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CASE REPORT

First report of tetracycline-resistant *Aeromonas veronii* infection in Amur catfish (*Silurus asotus*) cultured in Korea

Hyo Yeon Kim[1,2,†], Jin Woo Jun[3,†], Se Ra Lim[1], Seon Young Park[1], Jee Eun Han[4], Se Chang Park[2,*], Ji Hyung Kim[1,*]

[1]*Infectious Disease Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 34141, Korea*
[2]*Laboratory of Aquatic Biomedicine, Research Institute for Veterinary Science, College of Veterinary Medicine, Seoul National University, Seoul 08826, Korea*
[3]*Department of Aquaculture, Korea National College of Agriculture and Fisheries, Jeonju 54874, Korea*
[4]*Laboratory of Aquatic Biomedicine, College of Veterinary Medicine, Kyungpook National University, Daegu 41566, Korea*

Abstract: Mass mortality in commercially cultured Amur catfish (*Silurus asotus*), showing symptoms of dermal ulcerations, occurred on a private farm in Mar 2019 in Korea. β-hemolytic bacteria were isolated from the ulcers and kidneys of the fish and identified as *Aeromonas veronii*. The isolate was resistant to tetracycline and possessed cytotoxic heat-labile enterotoxin (*aerolysin*/*hemolysin*). We investigated the genetic determinants associated with tetracycline resistance, and the isolate has been confirmed to simultaneously possess *tetA* and *tetE* genes. This is the first report on the occurrence of tetracycline-resistant *A. veronii* infection related to mass mortality in commercially cultured Amur catfish in Korea.

Keywords: Amur catfish (*Silurus asotus*), *Aeromonas*, tetracycline resistance, *tetA*, *tetE*

*Corresponding author
Ji Hyung Kim
Infectious Disease Research Center, Korea Research Institute of Bioscience and Biotechnology, 125 Gwahak-ro, Yuseong-gu, Daejeon 34141, Korea
Tel: +82-42-879-8272
Fax: +82-42-879-8498
E-mail: kzh81@kribb.re.kr
Se Chang Park
Laboratory of Aquatic Biomedicine, Research Institute for Veterinary Science, College of Veterinary Medicine, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 08826, Korea
Tel: +82-2-880-1282
Fax: +82-2-880-1213
E-mail: parksec@snu.ac.kr
†The first two authors contributed equally to this work.
ORCID
Hyo Yeon Kim
https://orcid.org/0000-0002-3507-0311
Jin Woo Jun
https://orcid.org/0000-0002-0318-6889
Se Ra Lim
https://orcid.org/0000-0002-8915-3876
Seon Young Park
https://orcid.org/0000-0002-1351-7557
Jee Eun Han
https://orcid.org/0000-0002-2905-7524
Ji Hyung Kim
https://orcid.org/0000-0002-7921-2625
Se Chang Park
https://orcid.org/0000-0001-9821-387X

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Amur catfish (or Korean catfish, *Silurus asotus*) belonging to Siluridae, is widely distributed throughout East Asia including Korea, and is a commercially important freshwater fish in Korean aquaculture [1]. Up to recent, several bacterial pathogens such as *Aeromonas hydrophila*, *A. veronii*, *Edwardsiella ictaluri*, and *Vibrio ordalii* have been reported to be implemented in the mass mortality of the cultured Amur catfish in Korea [1,2].

Aeromonas spp. are ubiquitous gram-negative bacilli found in various aquatic environments, with several species identified as pathogens in aquatic animals, including fish, reptiles, and amphibians [3]. Among the major fish-pathogenic *Aeromonas* species, *A. veronii* is associated with Epizootic Ulcerative Syndrome (EUS) and Motile Aeromonas Septicemia (MAS) of fish, with disease outbreaks caused by the bacterium increasing in the recent years [4]. In Korea, several disease outbreaks associated with *A. veronii* have been reported in cultured Israeli carp (*Cyprinus carpio*) [5,6], the Japanese eel (*Anguilla japonica*) [7,8], and the Amur catfish [2]. In addition, the emergence of *Aeromonas* strains resistant to commercial antibiotics commonly used in aquaculture, has raised serious worldwide concerns. The acquisition of resistance to tetracycline and quinolones in fish-pathogenic *Aeromonas* spp., has confirmed in Korean aquaculture [9,10]. Although *A. veronii* isolated from Korean environmental water already possessed tetracycline resistance [10], the resistance mechanisms in fish-pathogenic isolates remain uninvestigated.

Herein, we report a case of mass mortality in commercially cultured Amur catfish (*S. asotus*) caused by the *A. veronii*. From the isolate, the acquisition of tetracycline resistance was verified, and the resistance-related genetic determinants identified. This is the first report of the occurrence of tetracycline-resistant *A. veronii* infection related to mass mortality in commercially cultured Amur catfish in Korea.

