

Efficacy and safety of norfloxacin for the control of bacterial diseases in eel (*Anguilla japonica*)

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Abstract : A study on quinolone antibacterial, norfloxacin, was performed to apply for the control of bacterial diseases in eel (*Anguilla japonica*). Norfloxacin was proved excellent in antibacterial activity and sensitivity against fish bacterial pathogens when compared with the existing antibacterials and antibiotics. And any side effect was not observed during the period of indicated use.

An outline of minimal inhibitory concentration was 0.03~0.1 μ g/ml, TLm_{48h} value was 3,500mg/l. The residual time of the day in fish body was less than 17 days and any pathological changes were not observed.

The study has revealed that norfloxacin can be applied to treat some fish bacterial disease by the dosage of 100g/day/ton of fish body weight for about 3 days perorally. Further, norfloxacin may be used for the control of bacterial pathogens in eel.

Key words : norfloxacin, efficacy, safety, *Anguilla japonica*.

Introduction

There are so many antibacterial agents used for fisheries, but now most of them are not effective against fish bacterial pathogens because many aquaculturists abuse and misuse drugs for the treatment of bacterial diseases, which prevails due to high-density rearing and over-supply of food in Korea. So resistance of bacteria to chemotherapeutics has be-

come a problem and inflicts a loss on aquaculture¹.

In the 1980's, a new group of quinolones (ofloxacin, enoxacin, norfloxacin, ciprofloxacin, tosufloxacin, pefloxacin) was developed and has been examined its possibility of the application for fisheries. These new quinolones have been very effective to *Vibrio anguillarum*, *Pasteurella piscicida*, *Edwardsiella tarda* and *Enterococcus seriolicida*². Sarafloxacin has shown its high efficacy in a bath treatment against *Aeromonas salmonicida* infection in chinook salmon, *Oncorhynchus*

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*tshawytscha*³. Resistance of pathogens against new quinolones is lower than that against the earlier quinolones (oxolinic acid, nalidixic acid, promidic acid and flumiquine)².

Norfloxacin [1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid; C₁₆H₁₈FN₃O₃] is the derivative of nalidixic acid and has a high antibacterial effect against Gram-negative and Gram-positive bacteria, including *Mycoplasma* spp.⁴. Norfloxacin interferes DNA gyrase, which synthesizes bacterial DNA. The appearance of the resistant strain is very rare and norfloxacin shows clear effectiveness against these bacteria.

The minimum inhibitory concentration (MIC) of norfloxacin against selected fish pathogens (*Vibrio anguillarum*, *Pseudomonas fluorescens*, *P. putida*, *Pasteurella piscicida*, *Edwardsiella tarda*, *Aeromonas salmonicida*, *A. hydrophilla* and *Enterococcus seriolicida*) is very low⁵.

There have been many researches about some newer quinolones against fish diseases in Japan and U.S.A.. Already in Korea, ciprofloxacin and norfloxacin have been used for hog and poultry farming, not only for man. So we investigated the efficacy and safety of norfloxacin for the control of bacterial diseases in eel (*Anguilla japonica*).

Materials and Methods

Antimicrobial agents : A 99.13%-active compound of norfloxacin (Korea Vetchem Co. Ltd., Seoul, Korea) was used in a disc diffusion test and MIC assay, and a commercial form of 10% norfloxacin was used in a challenge test and field trials.

Fishes : The healthy eel, *Anguilla japonica* (mean body length 9.3~11cm, mean body weight 8.5~9.6g) were collected from fish farms each time by 50~100 fish at random for experiment. Fish were kept in aquaria (120×60×40cm, 80×40×30cm, 60×40×30cm), equipped with aeration system, recircular system and light. Water temperature was maintained at 25±2°C and feed were given twice in a day.

Bacteria : Six bacterial strains of *Edwardsiella tarda*, *Aeromonas salmonicida* and *Vibrio anguillarum*, isolated from clinically diseased fish in Korea and Japan (Table 1), were used in this study.

Table 1. Bacterial strains used in this study

Species	Strains	Year reported	Source fish	Country
<i>Vibrio anguillarum</i>	HEO926	1992	Eel	Korea
	PB15*	1987	Ayu	Japan
<i>Aeromonas salmonicida</i>	HEO929	1992	Eel	Korea
	NCMB1102*	1985	Carp	Japan
<i>Edwardsiella tarda</i>	E22*	1972	Eel	Japan
	HEO939	1993	Eel	Korea

* : Supplied by H. Wakabayashi, University of Tokyo, Japan.

