J. of Practical Agriculture & Fisheries Research
2020 Dec. : 22(2): 51-58
ISSN 2234-1129

Isolation and Characterization of Antibiotic Resistant Vibrio Strains from Japanese Eel (*Anguilla Japonica*) Cultured in Korea

국내산 양식 뱀장어에서 항생제 내성 비브리오 세균 분리 및 특성

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Abstract

Continuous mortality in commercially cultured Japanese eel (*Anguilla japonica*), showing symptoms of dermal ulcerations and focal hemorrhages on the body, occurred on a private farm in November, 2019 in Korea. A series of mortality had been described in one local eel culture farm from November to December in 2019. From the three cases, three isolates of *Vibrio* spp. were recovered from the blood, ascitic fluid, and kidney of the dead fish, respectively. Based on the 16S rRNA sequence comparisons, the *Vibrio* isolates from the 1st and 3rd cases (strain named 1E1-2 and 3K1-2) were identified as *V. fluvialis* and the isolate from the 2nd case was identified as *V. plantisponsor* (strain named 2A3-1). Moreover, the 16S rRNA-based phylogenetic analysis revealed that strain 1E1-2 and 3K1-2 were most similar to *V. fluvialis* NBRC 103150^T, and strain 2A3-1 was most similar to *V. plantisponsor* NBRC103148^T. According to the results of the antibiotic resistance determination, *V. fluvialis* 1E1-2 showed intermediate resistance to tetracycline and chloramphenicol, and was resistant to trimethoprim-sulfamethoxazole. *V. plantisponsor* 2A3-1 showed intermediate resistance to ciprofloxacin and levofloxacin, and was resistant to trimethoprim-sulfamethoxazole. *V. fluvialis* 3K1-2 showed intermediate resistance to tetracycline, and was resistant to ampicillin and trimethoprim-sulfamethoxazole. These results have provided the evidences on the occurrence of antibiotic-resistant *Vibrio* infection in commercially cultured Japanese eels are present in Korea.

Key words : Japanese eel, Antibiotic-resistant *Vibrio, V. fluvialis, V. plantisponsor*

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Ⅰ. Introduction

It is known that the Japanese eel (*Anguilla japonica*) has a complicated life cycle and spawning migration (Tsukamoto *et al.*, 2003). It spawns far offshore in the ocean, returns to freshwater growth habitats, and then migrates back to its spawning area in the ocean (Jun *et al.*, 2020). This species is one of the most important cultured fish in East Asia, especially Korea, because of its high market value and the increasing demand for consumption (Lee *et al.*, 2003). In 2019, eel production in Korea accounted for more than 30% of the total Korean freshwater fisheries production, and approximately 70% of the total in monetary value (Korean Statistical Information Service, 2019).

Vibriosis is a serious fish disease caused by bacteria belonging to the genus *Vibrio* (Austin and Zhang, 2006). It has been known that some *Vibrio* species can infect a wide range of fish and invertebrates, and also cause mass mortality worldwide (Austin and Zhang, 2006). Among the *Vibrio* species, *V. vulnificus* and *V. anguillarum* have been known to be common bacterial pathogens in eel aquaculture (He et al., 2020; Zheng et al., 2019).

Herein, we report a series of mass mortality cases in commercially cultured Japanese eel caused by other *Vibrio* species. This is a meaningful case report of the occurrence of antibiotic-resistant *Vibrio* infection in commercially cultured Japanese eels in Korea.

Ⅱ. Materials and Methods

1. Cases:

From November to December in 2019, a series of disease outbreaks occurred on a private Japanese eel culture farm located in Jeollanam-do Province, Republic of Korea.

① 1st case: Hundreds of dead fish in each water tank were found for 6 days. Commercial antibiotics were applied to culture tank. Mortality seemed to decrease for 2 days.

② 2nd case: The number of dead fish rebounded from 2 days after antibiotics treatment. NaCl salinity (ca. 1.0%) treatment were applied to culture tank for 24 h. Fish were observed for 5-day post treatment for progressive change.

③ 3rd case: The mortality gradually increased after NaCl salinity treatment, presenting similar symptoms.

