한국환경보건학회지, 제36권 제6호(2010)
J. Env. Hlth. Sci., Vol. 36, No. 6, pp 496~501(2010)

Antimicrobial Efficacy of the Disinfectant Solution Nanoxil® Against Fish Pathogenic Bacteria

Chun-Nam Cha* · Won-Chul Jung · Yeo Eun Lee ·**
Chang-Yeul Yoo* · Suk Kim · Hu-Jang Lee†**

Research Institute of Live Sciences, College of Veterinary Medicine,
Gyeongsang National University, Chinju 600-701, Korea
**Engineering Research Institute, Department of Industrial Systems Engineering,*
Gyeongsang National University, Chinju 600-701, Korea
***Department of Environmental Health, Graduate School of Public Health,*
Gyeongsang National University, Chinju 660-751, Korea
****Department of Computer Information, Gyeongnam Provincial Namhae College,*
Namhae 668-801, Korea

(Received August 24, 2010/Revised October 30, 2010/Accepted December 16, 2010)

ABSTRACT

Fish pathogenic bacteria are a considerable danger of farmed fish and a source of economic loss in the fish farming industry. In this study, Nanoxil® was compared to hydrogen peroxide and a silver colloid in terms of disinfection efficacy against *E. tarda*, *V. anguillarum* and *S. iniae*. A bactericidal efficacy test conducted by a 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. Nanoxil® and test bacteria were diluted with distilled water (DW), hard water (HW) or an organic matter suspension (OM) according to the treatment condition. Under the OM condition, the bactericidal activity of Nanoxil® against *E. tarda* exhibited a lowered efficacy compared to that under the DW and HW conditions. Nanoxil® at 500 fold (dilutions on) under all of the conditions demonstrated a high bactericidal efficacy against *S. iniae*. As Nanoxil® possess bactericidal efficacy against fish pathogenic bacteria such as *E. tarda*, *V. anguillarum* and *S. iniae*, this disinfectant solution can be used to limit the spread of fish bacterial diseases.

Keywords: Nanoxil®, *Edwardsiella tarda*, *Vibrio anguillarum*, *Streptococcus iniae*, Disinfectant efficacy

Bacterial diseases occur in cultured fish and are responsible for heavy stock mortality in Korea. Diseases such as edwardsiellosis, streptococosis and vibriosis currently prevalent in cultured eel, flounder, rockfish and sea bass.[1-3]

Edwardsiella tarda (*E. tarda*) is a Gram-negative bacterium of the family *Enterobacteriaceae* and is the causative agent of edwardsiellosis and leads to extensive losses in many commercially important freshwater and marine fish worldwide.[4] *E. tarda* has been associated with diseases in a diverse array of fish species and human diseases.[5]

Generally, edwardsiellosis in fish is characterized by the loss of pigmentation and a swollen abdomen filled with ascitic fluid, and small white nodules may be observed in the gills and internal organs.[6] In human, diseases related with *E. tarda* have been reported such as gastroenteritis, septicemia, wound infection, and cellulitis.[7,8]

Vibrio anguillarum (*V. anguillarum*) is a highly motile, Gram-negative, curved rod bacterium and is the aetiological agent of vibriosis, a fatal hemorrhagic septicemia in fish.[9] Fish infected with *V. anguillarum* display skin discoloration and

†Corresponding author : College of Veterinary Medicine, Gyeongsang National University
Tel: 82-55-751-6642, Fax: 82-55-751-5803
E-mail: hujang@gnu.ac.kr

erythema around the mouth and fins. Necrotic lesions are observed in the abdominal muscle.[10)]

Mortality rates for infected fish populations may range from 30% to as high as 100%.[6)] Vibriosis has resulted in severe economic losses to aquaculture worldwide and affects many farm-raised fish.[10-12)]

Streptococcus iniae (*S. iniae*) is a hemolytic, Gram-positive pathogen in wild and cultured fish species worldwide. *S. iniae* has been shown to cause meningoencephalitis in fish grown by aquaculture and may colonize the surface of fish or cause invasive disease associated with 30 to 50 percent mortality in affected fishes.[13)] The estimated annual impact of infection by *S. iniae* on the aquaculture industry reached US $100 million globally.[14)]

