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Temperature-Dependent In Vitro Antimicrobial Activity of Four 4-Quinolones and Oxytetracycline against Bacteria Pathogenic to Fish

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The in vitro antimicrobial activities of oxolinic acid, flumequine, sarafloxacin, enrofloxacin, and oxytetracycline against strains of bacteria pathogenic to fish (*Aeromonas salmonicida* subsp. *salmonicida*, atypical *A. salmonicida*, *Vibrio salmonicida*, *Vibrio anguillarum*, and *Yersinia ruckeri*) were determined at two different incubation temperatures, 4 and 15°C, by a drug microdilution method. The main objective of the study was to examine the effect of incubation temperature on the in vitro activities of 4-quinolones and oxytetracycline against these bacteria. When tested against *A. salmonicida* subsp. *salmonicida*, all of the quinolones examined had MICs two- to threefold higher at 4°C than at 15°C. Similarly, 1.5- to 2-fold higher MICs were recorded for all of the quinolones except sarafloxacin at 4°C than at 15°C when the drugs were tested against *V. salmonicida*. In contrast to those of the quinolones, the MICs of oxytetracycline were two- to eightfold lower at 4°C than at 15°C against all of the bacterial species tested. Of the antimicrobial agents tested against the bacterial species included in the study, enrofloxacin was the most active and oxytetracycline was the least active. Sarafloxacin was slightly more active than flumequine and oxolinic acid, especially against oxolinic acid-resistant *A. salmonicida* subsp. *salmonicida* strains.

In recent years, the 4-quinolone antimicrobial compounds oxolinic acid and flumequine, in addition to oxytetracycline, and sulfadiazine-trimethoprim, have been the most frequently used antimicrobial agents in Norwegian aquaculture (18, 23).

Substantial development of oxolinic acid and tetracycline resistance in bacteria pathogenic to fish (2, 19, 29, 31) has led to a need for new antimicrobial drugs for control of bacterial fish diseases in aquaculture. Aminopenicillins, several sulfonamide-trimethoprim combinations, chloramphenicol analogs such as thiamphenicol and florfenicol, and potent fluoroquinolones have all been proposed and tested as potential new drug candidates (2, 3, 12, 16, 21, 24, 25, 30).

Several of the new fluoroquinolones generated during the last decade (32) show increased inhibitory (6, 15) and bactericidal (4) activities in vitro against many bacterial pathogens, compared with the old 4-quinolones, such as nalidixic acid and oxolinic acid. Studies have also revealed that the newer compounds show increased potency (2, 25), as well as more effective bactericidal activity (3, 21), against bacteria pathogenic to fish.

Along the Norwegian coastline, there are significant seasonal and geographic seawater temperature variations, from just above zero in the winter to well above 18°C in the summer at the same site. The most common bacterial fish diseases in Norway, furunculosis, vibriosis, cold water vibriosis, bacterial kidney disease, yersiniosis, and infections caused by different strains of atypical *Aeromonas salmonicida*, may all occur at a wide range of temperatures.

The general influence of temperature on the pharmacokinetic properties of drugs in fish is well known (9, 10, 11, 13). However, less attention has been paid to the possible impact of temperature on the antimicrobial susceptibilities of specific bacterial fish pathogens.

The aims of this study were to examine the influence of incubation temperature on the in vitro activities of 4-quinolones and oxytetracycline against fish-pathogenic bacteria; to compare the potencies of oxolinic acid, three different fluoroquinolones, and oxytetracycline against common bacteria pathogenic to fish; and to observe whether cross-resistance occurs between old 4-quinolones and new fluoroquinolones.

MATERIALS AND METHODS

Bacterial strains. Ninety-two bacterial strains representing five bacterial species or subspecies (*A. salmonicida* subsp. *salmonicida*, atypical *A. salmonicida*, *Vibrio salmonicida*, *Vibrio anguillarum*, and *Yersinia ruckeri*) were included in the study. The strain collection consisted of 85 clinical isolates, five type strains (*A. salmonicida* subsp. *salmonicida* ATCC 14174, *A. salmonicida* subsp. *achromogenes* NCMB 1110, *V. anguillarum* ATCC 14181, *V. salmonicida* NCMB 2262, and *Y. ruckeri* ATCC 29473), and two laboratory-derived resistant clones.

