FULL PAPER *Bacteriology*

High Prevalence of *Bla$_{CTX-M}$* Group Genes in *Aeromonas dhakensis* Isolated from Aquaculture Fish Species in South Korea

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ABSTRACT. The prevalence of resistant genes against β-lactams in 119 *Aeromonas* strains was determined. A large number (99.2%) of the present fish strains were resistant to one or more β- lactams including ceftiofur, amoxicillin-clavulanic acid, ampicillin, piperacillin and cefpodoxime. Among antibiotic resistance phenotypes, the simultaneous resistance to all β-lactams occurred in 25.2% (n=30) of all strains, which consisted of 18 strains of *A. dhakensis*, 8 strains of *A. caviae*, 2 strains of *A. hydrophila* and only one strain of *A. veronii*. For exploring genetic background of the antibiotic resistances, multiple PCR assays were subjected to detect β-lactamase-encoding genes, *bla$_{TEM}$*, *bla$_{OXA-B}$* and *bla$_{CTX-M}$*. In the results, the *bla$_{TEM-1}$* gene was harbored in all strains, whereas only 3 strains harbored *bla$_{OXA}$* gene. In the case of *bla$_{CTX-M}$* gene, the gene was detected in 21.0% (25 out of 119) of all strains, which countered with 80% (20 out of 25) of *A. dhakensis*, 8% (2 out of 25) of *A. caviae* and 12% (3 out of 25) of *A. hydrophila*. In addition, most of the *bla$_{CTX-M}$* positive strains showed simultaneous resistance to all β-lactams (18 out of 30 strains). In sequence analysis for *bla$_{CTX-M}$* genes detected, they were CTX-M group 1-encoding genes including *bla$_{CTX-M-33}$* from 3 eel strains of *A. dhakensis*. Therefore, *A. dhakensis* obtained from cultured fish could represent a reservoir for spreading genes encoding CTX-M group 1 enzymes and hence should be carefully monitored, especially for its potential risk to public health.

KEY WORDS: *Aeromonas aquariorum*, *Aeromonas dhakensis*, aquaculture, β-lactams, *bla$_{CTX-M-1}$* group

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Since *Aeromonas dhakensis*, formerly called *A. aquariorum,* was firstly detected in ornamental fish [13], the bacterium has been recognized in retrospective studies to be an important pathogenic species within the *Aeromonas* genus because of its high prevalence in human clinical strains [6, 15, 16, 20]. However, there is less information about the prevalence and characteristics of *A. dhakensis* in veterinary aquatic medicine than human medicine [11, 22].

Aeromonas spp. can produce various β-lactamases for conferring resistance to β-lactams. Amber class-B, -C and -D β-lactamases have been known to be chromosomally mediated β-lactamases in *Aeromonas* spp. [8, 12, 15, 20, 21]. Of these β-lactamases, *cphA* and its related genes encoding class-B β-lactamases have shown species-specific distributions. In the case of class-A β-lactamases, the previous studies have shown different prevalence for genes encoding TEM-type β-lactamases in *Aeromonas* spp. according to isolation sources [2, 3, 14, 15]. In contrast, CTX-M encoding genes have been identified in only three previous studies, which found *A. caviae* and/or *A. hydrophila* producing CTX-M-3 and CTX-M-15 β-lactamases [5, 12, 21]. In addition, another previous study did not detect *bla$_{CTX-M}$* in a collection of human clinical *A. dhakensis* strains, whereas *bla$_{TEM}$* and *bla$_{MOX}$* genes were detected at high rates in the strains [15]. However, most previous studies aimed at detecting genetic backgrounds have employed very limited human clinical *Aeromonas* strains [15, 20, 21]. There is little information in the literature about the prevalence to β-lactamases-encoding genes in *Aeromonas* strains from aquaculture, which involves ecosystems that can include various global factors associated with selective and/or co-selective resistance to antibiotics.

The present study examined the resistance to β-lactams, amoxicillin, amoxicillin/clavulanic acid (AmC), piperacillin (PIP), ceftiofur (Tio) and cefpodoxime (Pod) in 119 strains from fish identified at the *Aeromonas* spp. level. To determine the prevalence to genetic determinants of resistance against β-lactams in fish clinical *Aeromonas* strains, a total of 119 strains of genus Aeromonas were evaluated for resistance to β-lactams. For exploring genetic background of the antibiotic resistances, multiple PCR assays were subjected to detect β-lactamase-encoding genes *bla$_{TEM}$*, *bla$_{OXA-B}$* and *bla$_{CTX-M}$*.