S. iniae has also been reported to cause the fulminant soft tissue infection in human.[13,15)]

Bacterial diseases in farmed fish are one of major factors to cause economic loss of fish farming. The stress on fish caused by intensive farming practices and the development of antibiotic-resistant bacteria are among the major reasons for the increased frequency of bacterial disease outbreaks.[12)] Highly hygienic measure including the use of disinfectant is very effective for successful control of bacterial diseases, fungi and ectoparasites in farmed fish.[16)] Several disinfectants including iodophores, salts, organic chlorocompounds, aldehydes, hydrogen peroxide, quaternary ammonium compounds and antiseptic dyes are used for decontamination after outbreaks of farmed fish diseases.[16-18)] But, efficacy of the disinfectant composed of hydrogen peroxide and colloidal silver has never been examined against bacterial fish diseases. Therefore, this study was carried out to examine bactericidal efficacy of the disinfectant solution against *E. tarda*, *V. anguillarum* and *S. iniae*.

II. Materials and Methods

1. Bacteria and culture

The test bacteria, *E. tarda* (KCTC 12267), *V. anguillarum* (KCTC 2911), and *S. iniae* (KCTC 3657) were obtained from the Korean Collection for Type Cultures (KCTC, Seoul, Korea). The strains were cultured in Brain Heart Infusion broth (BHIB) supplemented with 1.5% (w/v) NaCl for 24 hr at 25°C under constant agitation.

2. Disinfectant

The active ingredients for Nanoxil®, the tested disinfectant solution, are hydrogen peroxide (30% v/v) and colloidal silver (0.03% w/v). Nanoxil® was provided by G. P. I. Co. (Changwon, Korea). The disinfectant solution was stored in the dark in room temperature and prepared for dilution on the day of evaluation. Determination of the antimicrobial efficacy of the disinfectant was based on National Veterinary Research & Quarantine Service Regulation No. 30, Korea.

3. 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 $MgCl_2\cdot$

Table 1. Experimental design for the determination of the bactericidal efficacy of Nanoxil®

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.

$6H_2O$ 0.139 g into 1 *l* 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/m*l* viable organisms with the same kind of diluents of treatment condition.

4. Experimental procedures

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. 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, the mixture was neutralized by 1:10 dilution of Nutrient broth (Becton Dickinson & Co., MD, USA) at 37°C. 0.1 m*l* of the neutralized reaction mixture was subcultured into 10 m*l* 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 were confirmed. The final valid dilution 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.

III. Results and Discussion

Table 2 shows the final valid dilution of Nanoxil® composed to hydrogen peroxide and colloidal silver. On DW condition, *E. tarda*, *V. anguillarum* and *S. iniae* were completely inactivated with 150, 50, and 500 fold dilutions of the disinfectant, respectively. When the bactericidal effect on HW condition was evaluated, the antibacterial activity of the disinfectant showed on 100, 50 and 500 fold

Table 2. Final valid dilution of Nanoxil® against *Edwardsiella tarda*, *Vibrio anguillarum* and *Streptococcus iniae*

Bacterial strains	Dilution times	Treatment condition*								
		DW			HW			OM		
		1	2	3	1	2	3	1	2	3
Edwardsiella tarda	250	○	○	○	○	○	○	○	○	○
	200	○	○	○	○	○	○	○	○	○
	150	×	×	×	○	○	×	○	○	○
	100	×	×	×	○	×	×	○	○	○
	50	×	×	×	×	×	×	×	×	×
	Valid dilution	150			100			50		
Vibrio anguillarum	90									
	70	○	○	×	○	○	○	×	○	○
	50	×	×	×	×	×	×	×	×	○
	30	×	×	×	×	×	×	×	×	×
	10	×	×	×	×	×	×	×	×	×
	Valid dilution	50			50			50		
Streptococcus iniae	700	○	○	○	○	○	O	○	○	○
	600	×	○	○	○	○	○	○	○	○
	500	×	×	×	O	×	×	O	×	×
	400	×	×	×	×	×	×	×	×	×
	300	×	×	×	×	×	×	×	×	×
	Valid dilution	500			500			500		

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

dilutions against *E. tarda*, *V. anguillarum* and *S. iniae*, respectively. With the investigation of the bactericidal effect of the disinfectant on OM condition, *E. tarda*, *V. anguillarum* and *S. iniae* were inactivated on 50, 50 and 500 fold dilutions, respectively. Because 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 lowered efficacy against three fish pathogenic bacteria compared with DM or HW conditions.

