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Isolation and Identification from Korean Olive Flounders (*Paralichthys olivaceus*) Showing Abnormal Swimming Behavior, and Sstudy of Antibiotic Susceptibility

이상유영 증상 국내산 양식 넙치에서의 슈도알테로모나스 분리·동정 및 항생제 감수성 연구

H. Kwon
권혜민
한국생명공학연구원
감염병연구센터[1]
hena0922@kribb.re.kr

S. Yang
양소리
한국농수산대학
어류양식학과[2]
didthfl0204@naver.com

J. H. Kim
김지형
한국생명공학연구원
감염병연구센터[1]
kzh81@kribb.re.kr

J. W. Jun*
전진우
한국농수산대학
어류양식학과[2]
advancewoo@snu.ac.kr

Abstract

Olive flounders which were cultured in commercial fish farm showed abnormal swimming behavior in November 2020. Then, gradual mortality was observed in the fish farm. During the diagnosis, bacterial strain KNCFKW-PN1 was isolated from the kidney of the dead fish. Based on the sequence of *gyrase B subunit* gene, KNCFKW-PN1 was proved to be *Pseudoalteromonas nigrifaciens*, showing 99.59% nucleotide identity with that of *P. nigrifaciens* LMG 2227^{T}. According to the result of antibiotic susceptibility test, *P. nigrifaciens* KNCFKW-PN1 showed intermediate resistance to ciprofloxacin, and was resistant to ampicillin, cefepime, cefotaxime, ceftazidime, and amikacin. This is the first report of the isolation of multiple-antibiotic-resistant *P. nigrifaciens* from olive flounder.

Key words : Olive flounder, Multiple-antibiotic-resistant, *Pseudoalteromonas nigrifaciens*

*교신저자
1 Infectious Disease Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 34141, Korea
2 Department of Aquaculture, Korea National College of Agriculture and Fisheries, Jeonju 54874, Korea

Ⅰ. Introduction

Olive flounder (*Paralichthys olivaceus*), a marine large-tooth flatfish inhabits the north-western Pacific Ocean. This species is one of the most valuable cultured fish in East Asia, especially Korea (Seikai, 2002). In 2020, it accounted for more than 50% of the total marine fish aquaculture in Korea, and 65% of the total in monetary value (Korean Statistical Information Service, 2021).

The genus *Pseudoalteromonas* was characterized in 1995, which is an aquatic organism found in marine environments (Yu *et al.*, 2018). *Pseudoalteromonas* is known to exist in a variety of marine lives: *P. tunicate* isolated from sea lions, *P. citrea* isolated from sponges, and *P. nigrifaciens* isolated from mussels (Yu *et al.*, 2018). *P. nigrifaciens* is known to be mainly pathogenic to the sea cucumber (*Apostichopus japonicus*), causing skin ulcer syndrome (Jiang *et al.*, 2017).

The aim of the present study was to isolate and characterize antibiotic resistant *P. nigrifaciens* from commercially cultured olive flounders in Korea. This is the first report of the isolation of *P. nigrifaciens* from olive flounders.

Ⅱ. Materials and Methods

1. Cases:

Olive flounders were cultured in a private fish farm equipped with an aeration system and water temperature from 18 to 22℃ in Jeollanam-do province, Republic of Korea. In November 2020, some fish showed abnormal swimming behavior, and then gradual mortality. The mortality rate increased to 1% per day and antibiotic treatment started.

2. Isolation and identification of the causative bacteria:

Parasitological examinations were performed for the post-mortem analysis. Sterile swabs from the ascitic fluid and kidney of the dead fish were streaked onto LB agar supplemented with 1% NaCl to isolate the causative bacteria, and the innoculated

plate was incubated at 25℃. From the bacterial isolate, its genomic DNA was extracted by the DNeasy Blood & Tissue Kit (Qiagen, Germany), following the manufacturer's instruction. The isolated bacteria were identified based on the sequencing and phylogenetic analyses of the *gyrase B subunit* (*gyrB*) gene. The *gyrB* gene was amplified using the up1E (5'-GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAARTTYRA-3') and up2AR (5'-AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGYCAT-3') primer sets following the PCR condition of Pascual et al. (2010). Then, the amplified *gyrB* gene of the isolate was 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). The obtained *gyrB* sequence of the isolate was preliminarily compared with other bacterial genus in the GenBank database by BLAST searches (www.ncbi.nlm.nih.gov/BLAST) and aligned with representative sequences from various type strains of *Pseudoalteromonas* 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 X (v10.0) (Kumar et al., 2018). A Maximum-Likelihood 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 isolate 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