MATERIALS AND METHODS

Aeromonas strains: One hundred and nineteen strains were isolated from eel (n=70), pet fish (n=36) and koi carp (n=13). The eel strains were identified at the species level in our previous study by phylogenetic analysis using house-

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Table 1. Resistance to five different β-lactams according to *Aeromonas* spp. used in the present study

Species	No. of resistant strain against				
	AM	AmC	PIP	Pod	Tio
A. veronii (n=49)	48 (1)*	16 (16)	38 (1)	2 (2)	12 (3)
A. dhakensis (n=23)	23 (0)	22 (20)	22 (1)	18 (7)	21 (2)
A. hydrophila (n=21)	21 (0)	16 (14)	17 (3)	2 (2)	10 (3)
A. caviae (n=16)	16 (0)	15 (6)	14 (2)	9 (4)	15 (1)
A. jandaei (n=5)	5 (0)	2 (2)	5 (1)	0	2 (1)
A. media (n=2)	2 (0)	2 (1)	2 (0)	1 (0)	2 (0)
A. allosaccharophila (n=2)	2 (0)	0	2 (0)	0	0
A. trota (n=1)	1 (1)	0	1 (0)	1 (0)	1 (0)
Total (n=119)	118 (2)	73 (59)	101 (8)	33 (15)	63 (10)

* Parenthesized numbers indicate the number of strains with intermedate resistance.

keeping genes (gyrB and rpoD) [11, 22]; they consisted of *A. dhakensis* (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 pet fish strains were identified as 5 *Aeromonas* spp.—*A. veronii* (*n*=31), *A. allosaccharophila* (n=2), *A. dhakensis* (n=1), *A. hydrophila* (n=1) and *A. jandaei* (n=1)—by phylogenetic analysis using *gyrB* gene sequences. The same identification method was applied for the koi carp strains, which consisted of *A. hydrophila* (n=8) and *A. veronii* (n=5). All strains were stored at −70°C using Cryocare Bacteria Preservers (Key Scientific Products, Stamford, TX, U.S.A.) until used for antimicrobial susceptibility tests and the detection of β-lactamase-encoding genes (bla_{TEM}, bla_{OXA-B} and bla_{CTX-M}).

Antimicrobial susceptibility test: The antimicrobial susceptibility test (AST) was implemented using the VITEK 2 system with a veterinary susceptibility test card for Gram-negative bacteria (AST-GN38) (BioMérieux, Lyon, France), according to the manufacturer's instructions. The AST-GN38 card contains the following β-lactam antimicrobial agents as dehydrated substances at the indicated concentrations: ceftiofur (Tio)—1 and 2 μg/m*l*; amoxicillin and clavulanic acid (AmC)—4/2, 16/8 and 32/16 μg/m*l*; ampicillin (AM)—4, 8 and 32 μg/m*l*; cefpodoxime (Pod)—0.5, 1 and 4 μg/μ*l*; and piperacillin (PIP)—4, 16, 32 and 64 μg/m*l*. The cutoff for resistance to each antimicrobial agent was designated as a resistance of greater than "intermediate" as defined by the criteria of the VITEK 2 system (version 04.01) based on the CLSI M100-S18 [19].

Detection of β-lactam resistance genes: The genomic DNA from strains was purified using the AccuPrep genomic extraction kit (Bioneer, Seoul, Korea). The β-lactamase-encoding genes, bla_{TEM}, bla_{OXA-B} and bla_{CTX-M}, were subjected to PCR assays using the same primers and conditions as used in previous studies [8, 18]. The specificity for each PCR assay was demonstrated by direct sequencing analysis using the same primer sets for amplification on randomly selected PCR products. However, all PCR products for the bla_{CTX-M} gene were sequenced at the Macrogen Service Center (Seoul, Korea). The generated sequences (277 to 704, based on the *E. coli* numbering of the $bla_{CTX-M-3}$ gene; accession number AB231615) were aligned with those of well-known bla_{CTX-M} genes (GenBank) according to CTX-M-1, −2, −8/25 and −9 groups. Genetic distances were determined using the Kimura two-parameter models, and neighbor-joining phylogenetic trees were constructed using the MEGA5 program, according to the previously reported method [22].

