. 2000, Eleraky et al. 2002). In hospitals, they are used for the disinfection of medical instruments and hands (Romanelli et al. 2000, Wutzler et al. 2000). A previous report using brine shrimp revealed that chlorine dioxide effectively controlled *Vibrio parahaemolyticus* in seawater (Puente et al. 1992).

Expts 1 and 2 showed that STEL water had a direct virucidal effect against WSSV in challenge tests with 2- and 5-mo-old shrimp. Protection also resulted if aquarium water was pre-treated with STEL before addition of WSSV. These laboratory results suggest that it would be worthwhile determining whether continuous STEL addition to seawater in shrimp farms could be an effective way of preventing WSSV disease outbreaks.

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