

Antibacterial Effect of Chloramphenicol, Thiamphenicol and Florfenicol against Aquatic Animal Bacteria

Shu-Peng HO, Tain-Yao HSU, Ming-Hui CHEN¹⁾ and Way-Shyan WANG*

Department of Veterinary Medicine, National Chung-Hsing University, and ¹⁾The Livestock Disease Control Center of Taichung City, Taichung, 402, Taiwan

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ABSTRACT. The minimum inhibitory concentration (MIC) was measured to evaluate the antibacterial activities of chloramphenicol (CP), thiamphenicol (TP) and florfenicol (FFC) against the aquatic bacterial isolates from soft-shell turtles, fish and shellfish. Amoxicillin (AMPC), oxytetracycline (OTC) and oxolinic acid (OA) were included to compare with above protein synthesis inhibitors. The results showed that the order of MIC range of the isolates from soft-shell turtles for tested drugs was OA>FFC, CP>TP>AMPC, OTC. The percentage of the resistant strains indicated that OA was the lowest (7.14%) and OTC was the highest (85.07%). The order of antibacterial activity against the isolates from fish was OA>FFC>CP >AMPC>OTC>TP. The percentage of the resistant strains revealed that OA (13.64%) and OTC (80.91%) were the lowest and the highest, respectively. For the isolates from shellfish, the order of antimicrobial activity was OA>CP, FFC>AMPC, OTC, TP. TP showed the greatest percentage of the resistant strains (58.7%), but that of OA was the lowest (4.35%). The most common resistant patterns of the isolates from turtles, fish and shellfish were AMPC-OTC, CP-TP-AMPC-OTC, and FFC-CP-TP-AMPC-OTC, respectively. There were partially-complete resistance of the resistant isolates among CP, TP and FFC. The findings indicated that previous treatment might affect the choice of drug to use for aquatic bacterial diseases.—KEY WORDS: antimicrobial susceptibility, aquatic bacteria, bacterial resistance, minimum inhibitory concentration.

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The use of antibacterial compounds in fisheries was a highly regarded issue because of the possibility of tissue deposition and development of the resistant feature of the bacteria [5, 14]. Chloramphenicol (CP), a broad-spectrum antimicrobial agent, was used widely in fisheries of Taiwan to control bacterial diseases. However, CP was banned for use in food-producing animals in USA and Europe because of its side effect related to aplastic anemia in human [23]. Besides, its bitterness also limited its clinical use by oral administration. After then, thiamphenicol (TP) was developed. In the investigative report by Yunis and Gross [22], it showed that TP had not been linked to aplastic anemia, as seen in CP. But, the antibacterial potency of TP was less than that of CP and this had limited its use [16, 20]. Florfenicol (FFC), a fluorinated derivative of TP, had broad antibacterial spectrum similar to CP and stronger than TP *in vitro*. Moreover, FFC showed activity against CP-resistant strains, such as *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Salmonella* Typhimurium and *Staphylococcus aureus* [16]. In fact, with respect to the pharmacokinetics and broad spectrum of activity [1], FFC could be a good antimicrobial agent to replace CP which is limited by its toxicity. In this study, the susceptibility of aquatic animal bacteria in Taiwan to CP, FFC and TP in comparison with amoxicillin (AMPC), oxolinic acid (OA) and oxytetracycline (OTC), was investigated. The results can reveal the resistant models of bacteria and provide information for owners of fish farm to control the bacterial

diseases.