Five dead fish (average body length 448mm, average body weight 1,266g) 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 19.5 to 20.5℃. The symptoms were characterized by the presence of diffuse bleeding on the skin around the jaw and focal hemorrhages on the abdominal regions of the body. Accumulation of ascitic fluid in the peritoneal cavities and hemorrhages on the kidney were observed in examined fish (Fig. 1).

Fig. 1. Ascitic fluid and hemorrhage on the kidney of olive flounder

Parasitological examinations did not reveal the presence of external or gill parasites on the fish. Suspected common colonies were re-streaked onto LB agar and incubated at 25℃ for 24 h. Based on the results of *gyrB* gene comparision and phylogenetic analysis, the isolate KNCFKW-PN1 was identified to be *P. nigrifaciens* (Fig. 2).

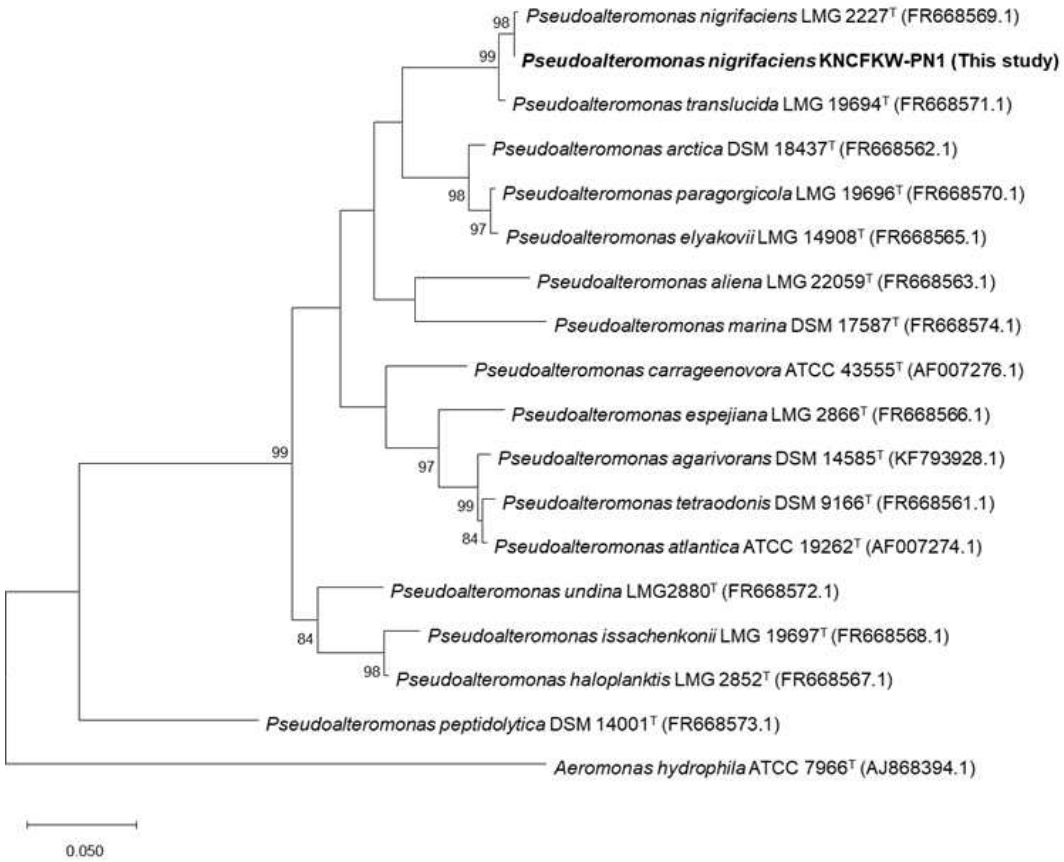

Fig. 2. Maximum-likelihood tree based on the *gyrB* gene sequences of isolate KNCFKW-PN1 to some representative type strains of *Pseudoalteromonas* species. The scale bar represents 0.05 nucleotide substitutions per site

