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Original Article

Molecular characterization of tetracycline- and quinolone-resistant *Aeromonas salmonicida* isolated in Korea

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The antibiotic resistance of 16 *Aeromonas* (*A.*) *salmonicida* strains isolated from diseased fish and environmental samples in Korea from 2006 to 2009 were investigated in this study. Tetracycline or quinolone resistance was observed in eight and 16 of the isolates, respectively, based on the measured minimal inhibitory concentrations. Among the tetracycline-resistant strains, seven of the isolates harbored *tet*A gene and one isolate harbored *tet*E gene. Additionally, quinolone-resistance determining regions (QRDRs) consisting of the *gyr*A and *par*C genes were amplified and sequenced. Among the quinolone-resistant *A. salmonicida* strains, 15 harbored point mutations in the *gyr*A codon 83 which were responsible for the corresponding amino acid substitutions of $Ser^{83} \rightarrow Arg^{83}$ or $Ser^{83} \rightarrow Asn^{83}$. We detected no point mutations in other QRDRs, such as *gyr*A codons 87 and 92, and *par*C codons 80 and 84. Genetic similarity was assessed via pulsed-field gel electrophoresis, and the results indicated high clonality among the Korean antibiotic-resistant strains of *A. salmonicida*.

Keywords: *Aeromonas salmonicida*, minimal inhibitory concentration, pulsed-field gel electrophoresis, quinolone-resistance determining region, tetracycline-resistance

Introduction

Aeromonas (*A.*) *salmonicida* is a pathogen that causes furunculosis and bacterial septicemia in a broad variety of fish, and is thus responsible for significant economic losses in the global aquaculture industry [37]. Recently, antibiotic-resistant *A. salmonicida* strains have been recognized as a

serious concern owing to their potential health risks to humans and animals [32,33]. Among the antibiotics utilized in the treatment of furunculosis, both tetracycline and quinolone resistance have been widely documented [10,31]. Tetracycline-resistant strains of *A. salmonicida* are suspected to be the source of tetracycline resistance dissemination in the aquatic environment because *tet* genes, the determinants of tetracycline resistance, are generally encoded on plasmids [1,32,33]. Quinolone resistance is a potential public health threat since quinolones are also utilized for the treatment of *Aeromonas* infections in humans [15,16]. Quinolone resistance in Gram-negative bacteria is primarily attributable to mutations in the quinolone-resistance determining regions (QRDRs) consisting of the *gyr*A and *par*C genes, which are the subunits of the target enzymes of quinolones, DNA gyrase subunit A and topoisomerase IV, respectively [2]. The presence of the *qnr* gene which is associated with the plasmid-mediated quinolone-resistance, or efflux pumps is also known to be associated with mid- to low-level quinolone resistance [6,30].

Antibiotic resistance has been previously reported in several aquatic bacteria isolated in Korea including *Edwardsiella tarda* [17], *Streptococcus iniae*, and *Streptococcus parauberis* [29]. However, the antibiotic resistance of *Aeromonas* spp. has not previously been addressed. Therefore, in this study we evaluated the antimicrobial susceptibility and clonal relationship in *A. salmonicida* isolated from both cultured fish and the environmental water in Korea. In particular, the genetic determinants of tetracycline and quinolone resistance were assessed via (i) the detection of *tet*A to E, (ii) the detection of plasmid-encoded *qnr* genes, and (iii) the analysis of point mutations in QRDRs.

Materials and Methods

Bacterial isolation and culture conditions
Between 2006 and 2009, sixteen strains of *A. salmonicida*

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were isolated from a variety of samples from fish and sewage water from two private aquariums and three salmonid farms in Korea (Table 1). Two reference strains were purchased from the American Type Culture Collection (ATCC, USA): *A. salmonicida* subsp. *salmonicida* ATCC 33658 (ASS) and *A. salmonicida* subsp. masoucida ATCC 27013 (ASM). *A. salmonicida* isolates were first screened using a Vitek System 2 (bioMérieux, France). All strains of *A. salmonicida* were stored in tryptic soy broth (Difco, USA) with 10% glycerol at -80°C and sub-cultured for 48 h on tryptic soy agar (Difco, USA) at 22°C. To assess strain purity, single colonies were selected and sub-cultured three times, and the resulting bacterial cells were harvested for further experiments.