In March 2019, mass mortality in catfish occurred on a private catfish farm located in Jeollabuk-do Province, Republic of Korea. Prior to the onset of the disease, a batch of catfish in juvenile stage (14 days after hatching) were transferred to a culture tank and maintained at 20 ± 2ºC. The water quality

parameters including dissolved oxygen, pH, and ammonia were appropriately maintained. The onset of disease symptoms was first observed four days post transfer into the culture tank, while cumulative mortality reported around six days after the transfer. The symptoms were characterized by the presence of severe, open dermal ulcers with focal hemorrhages on the head, middle of the body, and the dorsal regions of the fish, similar to the typical symptoms in EUS [4]. Dead or moribund fish (average body length 77.0 ± 2.6 mm, average body weight 3.7 ± 0.5 g) were submitted to the Infectious Diseases Research Center, Korea Research Institute of Bioscience and Biotechnology for investigation of etiological agents.

During the post-mortem analysis, several deep ulcerations that extended through muscle tissue were reported, and a weak accumulation of ascitic fluid in the peritoneal cavities was observed (Fig. 1). Parasitological examinations did not reveal the presence of external or gill parasites on the catfish. Sterile swabs from kidney and skin ulcers were streaked onto 5% sheep blood agar plates (BAPs) (Synergy innovation, Korea) and incubated at 20ºC for 24 h. Similar β-hemolytic bacterial growth was observed in plates streaked with kidney and skin ulcer swabs. The colonies were repeatedly sub-cultured onto BAPs until pure isolates were obtained, and the isolated β-hemolytic bacteria were identified using

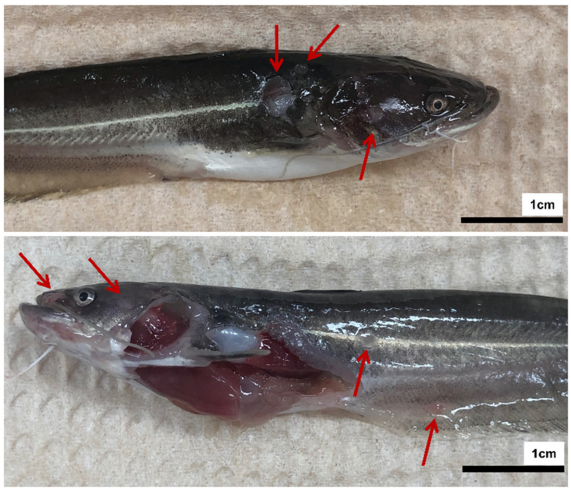

Fig. 1. *Aeromonas veronii* infected juvenile Amur catfish (*Silurus asotus*) showing severe, open dermal ulcerations (red arrows) on the head, on the middle of the body, and on the dorsal regions of the fish. Scale bars indicate one centimeter.

Table 1. List of polymerase chain reaction primers used in this study

Primer name	Sequence (5'-3')	Reference
Bacterial identification		
16s rRNA		
785F	GGATTAGATACCCTGGTA	Universal primer
907R	CCGTCAATTCMTTTRAGTTT	
rpoB		
Pasrpob-L	GCAGTGAAAGARTTCTTTGGTTC	[11]
Rpob-R	GTTGCATGTTNGNACCCAT	
Tetracycline resistance		
tetA_F	GCTACATCCTGCTTGCCTTC	[9]
tetA_R	GCATAGATCGCCGTGAAGAG	
tetB_F	TCATTGCCGATACCACCTCAG	
tetB_R	CCAACCATCATGCTATTCCATCC	
tetC_F	CTGCTCGCTTCGCTACTTG	
tetC_R	GCCTACAATCCATGCCAACC	
tetD_F	TGTGCTGTGGATGTTGTATCTC	
tetD_R	CAGTGCCGTGCCAATCAG	
tetE_F	ATGAACCGCACTGTGATGATG	
tetE_R	ACCGACCATTACGCCATCC	
Virulence factors		
alt_F	AAAGCGTCTGACAGCGAAGT	[12]
alt_R	AGCGCATAGGCGTTCTCTT	
ast_F	ATCGTCAGCGACAGCTTCTT	
ast_R	CTCATCCCTTGGCTTGTTTTTAC	
aerA/haem_F	CCTATGGCCTGAGCGAGAAG	
aerA/haem_R	CCAGTTCCAGTCCCACCACT	

rRNA, ribosomal RNA.

