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Antimicrobial Effects of Chemical Disinfectants on Fish Pathogenic Bacteria

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Abstract This study was to examine the potential disinfection efficiencies of 10 compounds by determining their antimicrobial capacity and ichthyotoxicity. Antimicrobial effects against *Vibrio* sp., *Edwadsiella tarda*, *Streptococcus* sp., and *Staphylococcus* sp. were tested using 10 different disinfectants; hydrogen peroxide, sodium hypochlorite, chlorine dioxide, povidon iodine, formaldehyde, glutaraldehyde, quaternary ammonium compounds (QACs), didecyl dimethyl ammonium chloride (DDAC), *ortho*-dichlorobenzen, and copper sulfate. Chlorine dioxide (ClO_2) containing 5% ClO_2 and copper sulfate had no effects on bactericidal activity, while the other disinfectants resulted in 99.99% bactericidal activity against 4 strains of fish pathogenic bacteria. The ichthyotoxicity of the 10 disinfectants was investigated using 3 kinds of fish species; flounder (*Paralichthys olivaceus*), rockfish (*Sebastes pachycephalus*), and black sea bream (*Acanthopagrus schlegelii*). Median lethal concentration (LC_{50}) values of the 10 disinfectants were estimated to determine toxicity ranges of the doses within 24 hr. Among test disinfectant solutions, hydrogen peroxide showed the highest LC_{50} in flounder (201.3), rockfish (269.7), and black sea bream (139.3 ppm). DDAC revealed the lowest LC_{50} in flounder (2.1), rockfish (1.0), and black sea bream (1.5 ppm). These results suggest that DDAC, quaternary ammonium compounds, glutaraldehyde, and sodium hypochlorite are effective disinfectants for fish and bacterial species examined in this study.

Keywords: disinfectant, fish pathogenic bacteria, *Vibrio*, *Edwardsiella*, *Streptococcus*, *Staphylococcus*

Introduction

Aquaculture has become an important industry in Korea, where economically important marine-cultured fish include olive flounder (*Paralichthys olivaceus*), rockfish (*Sebastes pachycephalus*), sea bream (*Pagrus major*), and black sea bream (*Acanthopagrus schlegelii*). Along with the rapidly expanding Korean aquaculture industry, the number of diseases caused by pathogens such as bacteria, viruses, fungi, and parasites have increased. Fish farmers have experienced substantial economic losses due to heavy stock mortalities as a result of these pathogens (1-4). In particular, bacterial infectious diseases such as Vibriosis, Photobacteriosis, Streptococcosis, and Edwardsiellosis have led to massive losses in cultured aquatic animals during the last few years, becoming a major concern of aquatic farms (4-8). In Korea, the use of hydrogen peroxide, chlorine dioxide, povidone iodine, chloramine T, and wood vinegar have been permitted as disinfectants in aquaculture. Formalin has also been permitted as an external parasiticide since November 2006.

In order to prevent the transmission of various infectious pathogens, effective protocols are required in farms for disinfecting field equipment such as boots, nets, and measuring instruments, with disinfectants often being used as biocidal agents in aquaculture and laboratory facilities (9-11). Chemical disinfectants are also widely used for controlling bacterial infections (12). For example, formalin and iodophors are the most widely used disinfectants in aquaculture. Moreover, organic iodine compounds have also been recommended in disinfection procedures for eggs and equipment, whereas hydrogen peroxide has been used to destroy fish pathogens (13-17). Disinfectants are divided into several groups such as oxidizing agents containing peroxides and halogens, and reducing agents containing formaldehydes, acids, alkalis, alcohols, and phenols. In general, disinfectants play important roles in cleaning of equipments such as aquariums, nets, and farming instruments, in order to prevent pathogens from spreading to the wild. An ideal disinfectant should work rapidly, be safe for human operators, pose no risk to the environmental, and be easily available. Furthermore, any residual concentration should have no lasting harmful effect on fish. The needs for comparative toxicological researches on cultured fish increased because large quantities of disinfectants are needed to disinfect tools and eggs and to exterminate parasites. However, little research work has been reported to evaluate the bactericidal effects and ichthyotoxicity of disinfectants used in aquafarm.

The aim of this study was to examine the potential disinfection efficiencies of 10 commercial compounds by determining their bactericidal capacity and ichthyotoxicity. Bactericidal capacity and ichthyotoxicity were evaluated by counting colonies grown on plates and by median lethal concentration (LC_{50}) values after treatment with each disinfectant.