Except for eight isolates of *A. salmonicida* subsp. *salmonicida*, which were received from the Marine Laboratory, Aberdeen, Scotland, all clinical isolates were derived from diseased Norwegian farmed salmonid fish from 1985 to 1990. The strains were identified as described by Martinsen et al. (24). According to previous antimicrobial disc diffusion test results, approximately one-half of the *A. salmonicida* subsp. *salmonicida* strains had a known history of quinolone or tetracycline resistance. With reference to the MICs of oxolinic acid against *A. salmonicida* subsp. *salmonicida* obtained in this study, the *A. salmonicida* subsp. *salmonicida* strains were grouped by the criterion of Tsoumas et al. (31).

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The strains whose oxolinic acid MICs were less than 1.0 μg/ml (the cutoff MIC for oxolinic acid) were classified as oxolinic acid susceptible, and those having oxolinic acid MICs equal to or higher than 1.0 μg/ml were classified as oxolinic acid resistant. By using previously encountered susceptibility and pharmacokinetic data for enrofloxacin as presented by Tsoumas et al. (31) and Bowser et al. (13), the cutoff MIC of enrofloxacin was determined to be 0.5 μg/ml.

During these in vitro susceptibility studies, occasional growth of test strains was observed in assay wells containing antimicrobial agents at concentrations above the MICs of all of the drugs tested. Two such drug-resistant *A. salmonicida* subsp. *salmonicida* strains were selected for further studies. Each was selected spontaneously in the presence of sarafloxacin or oxytetracycline, respectively, at concentrations 4 and 16 times above the recorded MICs for the susceptible parent strains.

Antimicrobial agents. Enrofloxacin was obtained from Bayer AG, Leverkusen, Germany, and sarafloxacin was from Abbott Laboratories, North Chicago, Ill., while flumequine, oxolinic acid, and oxytetracycline, were purchased from Sigma Chemical Co., St. Louis, Mo.

Antimicrobial solutions. Antibacterial solutions were made as described by Martinsen et al. (24), by dissolving the quinolones in 0.1 N NaOH and oxytetracycline in distilled water before addition of modified Mueller-Hinton broth (24). The antimicrobial agent concentrations in the test wells before inoculation ranged from 0.0005 to 512 μg/ml.

Bacterial inocula. Single bacterial colonies obtained from pure cultures on blood agar plates (blood agar base [Difco Laboratories, Detroit, Mich.] containing 5% citrated bovine blood and 2% NaCl) incubated aerobically at 15°C for 48 h were used. Each strain was inoculated into tubes containing 10 ml of modified Mueller-Hinton broth and incubated at 15°C on a roller drum (Bellco Glass Inc., Vineland, N.J.) at 25 rotations per min. Strains were incubated aerobically as follows: *Y. ruckeri* for 24 h, *V. anguillarum* and *A. salmonicida* subsp. *salmonicida* for 48 h, and *V. salmonicida* and atypical *A. salmonicida* for 72 h. By using McFarland nephelometer standard no. 1 (22), followed by consecutive dilutions in 0.9% saline, the inoculum was diluted to approximately 5×10^5 CFU/ml before transfer to microtiter tray wells.

MIC assay. MICs were determined at 4 and 15°C by a broth microdilution method (24, 26). After inoculation, the test wells contained antimicrobial agent concentrations ranging from 256 to 0.00025 μg/ml, and the final bacterial test inoculum was 2.5×10^5 CFU/ml in a total volume of 100 μl. The microtiter trays were sealed with transparent plastic covers and incubated aerobically for 6 days. Four identical trays were made for each antimicrobial agent, of which two were incubated at 4°C and two were incubated at 15°C.

Before the MICs were read, the trays were shaken at 350 rotations per min for 8 min on a shaker (IKA Schüller MTS 4; Janke & Kunkel GmbH & Co., IKA Labortechnik, Staufen, Germany), followed by centrifugation (Heraeus Digifuge; Heraeus-Christ GmbH, Osterode, Germany) at $1,200 \times g$ for 10 min. Each MIC was determined as the lowest antimicrobial agent concentration which inhibited visible bacterial growth.

Statistical analyses. The Wilcoxon signed-rank test (8) was used for statistical comparison of MICs at the two incubation temperatures and between different antimicrobial agents. The level of significance used was $\alpha = 0.05$.