On OM condition, the bactericidal activity of the disinfectant lowered efficacy against *E. tarda* compared with DM or HW conditions. A bactericidal effect of the disinfectant against *V. anguillarum* and *S. iniae* showed the same potency on all conditions. However, the bactericidal effect of the disinfectant against *V. anguillarum* lowered compared with that against *E. tarda* and *S. iniae* on DW or HW conditions. When comparing the results of the disinfectant against three fish pathogenic bacteria in the present study, the bactericidal effect of Nanoxil® was the highest efficacy against *S. iniae* on all conditions.

Jin *et al*. (2010) reported that bath treatment with hydrogen peroxide at the concentration of 200 μg/m*l* showed higher efficacy against *Philasterides dicentrarchi* compared to formalin at the same dose.[19] And Rach *et al*. (2000) reported that bath treatment with hydrogen peroxide administered at concentrations of 56-230 mg/*l* as a 30 min exposure was effective in the control of bacterial gill disease.[20] In the research of Han *et al*., *E. tarda* strains were inhibited by discs containing different concentrations of H_2O_2 and also showed effervescence in the presence of 3% H_2O_2.[1] In the research for the bactericidal activity by Boesen *et al*. (2001), it was reported that the bactericidal activity of 1 hr exposure to 20 μM hydrogen peroxide showed against *V. anguillarum*.[21] Treasurer and Grant (1997) reported that the reduction in mobile sea lice numbers on salmon in 20 min cage treatment of hydrogen peroxide at 68°C varied from 43 to 100% depending on louse developmental stage, with greatest reduction in numbers of preadults.[22] Kim *et al*. (2009) reported that most of the minimum inhibitory concentrations against 30 fish pathogenic bacteria including *E. tarda* and *Vibrio spp*. were less than 40 μ*l*/*l* hydrogen peroxide.[23]

The scientific literature points to the wide use of silver in numerous applications. It is well established that silver nanoparticles are known for their strong antibacterial effects for a wide array of viruses, bacteria and fungi.[24]

Lee *et al*. (2005) showed that silver nanoparticle loaded hydrogen-bonded multilayers assembled on planar and curved supports such as magnetic microspheres show excellent antibacterial properties.[25]

In the research for antimicrobial effect of silver colloid by Zhang *et al*. (2008),[26] it was reported that the inhibition ratios of the bacteria including *Escherichia coli* and *Staphylococcus aureus* reached up to ca. 98% at the low silver content of ca. 2.0 μg/m*l*.

According to a previous paper by Maass (2008),[27] silver nanoparticles including colloidal silver do not remain nanosize when they come in contact with normal environmental samples such as solil and water, but they agglomerate to form more larger silver particles which is non-toxic and have no history of being harmful to the environment and aquatic life.

When added to water, hydrogen peroxide breaks down into oxygen and water over time, and the formation of these by-products is one reason that hydrogen peroxide is considered to be relatively safe for the environment. Hydrogen peroxide is using in aquaculture against numerous external fish-disease-causing organisms.[28]

In the present study, disinfectant efficacy of Nanoxil® has limitation that the results are based on *in vitro* test. Organic material in suspension (OM condition) could not represent all possible parameters of fish pathogenic bacteria contaminated in fish farm environments.

As the efficacy of Nanoxil® against *E. tarda*, *V. anguillarum* and *S. iniae* was investigated *in vitro*, a control field trial are required to determine whether use of Nanoxil® will be able to reduce new fish pathogenic bacteria infection ratio in fish farm area.

Acknowledgements

This work was financially supported by G. P. I. Co., Ltd. (Changwon, Korea).

