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Antibiotic and heavy-metal resistance in motile *Aeromonas* strains isolated from fish

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***Aeromonas* spp. have been recognized as important pathogens causing massive economic losses in the aquaculture industry. This study examined the resistance of fish *Aeromonas* isolates to 15 antibiotics and 3 heavy metals. Based on the results, it is suggested that selective antibiotheraphy should be applied according to the *Aeromonas* species and the cultured-fish species. In addition, cadmium-resistant strains were associated with resistance to amoxicillin/clavulanic acid, suggesting that cadmium is a global factor related to co-selection of antibiotic resistance in *Aeromonas* spp.**

Key words: *Aeromonas* spp., antibiotic resistance, heavy-metal resistance, aquaculture, multi-antibiotics resistance.

INTRODUCTION

Motile *Aeromonas* spp. is widely distributed in aquatic environments and is a member of the bacterial flora in aquatic animals (Roberts, 2001; Janda and Abbott, 2010). In aquaculture, the bacterium is an emergent pathogen for motile *Aeromonas* septicemia, which causes massive economic losses when cultured fish are in stressful environments (Roberts, 2001). Although many studies have investigated vaccine development for preventing this disease, there is no vaccine available for aquaculture use (Sommerset et al., 2005), which has led to antibiotheraphy being selected as the best way for controlling infection due to *Aeromonas* spp. in the industry (Roberts, 2001). However, veterinarians encounter certain problems related to this approach, including a high diversity of *Aeromonas* spp. and the presence of multi-antibiotics resistance (MAR) strains.

Recent phylogenetic analysis has revealed high taxonomical complexities in the genus *Aeromonas*, with resulting ramifications in *Aeromonas* spp. (Janda and Abbott, 2010; Martinez-Murcia et al., 2011). *Aeromonas hydrophila*, *Aeromonas sobria* and *Aeromonas veronii* have previously been recognized as major pathogens in human and aquatic medicine, and some new species, especially *Aeromonas aquariorum*, are now frequently isolated from human clinical samples (Figueras et al., 2009; Aravena-Roman et al., 2011; Puthucheary et al., 2012). Our previous study found that *A. aquariorum* and *A. caviae* were major pathogens for aeromoniasis of eel (*Anguilla japonica*) (Yi et al., 2013). However, there is less information available on antibiotic resistance

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at the species level of the pathogens in aquatic medicine than in human medicine under critical phylogenetic identification procedures. Recently, many studies have shown emergency of MAR strains is *Aeromonas* spp. from clinical and aquatic environmental samples (Schmidt et al., 2000; Verner-Jeffreys et al., 2009). However, little information has been known about resistance to antibiotics and heavy metals resistances among clinical *Aeromonas* strains in Korea.The antibiotic resistance could result from antibiotic selection pressure in bacteria. On the other hand, heavy metals have been suggested as contributing to co-selection of antibiotic resistance by molecular mechanism such as cross-resistance, co-resistance and co-regulatory resistance (Baker-Austin et al., 2006; Seiler and Berendonk, 2012; Tacão et al., 2013). In addition, heavy metal resistant strains reportedly have a propensity for the MAR phenotype (Akinbowale et al., 2007; Matyar et al., 2010). In fact, there has been a little information on co-selection of antibiotic and heavy metal resistance in *Aeromonas* spp. The aim of this study was to determine the antibiotic resistance patterns at the species level in a collection of *Aeromonas* strains from eel, koi carp and pet fish in order to establish an antibiotheraphy regime for use in aquatic medicine under emergency situations related to infection. In addition, we explored the relationships between heavy metal tolerance and antibiotic resistance by comparing antibiotic resistance patterns between heavy-metal-resistant and heavy-metal-susceptible strains.