MALDI-TOF MS (Macrogen Inc., Korea) and 16S ribosomal RNA (rRNA) sequencing (Macrogen Inc.). The obtained results indicated that the isolates belong to the genus *Aeromonas*, but failed to discriminate to the species level. Therefore, the *rpoB* gene of the isolates that encodes the β-subunit of the DNA-dependent RNA polymerase was amplified and sequenced using the primers Pasrpob-L/Rpob-R [11] (Table 1). The obtained sequences were compared to the type strains of the *Aeromonas* spp. in the GenBank database. The *rpoB* sequences were identical between our isolates and most similar to *A. veronii* ATCC35624^T (98.4% nucleotide identity) among the species in the *Aeromonadaceae* (Fig. 2). Based on the results of 16S rRNA and *rpoB* comparisons, the β-hemolytic bacterial isolates were identified as *A. veronii*. Here, the simultaneous isolation of *A. veronii* from the ulcers and kidneys of the catfish clearly indicates that the same bacteria caused MAS and EUS. The kidney-isolated *A. veronii* strain was designated AVNIH1 and used for further analysis in this study.

Biochemical characteristics of the *A. veronii* strain AVNIH1 were analyzed using the API 20E system (bioMérieux, France) following the manufacturer's protocol. The result was compared to *A. veronii* strain KC-1109, previously implicated in the mortality of adult catfish cultured in Korea [2], as well as ATCC 9071 and ATCC 35624^T, and the results are shown in Supplementary Table 1. The results showed that overall the biochemical phenotype of strain AVNIH1 was very similar to those of strain KC-1109 and ATCC 9071 but differed from ATCC 35624^T. Based on the positive results of the AMY test (oxidation of amygdalin), our isolate was distinct from strain KC-1109 and ATCC 9071.

To evaluate the pathogenic potential of the *A. veronii* isolate, the presence of *Aeromonas*-specific virulence-related genes coding for cytotoxic heat-labile enterotoxin (*aer*, also known as *aerolysin*/*hemolysin*), cytotonic heat-labile enterotoxin (*alt*), and a cytotonic heat-stable enterotoxin (*ast*) were determined by polymerase chain reaction (PCR) analyses [12] (Table 1). The isolate only possessed the *aer* gene, almost identical (> 99% amino acid identity) to those from other fish-pathogenic *Aeromonas* spp., including *A. veronii* strains X11 (CP024930) and AKEL/SRLAAH/2017 (MH757092). Aerolysin was reported to be one of the major virulent factors in the pathogenesis of *A. veronii*-associated fish diseases, and the aerolysin-mediated aerotaxis has been determined as a causal factor in MAS and EUS [13]. Based on these results, the isolated *A. veronii* strain AVNIH1 demonstrated a strong possibility of causing EUS in this case, and also possessed a pathogenic potential in other fish species.

Antimicrobial susceptibility tests for the *A. veronii* isolate were performed using the disk diffusion method, using a total of 20 antimicrobial agents (Oxoid Ltd., UK), belonging to 9 different classes according to the guidelines of the Clinical and Laboratory Standards Institute [14]. According to the results of the disc diffusion test, the isolate was intermediate to imipenem (10 μg) and ciprofloxacin (10 μg), and resistant to tetracycline (30 μg) (Table 2). The minimum inhibitory concentration (MIC) of *A. veronii* strain AVNIH1 against tetracycline (256-0.015 μg) were further determined using MIC Evaluator strip (Oxoid Ltd.), and the MIC value was estimated to be 16 μg. To determine the genetic determinants associated with resistance to tetracycline, a multiplex PCR assay was conducted as previously described [9] to amplify the five tetracycline resistant genes (*tetA* to *tetE*) (Table 1). A total of two PCR amplicons were detected from *A. veronii* strain AVNIH1, and the sequencing analyses revealed that the isolate simultaneously possessed *tetA* and *tetE* as its genetic determinants associated with resistance to tetracycline. The newly identified *tetA* and *tetE* in *A. veronii* strain AVNIH1 were identical to those were found in *A hydrophila*

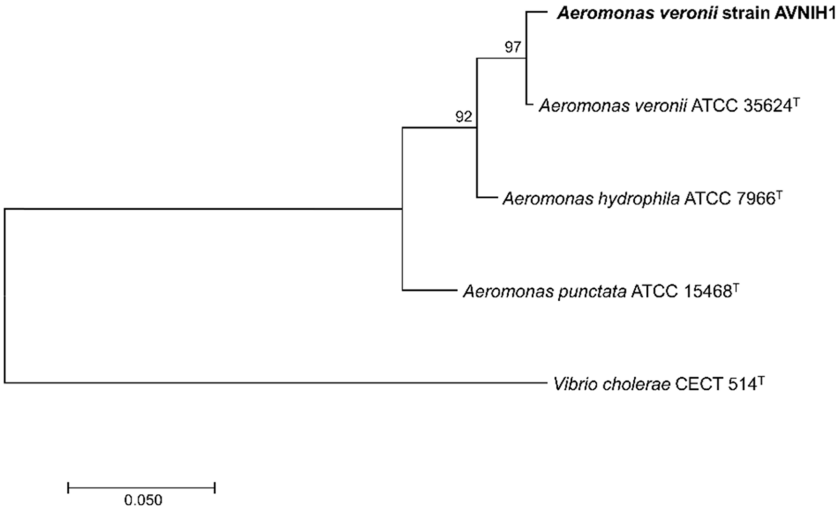

Fig. 2. Maximum-likelihood tree based on the nucleotide sequences of *rpoB* in *Aeromonas veronii* strain AVNIH1 to some representative type strains of *Aeromonas* species and the outgroup *Vibrio cholerae* CECT 514^T. The scale bar represents 0.05 nucleotide substitutions per site.