Materials and Methods

Preparation of disinfectants The commercial chemical products, hydrogen peroxide (H_2O_2 35%, pH 1.3, Hansol Co., Seoul, Korea), sodium hypochlorite (NaClO 1.5%, pH 12.1, Samchun Co., Seoul, Korea), chlorine dioxide (ClO_2

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5%, pH 5.0), povidon iodine [$(C_6H_9NO)nI$ 10%, Samyang Chem., Seoul, Korea], formalin (CH_2O 35%, Duksan, Co., Seoul, Korea), glutaraldehyde ($C_5H_8O_2$, Samyang Chem.), quaternary ammonium compounds (QACs, formula of NH_4Cl, Samwoo Chem. Co., Seoul, Korea), didecyl dimethyl ammonium chloride (DDAC, Samwoo Chem.), *ortho*-dichlorobenzen ($C_6H_4Cl_2$, Samwoo Chem.), and copper sulfate ($CuSO_4 \cdot 5H_2O$, Sigma-Aldrich. St. Louis, MO, USA), were purchased from the commercial market.

Bacteria preparation *Vibrio* sp., *Edwardsiella tarda, Streptococcus* sp., and *Staphylococcus* sp. were isolated from spleen and kidney of cultured flounder *Paralichyhus olivaceus,* suffering from high mortality at a fish farm in Yeosu during 2003-2005. Bacteria were isolated and identified according to the methodology of Lee *et al.* (18). Bacteria were incubated at 20°C for 12 hr using a brain heart infusion (BHI) broth (Difco, Detroit, MI, USA) with 2% NaCl prior to use in bactericidal experiments.

Bactericidal reaction Bacteria culture was diluted in phosphate buffered saline (PBS) to achieve bacterial concentration of 10^7 CFU/mL. Bacterial culture was mixed with concentration of 25, 50, 200, 400, 800, 1,600, and 3,200 ppm of disinfectant in a 1:1 ratio. After incubation for 20 and 60 min at 20°C, the supernatants were removed by centrifugation and pellets were resuspended in 0.2 mL PBS. The bactericidal effects of disinfectants were measured by counting cells, which were incubated on the BHI 1.5% agar plates for 24 hr at 20°C.

LC_{50} of fish against disinfectants Flounder (*Paralichthys olivaceus*), rockfish (*Sebastes pachycephalus*), and black sea bream (*Acanthopagrus schlegelii*) were collected from a farm and acclimatized in a 100-L aquarium tank in the lab. The specimens were transferred to a 20-L aquarium in different groups for experimentation. The LC_{50} experiments were designed to evaluate acute toxicity according to protocol of Hall and Golding (19), where LC_{50} value is the median lethal concentration of a material in rearing water that will kill 50% of the tested fish when administered as single exposure. Briefly, the fish were divided into 3 groups, each containing 20 individuals. The 10 chemical disinfectants were applied to the fish as an immersion challenge in order to measure LC_{50} and the data were analyzed by probit analysis (19). The experiments were performed in triplicate.

Results and Discussion

All chemical disinfectant treatments exhibited 99.99% mortality in at least 1 concentration level, except for the stabilized chlorine dioxide and copper sulfate, which was ineffective at all tested concentrations (Table 1). The 35% hydrogen peroxide was effective against *Vibrio* sp. and *E. tarda* at 1,600 ppm. Hydrogen peroxide was also effective against *Streptococcus* sp. and *Staphylococcus* sp. up to the 3,200 ppm level after exposures for 20 and 60 min, respectively. Minimum concentrations of sodium hypochlorite solution were dependent on the fish pathogens, working at 11.5% against *E. tarda*; at 400 ppm for 60 min against *Streptococcus* sp.; at 800 ppm for 20 min against *Vibrio*, *E. tarda*, and *Streptococcus*; and at over 1,600 ppm for 20 min against *Staphylococcus*. Stabilized chlorine dioxide (5%) was ineffective at 3,200 ppm for 20 min, although it showed near 90% mortality at 1,600 ppm for 60 min. Povidon iodine was effective against *E. tarda* at a concentration of 800 ppm for 20 min, at 1,600 ppm for 20 min against *Vibrio* sp., and at over 3,200 ppm for 20 min against *Staphylococcus*. The formalin solution (35%) only showed biocidal effectiveness against *Vibrio* sp. and *E. tarda* at 3,200 ppm for at least 20 min. Glutaraldehyde (10%) was effective against *E. tarda* and *Staphylococcus* sp. at 200 ppm for 60 min, and at over 400 ppm for 20 min against all tested bacterial species. The mixed QACs, containing a formula of NH_4Cl [2.250% octyldecyl dimethyl ammonium, 1.125% dioctyl dimethyl ammonium chloride, 1.125% DDAC, and 3.000% alkyl dimethyl benzyl ammonium as available compounds (total 7.5%)], were effective against *Streptococcus* sp. and *Styphylococcus* sp. at 200 ppm for 60 min, and against all tested bacteria at over 400 ppm for 20 or 60 min.