RESULTS

The in vitro antimicrobial activities of enrofloxacin, sarafloxacin, flumequine, oxolinic acid, and oxytetracycline against 92 bacterial strains at 15 and 4°C are shown in Table 1. Individual MICs are the means of replicate results done twice for each temperature. At both temperatures, enrofloxacin was the most active agent ($P < 0.001$) against all of the bacterial species included in the study, whereas oxytetracycline was the least active ($P < 0.001$). Generally, sarafloxacin was slightly more active than flumequine and oxolinic acid ($P = 0.005$), especially against oxolinic acid-resistant strains of *A. salmonicida* subsp. *salmonicida* ($P < 0.001$).

When tested against *A. salmonicida* subsp. *salmonicida*, all of the quinolones had two- to threefold higher MICs at 4°C than at 15°C. These temperature-dependent differences in antimicrobial activity were statistically significant ($P < 0.001$). Likewise, statistically significant 1.5- to 2-fold higher MICs were obtained at 4°C than at 15°C for all of the quinolones except sarafloxacin when the drugs were tested against *V. salmonicida* (for enrofloxacin, $P = 0.014$; for flumequine, $P = 0.041$; for oxolinic acid, $P = 0.008$). There were no statistically significant differences in the MICs of the four quinolones at the two incubation temperatures against atypical *A. salmonicida*, *V. anguillarum*, and *Y. ruckeri*. However, against *Y. ruckeri*, an analogous temperature dependency was noticed.

In contrast to those of the quinolones, the MICs of oxytetracycline were much lower at 4°C than at 15°C. The in vitro antimicrobial activity of oxytetracycline showed a statistically significant two- to eightfold increase at 4°C compared with 15°C against all of the bacterial species tested (for *A. salmonicida* subsp. *salmonicida*, $P < 0.001$; for atypical *A. salmonicida*, $P = 0.008$; for *V. anguillarum*, $P = 0.002$; for *V. salmonicida*, $P = 0.006$; for *Y. ruckeri*, $P = 0.016$). The most oxytetracycline-susceptible strains, irrespective of bacterial species, demonstrated the greatest temperature dependency.

Figure 1 shows the MIC ranges of enrofloxacin, sarafloxacin, flumequine, oxolinic acid, and oxytetracycline against oxolinic acid-resistant and oxolinic acid-susceptible strains of *A. salmonicida* subsp. *salmonicida* at 15 and 4°C. According to the criteria proposed by Tsoumas et al. (31), there were 22 oxolinic acid-resistant and 22 oxolinic acid-susceptible strains. All of the strains resistant to oxolinic acid demonstrated reduced susceptibility to the other quinolones tested. Four of these strains were also highly resistant to oxytetracycline.

Enrofloxacin and, to a lesser extent, sarafloxacin were more active than oxolinic acid and flumequine against oxolinic acid-resistant strains. According to the proposed enrofloxacin cutoff MIC (0.5 μg/ml), 31 (70.5%) and 38 (86.4%) of the *A. salmonicida* subsp. *salmonicida* strains were susceptible to enrofloxacin at 4 and 15°C, respectively. Enrofloxacin was also the most active drug against oxolinic acid-susceptible strains. The differences in activity among quinolones were most pronounced against oxolinic acid-resistant strains. Furthermore, oxytetracyclin resistance was observed independently of quinolone resistance. Figure 1 also shows the dispersion of the MICs of all of the antimicrobial agents tested against *A. salmonicida* subsp. *salmonicida* at 15 and 4°C. A distinct separation between MICs obtained against oxolinic acid-susceptible and oxolinic acid-resistant strains was demonstrated for all of the quinolones tested, since no overlap of the MICs of the different quinolones was observed.

TABLE 1. Comparative in vitro activities of enrofloxacin, sarafloxacin, flumequine, oxolinic acid, and oxytetracycline at 15 and 4°C against bacteria pathogenic to fish