Antimicrobial susceptibility test

Antimicrobial susceptibility tests were conducted via broth micro-dilution methods according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI), and ASS was utilized as a quality control bacterial strain [7,8]. Since cut-off values have not been determined for all antibiotics, three references [7,8,24] were used for interpretation, as was the case in other previous reports [2,5,29]. Seven antimicrobials were diluted in following ranges: ampicillin (0.06 to 32 μg/mL), enrofloxacin (0.002 to 4 μg/mL), florfenicol (0.12 to 64 μg/mL), gentamicin (0.06 to 32 μg/mL), oxolinic acid (0.004 to 8 μg/mL), oxytetracycline (0.03 to 16 μg/mL), and trimethoprim-sulfamethoxazole (0.03/0.6 to 2/38 μg/mL). All antimicrobials were purchased from Sigma-Aldrich (USA). The antimicrobials were serially diluted two-fold in cation-adjusted MHB (CAMHB; Difco, USA) and 100 μL volumes of the dilutions were placed into 96-well micro-titer plates. The inoculations were prepared as follows: 18 strains of *A. salmonicida* were adjusted to a McFarland value of 0.5 and diluted 10-fold with CAMHB. With the addition of 5 μL of inocula into each micro-titer wells, the final cell densities were adjusted to 5×10^5 CFU/ mL. In all cases, two control wells without antimicrobials or inocula were maintained. After 44 to 48 h of incubation at 22°C, the lowest concentration of antibiotics that visibly inhibited bacterial growth was defined as the minimal inhibitory concentration (MIC). The MIC results of *A. salmonicida* subsp. *salmonicida* were used to classify the strains as resistant or sensitive in accordance with the cut-off values established by Miller *et al.* [24] and the guidelines of M49-A [7] and M31-A3 [8].

DNA extraction and polymerase chain reactions (PCR)

Genomic DNA was extracted by harvesting the cells with sterile water followed by 10 min of boiling. After 3 min of centrifugation at 10,000 ×g, the supernatants were collected and 1 : 100 dilutions in sterile water were utilized as a PCR template. All isolates were confirmed to be *A. salmonicida* using Fer-3 and Fer-4 PCR primers [3].

Table 1. *Aeromonas* (*A.*) *salmonicida* strains used in this study

Name	Source	Isolated year	Bacterial identification			
			Vitek System 2	PCR [3]	PCR [4]	16S rRNA sequence
AS01	Cherry salmon (*Oncorhynchus masou masou*)	2006	*A.salmonicida*	+	+	subsp. *salmonicida*
AS02	Cherry salmon (*Oncorhynchus masou masou*)	2006	*A.salmonicida*	+	+	subsp. *salmonicida*
AS03	Crucian carp (*Carassius carassius*)	2006	*A.salmonicida*	+	−	subsp. *achromogenes*
AS04	Neon tetra (*Paracheirodon innesi*)	2007	*A.salmonicida*	+	+	subsp. *salmonicida*
AS05	Rainbow trout (*Oncorhynchus mykiss*)	2008	*A.salmonicida*	+	+	subsp. *salmonicida*
AS06	Rainbow trout (*Oncorhynchus mykiss*)	2008	*A.salmonicida*	+	+	subsp. *salmonicida*
AS07	Rainbow trout (*Oncorhynchus mykiss*)	2008	*A.salmonicida*	+	+	subsp. *salmonicida*
AS08	Rainbow trout (*Oncorhynchus mykiss*)	2008	*A.salmonicida*	+	+	subsp. *salmonicida*
AS09	Chum salmon (*Oncorhynchus keta*)	2008	*A.salmonicida*	+	+	subsp. *salmonicida*
AS10	Chum salmon (*Oncorhynchus keta*)	2008	*A.salmonicida*	+	+	subsp. *salmonicida*
AS11	Chum salmon (*Oncorhynchus keta*)	2008	*A.salmonicida*	+	+	subsp. *salmonicida*
AS12	Chum salmon (*Oncorhynchus keta*)	2009	*A.salmonicida*	+	+	subsp. *salmonicida*
AS13	Malma trout (*Salvelinus malma malma*)	2009	*A.salmonicida*	+	+	subsp. *salmonicida*
AS14	Malma trout (*Salvelinus malma malma*)	2009	*A.salmonicida*	+	+	subsp. *salmonicida*
AS15	Cherry salmon (*Oncorhynchus masou masou*)	2009	*A.salmonicida*	+	+	subsp. *salmonicida*
AS16	Sewage water	2007	*A.salmonicida*	+	−	subsp. *flounderacida*
ASS	*A.salmonicida* subsp. *salmonicida* ATCC 33658	−	*A.salmonicida*	+	+	subsp. *salmonicida*
ASM	*A.salmonicida* subsp. *masoucida* ATCC 27013	−	*A.salmonicida*	+	−	subsp. *masoucida*

