Isolations of *Shewanella* and *Aeromonas* Species from Silver European Eel Fish (*Anguilla anguilla* Linnaeus, 1758)

Gümüş Avrupa Yılan Balığı (*Anguilla anguilla* Linnaeus, 1758)'ndan *Shewanella* ve *Aeromonas* Türlerinin İzolasyonları

ABSTRACT

The aim of this study is to inform isolations of *Shewanella putrefaciens*, *Aeromonas sobria* and *Aeromonas veronii* from the silver European eel fish (*Anguilla anguilla*) caught from the Gulf of Antalya, Turkey. Three silver eel fish samples as freshly dead (mean weight 75 g) were obtained by a local fisherman. For presumptive identification of the bacterial strains, the strains were identified using a variety of phenotypic characteristics. The further identification of the strains was performed with PCR. Clinically, the silver eel samples showed pale gills, losses of scale, necrotic lesions on dorsal, hemorrhagies on the jaws, lateral and ventral sides of the body and around the anus and hemoragic anal fin. At necropsy, the fish had enlarged and pale spleen and the gastrointestinal tract was empty. After 72 hours, 10 bacterial strains were isolated. 6 strains as *Shewanella putrefaciens*, 2 strains as *Aeromonas sobria* and 2 strains as *Aeromonas veronii* were identified according to the phenotypical characteristics and PCR study results. The eel fish stocks in natural conditions are decreasing and this problem may be multifactorial such as disease, migration and overhunting. As a result of this study, *Aeromonas veronii* was first isolated from *Anguilla anguilla*.

Key Words: *Anguilla anguilla*, *Shewanella putrefaciens*, *Aeromonas sobria*, *Aeromonas veronii*, PCR

ÖZET

Bu çalışmanın amacı Türkiye, Antalya Körfezi'nden yakalanan gümüş Avrupa yılan balığı (*Anguilla anguilla*)'ndan *Shewanella putrefaciens*, *Aeromonas sobria* ve *Aeromonas veronii*'nin izolasyonlarını bildirmektir. Üç gümüş yılan balığı örneği (75 g) yeni ölmüş olarak yerel bir balıkçıdan temin edildi. Bakteriyel suşların varsayımsal tanımlanması için, suşlar bir dizi fenotipik özellikler kullanılarak tanımlandı. Suşların ileri tanımlanması PZR ile yapıldı. Klinik olarak, gümüş yılan balığı örnekleri solgun solungaçlar, pul kaybı, dorsalde nekrotik lezyonlar, çenelerde, vücudun yan ve ventral kısımlarında ve anüs etrafında kanamalar ve hemorajik anal yüzgeç gösterdi. Nekropside, balıklar büyümüş ve solgun dalağa sahip ve mide bağırsak kanalı boştu. 72 saat sonra, 10 bakteriyel suş izole edildi. Fenotipik özellikler ve PZR çalışma sonuçlarına göre 6 suş *Shewanella putrefaciens*, 2 suş *Aeromonas sobria* ve 2 suş *A. veronii* olarak tanımlanmıştır. Doğal koşullarda yılan balığı stokları azalmakta ve bu sorun hastalık, göç ve aşırı avlanma gibi çok faktörlü olabilmektedir. Bu çalışmanın bir sonucu olarak *Aeromonas veronii*, *Anguilla anguilla*'dan ilk kez izole edilmiştir.

Anahtar Kelimeler: *Anguilla anguilla*, *Shewanella putrefaciens*, *Aeromonas sobria*, *Aeromonas veronii*, PZR

INTRODUCTION

The genus *Anguilla* consists of about one hundred fish species; however, European eel (*Anguilla anguilla*, Linnaeus, 1758), Japanese eel (*A. japonica*, Tomminck and Schlegel, 1847) and American eel (*A. rostrata*, Lesueur, 1817) are mostly consumed eel fish species in several regions of the America, Japan and Europe countries (Caruso et al., 2014). The European eel enters European waters and some of these fish start an upstream migration to colonise at the glass eel stage. Then, they begin spawning migration back down to the sea at the silver stage (Simon, 2015).

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Jale Korun [a]
Yağmur Saadet Çelik [b]
Mesut Yılmaz [c]
Mehmet Gökoğlu [d]

Akdeniz University, faculty of aquaculture, Antalya, TURKEY
[a] ORCİD: 0000-0002-1930-9978
[b] ORCİD: 0000-0003-2877-0915
[c] ORCİD: 0000-0001-8799-3452
[d] ORCİD: 0000-0001-9723-8581

Correspondence
Jale Korun
Akdeniz Üniversitesi Kampüsü Su ürünleri fakültesi Antalya Türkiye
jalekorun@akdeniz.edu.tr

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But, population of the species has been in step decline (Dekker, 2003). All anthropogenic factors which decrease production and population of the silver eels in natural waters include chemical pollution, loss of habitat, fisheries, migration barriers and disease problems (Mariarty and Dekker, 1997; Dezfuli et al., 2014; Roland et al., 2014; ICES, 2017).