Microorganism (no. of strains) and antimicrobial agent	MIC (μg/ml)								P value (4 vs 15°C or 15 vs 4°C)[a]
	Range		For 50% of strains		For 90% of strains		Mean		
	15°C	4°C	15°C	4°C	15°C	4°C	15°C	4°C	
A. salmonicida subsp. *salmonicida* (44)									
Enrofloxacin	0.005–0.80	0.006–1.28	0.05	0.06	0.60	0.93	0.19	0.34	<0.001
Sarafloxacin	0.005–4.05	0.02–5.12	0.10	0.22	2.99	4.38	0.92	1.33	<0.001
Flumequine	0.02–4.80	0.05–8.00	0.27	0.32	2.40	5.12	0.96	2.00	<0.001
Oxolinic acid	0.01–4.48	0.04–12.80	0.06	0.16	2.88	6.40	1.07	2.62	<0.001
Oxytetracycline	0.40–>256.00	0.08–192.00	0.85	0.15	64.00	28.80	17.72	9.63	<0.001
Atypical *A. salmonicida* (8)									
Enrofloxacin	0.005–0.08	0.005–0.06	0.006	0.01	0.08	0.06	0.02	0.02	NS[b]
Sarafloxacin	0.009–0.32	0.009–0.19	0.02	0.02	0.32	0.19	0.05	0.04	NS
Flumequine	0.02–1.71	0.02–1.28	0.04	0.04	1.71	1.28	0.25	0.20	NS
Oxolinic acid	0.01–1.44	0.01–1.92	0.03	0.04	1.44	1.92	0.23	0.27	NS
Oxytetracycline	0.58–85.30	0.40–42.70	1.38	0.80	85.30	42.70	19.63	9.86	0.008
V. anguillarum (11)									
Enrofloxacin	0.001–0.08	0.001–0.06	0.005	0.008	0.06	0.04	0.02	0.02	NS
Sarafloxacin	0.008–0.16	0.009–0.16	0.03	0.02	0.16	0.08	0.05	0.04	NS
Flumequine	0.01–0.18	0.005–0.12	0.04	0.02	0.16	0.08	0.06	0.04	NS
Oxolinic acid	0.005–0.09	0.005–0.08	0.02	0.01	0.08	0.08	0.04	0.03	NS
Oxytetracycline	0.73–48.00	0.15–48.00	2.20	0.80	4.00	2.00	6.36	4.99	0.002
V. salmonicida (21)									
Enrofloxacin	0.005–0.10	0.005–0.16	0.04	0.05	0.08	0.09	0.05	0.06	0.014
Sarafloxacin	0.005–0.20	0.01–0.24	0.16	0.16	0.17	0.16	0.13	0.14	NS
Flumequine	0.01–0.32	0.01–0.48	0.08	0.08	0.24	0.36	0.12	0.15	0.041
Oxolinic acid	0.005–0.36	0.008–0.32	0.07	0.08	0.16	0.24	0.09	0.12	0.008
Oxytetracycline	0.90–64.00	0.15–64.00	1.70	0.50	48.00	48.00	12.83	10.95	0.006
Y. ruckeri (8)									
Enrofloxacin	0.01–0.03	0.005–0.02	0.02	0.01	0.03	0.02	0.02	0.01	NS
Sarafloxacin	0.02–0.05	0.02–0.06	0.02	0.03	0.05	0.06	0.03	0.04	NS
Flumequine	0.08–0.18	0.12–0.24	0.14	0.20	0.18	0.24	0.14	0.19	NS
Oxolinic acid	0.06–0.11	0.04–0.12	0.08	0.08	0.11	0.12	0.08	0.09	NS
Oxytetracycline	16.00–>256.00	9.20–>256.00	30.70	14.70	>256.00	>256.00	62.74	45.83	0.016

[a] Quinolones: higher MICs at 4°C than at 15°C. Oxytetracycline: higher MICs at 15°C than at 4°C.
[b] NS, not significant.

Table 2 shows the in vitro antimicrobial susceptibilities of the two laboratory-derived resistant clones of *A. salmonicida* subsp. *salmonicida* compared with those of the parent strains. The MICs of the quinolones against the sarafloxacin-induced resistant clone were 33 to 256 times higher than those against the parent strain, while there were no such changes in the MICs of oxytetracycline against that clone. Likewise, there was no change in the quinolone susceptibility of the oxytetracycline-induced resistant clone, while the MICs of oxytetracycline were 135 and 1,280 times higher at 15 and 4°C, respectively.

DISCUSSION

The finding of higher MICs at 4°C than at 15°C when the quinolones were tested against a panel of bacteria pathogenic to fish is in accordance with previous reports (2, 24). Barnes et al. (2) reported lower activity in vitro for six different quinolones, including enrofloxacin, sarafloxacin, and oxolinic acid, at 10°C than at 22°C against strains of *A. salmonicida*. Martinsen et al. (24) observed higher sarafloxacin MICs at 4°C than at 15°C against strains of *A. salmonicida* subsp. *salmonicida* and *Y. ruckeri*.