2. Isolation and identification of the causative bacteria:

Parasitological examinations were performed for the post-mortem analysis. Sterile swabs from the blood, ascitic fluid, and kidney of the dead fish were streaked onto tryptic soy agar (TSA; Becton, Dickinson and Company, USA) to isolate the causative bacteria, and the innoculated plate was incubated at 25℃ for 24 h. From the bacterial isolates, its genomic DNA was extracted by the DNeasy Blood & Tissue Kit (Qiagen, Germany), following the manufacturer's instruction. The 16S ribosomal RNA (rRNA) gene of the isolates were sequenced using an ABI PRISM Big Dye TM Terminator Cycle Sequencing Kit (Applied Biosystems, USA) at the Macrogen Genomic Division (Korea). Electrophoresis of sequencing reactions was performed using the Automatic Sequencer ABI 3730XL DNA Analyzer (Applied BioSystems).

In addition, the obtained 16S rRNA sequences of the isolates were aligned with representative sequences from each type strain of Vibrio species using ClustalX (version 2.1) (Larkin et al., 2007) and BioEdit Sequence Alignment Editor (version 7.1.0.3) (Hall, 1999). Then, the datasets were phylogenetically analyzed using the MEGA ver. 7.0 (Kumar et al., 2016). A neighbor-joining phylogenetic tree was constructed using a Jukes-Cantor distances matrix, and the reliability of the tree was assessed using 1,000 bootstrap replicates.

3. Antibiotic susceptibility test:

Antibiotic susceptibility of bacterial isolates was performed using the disk diffusion method, using antibiotic agents (Oxoid Ltd., UK). The sensitivity and resistance of isolated bacteria were determined according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2015).

Ⅲ. Results

In each case, five dead or moribund fish (average body length 407mm, average body weight 379g) were submitted to the Department of Aquaculture, Korea National College of Agriculture and Fisheries for diagnosis.

The quality of water was properly controlled and the water temperature ranged from 27.5 to 28.5℃. The symptoms were characterized by the presence of severe dermal ulcers with focal hemorrhages on the abdominal regions of the body, and accumulation of ascitic fluid in the peritoneal cavities (Fig. 1A, B, C, D, E). Bloody fluid in the peritoneal cavities and hemorrhages on intestine regions were observed in several examined fish (Fig. 1F).

Fig. 1. Diseased Japanese eel showing vibriosis symptoms

Parasitological examinations did not reveal the presence of external or gill parasites on the fish. Several bacterial strains which showed typical *Vibrio*-like colonies were isolated from examined fish in three cases. More than 75% of all bacterial strains isolated in three cases were found to belong to *Vibrio* sp.. As a result of 16S rRNA gene sequencing, the bacterial isolate from blood fluid of the fish which were submitted for the 1st case was identified to be *V. fluvialis*; the bacterial isolate from ascitic fluid of the fish (2nd case) was identified to be *V. plantisponsor*; the bacterial isolate from kidney of the fish (3rd case) was identified to be *V. fluvialis*. The 16S rRNA gene sequences of our isolates from the 1st case and 3rd case were most similar to that of *V. fluvialis* NBRC 103150^{T} (100% nucleotide identity; 99.86% nucleotide identity), and the 16S rRNA gene sequence of our isolate from the 2nd case was most similar to that of *V. plantisponsor* NBRC103148^{T} (100% nucleotide identity) (Fig. 2).

According to the results of the disk diffusion test, *V. fluvialis* 1E1-2 isolated from