QAC containing 10% DDAC were effective against all bacteria tested at 100 ppm for 20 or 60 min.

The mixed *ortho*-dichlorobenzene, containing 750 g of $C_6H_4Cl_2$/kg, 40 g of methanol/kg, and 50 g of cresol/kg, was effective against *Vibrio* sp., *E. tarda,* and *Staphylococcus* sp., and against all bacteria tested at over 800 ppm for 20 or 60 min.

Commercial copper sulfate (99%) was effective against *E. tarda* at 3,200 ppm for 60 min, but for all other bacteria it showed near 90% effectiveness at 3,200 ppm for 20 min. Copper sulfate was only effective at 3,200 ppm over 60 min exposure time.

The LC_{50} levels of the 10 chemical disinfectants were also tested on the aforementioned fish species (Table 2). Toxic concentration varied by each chemical as well as by species. The highest toxic concentration were found at 201.3, 269.7, and 139.3 ppm of hydrogen peroxide applied to flounder, rockfish, and black sea bream, respectively, while the lowest toxic concentrations of DDAC were at 2.1, 1.0, and 1.5 ppm, respectively. Variation for copper sulfate was both chemical and species specific.

The results of this investigation show that a number of chemical disinfectants can cause 99.99% mortality of fish pathogenic bacteria *in vitro*. Bactericidal effects were evaluated in duplicate using a range of chemical concentrations. The 10 chemical disinfectants used here were chosen because they are easily obtainable and often used in Korean farms.

In the case of hydrogen peroxide, the highest LC_{50} values were 201.3, 269.7, and 139.3 ppm in flounder, rockfish, and black sea bream, respectively. For DDAC, the lowest LC_{50} values were observed at 2.1, 1.0, and 1.2 ppm in flounder, rockfish, and black sea bream, respectively. The LC_{50} for flounder against 6 disinfectants (chlorine dioxide, formaldehyde, glutaraldehyde, QACs, DDAC, and copper sulfate) were higher than those for rockfish and black sea bream. In the cases of sodium hypochlorite and povidon iodine, the LC_{50} for flounder were lower than those of rockfish and black sea bream. Finally, it is difficult to maintain a residual concentration of chlorine due to the rising corrosion of temperature operating systems (16).

Active sodium hypochlorite was rapidly effective at 800 ppm, while at 400 ppm it required a minimum of 60 min