Subspecies were determined by *A. salmonicida* subsp. *salmonicida*-specific PCR with MIY1 and MIY2 primers [4,26] and confirmed by 16S rRNA sequencing at Macrogen (Korea). Two multiplex PCR procedures were conducted as previously described to amplify the five tetracycline resistant genes (*tet*A to E) [27] and to detect the *qnr* genes [5]. The QRDRs of the *gyr*A and *par*C genes were detected using the following primers: ASGYRA1, ASGYRA2, ASPARC3, and ASPARC4 [10]. The primers used in this study are shown in Table 2.

Sequence analysis

Sequencing was conducted by Macrogen (Korea) and the sequences were analyzed with the AlignX tool in the Vector NTI program (Invitrogen, USA). BLAST searches were conducted using both the blastn and blastx algorithms provided by the National Center for Biotechnology Information (NIH, USA).

Pulsed-field gel electrophoresis (PFGE)

Harvested bacterial cells were diluted with cell suspension buffer (100 mM Tris-HCl and 100 mM EDTA, pH 8.0) up to an optical density of 1.0 at 600 nm. A cell suspension volume of 100 µL was mixed with an equal volume of 1.6%

SeaKem Gold agar (FMC Corporation, USA) and solidified in a 100 µL plug mold. The plugs were then incubated for 2 h with 1 mg/mL of lysozyme (Sigma-Aldrich, USA) at 37°C and treated with 1 mg/mL of proteinase K (Sigma-Aldrich, USA) at 50°C for 8 h. DNA in the plugs was digested for 18 h with 30 U of *SpeI* (New England Biolabs, USA) at 37°C and electrophoresed in 1.0% SeaKem Gold agarose gel with a CHEF-Mapper III PFGE system (Bio-Rad, USA). The running conditions were 6 V/cm at 14°C for 22 h, and the pulse times were 1.5 to 25 sec. The Lambda ladder PFG marker (New England Biolabs, USA) was included as a size marker. The gels were stained with ethidium bromide and photographed under UV trans-illumination. The genetic relationships among isolates were analyzed with Bionumerics software (Applied Maths, Belgium) and the clusters were determined using the UPGMA algorithm with the 70% Dice-coefficient of similarity (2.0% position tolerance).

Results

Bacterial identification

The 16 isolates were successfully identified as *A. salmonicida* using Vitek System 2 and species-specific PCR

Table 2. PCR primers used in this study

Name	Sequences (5′ to 3′)	Target gene	References
Fer-3	CGGTTTTGGCGCAGTGACG	*fst*A	[3]
Fer-4	AGGCGCTCGGGTTGGCTATCT		
MIY1	AGCCTCCACGCGCTCACAGC	Asal-3	[4, 26]
MIY2	AAGAGGCCCCATAGTGTGGG		
*tet*AF	GCTACATCCTGCTTGCCTTC	*tet*A	[27]
*tet*AR	GCATAGATCGCCGTGAAGAG		
ClassB *tet*AF	TCATTGCCGATACCACCTCAG	*tet*B	
ClassB *tet*AR	CCAACCATCATGCTATTCCATCC		
ClassC *tet*AF	CTGCTCGCTTCGCTACTTG	*tet*C	
ClassC *tet*AR	GCCTACAATCCATGCCAACC		
ClassD *tet*AF	TGTGCTGTGGATGTTGTATCTC	*tet*D	
ClassD *tet*AR	CAGTGCCGTGCCAATCAG		
ClassE *tet*AF	ATGAACCGCACTGTGATGATG	*tet*E	
ClassE *tet*AR	ACCGACCATTACGCCATCC		
ASGYRA1	CCATGAGCGTGATCGTAGGA	*gyr*A	[10]
ASGYRA2	CTTTGGCACGCACATAGACG		
ASPARC3	CAGCGGCGCATCATCTAC	*par*C	
ASPARC4	GGATATCGGTGGCCATGC		
qnrAm-F	AGAGGATTTCTCACGCCAGG	*qnr*A1 to *qnr*A6	[5]
qnrAm-R	TGCCAGGCACAGATCTTGAC		
qnrBm-F	GGMATHGAAATTCGCCACTG	*qnr*B1 to *qnr*B6	
qnrBm-R	TTTGCYGYYCGCCAGTCGAA		
qnrSm-F	GCAAGTTCATTGAACAGGGT	*qnr*S1 to *qnr*S2	
qnrSm-R	TCTAAACCGTCGAGTTCGGCG		