RESULTS

Table 1 shows proportion of resistance to five different β-lactams according to *Aeromonas spp*. AM and PIP showed resistance in 99.2% and 84.9% of all *Aeromonas* strains, respectively. Major species (*A. veronii*, *A. hydrophila*, *A. caviae* and *A. dhakensis*) revealed variable resistance levels to the other β-lactams. Of these β-lactams, the most resistant species to Pod was *A. dhakensis* (78.3%), followed by *A. caviae* (56.3%), *A. hydrophila* (9.5%) and *A. veronii* (4.1%). In addition, Pod resistant strains (27.7% of 119 strains) were simultaneously resistant to Tio. Tio resistant strains were observed in 52.9% of all strains including 21 strains of *A. dhakensis* (91.3%), 15 strains of *A. caviae* (93.8%), 10 strains of *A. hydrophila* (47.6%) and 12 strains of *A. veronii* (24.5%). In the case of AmC resistance, the prevalence was 61.3%. However, 89.0% (n=105) of AM-resistant strains (n=118) showed below intermediate resistance to AmC. When compared with *Aeromonas* spp., the decreased resistance was frequently observed in *Aeromonas* spp., except for *A. caviae* strains (Table 1).

Eleven different resistance patterns for β-lactams strains could be classified among all *Aeromonas* strains (Table 2). The predominant pattern was simultaneous resistance to all β-lactams (30 strains), followed by AM+PIP (29 strains), AM+AmC+PIP+Tio (23 strains), AM+AmC+PIP (12 strains) and only AM (9 strains). Of these patterns, simultaneous resistance to all β-lactams was the most frequently observed patterns for *A. dhakensis*.

In investigation of genetic background to β-lactam resistances, all of the 119 strains harbored the bla_{TEM}, and sequences of random PCR products had 100% identity to gene encoding TEM-1-type β-lactamase (GenBank No. KJ933392). The bla_{OXA} gene was encountered in three strains: one *A. allosaccharophila* strain from pet fish and two *A. caviae* strains from eel. In the case of bla_{CTX-M} gene,

Table 2. Prevalence of *bla*$_{CTX\text{-}M\text{-}1}$ group in Aeromonas spp. according to resistance patterns of β-lactams

Resistance pattern	Detection of *bla*$_{CTX\text{-}M\text{-}1}$ group in *Aeromonas* spp. (number of strains)								
	A. dhakensis	*A. caviae*	*A. hydrophila*	*A. veronii*	*A. jandaei*	*A. trota*	*A. allosaccharophila*	*A. media*	Total
None	0	0	0	0 (1)	0	0	0	0	0 (1)
AM	0	0	1 (1)	0 (8)	0	0	0	0	1 (9)
AM+AmC	0	0	0 (3)	0 (2)	0	0	0	0	0 (5)
AM+PIP	1 (1)*	1 (1)	0 (2)	0 (20)	0 (3)	0	0 (2)	0	2 (29)
AM+PIP+Tio	0	0	0 (2)	0 (3)	0	0	0	0	0 (5)
AM+AmC+Tio	1 (1)	0 (1)	0	0	0	0	0	0	1 (2)
AM+AmC+PIP	1 (1)	0	0 (5)	0 (6)	0	0	0	0	1 (12)
AM+AmC+PIP+Tio	2 (2)	0 (5)	0 (6)	0 (7)	0 (2)	0	0	0 (1)	2 (23)
AM+PIP+Pod+Tio	0	0	0	0 (1)	0	0 (1)	0	0	0 (2)
AM+AmC+Pod+Tio	0	0 (1)	0	0	0	0	0	0	0 (1)
AM+AmC+PIP+Pod+Tio	15 #(18)	1 (8)	2 (2)	0 (1)	0	0	0	0 (1)	18 (30)
Total	20 (23)	2 (16)	3 (21)	0 (49)	0 (5)	0 (1)	0 (2)	0 (2)	25 (119)

*Nonparenthesized and parenthesized numbers indicate the number of strains carrying *bla*$_{CTX\text{-}M\text{-}1}$ group genes and showing each grouped phenotypic antimicrobial resistance, respectively. #3 out of 15 strains harbored *bla*$_{CTX\text{-}M\text{-}33}$ gene.