Table 1. Bactericidal percentage following treatments of commercial chemicals

Strains	Disinfectant[1)]	Treat time (min)	Treated concentration of disinfectants (ppm)							
			25	50	100	200	400	800	1,600	3,200
Vibrio sp.	Hydrogen peroxide	20	<90	<90	<90	<90	90	99	99.99	>99.999
		60	<90	<90	<90	90	99	99.99	>99.999	>99.999
	Sodium hypochlorite	20	<90	<90	<90	<90	99.9	99.999	>99.999	>99.999
		60	<90	<90	<90	<90	99.99	>99.999	>99.999	>99.999
	Chlorine dioxide	20	<90	<90	<90	<90	<90	<90	90	99.9
		60	<90	<90	<90	<90	<90	<90	90	99.9
	Povidon iodine	20	<90	<90	<90	<90	90	99.9	>99.999	>99.999
		60	<90	<90	<90	<90	90	99.99	>99.999	>99.999
	Formaldehyde	20	<90	<90	<90	<90	99	99.99	99.99	>99.999
		60	<90	<90	<90	<90	90	99.99	>99.999	>99.999
	Glutaraldehyde	20	<90	<90	99	99.99	99.999	99.999	>99.999	>99.999
		60	<90	90	99.9	99.99	>99.999	>99.999	>99.999	>99.999
	QACs	20	<90	<90	90	99.9	99.99	>99.999	>99.999	>99.999
		60	<90	<90	99	99.9	>99.999	>99.999	>99.999	>99.999
	DDAC	20	<90	90	99.9	>99.999	>99.999	>99.999	>99.999	>99.999
		60	<90	95	>99.999	>99.999	>99.999	>99.999	>99.999	>99.999
	Ortho-dichlorobenzene	20	<90	<90	90	99	99.9	99.999	>99.999	>99.999
		60	<90	<90	90	99.9	99.999	>99.999	>99.999	>99.999
	Copper sulfate	20	<90	<90	<90	<90	<90	<90	<90	90
		60	<90	<90	<90	<90	<90	<90	<90	90
Edwardsiella tarda	Hydrogen peroxide	20	<90	<90	<90	<90	90	99	>99,999	>99.999
		60	<90	<90	<90	90	99	99.9	>99.999	>99.999
	Sodium hypochlorite	20	<90	<90	<90	<90	99.99	>99.999	>99.999	>99.999
		60	<90	<90	<90	99	99.999	>99.999	>99.999	>99.999
	Chlorine dioxide	20	<90	<90	<90	<90	<90	<90	<90	99.99
		60	<90	<90	<90	<90	<90	<90	99	99.99
	Povidon iodine	20	<90	<90	<90	<90	99	99.999	>99.999	>99.999
		60	<90	<90	<90	90	99	99.999	>99.999	>99.999
	Formaldehyde	20	<90	<90	<90	90	99	99.95	>99.999	>99.999
		60	<90	<90	<90	90	99	99.99	>99.999	>99.999
	Glutaraldehyde	20	<90	<90	99	99.9	99.999	>99.999	>99.999	>99.999
		60	<90	<90	99.99	99.999	>99.999	>99.999	>99.999	>99.999
	QACs	20	<90	<90	99	99.9	>99.999	>99.999	>99.999	>99.999
		60	<90	<90	90	99.99	>99.999	>99.999	>99.999	>99.999
	DDAC	20	90	95	>99.999	>99.999	>99.999	>99.999	>99.999	>99.999
		60	90	95	>99.999	>99.999	>99.999	>99.999	>99.999	>99.999
	Ortho-dichlorobenzene	20	90	99	99.9	99.9	99.999	>99.999	>99.999	>99.999
		60	90	99	99.9	99.9	>99.999	>99.999	>99.999	>99.999
	Copper sulfate	20	<90	<90	<90	<90	<90	90	95	99
		60	<90	<90	<90	<90	<90	95	99.5	99.999

exposure time, and was ineffective below 200 ppm. Decreasing activation of sodium hypochlorite may be caused by combination with organic materials in the water used for dilution, as it can be inactivated by organic debris, which may reduce the disinfecting action of concentrations that are effective *in vitro*. Bloomfield and Miller (20) found substantial inactivation of sodium hypochlorite in the presence of blood.

DDAC was a highly effective disinfectant at 100 ppm. As such, farm workers could carry small amounts of concentrated solution to dilute with fresh or seawater. The use of DDAC as a safe disinfectant has increased within the agriculture and forestry industries (21). DDAC, hydrogen peroxide, and chlorines are produced during oxidative metabolism in cells, and are recognized as common bacteriostatic chemicals found in most environments. They are a potent source of reactive oxygen species (ROS) such as superoxide, perhydroxyl, and hydroxyl radicals, capable of causing lipid peroxidation and the destruction of protein and DNA, and the activation/inactivation of enzymes (22).