Higher MICs at 4°C than at 15°C could be explained by decreased drug diffusion into bacterial cells at lower temperatures (7, 17) and diminished drug availability at the site of action. An additional explanation is that the growth rate of these organisms is low at 4°C, making them less susceptible to the DNA gyrase-inhibitory activity of quinolones. A more hypothetical explanation could be that the affinity of the quinolones for the DNA gyrase complex is decreased at lower temperatures, resulting in less efficient inhibition of DNA synthesis (14, 27). In these cases, elevated levels of quinolone antimicrobial agents would be required for bacterial growth inhibition at low temperatures.

Lower MICs of oxytetracycline were obtained at 4°C than at 15°C against all of the bacterial species tested. Recently, corresponding results have been observed for tetracycline against *V. salmonicida* and for trimethoprim and sulfonamides against *A. salmonicida*, *V. anguillarum*, *V. salmonicida*, and *Y. ruckeri* (28). Lower MICs as a response to decreased incubation temperatures can hardly be explained

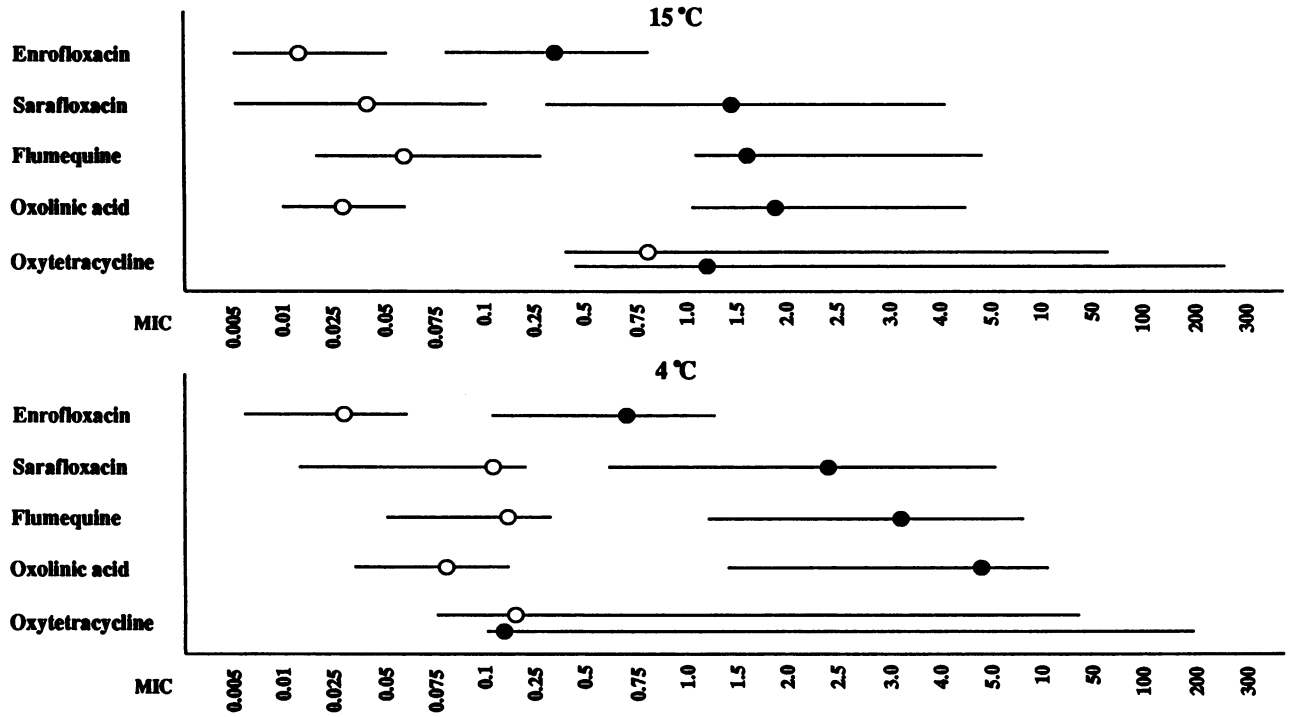

FIG. 1. MIC ranges (micrograms per milliliter) of enrofloxacin, sarafloxacin, flumequine, oxolinic acid, and oxytetracycline for oxolinic acid-susceptible (○) and -resistant (●) strains of *A. salmonicida* subsp. *salmonicida* at 15 and 4°C. Open circles indicate the MICs which inhibited 50% of the oxolinic acid-susceptible strains tested, and filled circles indicate the MICs which inhibited 50% of the oxolinic acid-resistant strains tested.

by membrane permeability variations. However, alterations in the energy-dependent active transport mechanisms for tetracyclines in bacterial membranes, or a temperature-dependent mode of action at the bacterial ribosome, could be possible explanations for the increased oxytetracycline susceptibility found at lower temperatures. These theoretical explanations require further investigation.