MATERIALS AND METHODS

Bacterial strains: All of the bacterial strains were isolated from the disease case or water of aquaculture, mainly in the central area in Taiwan from 1995 to 1998. The identification of isolates was performed by the methods described in "Bacterial Fish Pathogens" [4]. The number of strains isolated from soft-shell turtles including 30 *Aeromonas hydrophila*, 11 *Edwardsiella tarda*, 16 *Klebsiella* spp., 30 *Pseudomonas fluorescens*, 6 *Salmonella* spp. and 5 *Vibrio cholerae*. The strains isolated from fish including 11 *Aeromonas hydrophila*, 65 *Edwardsiella tarda*, 2 *Escherichia coli*, 2 *Flavobacterium ferrugineum*, 2 *Flexibacter* spp., 5 *Klebsiella* spp., 1 *Proteus* spp., 2 *Pseudomonas fluorescences*, 1 *Staphylococcus* spp., 12 *Streptococcus* spp. and 7 *Vibrio anguillarum*. The strains isolated from shellfish were all vibrio, including 23 *Vibrio anguillarum*, 2 *Vibrio alginolyticus*, 2 *Vibrio cholerae*, 3 *Vibrio damsela*, 4 *Vibrio harveyi*, 4 *Vibrio parahaemolyticus*, 1 *Vibrio salmonicida*, 3 *Vibrio tubiashii* and 4 *Vibrio vulnificus* strains.

All bacterial strains were stored in PROTECT, a product of bacterial preservers (Technical Service Consultants Ltd., Bury, Lancs, UK). Before each experiment for minimum inhibitory concentration (MIC), a single treated bead with stored bacterial strain was transferred to trypticase soy broth (TSB, Difco Laboratories, Detroit, MI, U.S.A.) and incubated at 25°C for 24 hr to reach the log growth phase of the isolates.

Antimicrobial agents: The tested antimicrobials included

* CORRESPONDENCE TO: WANG, W. S., Department of Veterinary Medicine, National Chung-Hsing University, 250 Kuo Kuang Rd., Taichung, 402, Taiwan.

chloramphenicol, thiamphenicol (Sigma Chemical Co., St. Louis, MO., U.S.A.) and florfenicol (Takeda Chemical Ind., Ltd., Tokyo, Japan). Amoxicillin, oxolinic acid and oxytetracycline (Sigma) were used for comparing with the above chemicals.

Minimum inhibitory concentrations (MICs): An agar dilution method [6] was used in this study. Distilled water was used for preparing the antibiotic standards, except oxolinic acid which was dissolved in 1 N NaOH. The sterile Mueller-Hinton Agar (Difco) was used as sensitivity test medium. A serial two-fold dilution was processed with distilled water, giving a series of tested concentrations from 100 µg/ml to 0.00195 µg/ml. The inoculum was 10⁵–10⁶ CFU/ml logarithmic organisms and incubated at 25°C for 24 hr. Plates without antimicrobial agents were incubated as controls. (After the incubation period, the MIC for each strain was recorded as the lowest concentration of antimicrobial agent which completely inhibited the growth.) The MICs of the antimicrobial agents were analyzed with the Duncan's multiple range test by the bacterial species of the isolates [18]. In addition, the MIC values for isolates from the same source against the different drugs were compared by Wilcoxon signed-rank test [7] or T-test (Least significant difference) [18]. The isolate was considered to be resistant to antimicrobials if the value of MIC was over 25 µg/ml.

RESULTS

The values of MICs for different species of isolates (n=12) against six antimicrobials are presented in Table 1. The MIC₅₀ and MIC₉₀ were minimum concentration of antimicrobials required to inhibit 50% and 90% of tested isolates, respectively. The data showed that OA was the most potent drug for the isolates tested. The MIC₅₀ values of OA for most isolates were less than 2 µg/ml; except for *Streptococcus* spp. (25 µg/ml). The MIC₅₀ of CP and FFC for most isolates were less than 25 µg/ml, except for *E. tarda* (50 µg/ml). For species not shown in Table 1, these six antimicrobials were effective against 2 *E. coli* isolates (MIC₅₀ 3.125 µg/ml). For *Salmonella* spp. (n=6), the MIC_{50s} of OA, CP, FFC, TP, AMPC and OTC were 3.125, 25, >100, >100, >100 and >100 µg/ml, respectively. The MICs of these six antimicrobials against *Proteus* spp. (n=1) were 25 µg/ml. The MICs of CP, FFC and TP against *Flavobacterium* spp. (n=2), *Flexibacter* spp (n=2), and *Staphylococcus* spp (n=1) were 3.125- >100, 0.78- >100, 3.125- >100 µg/ml respectively.