MATERIALS AND METHODS

The *Aeromonas* spp. used in this study comprised 117 strains isolated from diseased fish: 70, 11 and 36 strains isolated from eel, koi carp and imported pet fish, respectively. These isolates were well characterized by our previous phylogenetic analysis using *gyrB* and *rpoD* gene sequences (Kim et al., 2013; Yi et al., 2013). The 117 strains could be categorized as follows: 49 *Aeromonas veronii* strains (31 from pet fish, 13 from eel, and 5 from koi carp), 23 *A. aquariorum* strains (22 from eel and 1 from pet fish), 19 *A. hydrophila* strains (12 from eel, 6 from koi carp, and 1 from pet fish), 16 *A. caviae* strains (eel), 5 *Aeromonas jandaei* strains (4 from eel and 1 from pet fish), 2 *Aeromonas allosaccharophila* strains (pet fish), 2 *Aeromonas media* strains (eel), and 1 *Aeromonas trota* strains (eel). All strains were stored at -70°C using Cryocare Bacteria Preservers (Key Scientific Products).

The antibiotic susceptibility test (AST) was implemented using the Vitek2 system with a veterinary susceptibility test card for Gram-negative bacteria (AST-GN38), according to the manufacturer's instructions. Extended-spectrum β-lactamases (ESBL) producing test wells were loaded with cefepime (1 μg/ml), cefotaxime (0.5 μg/ml), or ceftazidime (0.5 μg/ml), or a combination thereof with clavulanic acid (4 μg/ml). Of 20 antibiotics, five different tests could not be evaluated for *Aeromonas* spp. because rifampicin, cefpirome, cephalexin, polymyxin B, and ESBL tests were not available for the species from Clinical and Laboratory Standards Institute (CLSI) criteria for resistance determination using the Vitek-2 AST system.

According to Matyar et al. (2008), the heavy-metal resistance of each strain was determined as the minimum inhibitory concentration (MIC) resulting from the agar dilution test using Mueller-Hinton medium containing Cd^{2+}, Cu^{2+} and Cr^{6+} at concen-trations ranging from 0.32 to 3200 μg/ml generated from $CdCl_2$, $CuSO_4$ and CrO_3, respectively. The *Escherichia coli* K-12 strain was used as the control; a tested strain was considered resistant if its MIC value was higher than that of the *E. coli* K-12 strain.

Levels of MAR to all isolates were quantified using the MAR index, defined as a/b, where 'a' represents the number of antibiotics to which the strain was resistant and 'b' represents the total number of antibiotics against which the individual isolate was tested (Krumperman et al., 1983). Overall MAR indexes were quantified as mean ± SD values for all strains grouped according to species and heavy-metal resistance. The obtained data were analyzed by SPSS (version 11.0) for Microsoft Windows. ANOVA and the t-test were used to identify significant differences in MAR values between groups, while Fisher's exact test was used to assess the significance of such differences. A probability value of $p<0.05$ was considered to be significant in all statistical analyses.

RESULTS

All of the *Aeromonas* strains were found to have the following overall percentage resistances for the indicated antibiotic agents: 99.2% to ampicillin (AM), 84.9% to piperacillin (PIP), 83.2% to tetracycline (TE), 80.7% to enrofloxacin (Eno), 59.7% to amoxicillin/clavulanic acid (AmC), 54.6% to sulfamethoxazole/trimethoprime (Sxt), 54.6% to ceftiofur (Tio), 44.5% to imipenem (Imi), 43.7% to marbofloxacin (Mar), 40.3% to nitrofurantoin (Nit), 34.5% to chloramphenicol (Chl), 26.9% to cefpodoxime (Pod), 24.4% to tobramycin (Tob), 15.1% to gentamicin (Gen) and 4.2% to amikacin (Ami). Table 1 lists the prevalence of resistant strains to 15 different antibiotic agents among major fish groups and *Aeromonas* spp. In the fish groups, AmC- and Chl-resistant strains were more frequently detected in fish strains cultured in Korea than in the imported pet fish strains. Eel strains were significantly more resistant to Pod and Tio, whereas resistance to Gen and Tob was more frequent among pet fish strains. The MAR index values did not differ significantly among fish groups.