Table 3. Minimal inhibitory concentrations (MICs), tetracycline resistance (*tet*) genes, mutations in quinolone-resistance determining regions (QRDRs) in the *A. salmonicida* strains

Strains	MIC (µg/mL)							*tet* gene	*gyr*A QRDR*						*par*C QRDR			
	AM	GM	SXT	FFC	OTC	ENR	OA		Codon 83	Aa^{83}	Codon 87	Aa^{87}	Codon 92	Aa^{92}	Codon 80	Aa^{80}	Codon 84	Aa^{84}
AS01	2	4	0.12	1	0.5	0.008	2 (R)	−[†]	**AGA**	**Arg**	GAC	Asp	TTG	Leu	AAA	Lys	CAC	His
AS02	2	4	0.12	2	0.5	0.008	2 (R)	−	**AGA**	**Arg**	GAC	Asp	TTG	Leu	AAA	Lys	CAC	His
AS03	32 (R)	2	0.06	1	0.06	0.004	2 (R)	−	AGT	Ser	GAC	Asp	TTG	Leu	AAA	Lys	CAC	His
AS04	0.25	2	0.12	0.5	0.12	0.015	1 (R)	−	**AGA**	**Arg**	GAC	Asp	TTG	Leu	AAA	Lys	CAC	His
AS05	0.12	4	0.12	2	1	0.004	2 (R)	−	**AGA**	**Arg**	GAC	Asp	TTG	Leu	AAA	Lys	CAC	His
AS06	0.25	4	0.12	1	1	0.004	1 (R)	−	**AGA**	**Arg**	GAC	Asp	TTG	Leu	AAA	Lys	CAC	His
AS07	0.25	2	0.12	1	1	0.008	2 (R)	−	**AGA**	**Arg**	GAC	Asp	TTG	Leu	AAA	Lys	CAC	His
AS08	0.25	2	0.12	1	1	0.004	1 (R)	−	**AGA**	**Arg**	GAC	Asp	TTG	Leu	AAA	Lys	CAC	His
AS09	0.12	4	0.12	2	>16 (R)	0.015	2 (R)	*tet*A	**AGA**	**Arg**	GAC	Asp	TTG	Leu	AAA	Lys	CAC	His
AS10	0.12	2	0.12	1	>16 (R)	0.004	1 (R)	*tet*A	**AGA**	**Arg**	GAC	Asp	TTG	Leu	AAA	Lys	CAC	His
AS11	0.12	2	0.12	1	>16 (R)	0.008	1 (R)	*tet*A	**AGA**	**Arg**	GAC	Asp	TTG	Leu	AAA	Lys	CAC	His
AS12	0.12	2	0.12	1	>16 (R)	0.004	1 (R)	*tet*A	**AGA**	**Arg**	GAC	Asp	TTG	Leu	AAA	Lys	CAC	His
AS13	0.12	1	0.12	1	>16 (R)	0.004	1 (R)	*tet*A	**AGA**	**Arg**	GAC	Asp	TTG	Leu	AAA	Lys	CAC	His
AS14	2	4	0.12	2	>16 (R)	0.004	2 (R)	*tet*A	**AGA**	**Arg**	GAC	Asp	TTG	Leu	AAA	Lys	CAC	His
AS15	4	4	0.12	2	>16 (R)	0.015	2 (R)	*tet*A	**AGA**	**Arg**	GAC	Asp	TTG	Leu	AAA	Lys	CAC	His
AS16	32 (R)	32 (R)	0.12	2	>16 (R)	>4 (R)	>8 (R)	*tet*E	**ATT**	**Asn**	GAC	Asp	TTG	Leu	**AAG**	Lys	CAC	His
ASS	0.12	0.25	0.03	1	0.12	0.004	0.008	−	AGT	Ser	GAC	Asp	TTG	Leu	AAA	Lys	CAC	His
ASM	32 (R)	0.5	0.12	1	0.12	0.03	0.015	−	−	−	−	−	−	−	−	−	−	−

*Nucleotide changes and corresponding amino acid substitutions are shown in bold. [†]Not amplified. AM: ampicillin, GM: gentamicin, SXT: trimethoprim-sulfamethoxazole, FFC: florfenicol, OCT: oxytetracycline, ENR: enrofloxacin, OA: oxolinic acid, R: resistance.

