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Prevalence of plasmid-mediated quinolone and tetracycline resistance genes in *Aeromonas* strains isolated from eel (*Anguilla japonica*) and ornamental fish

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This study investigated the genetic determinants of plasmid-mediated antibiotic resistance (PMAR) to quinolones and tetracycline in 106 *Aeromonas* strains isolated from eel (*Anguilla* japonica, 70 strains) and ornamental fish (36 strains) in Korea. Quinolones and tetracycline resistance phenotypes were found to be widely distributed throughout the both fish groups. However, the prevalence of *qnr* and *tet* genes was higher in ornamental fish strains than in eel strains (42.9% *vs*. 86.1% for *qnr* and 51.4% *vs*. 69.4% for *tet*). In addition, the profiling of the present genetic determinants revealed the dominance of *qnrS*, *tetA*, *tetE* and *tetE+qnrS* genes for eel strains but of *tetA+qnrS qnrS* and *tetE+qnrS* genes for ornamental fish strains. These results indicate that aquaculture and related industries could be a major threat to public health due to the possible spread of PMAR.

Key words: *Aeromonas* sp., Tetracycline, Quinolones, Genetic-resistance determinants, Aquaculture

Introduction

Motile *Aeromonas* spp. are autochthonous bacteria of aquatic environments and members of the bacterial flora in aquatic animals. These bacteria are also important pathogens causing septicemia and ulcerative diseases that are responsible for vast economic losses in the global aquaculture industry (Roberts, 2001; Janda and Abbott, 2010).

Quinolone (Qnr) and tetracycline (Te) antibiotics are the two most commonly used classes of antibiotics in aquaculture (Sapkota et al., 2008; Weir et al., 2012). The intensive use of antibiotics has resulted in the frequent emergence of antibiotic-resistance phenotypes in and around aquaculture environments due to the selective antibiotic pressure provided by aquatic farming (Sapkota et al., 2008; Heuer et al., 2009). Such antibiotics resistance is due to mutation of chromosomal DNA and/or the acquisition of genes related to plasmid-mediated antibiotic resistance (PMAR), which has led to considerable concern about the effects of aquaculture and related industries on public health, based on previous data showing a genetic relationship between resistance genes of fish and human bacterial origins and the distributions of various antibiotic-resistance genes on plasmids (Furushita et al., 2003; Sapkota et al., 2008).

Eel (*Anguilla japonica*) is common fresh water fish species that have been farmed for human consumption in Korea. In addition, ornamental fish are major aqua-

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culture imports as indoor-reared pet animal in Korea. However, few studies have investigated the prevalence of the plasmid-mediated determinants responsible for the Qnr and Te resistance of bacteria including *Aeromonas* spp. in aquaculture and related industries in Korea. The present study investigated the genetic determinants (*qnr* and *tet* genes) of PMAR to Qnr and Te in *Aeromonas* strains isolated from eel and ornamental fish in Korea.

Material and Method

Aeromonas strains

In total, 106 strains were investigated, comprising 70 strains isolated from eel (*Anguilla japonica*) and 36 strains isolated from ornamental fish in Korea. The 36 ornamental fish strains, which were collected during quarantine period of the imported pet fish, were kindly provided by the Animal, Plant and Fisheries Quarantine and Inspection Agency of Korea. The *Aeromonas* spp. were identified by phylogenetic analysis using *gyrB* or *rpoD* gene sequences, according to our previous study involving the same eel strains as in the present study (Yi et al., 2013). In the phylogenetic identification, the eel strains detected were *A. aquariorum* (*n*=22), *A. caviae* (*n*=16), *A. hydrophila* (*n*=12), *A. veronii* (*n*=13), *A. jandaei* (*n*=4), *A. media* (*n*=2) and *A. trota* (*n*=1). The ornamental fish strains detected consisted of *A. veronii* (*n*=29), *A. allosaccharophila* (*n*=2), *A. aquariorum* (*n*=1), *A. hydrophila* (*n*=1), *A. culicicola* (*n*=2) and *A. jandaei* (*n*=1). All strains were stored at -70°C using Cryocare Bacteria Preservers (Key scientific products) until used in antimicrobial susceptibility tests (ASTs) and PCR assays for the detection of plasmid-mediated genetic determinants to quinolones and tetracycline resistance.

Antimicrobial susceptibility testing

The AST was performed with the Vitek2 system using a veterinary susceptibility test card for Gram-negative bacteria (AST - GN38), according to the manufacturer's instructions. The AST - GN38 card contains the following antimicrobial agents as dehydrated substances at the indicated concentrations: enrofloxacin (Eno) - 0.25, 1, and 4 mg/ml; marbofloxacin (Mar) - 1 and 2 mg/ml; and tetracycline (Te) - 2, 4, and 8 mg/ml. Resistance to an antimicrobial agent was considered to be present when the resistance was greater than "intermediate" according to the criteria of the Vitek2 AST system.