Table 1 also showed antibiotic resistance and MAR indexes of major *Aeromonas* spp. Statistically significant differences in the prevalence of strains with resistance to AmC, Pod, Tio, Gen, Tob, Te, Chl and Sxt were observed among the *Aeromonas* spp. AmC-, Pod-, Tio- and Te-resistant strains were frequently detected in *A. caviae* and *A. aquariorum* strains. All *A. caviae* strains were susceptible to Imi. The levels of Gen and Tob resistance were higher in *A. veronii* strains than in the other *Aeromonas* spp. Chl and Sxt did not inhibit the growth of most *A. caviae* strains. The MAR index was highest in *A. caviae*, followed by *A. aquariorum*, *A. veronii* and *A. hydrophila*. This index differed significantly between *A. veronii* and both *A. caviae* and *A. aquariorum*, but not between *A. caviae* and *A. aquariorum* or between *A. hydrophila* and *A. veronii*.

The *E. coli* K-12 control strain exhibited MICs of 200, 1600 and 400 μg/ml to $CdCl_2$, $CuSO_4$ and CrO_3, respectively. Resistance to Cu was detected in only five

Table 1. Comparisons of the prevalence of resistance against 15 different antibiotic agents among different fish groups and major *Aeromonas* species.

Anibiotic	Conc. (μg/ml)	Eel (n=70)	Pet fish (n=36)	Koi (n=11)	p-value	*A. veronii* (n=49)	*A. caviae* (n=16)	*A. aquariorum* (n=23)	*A. hydrophila* (n=19)	p-value
AM	4—32	100	100	90.9	N/A	98.0	100.0	100.0	100.0	1
AmC	4/2—32/16	75.7	30.6	63.6	**<0.001**	32.7	93.8	95.7	78.9	**<0.001**
PIP	4—64	90	75	81.8	0.119	77.6	87.5	95.7	78.9	0.236
Pod	0.5—4	44.3	5.6	0	**N/A**	4.1	56.3	78.3	10.5	**<0.001**
Tio	1—2	70	27.8	27.3	**<0.001**	24.5	93.8	91.3	47.4	**<0.001**
Imi	2—8	47.1	44.4	36.4	0.852	36.7	0.0	91.3	57.9	**<0.001**
Ami	8—64	0	5.6	18.2	N/A	4.1	0.0	0.0	10.5	0.33
Gen	4—32	1.4	36.1	18.2	**<0.001**	26.5	0.0	0.0	10.5	**0.003**
Tob	8—64	10	47.2	27.3	**<0.001**	36.7	18.8	0.0	21.1	**0.002**
Eno	0.25—4	82.9	72.2	90.9	0.337	75.5	100.0	82.6	89.5	0.112
Mar	1—2	47.1	44.4	36.4	0.852	44.9	75.0	34.8	36.8	0.067
Te	2—8	82.9	91.7	72.7	0.222	79.6	100.0	100.0	73.7	**0.007**
Nit	16—64	35.7	47.2	36.4	0.507	36.7	62.5	34.8	42.1	0.304
Chl	4—32	42.9	19.4	36.4	**0.047**	18.4	87.5	17.4	57.9	**<0.001**
Sxt	1/19—16/304	60	52.8	27.3	0.122	49.0	93.8	43.5	47.4	**0.004**
MAR index		0.53±0.17	0.47±0.19	0.44±0.26		0.43±0.20	0.65±0.10 [a]	0.58±0.11 [a]	0.51±0.19	

[a]: Significantly different (p<0.05) from *A. veronii* strains; *AM, ampicillin; Amc, amoxicillin/clavulanic acid; PIP, piperacillin; Pod, cefpodoxime; Tio, ceftiofur; Imi, imipenem; Ami, amikacin; Gen, gentamicin; Tob, tobramycin; Eno, enrofloxacin; Mar, Marbofloxacin; Te, tetracycline; Nit, nitrofurantoin; Chl, chloramphenicol; Sxt, sulfamethoxazole/trimethoprime.