Table 1. Continued

Strains	Disinfectant[1)]	Treat time (min)	Treated concentration of disinfectants (ppm)							
			25	50	100	200	400	800	1,600	3,200
Streptococcus sp.	Hydrogen peroxide	20	<90	<90	<90	<90	<90	90	99.9	>99.999
		60	<90	<90	<90	<90	<90	90	99.99	>99.999
	Sodium hypochlorite	20	<90	<90	<90	<90	99	99.999	>99.999	>99.999
		60	<90	<90	<90	<90	99.999	>99.999	>99.999	>99.999
	Chlorine dioxide	20	<90	<90	<90	<90	<90	<90	<90	<90
		60	<90	<90	<90	<90	<90	<90	90	95
	Povidon iodine	20	<90	<90	<90	90	99	99.9	99.99	>99.999
		60	<90	<90	<90	90	99	99.9	99.99	>99.999
	Formaldehyde	20	<90	<90	<90	<90	99	99.9	99.99	>99.999
		60	<90	<90	<90	<90	99	99.9	99.99	>99.999
	Glutaraldehyde	20	<90	90	99.9	99.9	>99.999	>99.999	>99.999	>99.999
		60	90	99.5	99.95	99.99	>99.999	>99.999	>99.999	>99.999
	QACs	20	<90	90	99	99.99	>99.999	>99.999	>99.999	>99.999
		60	<90	90	99	99.999	>99.999	>99.999	>99.999	>99.999
	DDAC	20	99.9	99.99	>99.999	>99.999	>99.999	>99.999	>99.999	>99.999
		60	99.9	99.99	>99.999	>99.999	>99.999	>99.999	>99.999	>99.999
	Ortho-dichlorobenzene	20	<90	90	95	99	99,99	99.999	>99.999	>99.999
		60	<90	90	99	99.99	99.99	>99.999	>99.999	>99.999
	Copper sulfate	20	<90	<90	<90	<90	<90	<90	<90	90
		60	<90	<90	<90	<90	<90	<90	<90	90
Styphylococcus sp.	Hydrogen peroxide	20	<90	<90	<90	<90	<90	90	99.9	99.999
		60	<90	<90	<90	<90	<90	99	99.99	>99.999
	Sodium hypochlorite	20	<90	<90	<90	<90	90	99	>99.999	>99.999
		60	<90	<90	<90	<90	99	99	>99.999	>99.999
	Chlorine dioxide	20	<90	<90	<90	<90	<90	<90	<90	<90
		60	<90	<90	<90	<90	<90	<90	90	99.5
	Povidon iodine	20	<90	<90	<90	<90	90	95	99.9	>99.999
		60	<90	<90	<90	<90	90	95	99.99	>99.999
	Formaldehyde	20	<90	<90	<90	<90	<90	90	99.9	99.999
		60	<90	<90	<90	<90	<90	99	99.99	99.999
	Glutaraldehyde	20	<90	90	99.99	99.99	>99.999	>99.999	>99.999	>99.999
		60	<90	90	99.99	99.999	>99.999	>99.999	>99.999	>99.999
	QACs	20	<90	90	99	99.99	>99.999	>99.999	>99.999	>99.999
		60	<90	90	99.9	99.999	>99.999	>99.999	>99.999	>99.999
	DDAC	20	99.9	99.99	>99.999	>99.999	>99.999	>99.999	>99.999	>99.999
		60	99.9	99.99	>99.999	>99.999	>99.999	>99.999	>99.999	>99.999
	Ortho-dichlorobenzene	20	<90	90	90	99	99.99	>99.999	>99.999	>99.999
		60	<90	90	99	99.9	>99.999	>99.999	>99.999	>99.999
	Copper sulfate	20	<90	<90	<90	<90	<90	<90	<90	90
		60	<90	<90	<90	<90	<90	<90	<90	90

[1)]QACs, quaternary ammonium compounds; DDAC, didecyl dimethyl ammonium chloride.

Formaldehyde was also included in our experiments, and is used extensively as a bactericidal or insecticidal by fish farmers in Korea. Formaldehyde solution was effective at 3,200 ppm for at least 20 min of treatment (*Vibrio* sp. and *E. tarda*), and at 1,600 ppm for at least 60 min of treatment (*Vibrio* sp. and *E. tarda*), while lower concentrations were ineffective. Due to its residual toxicity at effective concentrations, formaldehyde solution is not recommended as a disinfectant for fish pathogenic bacteria (Table 1).

Ideally, treatments should not be harmful to the environment, and should be inexpensive and practical. For disinfecting equipment and personnel in fish farms, short exposure times are preferable. Therefore, a range of concentrations were tested for each reagent over a range of short exposure times. In conclusion, it is suggested that DDAC, QACs, glutaraldehyde, and sodium hypochlorite are effective disinfectants for fish and bacterial species examined in this study.

Table 2. LC_{50} of flounder, rockfish, and black sea bream following treatments of available chemical grades for 24 hr

Disinfectant[1)]	Mean of LC_{50} (ppm)		
	Flounder	Rockfish	Black sea bream
Hydrogen peroxide	201.3	269.7	139.3
Sodium hypochlorite	3.5	5.3	5.6
Chlorine dioxide	103.6	59.0	60.4
Povidon iodine	5.1	27.3	18. 8
Formaldehyde	142.2	94.1	61.2
Glutaraldehyde	61.1	33.0	20.8
QACs	3.4	2.6	2.8
DDAC	2.1	1.0	1.5
Ortho-dichlorobenzene	13.1	9.4	15.0
Copper sulfate	43	5.0	4.5

[1)]QACs, quaternary ammonium compounds; DDAC, didecyl dimethyl ammonium chloride.

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