prevalence of the gene showed differences according to *Aeromonas* spp. and β-lactam resistance patterns (Table 2). The *bla*$_{CTX\text{-}M}$ genes were detected in 25 strains including 20 of *A. dhakensis* (19 eel strains and 1 pet fish strain), 2 eel strains of *A. caviae* and 3 eel strains of *A. hydrophila*. The PCR products were subjected to phylogenetic analysis by direct sequencing analysis using the primer set for *bla*$_{CTX\text{-}M}$ consensus [18]. The constructed phylogenetic tree showed that 25 *bla*$_{CTX\text{-}M}$-positive strains were grouped with genes encoding CTX-M-1-group β-lactamases (Fig. 1). On the other hand, 3 eel strains of *A. dhakensis* were clustered with the gene encoding CTX-M-33 belonging to CTX-M-1 group. In addition, the genes were detected more among strains with resistance to all β-lactams (18/30) than among strains with other resistance patterns (Table 2 and Fig. 1).

DISCUSSION

The principal findings of the present study were twofold: (i)*A. dhakensis* could be a reservoir for genes encoding CTX-M-1-group β-lactamases in aquatic farming, and (ii) this is the first time that strains harboring *bla*$_{CTX\text{-}M\text{-}33}$ gene—which encodes a variant of CTX-M-15 β-lactamases—have been isolated in worldwide veterinary aquatic medicine and the Korean microbiology community.

There are many doubts about the resistance of *Aeromonas* spp. to β-lactams in aquaculture farming [11, 14, 17]. In general, most of Aeromonas strains were resistant to aminopenicillins (e.g., amoxicillin and ampicillin), regardless of the isolation sources [9, 17]. The resistance was also shown in most strains used in the present study. Therefore, the results were agreed with the previous hypothesis, suggesting the production of naturally occurring aminopenicillinase in *Aeromonas* spp. [9]. In addition, all of the present strains harbored the *bla*$_{TEM}$ gene encoding TEM-type β-lactamase. When compared with the previous studies [1, 8, 17], a particularly interesting finding of the present study was the frequent occurrence of strains with resistance to ceftiofur and cefpodoxime. Neither of these antibiotics is approved for aquaculture use, but they are both approved for veterinary use. Although its occurrence is rare, a previous study found resistance to both antibiotics in *Aeromonas* spp. and *Pseudomonas* spp. from rainbow trout (*Oncorhynchus mykiss*) [1]. It is possible that the resistance to both antibiotics is a consequence of their off-label use in aquaculture and/or anthropogenic contamination from other farmed areas .

Cefpodoxime-resistant strains were found to be simultaneously resistant to ceftiofur. The prevalence of resistance to both antibiotics differed among the *Aeromonas* spp.: Ceftiofur resistance was more prevalent in *A. caviae* and *A. dhakensis* than in other *Aeromonas* spp., and *A. dhakensis* was the predominant species for cefpodoxime resistance. These results could be related to differences in genetic determinants related to antibiotic resistance among *Aeromonas* spp. Previous studies have also frequently detected genes encoding CTX-M-type cephalosporinase in ceftiofur-resistant *Enterobacteriaceae* isolates from domestic animals [4, 10]. On the other hand, most of present strains were highly resistant to ampicillin, but they showed decreased resistance to combination of aminopenicillin and β-lactamase inhibitor. The behavior was observed in all *Aeromonas* spp. except *A. caviae* and was similar to extended-spectrum β-lactamase-producing strain [4, 5, 7, 10]. In these aspects, the present study subjected all strains to PCR assays in order to detect *bla*$_{CTX\text{-}M}$ genes, followed by sequencing analysis of the amplicons. The CTX-M-1 group-encoding genes were frequently detected in strains with resistance to all β-lactams, with the pattern commonly observed in *A. dhakensis* strains of eel. This result supports our expectation of different genetic backgrounds to β-lactams antibiotic resistance in *Aeromonas* spp. A previous study found no *bla*$_{CTX\text{-}M}$ genes, but frequently detected *bla*$_{MOX}$ genes in 47 clinical strains of *A. dhakensis* with high prevalence of resistances to amoxicillin and amoxicillin-clavulanic acid [15]. Therefore, it is unclear whether the distribution of *bla*$_{CTX\text{-}M\text{-}1}$-group genes is species specific, as for previous reports on *cphA*-related