Diseases observed in fish are classified as contagious and non-contagious diseases. Infectious agents cause contagious diseases and these agents include bacteria, viruses, microparasites such as protozoan parasites and macroparasites such as trematodes and nematodes. Non-contagious diseases are caused by a variety of factors including chemical contaminants and genetic factors (Johnson and Paull, 2011). Of the infectious agents, bacterial infections are important because of having potential threat for aquaculture production systems. These infections lead to serious ecomical fish losses. But, they have less important for wild fish populations (Chang and Liu, 2002; Haenen et al., 2013).

For rearing of *A. anguilla* for commercial purposes, the production of the species depends on collection of the elver and/or glass eels from natural stocks. But, any disease outbreak or infection on the wild eel population may have negative effect on the fish stock. Edwardsiellosis, vibriosis and *Aeromonas hydrophila* infections are bacterial infections and were reported from both wild and reared the European eel under the controlled culture conditions (Esteve et al., 1993; Chang and Liu, 2002; Fouz et al., 2006; Haenen et al., 2013). *Edwardsiella tarda* is one of the causative agents of edwardsiellosis (syn. red disease of eels). The infection affects seriously the eel population (Chang and Liu, 2002; Haenen et al., 2013). *Vibrio vulnificus* has three biotypes and strains of the biotype 2 are commonly associated with the eel infections (Kim et al., 2017). Esteve et al. (1993) reported disease outbreaks from an eel farm that was located in Spain in 1987 and 1988. They noticed that the etiology of the disease was complex because of isolations of *A. hydrophila*, *A. jandaei*, *Pseudomonas fluorescens* and *S. putrefaciens* from sick eel samples during epizootics and also routine survey studies.

Shewanella putrefaciens causes shewanellosis. The disease is generally characterized by necrotic and ulcerative lesions on the skin of the affected fish. Clinical findings of the disease are lethargy, swollen abdomen and necrosis of gills. At necropsy, gross lesions such as haemorrhages in spleen and petechiae on the swim bladder are observed (Paździor, 2016). Koziñska and Pekala (2004) isolated *S. putrefaciens* from common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) which showed skin discoloration and a few fatal cases. Altun et al. (2014) reported shewanellosis on gold fish (*Carassius carassius auratus*) and the authors informed that the fish had bilateral exophthalmia, swollen abdomen, ascites, pale liver and enlarged spleen.

S. putrefacienens is a Gram-negative facultative anaerobic bacterium. It was first isolated from butter in 1931 (Koziñska and Pekala, 2004; Vignier et al., 2013). The bacterium was first reported as a fish pathogen in 1985 from rabbit fish (*Siganus rivulatus*) that was cultured at sea (Saeed et al., 1987). Koziñska and Pekala (2004) first isolated *S. putrefaciens* from a routine survey study of carp farms in Poland in 2002. After this time, the authors faced the same bacterium carp and trout farms and they isolated *S. putrefaciens* as dominant with other *Aeromonas* species. In Turkey, Korun et al. (2009) reported *S. putrefaciens* from the cultured European sea bass (*Dicentrarchus labrax*) and then Altun et al. (2014) informed the pathogen from gold fish.

The genus *Aeromonas* comprises two bacterial groups. One group is non-motile psychrophilic *Aeromonas salmonicida* and the other group is mesophilic motile *Aeromonas* spp. This group includes fish pathogenic species such as *A. hydrophila*, *A. sobria*, *A. caviae*, *A. veronii* biogroup *sobria* and *A. veronii* biogroup *veronii* (Praveen et al. 2016; Stratev and Odeyemi, 2017). The motile *Aeromonas* species cause motile *Aeromonas* septicaemia which is termed as ulcer disease, red-sore or tail and fin rot disease. The clinical findings of the disease are haemorrhages, ulcerations, exophthalmia, ascites, pale liver and kidney (Stratev and Odeyemi, 2017). In Turkey, *A. hydrophila* was firstly reported from the farmed European eel in 1983 (Timur, 1983). From 1983 to

date, some reports of *Anguillicoloides crassus* infections of *A. anguilla* have been reported (Genç et al., 2005; Koyuncu et al., 2017).

The aim of the present study is to inform *Shewanella purefaciens*, *Aeromonas sobria* and *A. veronii* isolations from the silver European eel (*Anguilla anguilla*) samples from the Gulf of Antalya, Turkey.

MATERIAL AND METHOD

Fish samples and Bacterial Isolation

Three silver European eel (*A. anguilla*) samples as freshly dead (mean weight 75 g) were obtained by a local fisherman from the Gulf of Antalya, Turkey. Bacteriological and molecular studies were done at the research laboratory of the Akdeniz University, Faculty of Fisheries. For bacteriological study, inoculations from the head kidney, spleen and liver were made and inoculated onto Brain Hearth Infusion Agar (BHIA-S). This medium was supplemented with 1.5 % NaCl and the inoculated petri dishes were incubated at 24 ± 2 °C for 72 hours. The bacterial colonies were subcultured at the end of the incubation period time.

Phenotypical characterization of the isolates

For presumptive identification of the bacterial strains, the strains were identified using motility, Gram-staining technique, biochemical reaction, sugar fermentation, salt and temperature tolerance tests according to Seeley et al. (1991).