Toxicity test : TLM_{48h} value (medium tolerance limit = LC₅₀) was measured by Doudoroff's method and concentration of norfloxacin tested were based on preliminary tests (500~5000mg/l). Tests were performed in 10 l glass aquaria. Fish were observed of their external abnormality for 48h, and necropsy was conducted comparing with the control group after test. Subacute toxicity test was performed for 1 month with ten times higher than the recommended dosage(100mg/kg). Fish were fed with medicated feed which mixed with the test drug once in a day spontaneously.

Residual analysis of antibacterial : Drug-treated fish were preserved in a freezer until analysis. After thawing, fish bodies were ground and the meat juice were filtered by gauze for several times. These meat juice were used for residual analysis by EEC 4-plate method⁶.

Histopathological observation : After toxicity test, fish were killed instantly by decapitation. For histopathological observation, gills and internal organs were fixed in 10% neutral buffered formalin, and embedded in paraffin routinely. They were sectioned at 4~5µm thickness and stained with hematoxylin and eosin (H & E).

Antimicrobial sensitivity test : Disc diffusion test was performed by culturing the above bacterial strains in Trypticase Soy agar (Difco) with the discs of norfloxacin (1, 5, 10µg/disc). After incubating for 3 days at 23±2°C, the zone diameter (φ mm) of antibacterial inhibition was measured.

Minimum inhibitory concentration assay : Determination of MICs of norfloxacin against the bacterial strains was con-

ducted with serial twofold dilutions in microtiter wells^{7,8}. Two fold dilutions of norfloxacin in Trypticase Soy broth were inoculated with 10µl volumes of diluted broth cultures for 18-20 hours to give approximately 10⁵CFU/ml final inocula. After 18h incubation, MICs were determined as the lowest drug concentrations which prevented visible bacterial growth.

Determination of recommended dosage : About 500ml (4.5 × 10⁷CFU/ml) of Trypticase Soy broth culture of *E. tarda* was added to the aquaria, with 20 l of water and 50 individuals of eel. After confirming the outbreak of edwardsiellosis, the serial dosage of the test drug below TLM value (10, 50, 100, 200mg/kg of body weight) mixed uniformly with feed and feed oil were given to fish once in a day for 3 days.

Field trials : Clinical trials with norfloxacin were performed against some bacterial diseases (ulcer disease, edwardsiellosis and vibriosis) in the farms where the diseases had been occurring. In each of the fish farms one group (1,000 fish) was administered 100mg of norfloxacin/kg body weight once a day for 3 consecutive days while the other group (1,000 fish) was not medicated. Cumulative mortality was observed between medicated and non-medicated groups for 5 days after treatment.

Result

Toxicity test : The TLM value of the test drug was 3,500 mg/l in eel. Under the concentration below the TLM value, external appearance and swimming of fish were normal, but mucus secreting increased in proportion to concentration of the test drug.

After exposure below than TLM value for 1 month, no visible side effects and histopathological changes were observed.

Residual analysis : There are some differences according to bacterial species and pH. But it is thought that norfloxacin remains generally for about 16 days in fish bodies (Table 2).

Antimicrobial sensitivity test and MIC assay : All bacterial strains used in this study showed high sensitivity to norfloxacin (Table 3). The diameters of each zone tended to increase as the concentration of norfloxacin increased, but sufficient antibacterial efficacy was recognized at 1µg/disc. The MICs of norfloxacin with five bacterial strains were

Table 2. Zone diameters of antimicrobial inhibition after norfloxacin treatment for 40 days by EEC 4-plate method in eel

Days after treatment	Zone diameter(ϕ ; mm)					
	<i>B. subtilis</i>			<i>E. tarda</i>		
	pH 6.0	pH 7.2	pH 8.0	pH 6.0	pH 7.2	pH 8.0
1	20	24	26	40	43	40
2	20	27	25	40	40	43
3	20	23	28	32	35	41
4	19	20	24	30	33	37
5	18	20	24	28	29	36
6	17	18	20	19	25	32
7	16	17	20	18	24	29
8	16	15	19	16	19	25
9	16	18	19	14	18	28
10	16	18	19	14	17	22
11	15	17	18	15	16	22
12	13	17	16	12	14	18
13	13	16	14	12	13	16
14	12	13	12	*	12	15
15	*	12	12	*	12	13
16	*	12	*	*	*	12
17	*	*	*	*	*	*
18	*	*	*	*	*	*
19	*	*	*	*	*	*
20	*	*	*	*	*	*
22	*	*	*	*	*	*
24	*	*	*	*	*	*
26	*	*	*	*	*	*
28	*	*	*	*	*	*
30	*	*	*	*	*	*
32	*	*	*	*	*	*
34	*	*	*	*	*	*
36	*	*	*	*	*	*
38	*	*	*	*	*	*
40	*	*	*	*	*	*

* : Less than 12mm (zone diameter).