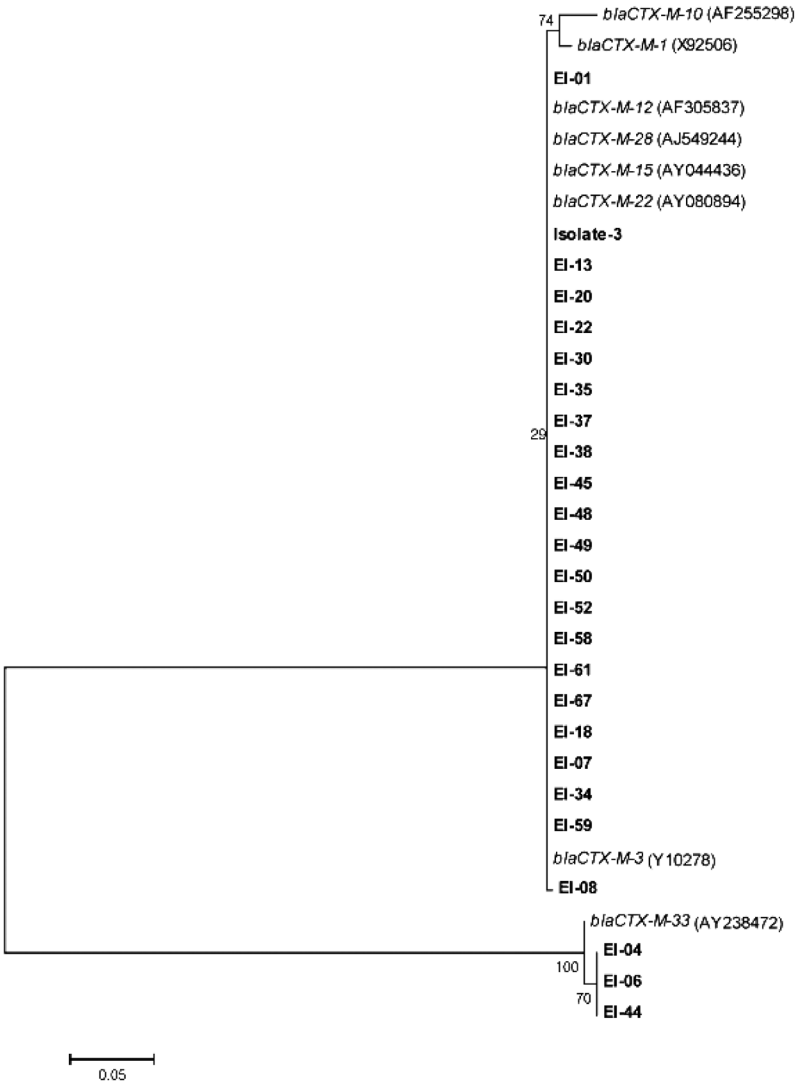

Fig. 1. Phylogenetic identification of $bla_{CTX\text{-}M\text{-}1}$-group gene sequences resulting from $bla_{CTX\text{-}M}$-positive strains. The identification was performed by alignments with major sequences belonging to $bla_{CTX\text{-}M\text{-}1}$-group genes in GenBank (accession numbers are within parentheses). The branch numbers refer to the percentage confidence as estimated by a bootstrap analysis with 1,000 replications.

genes in *Aeromonas* spp. [20]. Based on differences in the isolation sources between the present and previous studies [15], *A. dhakensis* is regarded as having different genetic backgrounds under selective pressure according to antibiotic use. In addition, AmpC producer might be *A. caviae* strains with high resistance to ampicillin and ampicillin-clavulanic acid in the present study.

To the best of our knowledge, no previous study has investigated the prevalence of $bla_{CTX\text{-}M}$ genes in aquatic cultured fish species. In addition, this study is the first to isolate the strains harboring the $bla_{CTX\text{-}M\text{-}33}$—a gene encoding a variant CTX-M-15 β-lactamases—from aquatic medicine after its first description in clinical *E. coli* isolates in Greece [7]. This study indicates that *A. dhakensis* could be the main carrier for spreading $bla_{CTX\text{-}M\text{-}1}$-group genes in aquatic farming, hence, its risk to public health needs to be determined and to be monitored seriously.

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