For *A. hydrophila* (n=41), the analyses of ranges of MICs among CP, FFC, OA and TP was not significant different by T test (p<0.05). The order of MIC values for *E. tarda* (n=76) was OA, FFC>CP>AMPC, OTC>TP (p<0.05). In fact, *E. tarda* was not susceptible to TP (MIC_{50,90} > 100 µg/ml). For *P. fluorescences* (n=32) and *Vibrio* spp. (n=58), the antimicrobial activity of OA, CP and FFC was better than that of TP (p<0.05). The MIC ranges of TP was significantly different between *A. hydrophila* and *E. tarda*

(Duncan's multiple range test p<0.05). The MIC ranges of OA, CP, FFC, AMPC and OTC were not significantly different among these six species. It showed that OA, CP and FFC were effective for these six species, but AMPC and OTC were not effective. In fact, the MIC_{50,90} of AMPC and OTC were greater than 50 µg/ml for *A. hydrophila*, *E. tarda*, and *Klebsiella* spp.

The MIC ranges of three different sources of isolates to six antimicrobial agents are demonstrated in Tables 2–4. The analysis with the Duncan's multiple-range test indicated that there were no significant difference in the MIC distribution of CP and FFC among the three sources of bacteria, but the isolates from fish and shellfish were more sensitive to AMPC and OTC than isolates from soft-shell turtles. TP was more effective against the isolates from soft-shell turtles and shellfish than those from fish (p<0.05). The isolates of shellfish were more sensitive to OA than the isolates of other 2 sources (p<0.05).

In the study of antimicrobial sensitivity of 98 isolates of the cultured soft-shell turtles, the MIC₅₀ for OA, FFC, CP, TP, AMPC and OTC was 0.78, 3.125, 3.125, 25, >100, and >100 µg/ml, respectively (Table 2). The result of the activity was ordered as OA>FFC, CP>TP>AMPC, OTC. The MIC₅₀ for OA, FFC, CP, TP, AMPC and OTC against the 110 isolates from fish was 0.39, 3.125, 12.5, 100, 50, and 50 µg/ml, respectively (Table 3). The order of antibacterial sensitivity of fish isolates was OA>FFC>CP>AMPC>OTC >TP. The value of MIC₅₀ of OA, CP, FFC, TP, AMPC and OTC against the 46 isolates from shellfish was 1.563, 3.125, 12.5, 12.5, 25, and 50 µg/ml, respectively (Table 4). The order of MIC ranges for shellfish was OA>CP>FFC> AMPC, OTC, TP (p<0.05).

The antimicrobial sensitivity of all isolates against six antimicrobials was shown in Table 5. OA had the greatest antibacterial activity among these six drugs (MIC₅₀=0.78 µg/ml). The CP and FFC showed the same MIC₅₀ (3.125 µg/ml). AMPC, OTC and TP showed the low activity (MIC_{50s} > 50 µg/ml). Moreover, the distribution of MIC values for all of the isolates (n=254) against six drugs was demonstrated as histograms in Fig. 1. The charts of AMPC, OTC and TP showed the tendency of drug-resistant development (more MIC values > 25 µg/ml). Although the CP and FFC had same MIC₅₀, there were more isolates' MIC values greater than 25 µg/ml for CP. In fact, FFC-resistant isolates (17%) were less than those of CP (38%).