In fish, the bioavailability and plasma drug levels of quinolones and oxytetracycline are reduced by low temperatures (9, 11, 13) and their therapeutic effects may, in turn, also be reduced. Regarding the quinolones, one might assume that the elevated MICs at low temperatures could further impair their clinical efficacy. On the other hand, the lower MICs of oxytetracycline could partly compensate for the reduced bioavailability at low temperatures, so that the outcome of oxytetracycline treatment might be less influenced by low water temperatures. Whether these theoretical considerations are of clinical relevance remains to be clarified.

On the basis of the criteria proposed by Tsoumas et al. (31), 22 strains of *A. salmonicida* subsp. *salmonicida* were resistant to oxolinic acid whereas only four strains were resistant to oxytetracycline. This difference probably reflects the abundant use of oxolinic acid for treatment of furunculosis in Norwegian aquaculture in recent years (18, 23), resulting in increased selection for oxolinic acid resistance (20).

Complete cross-resistance between quinolones has previously been demonstrated for various bacterial species (2, 5, 31). This was confirmed in the present study (Fig. 1) for the four 4-quinolones examined, as tested against strains of *A. salmonicida* subsp. *salmonicida*. However, the cross-resis-

TABLE 2. In vitro antimicrobial susceptibilities of two laboratory-derived resistant clones of *A. salmonicida* subsp. *salmonicida* compared with those of the parent strains

Drug[a] and strain	MIC (μg/ml) of:									
	Enrofloxacin		Sarafloxacin		Flumequine		Oxolinic acid		Oxytetracycline	
	4°C	15°C	4°C	15°C	4°C	15°C	4°C	15°C	4°C	15°C
Sarafloxacin										
3708/90	0.02	0.01	0.08	0.03	0.11	0.05	0.05	0.06	0.12	0.95
3708/90 clone	0.80	0.80	3.20	3.20	8.00	3.20	12.80	3.20	0.10	1.20
Oxytetracycline										
3895/90	0.70	0.30	2.40	1.80	4.27	1.60	4.00	2.00	0.15	1.90
3895/90 clone	0.80	0.60	2.40	1.60	2.80	2.40	4.80	2.40	192.00	256.00

[a] Used to induce resistance.

tance between the old 4-quinolones, such as oxolinic acid, and the new and more potent fluoroquinolones, such as enrofloxacin and sarafloxacin, might not be of clinical significance. Clinical resistance depends on the MIC for the bacterial strain together with the pharmacokinetic profile of the drug in the fish species treated. Consequently, strains showing decreased in vitro quinolone susceptibility might nevertheless be clinically susceptible to potent fluoroquinolones, as shown in Results for enrofloxacin against *A. salmonicida* subsp. *salmonicida*. However, more pharmacokinetic information is needed to determine whether the quinolone cross-resistance observed in our study is of clinical relevance for other fluoroquinolones.

Among bacteria pathogenic to fish, the frequency of spontaneous mutations leading to in vitro resistance against fluoroquinolones and other 4-quinolones is considered to be low (25, 30). In the present study, selection of resistant clones was noted in vitro for all five antimicrobial agents at concentrations of up to 16 times the MIC for the susceptible parent strain. Taking these observations into account, one might anticipate that therapeutic drug concentrations in plasma and tissue around 4 to 10 times the MIC are insufficient if selection of resistant mutants is to be minimized. During clinical treatment of susceptible strains, it would be desirable to have drug levels in plasma and tissue higher than the MICs expected for mutant strains to prevent their occurrence (1).

Further investigations are required to determine which quinolones are the most suitable for use in aquaculture. Various in vitro studies, including the present one, conclude that the new fluoroquinolones possess some advantages over the old 4-quinolones, such as oxolinic acid. It has been demonstrated that the fluoroquinolones are more potent (2, 3, 25), have lower resistance mutation frequencies (25, 30), and exhibit better in vitro bactericidal activity (3, 21) against bacterial species pathogenic to fish than do the old 4-quinolones. Only preliminary pharmacokinetic and efficacy studies with fish are available for the fluoroquinolones (11, 13, 16). Therefore, further pharmacokinetic and efficacy studies for each fluoroquinolone in both freshwater and marine environments at different temperatures are essential to evaluate the potential use of these substances in the treatment of bacterial diseases of fish.

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