strains: four eel strains for *A. aquariorum* and one pet fish strain for *A. allosaccarophila*. Resistance to Cr was detected in only one *A. veronii* strain from pet fish. In contrast to the low prevalence of resistance to Cr and Cu, Cd-resistant strains were common among the present strains (74.4%). Resistance was present in 61.2, 82.6, 85.7 and 75.0% of *A. veronii*, *A. aquariorum*, *A. hydrophila* and *A. caviae* strains, respectively, with its prevalence not differing significantly among *Aeromonas* spp. However, the prevalence of Cd resistance was significantly higher in strains from the fish cultivated in Korea than in those from the imported pet fish (82.7 *vs*. 40.8%). The prevalence of resistance to AmC, Pod, Tio, Imi and Chl tends to be higher for Cd-resistant than for Cd-susceptible strains. However, a significant difference was observed for AmC between Cd-resistant and Cd-susceptible strains (Table 2). The MAR index values did not differ significantly between the presence and absence of Cd resistance.

Table 2. Comparisons of the prevalence of resistant strains against 15 different antibiotic agents between Cd-resistant and -susceptible strains

Antibiotic	Cd-R (n=87)	Cd-S (n=30)	p
AM	98.9	100.0	1.000
AmC	67.8	43.3	**0.029**
PIP	82.8	90.0	0.557
Pod	32.2	16.7	0.107
Tio	58.6	30.0	0.322
Imi	48.3	40.0	0.526
Ami	2.3	6.7	0.271
Gen	11.5	20.0	0.354
Tob	20.7	30.0	0.321
Eno	79.3	86.7	0.431
Mar	44.8	46.7	1.000
Te	81.6	93.3	0.152
Nit	37.9	46.7	0.518
Chl	36.8	30.0	0.658
Sxt	52.9	60.0	0.531
MAR index	0.50±0.19	0.50±0.17	

DISCUSSION

Comparing our data with those obtained in other studies (Vila et al., 2002; Akinbowale et al., 2007; Aravena-Roman et al., 2012) is difficult due to differences in the antibiotics used and sources isolated. Moreover, there is limited information available on the prevalence of antibiotic – resistant strains in *Aeromonas* spp. isolated from aquatic farming, as accurately identified using housekeeping genes. As we expected, there were frequent emergences among the present strains to quinolones, Te, and Sxt regardless of the fish group. These antibiotics have been broadly used in both bath and oral therapies applied to cultured fish (including eels) in Korea. Although it is rare, there are also reports of the occurrence of resistance to these antibiotics in *Aeromonas* spp. isolated from clinical specimens for human, aquatic animals and environmental sources (Vila et al., 2002; Akinbowale et al., 2007; Aravena-Roman et al., 2012). However, we did not expect to find that the prevalence of strains with resistance to Gen, Tob, Te, Chl, and Sxt differed among *Aeromonas* spp. A survey of antibiotic use from the allied field of ornamental fish farming found aminoglycosides to be the fourth most frequently used drug type. In addition, Verner-Jeffreys et al. (2009) reported that 5.3, 31 and 61% of strains from ornamental fish were tolerant to Ami, Gen and Tob, respectively. In fact, 31 (63.3%) of the *A. veronii* strains included in the present study originated from imported ornamental fish, and 19 of the strains were resistant to one or more aminoglycosides. This could explain why aminoglycoside-resistant strains were detected frequently in *A. veronii* in the present study. In contrast, higher prevalences of Chl, Sxt and Te resistances were responsible for the characteristics of *A. caviae* and/or *A. aquariorum* strains isolated from eel and koicarp strains farmed in Korea (Kim et al., 2013; Yi et al., 2013). This discrepancy in the prevalence of antibiotics-resistant strains might be due to the resistance mechanism of *Aeromonas* spp. under antibiotic selection pressure induced by antibiotic use differing with the aquaculture environment. It could be that various global factors are relevant to the antibiotic resistance of bacteria.

