pISSN 1229-1153 *J. Fd Hyg. Safety*
Vol. 27, No. 3, pp. 290~294 (2012)

Journal of Food Hygiene and Safety
Available online at http://www.foodhygiene.or.kr

Bactericidal Efficacies of an Aquatic Disinfectant Tablet Composed to Calcium Hypochlorite Against *Vibrio anguillarum* and *Streptococcus iniae*

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(Received June 16, 2012/Revised June 24, 2012/Accepted July 8, 2012)

ABSTRACT - *Vibrio* spp. and *Streptococcus* spp. have caused a considerable disease of farmed fish and economic loss in fish farming and seafood industry. In this study, the efficacy of an aquatic disinfectant tablet composed to calcium hypochlorite was evaluated against *V. anguillarum* and *S. iniae*. A bactericidal efficacy test by broth dilution method was used to determine the lowest effective dilution of the disinfectant following exposure to test bacteria for 30 min at 4°C. An aquatic disinfectant tablet and test bacteria were diluted with distilled water (DW), hard water (HW) or organic matter suspension (OM) according to treatment condition. *V. anguillarum* on the DW, HW and OM condition was completely inactivated with 16,000 15,000 and 13,000 fold dilutions of the disinfectant, respectively. On the DW, HW and OM condition, *S. iniae* was absolutely inactivated with 17,000 16,000 and 14,000 fold dilutions of the disinfectant, respectively. As an aquatic disinfectant tablet possesses bactericidal efficacy against fish pathogenic bacteria such as *V. anguillarum* and *S. iniae* this disinfectant solution can be used to control the spread of fish infective bacterial diseases.

Key words: An aquatic disinfectant tablet, *Vibrio anguillarum*, *Streptococcus iniae*, Calcium hypochlorite, Disinfectant efficacy

The genus *Vibrio* includes gram-negative, facultative anaerobic, non-spore-forming bacilli that are oxidase positive and halophilic[1]. Several species of *Vibrio* can cause foodborne infection, usually associated with eating undercooked seafood[2]. Most disease-causing *Vibrio* strains are associated with gastroenteritis, but can also infect open wounds and cause septicemia[3,4]. It can be carried by numerous sea-living animals, such as crabs or prawns, and has been known to cause fatal infections in humans during exposure[5].

Vibrio anguillarum (*V. anguillarum*) is the causative agent of vibriosis, a deadly hemorrhagic and septicemic disease affecting various marine and fresh/brackish water fish, bivalves and crustaceans. In both aquaculture and larviculture, this disease is responsible for severe economic losses worldwide[6].

Streptococcus iniae (*S. iniae*) is a species of Gram-positive, sphere-shaped bacterium belonging to the genus *Streptococcus*[7]. Since its isolation from an Amazon freshwater dolphin in 1976, *S. iniae* has emerged as a leading fish pathogen in aquaculture operations worldwide, resulting in over $150 million in annual losses[8,9]. *S. iniae* infections have been reported in at least 27 species of cultured or wild fish from around the world[10]. *S. iniae* is highly pathogenic in cultured fishes with 30-50% mortality[11]. *S. iniae* can cause opportunistic infections in weakened or immunocompromised humans[12].

As *V. anguillarum* and *S. iniae* infections are becoming harder to control because of resistance to commonly used antibiotics, the effective cleaning and disinfection regimes are essential for the prevention of infections and outbreaks[13,14]. The cleaning and disinfectant regimes depend on the proper use of biocides, and there is the concern that the resulting increased use of biocides in fish farming, food production, and hospital settings, and the home could contribute to the selection of antibiotic-resistant strains as some mechanisms of biocide resistance also confer antibiotic resistance[15]. Biocides are often composed of a mixture of ingredients that act upon

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a wide range of cellular mechanisms and targets, which makes it difficult for bacteria to become resistant to biocides[16].

Vibriosis and *S. iniae* infection in cultured marine fishes and human may cause enormous economic loss[6,9]. The stress on cultured marine fishes caused by intensive farming systems, and the development of antibiotic-resistant bacteria is among the major reasons for the increased frequency of bacterial disease outbreaks[17]. Highly hygienic measures including the use of disinfectant are very effective for successful control of diseases from potential pathogens in cultured marine fishes[18]. Various disinfectants have been used for decontamination of cultured marine fishes and biological agents[19]. However, there is not the efficacy test for the disinfectant composed of calcium hypochlorite against *V. anguillarum* and *S. iniae*. Therefore, this study was carried out to examine bactericidal efficacy of a disinfectant tablet against *V. anguillarum* and *S. iniae*.