The percentage of the drug-resistant strains from soft-shell turtles against OTC, AMPC, TP, CP, FFC and OA was 85.07%, 80.61%, 58.16%, 27.55%, 20.41% and 7.14%, respectively (Table 2). The percentage of the drug-resistant strains from fish against OTC, AMPC, TP, CP, FFC and OA was 80.91%, 56.36%, 72.73%, 48.18%, 14.55% and 13.64%, respectively (Table 3). The percentage of the drug-resistant strains from shellfish against OTC, AMPC, TP, CP, FFC and OA was 52.17%, 45.65%, 58.7%, 34.78%, 13.04% and 4.35%, respectively (Table 4). These results indicated that these isolates of aquatic animal bacteria were most sensitive to OA. The percentage of the resistant

Table 1. Antimicrobial susceptibility of different species of isolates to six antimicrobial agents

Species (# of species)	Range of minimum inhibitory concentration ($\mu\text{g/ml}$) (MIC ₅₀ , MIC ₉₀)					
	Chloramphenicol	Florfenicol	Thiamphenicol	Amoxicillin	Oxolinic acid	Oxytetracycline
<i>Aeromonas hydrophila</i> (n=41)	0.39- >100 ^a (0.78, 50)	0.78- >100 ^a (0.78, 12.5)	^A 0.39- > 100 ^a (1.56, 50)	0.39- >100 ^b (>100, >100)	0.05- >100 ^a (0.39, 6.25)	1.56- >100 ^b (>100, >100)
<i>Edwardsiella tarda</i> (n=76)	0.78- >100 ^b (50, >100)	0.2- >100 ^a (6.25, 25)	^B 3.125- >100 ^d (>100, >100)	0.39- >100 ^c (>100, >100)	0.05-25 ^a (0.39, 6.25)	6.25- >100 ^c (100, >100)
<i>Klebsiella</i> spp. (n=21)	0.78-100 ^a (12.5, 100)	0.78-25 ^a (12.5, 25)	^{AB} 0.78- >100 ^b (100, >100)	0.2- >100 ^c (>100, >100)	0.05-100 ^a (1.56, 100)	25- >100 ^c (>100, >100)
<i>Pseudomonas fluorescences</i> (n=32)	0.78-100 ^a (3.125, 50)	0.78- >100 ^a (1.56, 25)	^{AB} 0.78- >100 ^b (100, >100)	1.56- >100 ^b (12.5, >100)	0.39-100 ^a (0.39, 12.5)	12.5- >100 ^b (50, >100)
<i>Streptococcus</i> spp. (n=12)	0.78- >100 ^a (3.125, 6.25)	0.78- >100 ^a (1.56, 12.5)	^{AB} 6.25- >100 ^{ab} (12.5, 100)	1.56- >100 ^{bc} (12.5, >100)	0.1- >100 ^{ab} (25, 100)	25- >100 ^c (>100, >100)
<i>Vibrio</i> spp. (n=58)	0.78-100 ^a (3.125, 25)	0.78-50 ^a (3.125, 50)	^{AB} 0.78- >100 ^b (12.5, 100)	0.2- >100 ^c (25, >100)	0.025-50 ^a (1.56, 6.25)	3.125- >100 ^{bc} (25, >100)

Different capital letters showed the values of MIC among different species of isolates were significantly different ($p < 0.05$) (Duncan's multiple range test).

Different small letters showed the values of MIC of different kind of antimicrobials against the species were significantly different ($p < 0.05$) (T test).

MIC₅₀: the minimal concentration that could inhibit 50% of tested isolates.

MIC₉₀: the minimal concentration that could inhibit 90% of tested isolates.

Table 2. Antimicrobial susceptibility of 98 of soft-shell turtle isolates to six antimicrobials

Antimicrobial agents	Range of MIC ($\mu\text{g/ml}$)	MIC ₅₀ ($\mu\text{g/ml}$)	MIC ₉₀ ($\mu\text{g/ml}$)	No. of antibiotic-resistant strains (%)
Chloramphenicol	0.39- >100 ^b	3.125	100	27 (27.55)
Florfenicol	0.78- >100 ^b	3.125	25	20 (20.41)
Thiamphenicol	0.39- >100 ^c	25	>100	57 (58.16)
Amoxicillin	1.56- >100 ^d	>100	>100	79 (80.61)
Oxolinic acid	0.025-100 ^a	0.78	6.25	7 (7.14)
Oxytetracycline	1.56- >100 ^d	>100	>100	84 (85.07)

Different letters showed the values of MIC between two groups were significantly different (Wilcoxon signed rank test), $p < 0.05$.