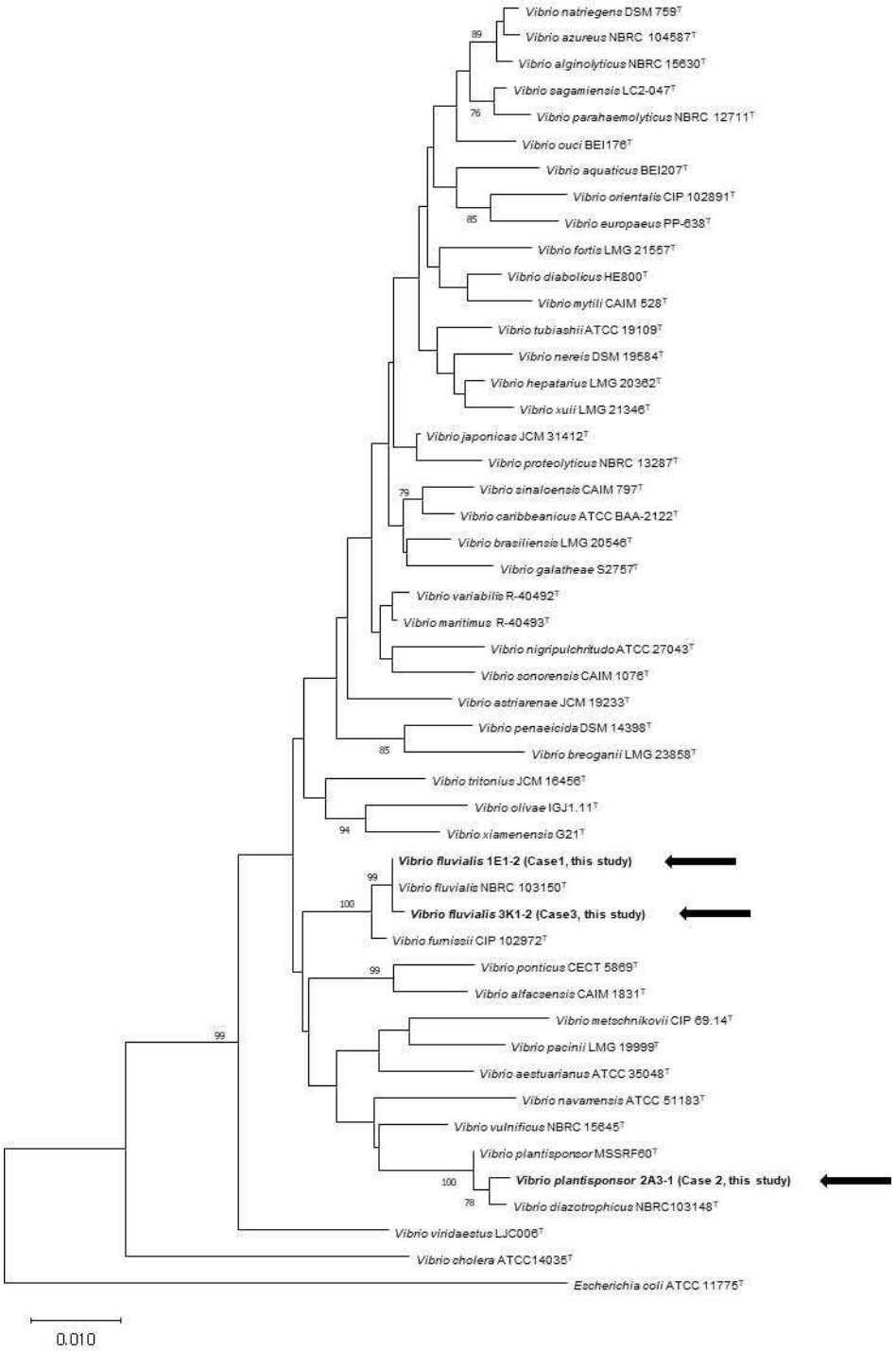

Fig. 2. Maximum-likelihood tree based on the 16S rRNA gene sequences of *Vibrio* isolates to some representative type strains of *Vibrio* species. The scale bar represents 0.01 nucleotide substitutions per site

Table 1. Antibiotic resistance profiles of *Vibrio* strains in this study

	Antimicrobial agent [disk content (μg)]											
	β-lactam	Cephems			Carb	Aminoglycosides		Te	Fluoroquinolones		Folate	P
Strains	AMP (10)	FEP(4) (30)	CTX(3) (30)	CAZ(3) (30)	IPM (10)	AK (30)	CN (10)	TE (30)	CIP (5)	LEV (5)	STX (1.25)	C (30)
V. fluvialis 1E1-2	S	S	S	S	S	S	S	I	S	S	R	I
V. plantisponsor 2A3-1	S	S	S	S	S	S	S	S	I	I	R	S
V. fluvialis 3K1-2	R	S	S	S	S	S	S	I	S	S	R	S