Materials and Methods

Bacteria and culture

The test bacteria, *V. anguillarum* (KCTC 2711) and *S. iniae* (KCTC 3657)) were obtained from the National Fisheries Research and Development Institute (Busan, Korea). The strains were maintained as frozen glycerol stock. *V. anguillarum* cells were cultured in tryptic soy broth (TSB) containing 1.5% agar and 1.0% NaCl at 25°C for 24-28 h. *S. iniae* cells were spread in brain heart infusion (BHI) broth containing 1.5% NaCl and incubated at 28°C for 24-48 h.

Disinfectant

The active ingredient for Easy-Wash®, the tested disinfectant tablet, is calcium hypochlorite (70% w/w). Easy-Wash® was provided by Dae Han New Pharm Co. (Seoul, Korea). The disinfectant tablet was stored in room temperature and prepared for dilution on the day of evaluation. Determination of the antimicrobial efficacy of the disinfectant was based on Animal, Plant and Fisheries Quarantine and Inspection Agency Regulation No. 2011-26, Korea.

Diluents and treatment condition

Testing was based on bactericidal effects of disinfectant diluents in three treatment conditions (distilled water (DW) condition, standard hard water (HW) condition, and organic matter (OM) condition), pathogen control (disinfectant negative control) and DW control (both disinfectant and pathogen negative control) in Table 1. HW, an ingredient of HW treatment condition, was made by adding anhydrous $CaCl_2$ 0.305 g and $MgCl_2 \cdot 6H_2O$ 0.139 g into one liter distilled water. Organic suspension, an ingredient of OM treatment condition, is a solution of 5% (w/v) yeast extract in HW. The test organisms were prepared by titration of each cultural broth into at least 10^8 CFU/ml viable organisms with the same kind of diluents of treatment condition.

Table 1. Experimental design for the determination of the bactericidal efficacy of an aquatic disinfectant tablet

Treatment condition*	Contents according to treatment condition**				
	DM	HW	OM	Disinfectant	Bacteria
DW condition	+	-	-	+	+
HW condition	-	+	-	+	+
OM condition	-	-	+	+	+
Bacteria control	-	+	-	-	+
DW control	+	-	-	-	+

*DW, distilled water; HW, standard hard water; OM, organic matter.
**+, presence; -, absence

Experimental procedures

For the efficacy test against *V. anguillarum*, an aquatic disinfectant tablet was diluted 12,800, 14,400, 16,000, 17,600 and 19,200 times with DW, 12,000, 13,500, 15,000, 16,500 and 18,000 times with HW, and 10,400, 11,700, 13,000, 14,300 and 15,600 times with OM, respectively. For the efficacy test against S. iniae, Easy-Wash® was also diluted 13,600, 15,300, 17,000, 18,700 and 20,400 times with DW, 12,800, 14,400, 16,000, 17,600 and 19,200 times with HW, and 11,200, 12,600, 14,000, 15,400 and 16,800 times with OM, respectively.

To verify the lowest effective dilution of the disinfectant, five serial dilutions of the disinfectant were prepared and placed at 4°C prior to test reaction. 2.5 ml of each disinfectant dilution was mixed with the same amount of test organism followed by contact time of 30 min at 4°C.

During this period, the mixture was shaken at 10 min interval. At the end of 30 min contact period, one ml of the mixture was neutralized with 9 ml of nutrient broth containing 5% inactivated horse serum (Becton Dickinson & Co., MD, USA) at 37°C. 0.1 ml of the neutralized reaction mixture was subcultured into 10 ml of recovery each cultural broth at 37°C for 48 h in incubator. The valid dilution was determined that the greatest dilution showing no growth in two or more in the five replicates was confirmed. The final dilution time was statistically determined by a median value among three valid dilution of the triplicate test, but each value of which should be within 20% experimental error.

Results

Table 2 shows the final valid dilution of an aquatic disinfectant tablet composed to calcium hypochlorite. On DW condition, *V. anguillarum* and *S. iniae* were completely inactivated with 16,000 and 17,000 fold dilutions of the

Table 2. Final valid dilution of an aquatic disinfectant tablet against *V. anguillarum* and *S. iniae*

Bacterial strains	Treatment condition*											
	DW				HW				OM			
	DT	1	2	3	DT	1	2	3	DT	1	2	3
V. anguillarum	12,800	×	×	×	12,000	×	×	×	10,400	×	×	×
	14,400	×	×	×	13,500	×	×	×	11,700	×	×	×
	16,000	×	×	○	15,000	×	×	×	13,000	○	×	×
	17,600	○	○	○	16,500	○	×	○	14,300	○	○	○
	19,200	○	○	○	18,000	○	○	○	15,600	○	○	○
	Valid		16,000		Valid		15,000		Valid		13,000	
S. iniae	13,600	×	×	×	12,800	×	×	×	11,200	×	×	×
	15,300	×	×	×	14,400	×	×	×	12,600	×	×	×
	17,000	×	○	×	16,000	○	×	×	14,000	×	×	○
	18,700	○	○	○	17,600	○	○	○	15,400	○	○	○
	20,400	○	○	○	19,200	○	○	○	16,800	○	○	○
	Valid		17,000		Valid		16,000		Valid		14,000	