MIC for isolate over 25 $\mu\text{g/ml}$ considered to be drug-resistant.

strains against OTC was the highest (Table 5). In addition, the most common resistant patterns of the isolates were AMPC-OTC (n=32), AMPC-OTC-TP (n=39) and AMPC-CP-OTC-TP (n=37) (Table 6). Investigating the resistant patterns of all aquatic animal bacteria, there were partially-complete resistance of the resistant isolates among CP, TP and FFC.

DISCUSSION

In comparing MICs of the six antimicrobials against all isolates, OA had the lowest MIC₅₀ values and percentage of the antibiotic-resistant strains. The low incidence of drug-

resistance could be contributed to its mechanism. The drug-resistance usually occurred because of the change of the DNA gyrase A subunit genes that mutated rarely (less than 10^{-9}) [10]. In addition, it was reported that the quinolone-resistant strains did not appear by way of transduction of R-plasmid [2]. Therefore, the OA-resistant strains appeared slowly and infrequently.

The percentage of antibiotic-resistant strains against OTC, TP and AMPC was very high. The high frequency of resistance to OTC may be associated with long term high dosage use. Although AMPC was used widely in veterinary and human medicine, it was recently introduced for therapeutic use in aquatic animals in Taiwan. The

Table 3. Antimicrobial susceptibility of 110 fish isolates to six antimicrobial agents

Antimicrobial agents	Range of MIC ($\mu\text{g/ml}$)	MIC ₅₀ ($\mu\text{g/ml}$)	MIC ₉₀ ($\mu\text{g/ml}$)	No. of antibiotic-resistant strains (%)
Chloramphenicol	0.2- >100 ^c	12.5	100	53 (48.18)
Florfenicol	0.2- >100 ^b	3.125	25	16 (14.55)
Thiamphenicol	0.78- >100 ^e	100	>100	80 (72.33)
Amoxicillin	0.1- >100 ^d	50	>100	62 (56.36)
Oxolinic acid	0.05- >100 ^a	0.39	50	15 (13.64)
Oxytetracycline	3.125- >100 ^{de}	50	>100	89 (80.91)

Different letters showed the values of MIC between two groups were significantly different (Wilcoxon signed rank test), $p < 0.05$.

Table 4. Antimicrobial susceptibility of 46 shellfish isolates to six antimicrobial agents

Antimicrobial agents	Range of MIC ($\mu\text{g/ml}$)	MIC ₅₀ ($\mu\text{g/ml}$)	MIC ₉₀ ($\mu\text{g/ml}$)	No. of antibiotic-resistant strains (%)
Chloramphenicol	0.78- 100 ^b	3.125	50	16 (34.78)
Florfenicol	0.78- 25 ^b	12.5	25	6 (13.04)
Thiamphenicol	1.56- >100 ^c	50	100	27 (58.70)
Amoxicillin	0.2- >100 ^c	12.5	>100	21 (45.65)
Oxolinic acid	0.025- >50 ^a	1.56	6.25	2 (4.35)
Oxytetracycline	3.125- >100 ^e	25	>100	24 (52.17)

Different letters showed the values of MIC between two groups were significantly different (Wilcoxon signed rank test), $p < 0.05$.

Table 5. Antimicrobial susceptibility of 254 aquatic isolates to six antimicrobial agents

Antimicrobial agents	Range of MIC ($\mu\text{g/ml}$)	MIC ₅₀ ($\mu\text{g/ml}$)	MIC ₉₀ ($\mu\text{g/ml}$)	No. of antibiotic-resistant strains (%)
Chloramphenicol	0.2- >100	3.125	25	96 (38)
Florfenicol	0.2- >100	3.125	25	42 (17)
Thiamphenicol	0.39- >100	50	>100	164 (65)
Amoxicillin	0.1- >100	100	>100	162 (64)
Oxolinic acid	0.025- >100	0.78	12.5	24 (9)
Oxytetracycline	1.56- >100	100	>100	197 (78)

AMPC-resistance of aquatic bacteria could be explained with interspecies transfer of resistant factors between resistant strains and sensitive strains [3, 9]. The drug resistance may cause the failure of disease control in fish farming.