The category 'S' means sensitive to antibiotic; 'I' means intermediate; 'R' means resistant.
AMP, Ampicillin; FEP, Cefepime; CTX, Cefotaxime; CAZ, Ceftazidime; IPM, Imipenem; AK, Amikacin; CN, Gentamicin; TE, Tetracycline; CIP, Ciprofloxacin; STX, Trimethoprim-sulfamethoxazole; LEV, Levofloxacin; C, Chloramphenicol

the 1st case showed intermediate resistance to tetracycline and chloramphenicol, and was resistant to trimethoprim-sulfamethoxazole. *V. plantisponsor* from the 2nd case showed intermediate resistance to ciprofloxacin and levofloxacin, and was resistant to trimethoprim-sulfamethoxazole. *V. fluvialis* from the 3rd case showed intermediate resistance to tetracycline, and was resistant to ampicillin and trimethoprim-sulfamethoxazole (Table 1).

IV. Discussion

Although there has been a previous report of vibriosis in eels in Korea (Kim *et al.*, 2011), it has not been reported that *V. fluvialis* or *V. plantisponsor* was isolated from Japanese eel farm. We were not able to be sure that *V. fluvialis* or *V. plantisponsor* was the major causative agent of the mortality of Japanese eel in this study. Both of *V. fluvialis* isolates in this study were not proved to be sensitive to tetracycline, which is commonly used to treat bacterial diseases in Korean aquaculture. It coincided with the progression of the disease after commercial antibiotics, tetracycline treatment at the 1st case. Also, the isolated strain *V. fluvialis* was shown to be resistant to ampicillin and trimethoprim -sulfamethoxazole. *V. fluvialis* is known to be an important cause of bloody diarrhea and causes wound infection with primary septicemia in immunocom-promised individuals from developed to underdeveloped countires (Igbinosa and Okoh, 2010). Although *V. fluvialis* clinical infection has not been reported in Korea, the incidence of antibiotic resistant strain can increase the risk of threatening public health. Therefore, more standardized guidelines for antibiotics use are necessary to prevent the dissemination of antibiotic resistant bacteria in Korean aquaculture.

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요약

2019년 11월, 국내의 뱀장어 양식장에서 양식 중이던 뱀장어가 피부궤양 및 피부의 점상출혈 소견을 보이며 지속적으로 폐사하였다. 일련의 폐사는 2019년 11월부터 12월까지 지속되었다. *Vibrio* 속 균주 1E1-2는 첫 번째 폐사 사례에서 폐사한 뱀장어의 출혈성 복수에서 분리되었고 균주 2A3-1는 두 번째 폐사 사례에서 폐사한 뱀장어의 복수에서 분리되었으며 균주 3K1-2는 세 번째 폐사 사례에서 폐사한 뱀장어의 신장에서 분리되었다. 16S rRNA gene 시퀀스 분석으로, 분리균주 1E1-2와 3K1-2가 *V. fluvialis* NBRC 103150^{T}와 가장 높은 유사도를 나타내며 *V. fluvialis*로 동정되었으며, 균주 2A3-1는 *V. plantisponsor* NBRC103148^{T}와 가장 높은 유사도를 나타내며 *V. plantisponsor*로 확인되었다. 항생제 감수성 실험 결과로, *V. fluvialis* 1E1-2는 tetracycline과 chloramphenicol에 대하여 중등도의 감수성을 보였고 trimethoprim-sulfamethoxazole에 대하여 내성을 나타내었다. *V. plantisponsor* 2A3-1는 ciprofloxacin과 levofloxacin에 대하여 중등도의 감수성을 나타내었고 trimethoprim-sulfamethoxazole에 대하여 내성을 나타내었다. *V. fluvialis* 3K1-2는 tetracycline에 대하여 중등도의 감수성을 나타내었고 ampicillin과 trimethoprim-sulfamethoxazole에 대하여 내성을 나타내었다. 이는 국내 양식 뱀장어에서의 항생제 내성 비브리오 감염증 사례로, 의미 깊은 보고라고 할 수 있다.

논문접수일 : 2020년 10월 30일
논문수정일 : 2020년 12월 1일
게재확정일 : 2020년 12월 4일