(Table 1). Among the various categories of biochemical tests in Vitek System 2, four (D-glucose, D-mannitol, and sucrose fermentation tests, and H_2S production test) which were known to be different between subspecies of *A. salmonicida* [14] were focused on in this study. The ASS strain and 14 isolates (AS01 to AS15 except AS03) were positive in the D-glucose and D-mannitol fermentation tests and negative in the sucrose fermentation and H_2S production tests. On the other hand, AS03 was positive only in a sucrose fermentation test and ASM was positive in all the four tests. Additionally, the AS16 strain showed different biochemical characteristics compared to the others; this strain was positive in the D-glucose, D-mannitol and sucrose fermentation tests, and negative in the H_2S production test.

Among the 16 isolates, 14 strains were confirmed by PCR to be *A. salmonicida* subsp. *salmonicida*. The other two strains were confirmed to be *A. salmonicida* subsp. *achromogenes* (AS03) and *A. salmonicida* subsp. *flounderacida* (AS16), as their 16S rRNA sequences showed 100% homology with the 16S rRNA gene of *A. salmonicida* subsp. *achromogenes* strain 870626-1/1C (GenBank accession No. AM296505.1) and *A. salmonicida* subsp. *flounderacida* strain HQ010320-1 (GenBank accession No. AY786177.1), respectively. All the isolates and reference strains of *A. salmonicida* used in this study are shown in Table 1.

MICs

The MIC values of the *A. salmonicida* isolates are shown in Table 3. Among the 16 Korean isolates, eight oxytetracycline-resistant strains and sixteen oxolinic acid-resistant strains were detected. Enrofloxacin resistance was noted in only one isolate (AS16). A total of 9 multidrug-resistant (MDR) strains were observed: 7 strains (AS09 to AS15) that were resistant to oxytetracycline and oxolinic acid, one strain (AS03) that was resistant to ampicilin and oxolinic acid, and one strain (AS16) that was resistant to five antibiotics (ampicillin, gentamicin, oxytetracycline, enrofloxacin, and oxolinic acid). Moreover, strain AS16 exhibited a high level of resistance to both enrofloxacin (≥ 4 µg/mL) and oxolinic acid (≥ 8 µg/mL) although the other 15 quinolone-resistant strains were susceptible to enrofloxacin (≤ 0.03 µg/mL) and showed low-level oxolinic acid resistance (1~2 µg/mL).

tet genes in *A. salmonicida* isolates

The *tet*A gene (211 bp) was detected in seven isolates (AS09 to AS15) while the *tet*E gene (744 bp) was detected in strain AS16 (Fig. 1). The amplified PCR products were sequenced and aligned with the *tet* gene sequences from
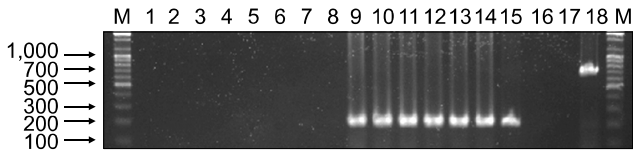
Fig. 1. Multiplex PCR assay of tetracycline resistance genes (*tet*A of 211 bp and *tet*E of 744 bp) in two reference strains and 16 isolates of *Aeromonas* (*A.*) *salmonicida.* Lane M: molecular mass marker; lane 1 to 18: strains AS01, AS02, AS03, AS04, AS05, AS06, AS07, AS08, AS09, AS10, AS11, AS12, AS13, AS14, AS15, ASS, ASM, and AS16, respectively. Marker sizes (bp) are indicated.