References

1. Acharya, M., Maiti, N. K., Mohanty, S., Mishra, P. and Samanta, M. : Genotyping of *Edwardsiella tarda* isolated from freshwater fish culture system. *Comparative Immunology, Microbiology and Infectious Diseases*, **30**, 33-40, 2007.
2. Agnew, W. and Barnes, A. C. : Streptococcus iniae: An aquatic pathogen of global veterinary significance and a challenging candidate for reliable vaccination. *Veterinary Microbiology*, **122**, 1-15, 2007.
3. Austin, B. and Austin, D. A. : Vibrionaceae representatives. In Bacterial Fish Pathogens: Disease in Farmed and Wild Fish, pp. 265-307, 2nd edition (edited by Austin, B. and Austin, D. A.), Ellis Horwood Ltd., Chichester, U.K., 1993.
4. Boesen, H. T., Larsen, M. H., Larsen, J. L. and Ellis, A. E. : *In vitro* interactions between rainbow trout (*Oncorhynchus mykiss*) macrophages and *Vibrio anguillarum* serogroup O2a. *Fish Shellfish Immunology*, **11**, 415-431, 2001.
5. Denkin, S. M. and Nelson, D. R. : Regulation of *Vibrio anguillarum empA* metalloprotease expression and its role in virulence. *Applied Environmental Microbiology*, **70**, 4193-4204, 2004.
6. Egidius, E. : Vibriosis: pathogenicity and pathology. *Aquaculture*, **7**, 15-28, 1987.
7. Han, H.-J., Kim, D.-H., Lee, D.-C., Kim, S.-M. and Park, S.-I. : Pathogenicity of *Edwardsiella tarda* to olive flounder, *Paralichthys olivaceus* (Temminck & Schlegel). *Journal of Fish Diseases*, **29**, 601-609, 2006.
8. Han, Y., Li, X., Qi, Z., Zhang, X. H. and Bossier, P. : Detection of different quorumsensing signal molecules in a virulent Edwardsiella tarda strain LTB-4. *Journal of Applied Microbiology*, **108**, 139-147, 2010.
9. Janda, J. M. and Abbott, S. L. : Infections associated with the genus *Edwardsiella*: the role of *Edwardsiella tarda* in human disease. *Clinicla Infecteous Diseases*, **17**, 742-748, 1993.
10. Jin, C. N., Harilkrishnan, R., Moon, Y. G., Kim, M. C., Kim, J. S., Balasundarm, C. and Heo, M. S. : Effectiveness of chemotherapeutants against scuticociliate Philasterides dicentrarchi, a parasite of olive flounder. *Veterinary Parasitology*, **168**, 19-24, 2010.
11. Kim, D.-G., Bae, J.-Y., Hong, G.-E., Min, M.-K., Kim, J.-K. and Kong, I.-S. : Application of the *rpoS* gene for the detection of *Vibrio anguillarum* in flounder and prawn by polymerase chain reaction. *Journal of Fish Diseases*, **31**, 639-647, 2008.
12. Kim, M. S., Cho, J. Y., Kim, D. H., Jeon, H. J. and Kim, E. O. : Toxicity, antibacterial and parasiticidal effects of hydrogen peroxide for Israel carp (*Cyprinus carpio*). *Journal of Fish Pathology*, **22**, 1-7, 2009.
13. Lee, D., Cohen, R. E. and Rubnet, M. F. : Antibacterial properties of Ag nanoparticle loaded multilayers and formation of magnetically directed antibacterial microparticles. *Langmuir*, **21**, 9651-9659, 2005.
14. Li, L., Rock, J. L. and Nelson, D. R. : Identification and characterization of a repeat-in-toxin gene cluster in *Vibrio anguillarum*. *Infection and Immunity*, **76**, 2620-2632, 2008.
15. Maass, G. J. : Siver nanoparticles: No threat to the environment. Colloidal Science Lab. Inc., New Jersey, USA, 2008.
16. Nelson, J. J., Nelson, C. A. and Carter, J. E. : Extraintestinal manifestations of *Edwardsiella tarda* infection: a 10l-year retrospective review. *The Journal of the Louisiana State Medical Society*, **161**, 103-106, 2009.
17. Plumb, J. A. : *Edwardsiella* septicaemia. In Bacterial diseases of fish, pp. 61-79, Inglis, V., Roberts, R. J., Bromage, N. R. (Editors), Blackwell Sci. Publ., Oxford, UK, 1993.
18. Rach, J. J., Gaikowski, M. P. and Ramsay, R. T. : Efficacy of hydrogen peroxide to control mortalities associated with bacterial gill disease infections on hatchery-reared Salmonids. *Journal of Aquatic Animal Health*, **12**, 119-127, 2000.
19. Ra, C.-H., Kim, Y.-J., Park, S.-J., Jeong, C.-W., Nam, Y.-K., Kim, K.-H. and Kim, S.-K. : Evaluation of optimal culture conditions for recombinant ghost bacteria vaccine production with the antigen of *Streptococcus iniae* GAPDH. *Journal of Microbiology and Biotechnology*, **19**, 982-986, 2009.
20. Shao, A. J. : Aquaculture pharmaceuticals and biologicals: current perspectives and future possibilities. *Advanced Drug Delivery Reviews*, **50**, 229-243, 2001.
21. Sharifuzzaman, S. M. and Austin, B. : Development of protection in rainbow trout (*Oncorhynchus mykiss*, Walbaum) to *Vibrio anguillarum* following use of the probiotic *Kocuria* SM1. *Fish and Shellfish Immunology*, **29**, 212-216, 2010.
22. Thune, R. L., Stanley, L. A. and Cooper, R. K. : Pathogenesis of Gram-negative bacterial infections in warm fish. *Annual Review of Fish Diseases*, **3**, 37-68, 1993.
23. Tolaymat, T. M., EI Badawy, A. M., Genaidy, A., Scheckel, K. G., Luxton, T. P. and Suidan, M. : An evidence-based environmental perspective of manufactured silver nanoparticle in syntheses and applications: a systematic review and critical appraisal of peer-reviewed scientific papers. *The Science of the Total Environment*, **408**, 999-1006, 2010.
24. Toranzo, A. E. and Barja, J. L. : Virulence factors of bacteria pathogenic for coldwater fish. *Annual Review of Fish Diseases*, **3**, 5-36, 1993.