The CP, TP and FFC inhibited the growth of the bacterial strains by means of inhibiting peptidyl transferase activity, specifically on 70 S ribosomes [19]. Among this structure group, FFC had the best antibacterial effect. The antimicrobial activity of TP was the lowest. This intrinsic factor may result in the high frequency of TP-resistance. In fact, it was reported that the strains resisted to CP and TP, in contrast, were susceptible to FFC [8]. The fluoride contained in FFC may have elevated the antibacterial activity against the pathogens [8, 13, 15, 21]. The range of MICs of FFC reported by Inglis and Richards [13] was 0.3–2.5 $\mu\text{g/ml}$. However, it was 2–8 $\mu\text{g/ml}$, even > 16 $\mu\text{g/ml}$ against *P. aeruginosa* as reported by Marshall *et al.* [15]. In this study, the strains isolated from aquatic animals in

Taiwan were less susceptible to FFC (MICs: 0.2–100 $\mu\text{g/ml}$). The wider range of FFC MICs may result from the expansive use of CP in Taiwan since CP-resistant isolates might be also less susceptible to FFC.

Ellis *et al.* [11] suggested that the resistance of bacteria to CP and TP was due to acetyltransferase type III (CAT_{III}). Products of CAT_{III}, the monoacetyl and diacetyl-CP that had low affinity for the peptidyltransferase, were not the inhibitors of protein synthesis [11]. It was reported that CP-resistant bacteria could express the *cat* gene was because CP stalled the ribosome, allowing the activation of its translation [12]. In bacteria not expressing CAT_{III}, the resistance mechanism was probably due to a barrier to drug permeability through cytoplasmic membrane [10, 17]. The alteration was not specific in the outer membrane so that it could develop the multi-drug resistance to unrelated antimicrobial agents [17].

It was found that the FFC-resistant isolates could also resist CP or TP, but the CP-resistant or TP-resistant isolates