GenBank. All amplfed *tet*A fragments in this study showed 100% homology with the *tet*A gene of pRAS1, a drug resistance plasmid of *A. salmonicida* (GenBank accession No. AJ517790.2). The *tet*E gene, which was detected in AS16, showed 100% homology with the *tet*E gene of *A. salmonicida* subsp. *salmonicida* A449 plasmid 4 (pAsa4; GenBank accession No. CP000645.1) and *A. salmonicida* plasmid pYA90644 (GenBank accession No. DQ366299.1).

qnr genes and codon mutations in the QRDRs of *A. salmonicida* isolates

The *gyr*A (663 bp) and *par*C (418 bp) genes of QRDRs were successfully amplified from all 16 isolates and the ASS reference strain (Table 3). The amplified products were sequenced and their corresponding amino acid sequences were aligned with the sequences of *gyr*A (GenBank accession No. L42453.1) and *par*C (GenBank accession No. AF473701.1) of *A. salmonicida* ATCC 14174. AS03 and ASS strains had no point mutations while the AS16 strain harbored a Ser[83]→Asn[83] substitution in *gyr*A codon 83 (Table 3). Other 14 isolates showed Ser[83]→Arg[83] substitutions in the same loci. Additionally, AS16 had a single nucleotide mutation (AAA→AAG) at the *par*C codon 80 without an amino acid substitution. No other substitutions were detected on *gyr*A codon 87 (Asp[87]) and 92 (Leu[92]), or *par*C codon 80 (Lys[80]) and 84 (His[84]). The *qnr* gene was not detected in any of the *A. salmonicida* strains in this study.

Strain typing by PFGE

All *A. salmonicida* strains utilized in this study were clustered into four types based the PFGE results (Fig. 2). The ASM, AS03, and AS16 were divided into type A, B, and C, respectively. The other 14 *A. salmonicida* subsp. *salmonicida* isolates and ASS were classified into the same cluster designated as type D.

Discussion

Based on Bergey's Manual of Determinative Bacteriology [14], at least three subspecies were identified among all 16
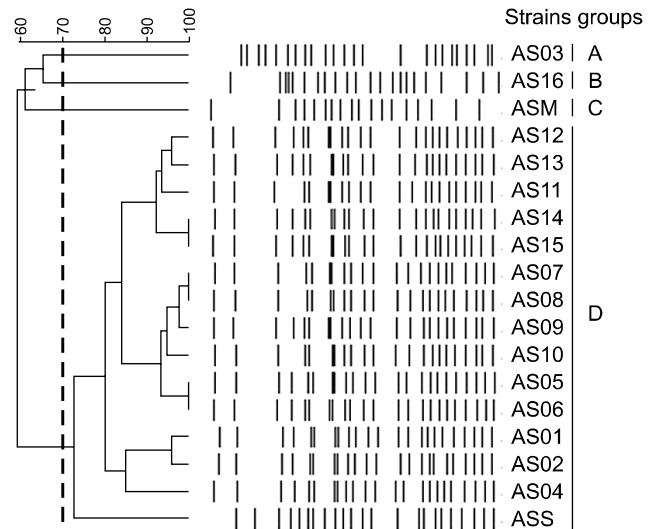

Fig. 2. Pulsed-field gel electrophoresis profiles of 18 *A. salmonicida* strains and UPGMA dendrogram. The vertical dotted line denotes a hypothetical node of 70% Dice coefficient of similarity (2.0% position tolerance).

isolates using the biochemical results of Vitek System 2. All of these interpretations were in concordance with the subspecies-specific PCR and 16S rRNA sequencing results. Interestingly, the AS16 strain that showed distinctive biochemical characteristics (D-glucose (+), D-mannitol (+), sucrose (+) and H_2S production ($-$)) was identified as the recently-reported *A. salmonicida* subsp. *flounderacida* (GenBank accession no. AY786177.1). Based on these results, we were able to confirm the presence of three subspecies of *A. salmonicida* (subsp. *salmonicida*, subsp. *achromogenes* and subsp. *flounderacida*) among those Korean isolates.

Considering the widespread use of tetracycline and quinolones in the aquaculture industry [12], the resistance of *A. salmonicida* to the two antibiotic classes was the focus of this study. According to the epidemiological cut-off values for *A. salmonicida* for oxytetracycline and oxolinic acid [24], eight oxytetracycline-resistant strains were detected, and sixteen isolates were oxolinic acid-resistant. Although enrofloxacin is one of the quinolones, like oxolinic acid [8], only one isolate (AS16) noted resistance to it. Interestingly, ampicillin resistance was detected only in three isolates (AS03, AS16, and ASM) although there have been some reports showing that *A. salmonicida* is naturally resistant to narrow-spectrum β-lactams [7]. One isolate showed resistance to gentamicin, and all strains were found to be susceptible to florfenicol and trimithoprim-sulfamethoxazole. Tetracycline resistance in *A. salmonicida* was strictly related to the presence of the *tet*A and *tet*E genes. These genes were also detected in other *A. salmonicida* strains from a variety of fish species in other countries [25,36]. The sequenced *tet*A

and *tet*E genes in this study showed 100% homology to *tet*A in pRAS1and *tet*E in pAsa4. Since the pRAS1 and pAsa4 plasmids can be transferred into or replicate within certain strains of *Escherichia coli* [31,35], it has been suspected that tetracycline resistance has been disseminated between various bacterial species. The location and transferability of the *tet*A and *tet*E genes in *A. salmonicida* clearly warrants further investigation.