25. Toranzo, A. E., Magarinõs, B. and Romalde, J. L. : A review of the main bacterial fish diseases in mariculture systems. *Aquaculture*, **246**, 37-61, 2005.
26. Treasurer, J. W. and Grant, A. : The efficacy of hydrogen peroxide for the treatment of farmed Atlantic salmon, *Salvo salar* L. infested with sea lice (Copepoda: Caligidae). *Aquaculture*, **148**, 265-275, 1997.
27. Verner-Jeffreys, D. W., Joiner, C. L., Bagwell, N. J., Reese, R. A., Husby, A. and Dixon, P. F. : Development of bactericidal and virucidal testing standards for aquaculture disinfectants. *Aquaculture*, **286**, 190-197, 2009.
28. Weinstein, M. R., Litt, M., Kertesz, D. A., Wyper, P., Rose, D., Coulter, M., McGeer, A., Facklam, R., Ostach, C., Willey, B. M., Borczyk, A. and Low, D. E. : Invasive infections due to a fish pathogen, *Streptococcus iniae*. *The New England Journal of Medicine*, **337**, 589-594, 1997.
29. Weinstein, M. R., Low, D. E., McGeer, A., Willey, B., Rose, D., Coulter, M., Wyper, P., Borczyk, A. and Lovgren, M. : Invasive infection due to Streptococcus iniae: A new or previously unrecognized disease. *Canada Communicable Disease Report*, **22**, 129-132, 1996.
30. Yanong, R. P. E. : Use of hydrogen peroxide in finfish aquaculture - FA 157. IFA extention, University of Florida, USA, 2008.
31. Zhang, Y., Peng, H., Huang, W., Zhou, Y. and Yan, D. : Facile preparation and characterization of highly antimicrobial colloid Ag or Au nanoparticles. *Journal of Colloid Interface Science*, **325**, 371-376, 2008.