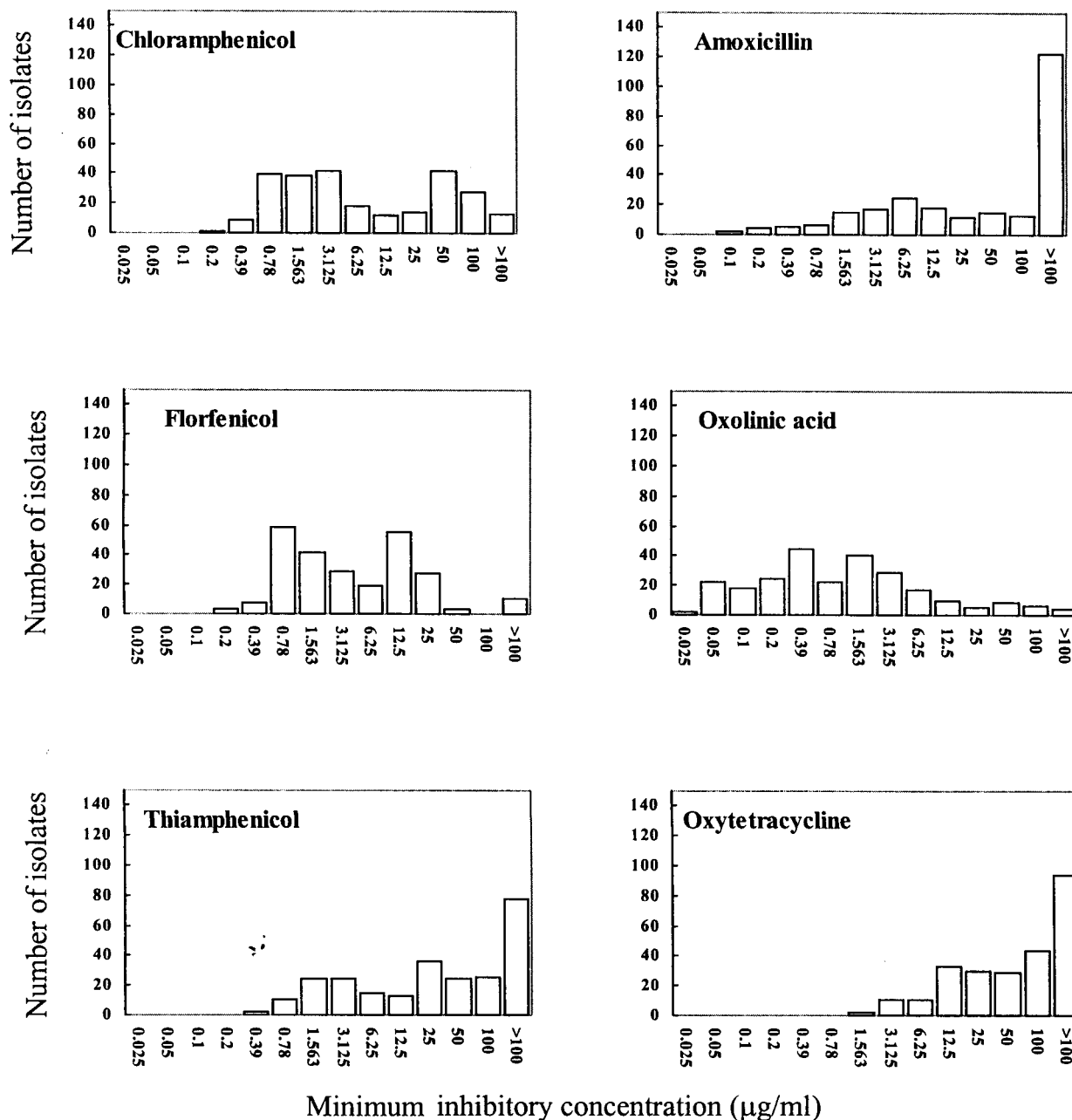


Fig.1. Histograms describing the distribution of minimum inhibitory concentration of six antimicrobial agents against 254 aquatic bacteria isolates.

did not always resist FFC. On the other hands, when the isolates were TP-resistant strains, they seldom could resist CP or FFC. In summary, there was a partially-complete cross-resistance among CP, FFC and TP same as the drug resistance between 4-quinolones [2]. This finding indicates that the choice of antimicrobial agents becomes important for effectively controlling bacterial pathogens of aquaculture.

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Table 6. Resistance patterns of aquatic bacteria against six antimicrobial agents

Pattern of resistance	Number of isolates			
	Total	Soft-shell turtles	Fish	Shellfish
AMPC	10	5	3	2
OA	2		1	1
OTC	11		9	2
TP	13	6	2	5
AMPC•OTC	32	31	1	
CP•TP	2		2	
OA•OTC ^r	7		7	
OA•TP	1		1	
OTC•TP	12	3	7	2
AMPC•CP•OTC	2	1	1	
AMPC•CP•TP	5		5	
AMPC•OA•OTC	1		1	
AMPC•OTC•TP	39	21	15	3
CP•OTC•TP	10		10	
OA•OTC•TP ^r	2		2	
AMPC•CP•OTC•TP	37	6	20	11
AMPC•OA•OTC•TP	2	1	1	
CP•FFC•OTC•TP	3	3		
AMPC•CP•FFC•OTC•TP	25	11	9	5
CP•FFC•OA•OTC•TP	3	3		
AMPC•CP•FFC•OA•OTC•TP	9	3	6	

MIC for isolate over 25 µg/ml considered to be drug-resistant.

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