The investigated *Aeromonas* spp. was susceptible to all the tested antibiotics except aminopenicillins. *Aeromonas* spp. has been considered to be a naturally occurring phenotype for resistance to ampicillin, and hence we expected the prevalence of ampicillin-resistant strains to be significantly higher among the present strains. However, some unexpected higher prevalence rates were observed for the resistance to AmC, Pod, Tio and Imi among eel strains, of which major species were *A. aquariorum* and/or *A. caviae*. Since Pod, Tio, and Imi are used as off-label drugs in aquaculture worldwide (including Korea), these antibiotics have rarely been used in aquatic animal farming. Some *Aeromonas* strains with resistance to these antibiotics have been found in rainbow-trout and shrimp farming. The authors of these previous reports have speculated that the resistances result from an inflow of the antibiotic from domestic animal farming to aquatic farming (Akinbowale et al., 2007; Matyar et al., 2010). However, those studies found very low prevalences of antibiotics when compared with our data. The differences among fish groups might be due to variousnon-antibiotic factors (e.g. cadmium) driving the co-selection of antibiotic resistance according to different methods of fish management.

A. aquariorum was the predominant species among Imi-resistant strains, followed by *A. hydrophila* and *A. veronii*. However, all *A. caviae* strains were susceptible to Imi. These observations are supported by previous studies (Rossolini et al., 1995; Wu et al., 2012) showing species-related distributions of *cphA* and related carbapenemase-encoding genes; for example, the genes were found in *A. aquariorum*, *A. hydrophila* and *A. veronii* but not in most *A. caviae* strains. In addition, the simultaneous resistance to all β-lactams was observed in 18 and 3 strains of *A. aquariorum* and *A. hydrophila*, respectively, which might be due to three chromosomally coordinately controlled β-lactamase genes for penicillinnase, cephalosporinase and carbapenemase, as previously reported in *A. sobria* (Walsh et al., 1995). On the other hand, the β-lactams resistance of *A. caviae* might be mediated by Class-C AmpC-type cephalosporinases, such as CAV-1 and MOX-4, that were previously identified in *A. caviae* strains (Fosse et al., 2003; Ye et al., 2010). The cephalosporinase could hydrolyze broad and extended-spectrum cephalosporin but not carbapenem; in addition, it is not inhibited by clavulanic acid. Therefore, our data might reflect that the different antibiotic resistances among *Aeromonas* spp. are due to the differences in their genetic backgrounds.

The method of antibiotic susceptibility testing applied in this study resulted in the frequent detection of MAR strains in *A. caviae* and *A. aquariorum*. This contrasts with previous studies showing frequent occurrences in *A. hydrophila* strains isolated from aquatic farming (Saavedra, 2004; Akinbowale et al., 2007). This discrepancy might be due to inaccuracies in the species-level identification, and differences in etiological species for aeromoniasis in the aquatic animals and antibiotics investigated. MAR *A. caviae* and *A. aquariorum* strains could be major pathogens for opportunistic infections in antibiotic treatments for controlling primary bacterial diseases in Korea aquaculture.

Heavy metals have been known to be selective agents for antibiotic resistance due to them sharing common structural and functional characteristics of efflux pumps associated with antibiotic and heavy-metal resistance (Nies, 2003; Baker-Austin et al., 2006; Hacioglu et al., 2013). To our knowledge, the present study is the first to show co-selection of Cd and AmC resistance among a collection of *Aeromonas* strains according to fish groups. In addition, the prevalence of Tio and Pod resistances tended to be higher among Cd-resistant strains than among Cd-susceptible strains ($p>0.05$). These results suggest that cadmium could be a suitable inducer for the production of inducible β-lactamases and/or co-selective efflux pumps related to resistance to β-lactams and β-lactamase inhibitors, at least in *Aeromonas* spp. In disagreement with previous studies showing a relationship between MAR and heavy-metal resistance (Akinbowale et al., 2007; Matyar et al., 2010), we found that Cd had no effect on the occurrence of MAR strains in *Aeromonas* strains.

Put together, these observations indicate that veterinarians need to carefully consider the possibility of secondary infection of these MAR strains during antibiotic treatments. In addition, the various antibiotic resistance patterns in the present study indicate the need for antibiotic susceptibility testing before applying antibiotics during an *Aeromonas* outbreak. In addition, Cd could be a global factor for co-selection for resistance to β-lactams, especially AmC.

Conflict of interest

There are no potential conflicts of interest.

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