Table 2. Antibiotic resistance profiles of *Aeromonas veronii* strain AVN1H1

Antimicrobial agents	Disk diffusion	MIC (μg/mL)
Piperacillin-tazobactam (110 μg)	S	ND
Cefepime (30 μg)	S	ND
Cefotaxime (30 μg)	S	ND
Ceftazidime (30 μg)	S	ND
Cefuroxime (30 μg)	S	ND
Imipenem (10 μg)	I	ND
Meropenem (10 μg)	S	ND
Aztreonam (30 μg)	S	ND
Amikacin (30 μg)	S	ND
Gentamicin (10 μg)	S	ND
Tetracycline (30 μg)	R	R (16)
Ciprofloxacin (5 μg)	I	ND
Levofloxacin (5 μg)	S	ND
Chloramphenicol (30 μg)	S	ND
Trimethoprim-sulfamethoxazole (25 μg)	S	ND

ND, not done; MIC, minimum inhibitory concentration.

strain 23-C-23 plasmid (CP038466) and *A. veronii* strain MS-18-37 chromosome (CP033604), respectively.

Although *A. veronii* infections, which caused epidermal exfoliation and muscular necrosis in cultured adult Amur catfish, have been previously reported in Korea [2], the isolated strain KC-1109 did not demonstrated a resistance to tetracycline, which is commonly used to treat bacterial diseases in Korean aquaculture. However, we were able to detect phenotypical tetracycline resistance and also identify its genetic determinants (*tetA* and *tetE*) from *A. veronii* strain AVNIH1, suggesting that catfish-pathogenic *A. veronii* in Korea have already acquired resistance to the tetracycline. In actuality, the presence of tetracycline resistance genes (*tetA*) in *A. veronii* with environmental origin have already been reported [10]; however, simultaneous acquisition of the genetic determinants (*tetA* and *tetE*) in the bacteria have not been reported in Korea yet. Of the currently recognized zoonotic *Aeromonas* species, *A. veronii* has been reported to be implicated in clinical infections in Korea [15]. Therefore, the acquisition of antibiotic-resistance genes in this *Aeromonas* species may present as a serious potential public health risk, necessitating stricter guidelines for the use of tetracycline to prevent the dissemination and acquisition of tetracycline resistance in Korean aquaculture.

Culture deposition and nucleotide sequence accession No.

The *rpoB*, *act*, *tetA*, and *tetE* nucleotide sequences of *A. veronii* strain AVNIH1 have been deposited in the GenBank database under accession numbers MN047444, MN115385, MN047445, and MN047446, respectively. A living axenic culture of *A. veronii* strain AVNIH1 has been deposited in the Korean Culture Center of Microorganisms (KCCM) as KCCM 90343.

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Supplementary Table 1. Biochemical characteristics of *Aeromonas veronii* strain AVN1H1 based on the API 20E test

Strains	*A. veronii* strain AVNIH1	*A. veronii* strain KC-1109	*A. veronii* ATCC 9071	*A. veronii* ATCC 35624^T
ONPG	+	+	+	−
ADH	+	+	+	−
LDC	−	−	−	+
ODC	−	−	−	+
CIT	+	+	+	+
H_2S	−	−	−	−
URE	−	−	−	−
TDA	−	−	−	−
IND	−	−	−	−
VP	+	+	+	+
GEL	+	+	+	+
GLU	+	+	+	+
MAN	+	+	+	+
INO	−	−	−	−
SOR	−	−	−	−
RHA	−	−	−	−
SAC	+	+	+	+
MEL	−	−	−	−
AMY	+	−	−	−
ARA	−	−	+	−

ONPG, β-galactosidase; ADH, arginine dihydrolase; LDC, lysine decarboxylase; ODC, ornithine decarboxylase; CIT, citrate utilization; H_2S, H_2S production; URE, urease; TDA, tryptophane deaminase; IND, indole production; VP, Voges-Proskauer; GEL, gelatinase; GLU, glucose; MAN, mannitol; INO, inositol; SOR, sorbitol; RHA, rhamnose; SAC, saccharose; MEL, melibiose; AMY, amygdalin; ARA, arabinose.