According to the result of the disk diffusion test, *P. nigrifaciens* KNCFKW-PN1 isolated from the study showed intermediate resistance to ciprofloxacin, and was resistant to ampicillin, cefepime, cefotaxime, ceftazidime, and amikacin (Table 1).

Table 1. Antibiotic susceptibility of *P. nigrifaciens* in this study

	Antimicrobial agent [disk content (μg)]											
	β-lactam	Cephems			Carb	Aminoglycosides		Te	Fluoroquinolones		Folate	P
Strains	AMP (10)	FEP (30)	CTX (30)	CAZ (30)	IPM (10)	AK (30)	CN (10)	TE (30)	CIP (5)	LEV (5)	STX (1.25)	C (30)
P. nigrifaciens KNCFKW-PN1												

The category of antibiotic susceptibility is indicated as follows: dark gray, resistant; light gray, intermediate; white, susceptible.
AMP, Ampicillin; FEP, Cefepime; CTX, Cefotaxime; CAZ, Ceftazidime; IPM, Imipenem; AK, Amikacin; CN, Gentamicin; TE, Tetracycline; CIP, Ciprofloxacin; LEV, Levofloxacin; STX, Trimethoprim-sulfamethoxazole; C, Chloramphenicol

Ⅳ. Discussion

Although there have been previous reports of *P. nigrifaciens* isolated from the mussel or sea cucumber (Ivanova et al., 1996; Jiang et al., 2017), there is no report of isolation of *P. nigrifaciens* from olive flounder. In this study, we found no evidence to support that *P. nigrifaciens* was the major causative agent of the mortality of olive flounder. Also, the result of antibiotic susceptibility test showed that *P. nigrifaciens* KNCFKW-PN1 was sensitive to tetracycline, which is one of the most commonly used antibiotics in Korean aquaculture. The result coincided with this case that no mortality was observed and the condition of fish started to improve after oxytetracycline (OTC) treatment. However, *P. nigrifaciens* KNCFKW-PN1 was resistant to several antibiotics such as ampicillin, cefepime, cefotaxime, ceftazidime, and amikacin, which reveals that the isolated strain was multiple-antibiotic-resistant bacterial strain. Considering the *Pseudoalteromonas* genus exists ubiquitously in marine environments (Yu et al., 2018), the incidence of multiple-antibiotic-resistant *Pseudoalteromonas* strain can increase the risk of public health. It is believed to be necessary to set more standardized guideline which is useful for antibiotics use in Korean aquaculture.

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요약

2020년 11월, 국내의 넙치 양식장에서 양식 중이던 넙치가 이상 유영 소견을 보이다가, 지속적으로 폐사하였다. 질병 진단 과정 중, 폐사어의 신장에서 세균(KNCFKW-PN1)이 분리되었다. *gyrase B subunit* 유전자의 시퀀스 분석 결과, KNCFKW-PN1 분리주는 기존에 보고된 LMG 2227^{T} 균주의 해당 유전자 시퀀스와 99.59% 유사도를 보여 *Pseudoalteromonas nigrifaciens* 로 동정이 되었다. 항생제 감수성 실험 결과에 따르면, KNCFKW-PN1 분리주는 ciprofloxacin에 대하여 중등도의 내성을 나타내었고, ampicillin, cefepime, cefotaxime, ceftazidime, amikacin에 내성을 나타내었다. 본 사례는 다제 내성 *Pseudoalteromonas nigrifaciens* 세균이 넙치로부터 분리된 최고의 보고이다.

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