Detection of qnr and tet genes

The prevalence rates of *qnr* and *tet* genes in the present strains were investigated by PCR assays using previously described methods (Akinbowale et al., 2007; Cattoir et al., 2007). Briefly, the genomic DNA from strains was purified using the AccuPrep® genomic extraction kit (Bioneer, Korea). The plasmid-mediated quinolone-resistance (PMQR) genes, such as *qnrS*, *qnrA*, and *qnrB*, were analyzed using multiplex PCR with primers designed in a previous study (Cattoir et al., 2007). A single PCR assay was able to detect *tetA* - *tetE* and *tetM* genes from the present strains according to the method described by Akinbowale et al. (2007). All PCR assays were carried out in 25 ml of AccuPrep® PCR PreMix with 1 ml of bacterial genomic DNA (30 ~ 40 ng), 1 ml of each forward and reverse primer at 10 mM, and sterile water for adjusting the final volume. The PCR amplicons were separated on a 1.5% (w/v) agarose gel containing 0.05 mg/l ethidium bromide. The specificity of each PCR assay was confirmed by sequencing analysis of randomly selected PCR amplicons.

Results

The Eno, Mar, and Te resistance phenotypes were observed in 80.2%, 46.2% and 94.3% of the 106 strains, respectively. Regarding Qnr resistance, forty-nine Mar resistant strains were simultaneously resistant to Eno whereas 30 strains were resistant to only Eno. However, there were the similar levels in

resistance rates of both Qnrs between eel and ornamental fish strains (81.4% *vs*. 77.8% for Enr and 47.1% *vs*. 44.4% for Mar). In the case of Te-resistance phenotypes, its resistance was more frequently detected in ornamental fish (Table 1).

In PCR assays for detecting genetic determinants to Qnr and Te resistances (Table 2), 80.2% out of 106 strains possessed at least one of resistance genes tested for the present study. The *qnr* genes were detected in 42.9% and 86.1% from eel and ornamental fish strains, respectively. The most prevalent *qnr* type was *qnrS* among all strains. Among 83 Eno-resistant strains, 51 strains harbored 2 *qnr* genes: *qnrS* (60 strains) and *qnrA* (1 *A. aquariorum* strain of eel origin). No *qnr* genes used for the present study could be detected in 32 Eno-resistant strains. On the other hand, *qnr* gene was able to be detected in 10 out of 23 Eno-susceptible strains. It occurred more frequently in ornamental fish compared with eel strains. The *tet* genes were detected in 64.2% of all strains, comprising 40 eel strains and 28 ornamental fish strains. In eel strains, the most prevalent *tet* gene was *tetE* (37.3%), followed by *tetA* (23.5%). However, ornamental fish strains exhibited the highest detection rate for *tetE* (50%). The *tet* genes were detected in 70.3% of Te-resistant strains but it was also detected in 26.7% of Te-susceptible strains.

Table 3 indicates the distribution of *qnr* and *tet* genes in the eel and ornamental fish strains. Of the 106 strains, 85 strains harbored at least one genetic determinant for Qnr and Te resistance. In addition, 2 or more genetic determinants co-existing in one strain were found in 45.9% of the 85 genetic-determinant-positive strains. Comparisons of the distribution of genetic determinants with fish groups revealed that *qnrS* gene-harboring strains were dominant among eel strains, followed by single *tetA* and *tetE* and *tetE*+*qnrS*. In the case of ornamental fish strains, the prevalence order was *tetA*+*qnrS* followed by single *qnrS* and *tetE*+*qnrS*. The *tetM* gene was detected in only two strains of ornamental fish, and only in combination with other genetic determinants.

Table 1. Resistance to quinolones and tetracycline in *Aeromonas* strains from eel and ornamental fish origins

Sources	*Aeromonas* species	Numbers of strains	Numbers (%) of resistant strains		
			Eno	Mar	Te
Eel	*A. aquariorum*	22	18 (81.8)	8 (36.4)	22 (100)
	A. caviae	16	16 (100)	12 (75.0)	16 (100)
	A. trota	1	0	0	1 (100)
	A. hydrophila	12	9 (75.0)	3 (25.0)	7 (58.3)
	A. jandaei	4	1 (50.0)	1 (50.0)	1 (50.0)
	A. media	2	2 (100)	2 (100)	2 (100)
	A. veronii	13	10 (76.9)	6 (46.2)	8 (61.5)
	Sub-total	70	57 (81.4)	33 (47.1)	58 (82.9)
Ornamental fish	*A. aquariorum*	1	1 (100)	0	1 (100)
	A. allosaccharophila	2	1 (50.0)	0	1 (50.0)
	A. culicicola	2	2 (100)	2 (100)	2 (100)
	A. hydrophila	1	1 (100)	0	1 (100)
	A. jandaei	1	1 (100)	0	1 (100)
	A. veronii	29	22 (75.9)	14 (48.3)	27 (93.1)
	Sub-total	36	28 (77.8)	16 (44.4)	33 (91.7)
Total		106	85 (80.2)	49 (46.2)	91 (94.3)