*DW, distilled water; HW, standard hard water; OM, organic matter; DT, dilution time.
○ , growth; ×, growth inhibition

disinfectant, respectively. When the bactericidal effect on HW condition was evaluated, *V. anguillarum* and *S. iniae* were completely inactivated with 15,000 and 16,000 fold dilutions of the disinfectant, respectively. With the investigation of the bactericidal effect of the disinfectant on OM condition, *V. anguillarum* and *S. iniae* were completely inactivated with 13,000 and 14,000 fold dilutions of the disinfectant, respectively. Because the organic material interferes with efficacy by either inactivating the disinfectant or blocking it from surface contact, the bactericidal activity of the disinfectant on the OM condition was lowered against fish pathogenic bacteria compared with DM or HW conditions.

Comparing the results of the disinfectant against two pathogenic bacteria in the present study, the bactericidal effect of an aquatic disinfectant tablet against *S. iniae* was higher than that against *V. anguillarum* on all experimental conditions.

Discussion

An aquatic disinfectant tablet is a potential antibacterial disinfectant which was composed of calcium hypochlorite. Calcium hypochlorite is widely used for water treatment and as a bleaching agent. And this compound is considered to be relatively stable and has greater available chlorine than sodium hypochlorite[20].

Calcium hyphochlorite has been used for water sanitization as an alternative of chlorine-based sanitizer. Aqueous calcium hypochlorite has a much higher oxidation capacity than chlorine due to the formation of hypochlorous acid[21]. Bacterial inactivation by hypochlorous acid is the result of inhibition of bacterial DNA replication[22]. Yousuf *et al*.[23] previously reported that calcium hypochlorite was the most effective than trisodium phosphate, sodium carbonate, and organic acids in reduction of total bacterial load as well as elimination of harmful bacteria in shrimp and prawn, and after treatment of 10 ppm calcium hypochlorite, the count of *Vibrio* spp. in shrimp and prawn reduced 0.23$\log_{10}$ CFU/g to zero. Kim *et al*.[24] reported that antimicrobial effects against *Vibrio* spp., *Edwadsiella tarda*, *Streptococcus* spp., and *Staphylococcus* spp. were tested using 10 different disinfectants including hyphochlorite salts. Hypochlorite salts resulted in 99.99% bactericidal activity against four strains of fish pathogenic bacteria. Abdel-Rahman *et al*.[25] reported that when Sprague-Dawley rats were administered hypochlorous acid at 0, 1, 10 or 100 mg/l daily in drinking water for one year, no significant chloroform concentrations were observed in rat blood at 12 months. In addition, OECD SIDS[26] reported that the acute oral toxicological test of calcium hypochlorite was carried out using Wister-derived albino rats dosed at 890, 1000, 1120, and 1260 mg/kg body weight, and with the results of the study, the LD_{50} of calcium hypochlorite was calculated to be 790 mg/kg.

With the consideration of previous studies, an aquatic disinfectant tablet is a more effective and safe disinfectant than chlorine and other treatments like ozone and beam irradiation against fish pathogenic bacteria.

In this study, disinfectant efficacy of an aquatic disinfectant tablet has limitation that the results are based on *in vitro* test. Organic material in suspension (OM condition) could not represent all possible parameters of *Vibrio* spp. and *Streptococcus* spp. contaminated fish farm and seafood-industry

environments.

As the efficacy of an aquatic disinfectant tablet against *V. anguillarum* and *S. iniae* was investigated *in vitro*, a controlled field trial is required to determine whether use of an aquatic disinfectant tablet will be able to reduce new pathogenic bacteria infection in fish farm and seafood industry area.

Conclusions

In cultured marine fish farm and seafood industry, vibriosis and *S. iniae* infection were very important diseases because of high mortality for farmed fish, zoonoses and economic loss. In the study of the bactericide efficacy test of an aquatic disinfectant tablet, the results suggest that an aquatic disinfectant tablet has potential bactericidal activity against *V. anguillarum* and *S. iniae*. So, an aquatic disinfectant tablet composed to calcium hypochlorite can be used to control the spread of fish pathogenic bacterial diseases.

Acknowledgements

This work was financially supported by Dae Han New Pharm Co., LTD. (Seoul, Korea).

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