Table 3. Sensitivity of fish bacterial strains to norfloxacin

Bacterial strains		Inhibitory zone diameter (mm) at a norfloxacin concentration($\mu\text{g}/\text{disc}$) of :		
		1	5	10
<i>V. anguillarum</i>	HEO926	36	42	40
<i>V. anguillarum</i>	PB 15	30	40	44
<i>A. salmonicida</i>	IIEO929	32	32	35
<i>A. salmonicida</i>	NCMB1102	44	44	48
<i>E. tarda</i>	E 22	26	30	33
<i>E. tarda</i>	HEO939	20	32	32

Table 4. Minimum inhibitory concentration of norfloxacin to fish bacterial pathogens

Strains		Mic($\mu\text{g}/\text{ml}$)
<i>A. salmonicida</i>	HEO929	0.03
<i>A. salmonicida</i>	NCMB1102	0.03
<i>E. tarda</i>	E 22	0.10
<i>E. tarda</i>	HEO939	0.10
<i>V. anguillarum</i>	HEO926	0.05
<i>V. anguillarum</i>	PB 15	0.05

between 0.03–0.1 $\mu\text{g}/\text{ml}$ (Table 4).

Laboratory challenge : Cumulative mortality in the *E tarda* -infected control group rose rapidly to 82 and 86% on day 10

Table 5. The cumulative mortality through 10 days after norfloxacin treatment in eel infected with *E tarda* HEO939

Treatment group	Number of dead/total (% mortality)	
	Trial 1	Trial 2
Non-medicated group, control	41/50(82)	43/50(86)
Norfloxacin, 10g/ton*	40/50(80)	42/50(84)
Norfloxacin, 50g/ton	28/50(56)	31/50(62)
Norfloxacin, 100g/ton	4/50(8)	5/50(10)
Norfloxacin, 200g/ton	2/50(4)	2/50(4)

* : g/ton of fish body weight.

in two trials, respectively (Table 5). Mortality in groups receiving 10 and 50mg norfloxacin/kg body weight also rose rapidly, although more slowly than the control group. Cumulative mortalities in groups receiving 100 and 200mg/kg were very similar, and they were lower than in the control group.

Field Trial : Edwardsiellosis, vibriosis and ulcer diseases that occurred in three fish farms were treated with norfloxacin (100mg/kg of fish body weight, based on laboratory trials) administered for 3 days. Final cumulative mortality on day 5 after treatment ranged from 6 to 12% in medicated fish and 24 to 38% in nonmedicated fish (Table 6). Feeding improved as the daily mortality of fish declined.

Table 6. Mortality of eel infected with bacterial pathogens and fed 100mg/kg of norfloxacin for 3 days on commercial farms

Diseases	Cumulative mortality(%) on the fifth day after treatment* ¹	
	Nonmedicated group* ²	Medicated group* ²
Ulcer disease	26	8
Vibriosis	38	12
Edwardsiellosis	24	6

*¹ : Number of replication(n) = 3.

*² : A thousand fish of each group was tested.

Discussion

Through the acute toxicity test, no histopathological lesions were observed at the recommended dosage of the test drug (100mg/kg). But, when over-dosage of the drug was diluted to water, toxicological effect and physical stimuli by the saturated sediments against gill were observed.

In residual analysis, the residual time of fish body was less than 17 days. If the zone diameter of antibacterial inhibition was about 1mm, it would be thought that the antibacterials remain below 0.1ppm in the bodies⁶. So it is desirable that the withdrawal time for mirror carp, eel and flounder must be longer than 17 days against norfloxacin.

In vitro sensitivity test showed that norfloxacin was very effective against all bacterial strains. Most antimicrobial

agents currently being used are not effective against fish bacterial pathogens because of their abuse by many aquaculturists. However, norfloxacin is thought to be very sensitive to fish bacterial pathogens¹.

And the antimicrobial agent used in this study confirmed remarkable treatment effect against many fish bacterial diseases - vibriosis, edwardsiellosis and ulcer disease. After treatment by norfloxacin the experimental group showed remarkable recovery, but the control group fish showed constant mortality.

In field trial, the recommended dosage was used by oral administration and remarkable effects were confirmed. Namely, 100g/ton of fish body weight was prescribed once a day for 3 day treatment period. But according to the species and the environmental condition, dosage needs to be readjusted¹⁰.

So we can generalize these facts as follows; The test drug is confirmed to have remarkable efficacy against most fish bacterial pathogens of cultured eel while the existing antibacterials have considerable resistance. And prominent decreased mortality in clinical treatments was observed at the recommended dosage (100mg/kg) against most bacterial diseases.

We believe that the data presented in this report and the accompanying comments support norfloxacin is an effective antibacterial for the treatment of bacterial diseases of cultured eel.

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