Despite the high levels of activity of quinolones against *Aeromonas* species [16,18], the number of quinolone-resistant *Aeromonas* strains has increased [10,34]. In this study, all 16 isolates showed resistance to oxolinic acid, and the AS16 strain was resistant to enrofloxacin. Genetic analysis through sequencing QRDRs revealed that these resistant strains except AS03 harbored point mutations in *gyr*A codon 83. The AS01 to AS15 (except AS03) strains had $Ser^{83} \rightarrow Arg^{83}$ substitutions and the AS16 strain harbored a $Ser^{83} \rightarrow Asn^{83}$ substitution. In particular, the AS16 strain having a $Ser^{83} \rightarrow Asn^{83}$ substitution exhibited high-level resistance to both oxolinic acid and enrofloxacin. It is well known that quinolone resistance is principally related to mutations of the QRDRs, particularly in *gyr*A codons 83, 87, and 92, and in *par*C codons 80 and 84 [10,11]. In addition to previous studies that found $Ser^{83} \rightarrow Ile^{83}$ and $Ser^{83} \rightarrow Val^{83}$ substitutions on *gyr*A codon 83 in strains of *Aeromonas* [2,11,28], here we report two more putative substitutions that might contribute to quinolone resistance. Moreover, based on our results, amino acid substitutions on *gyr*A codon 83 may affect the level and spectrum of quinolone resistance in *A. salmonicida*.

In addition to the point mutations in the QRDRs, acquisition of *qnr* genes or efflux pumps also contribute to quinolone resistance [6,30]. In this study, the AS03 strain showed low-level resistance to oxolinic acid without mutations in the QRDRs. Since no *qnr* genes were detected, further investigation should be performed for determining whether this strain contains other quinolone-resistant mechanisms such as the efflux pump. The other possibility that could explain the differences in quinolone resistance between the isolates might be subspecies-specific natural resistance mechanisms that have yet to be elucidated; AS03 (*A. salmonicida* subsp. *achromogenes*) and AS16 (*A. salmonicida* subsp. *flounderacida*) were different subspecies from *A. salmonicida* subsp. *salmonicida* strains.

The PFGE results of this study typed isolates at the subspecies level. The genetic heterogeneity between subspecies was consistent with previous reports that described differences between typical and atypical *A. salmonicida* strains [9,13]. Interestingly, 14 *A. salmonicida* subsp. *salmonicida* strains isolated from Korea were found to be distinct from ASS although they were included in the same cluster; this result suggests geographical differences in the distribution of *A. salmonicida*.

Besides the subspecies classification, most of biochemical

results and MDR patterns of the *A. salmonicida* strains also concurred with the PFGE types. For example, AS16 (type B) was identified as *A. salmonicida* subsp. *flunderacida* and resistant to the highest number of antibiotics including ampicillin, gentamicin, oxytetracycline, enrofloxacin, and oxolinic acid. Strains in other PFGE types (type A and D) were identified as *A. salmonicida* subsp. *achromogenes* or subsp. *salmonicida* that were resistant to one or two antibiotics. On the other hand, the similar PFGE pattern of the tetracycline-resistant and susceptible *A. salmonicida* subsp. *salmonicida* strains within type D appears to imply horizontal transfer of *tet* genes among these isolates. These results suggest a potential risk of the spread of MDR strains or dissemination of antimicrobial resistance genes in the Korean aquatic industry.

Thus far, only a few antibiotics are approved for use in animals in the worldwide aquatic industry [19,24]; nevertheless, antibiotic resistance is expected to continue to become more frequent [20-23]. The detection of MDR in Korean strains of *A. salmonicida* suggests that antibiotic resistance in aquaculture can pose a risk to both humans and animals. Thus, stricter guidelines for the use of tetracycline and quinolones will be necessary to prevent the dissemination and acquisition of antibiotic resistance in aquaculture.

Acknowledgments

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