Table 2. Detection of *qnr* and *tet* genes according to resistance of quinolones and tetracycline in *Aeromonas* strains from eel and ornamental fish origins

Eno	Eel			Ornamental fish			Total		
	Resistant	Susceptible	Sub-total	Resistant	Susceptible	Sub-total	Resistant	Susceptible	Total
No. of strain	57	13	70	26	10	36	83	23	106
No (%). of detection	28 (49.1)	2 (15.4)	30 (42.9)	23 (88.5)	8 (80.0)	31 (86.1)	51 (61.4)	10 (43.5)	61 (57.5)
Te	Resistant	Susceptible	Sub-total	Resistant	Susceptible	Sub-total	Resistant	Susceptible	Total
No. of strain	58	12	70	33	3	36	91	15	106
No (%). of detection	38 (65.5)	2 (16.7)	40 (57.1)	26 (78.8)	2 (66.7)	28 (77.8)	64 (70.3)	4 (26.7)	68 (64.2)

Table 3. Profiling *qnr* and *tet* determinants in *Aeromonas* strains from eel and ornamental fish

Profiles of resistance gene	No (%). of strain isolated from fish sources		
	Eel (*n*=70)	Ornamental fish (*n*=36)	Total (*n*=106)
tetA	8 (11.4)	2 (5.6)	10 (9.4)
tetC	1 (1.4)	0	1 (0.9)
tetD	2 (2.9)	0	2 (1.9)
tetE	8 (11.4)	1 (2.8)	9 (8.5)
qnrS	15 (21.4)	9 (25.0)	24 (22.6)
tetA, qnrA	1 (1.4)	0	1 (0.9)
tetA, qnrS	3 (4.3)	13 (36.1)	16 (15.1)
tetB, tetE	1 (1.4)	0	1 (0.9)
tetC, tetE	1 (1.4)	0	1 (0.9)
tetC, qnrS	0	1 (2.8)	1 (0.9)
tetD, qnrS	2 (2.9)	0	2 (1.9)
tetE, qnrS	7 (10.0)	6 (16.7)	13 (12.3)
tetA, tetM, qnrS	0	1 (2.8)	1 (0.9)
tetC, tetE, qnrS	2 (2.9)	0	2 (1.9)
tetA, tetD, tetM. qnrS	0	1 (2.8)	1 (0.9)

Discussion

Antibiotics are frequently used as both oral and bath medications for controlling various bacterial diseases in aquaculture worldwide. A previous survey found that Qnr and Te are frequently used in ornamental fish farming (Weir et al., 2012). A literature review of the antibiotic resistance to *Aeromonas* spp. of ornamental fish origin found that the prevalence rates of Qnr and Te resistant *Aeromonas* strains were within the ranges of 51.8-85.0% and 81.8-91.0%, respectively (Jongjareanjai et al., 2009; Verner-Jeffreys et al., 2009; Weir et al., 2012). Similar prevalence rates have been found for eel *Aeromonas* spp. isolated from fish farmed in Korea (Kim et al., 2011; Han et al., 2012a, 2012b). Therefore, it seems that antibiotic-resistant *Aeromonas* strains are widely distributed in aquatic farming worldwide, including in Korea. However, there was a discrepancy between the detection rates of resistance phenotypes and genetic

determinants in the strains included in the present study. Previous studies have shown that the acquisition of PMQR genes confers low-level resistance to quinolones and enhance the resistance level to quinolones by mutations of quinolone resistance determining regions (QRDRs) in *Aeromonas* strains (Gibson et al., 2010; Kim et al., 2011; Han et al., 2012a). In addition, various *tet* genes including those analyzed in the present study have been identified as genetic determinants for Te resistance. Therefore, numerous genetic determinants might contribute to the overall resistance to Qnr and Te among the present strains.

There have been many reports about the frequent emergence and spread of antibiotic-resistant strains in aquaculture and related industries. However, there is little information about the prevalence of genetic determinants in aquaculture (Verner-Jeffreys et al., 2009). The present results revealed clear differences in the profiles of genetic determinants for PMAR to quinolones and Te between eel and ornamental fish *Aeromonas* strains. In addition, the prevalence of *qnr* genes were higher in the present fish strains than in *Enterobacteriaceae* strains from clinical, food, and poultry samples in Korea (Kim et al., 2010; Park et al., 2010; Tamang et al., 2012). Although the prevalence of *tet* genes could differ with the bacterial species, *tetM* gene has been rarely detected in *Aeromonas* strains from aquaculture or its natural environment. Therefore, based on the present and previous studies, aquaculture and related industries play an important role in the spread of plasmid-mediated resistance.

In conclusion, quinolones and Te resistant strains were found to be very common in the present study regardless of the isolation source. However, the genetic determinants were specifically distributed between eel and ornamental fish *Aeromonas* strains, indicating the geographical variations. Therefore, aquaculture and related industries could be a major threat to public health due to the possible spread of antibiotic-resistant strains or genes.

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