PCR (Polymerase Chain Reaction)

Bacterial DNA was extracted using DNA extraction kit (Thermo Scientific) according to the manufacturer's instructions. The bacterial genomic DNA was adjusted by 10 mM Tris-EDTA buffer up to volume 200 µl and stored at -20 °C. The universal primers B27F (5′-GGTTACCTTGTTACGACTT-3′) and U1492R (5′-GGTTACCTTGTTACGACTT-3′) were synthesized (Macrogen Inc.) and used for amplification of the 16S ribosomal DNA gene (Chu and Lu, 2005; Liu et al., 2013; Liu et al., 2014). 5 µl of genomic DNA solution in Tris-EDTA buffer was added to 45 µl of a PCR mixture consisting 2 x mix (Qiagen), 1 µl of each primer (10 nmol) and sterile water added up to 50 µl. The amplification was carried out in the thermocycler (Kyratec SC-200), iniated by 10 min of denaturation at 95 °C and then carried out for 35 cycles, with 1 cycle consisting of 45 sec of denaturation at 95 °C, 45 sec of annealing at 60 °C and 2.5 min extension at 72 °C. The reaction was lasted by heating at 72 °C for 10 min after the last cycle. A negative control with all the reaction components except the template DNA was included with each test run. Twenty microliters of the PCR reaction products were then analyzed by gel electrophoresis in 1% agarose at 8 V/cm (Figure 1). 1 kb of DNA ladder (Thermo Scientific) was used as a marker. Before sequence analysis of the PCR products, the products were purified by gel extraction kit (Machery-Nagel) used according to the manufacturer's instructions and the sequences were subjected to BioEdit V 7.2.5. program (Hall, 1999) to assemble the fragments which were compared to 16S ribosomal DNA sequences in the GenBank database using the BLASTN algorithm (Chu and Lu, 2005; Liu et al., 2014).

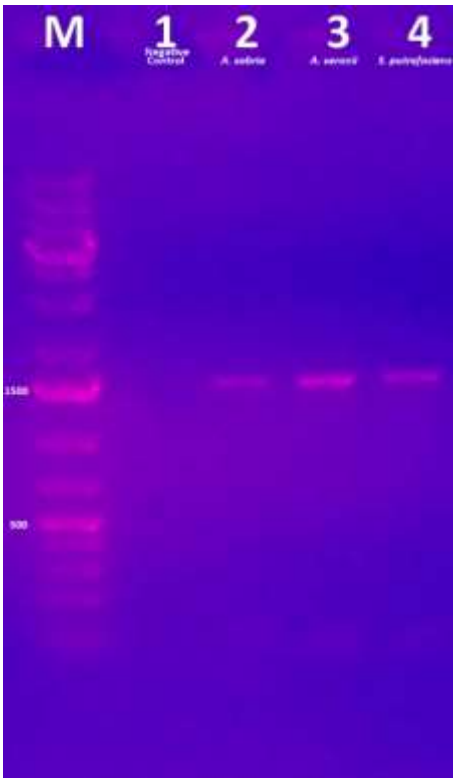

Figure 1. PCR products in gel electrophoresis (1% agarose)

RESULTS

The freshly dead silver eel samples had pale gills, losses of scale, necrotic lesions on dorsal,

hemorrhagies on the jaws, lateral and ventral sides of the body and around the anus and hemorrhagic anal fin (Figures 2 and 3). At necropsy, the fish had enlarged and pale spleen. The liver was pale and the gastrointestinal tract was empty.

Figure 2. Scale losses (arrowed) and necrotic lesions on the skin in silver eel

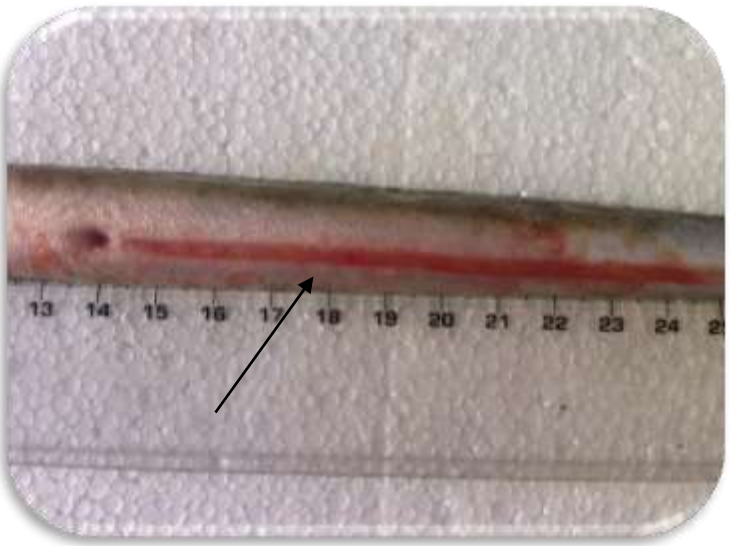

Figure 3. Hemorrhagic anal fin (arrowed) and hemorrhagies around the anus and ventral part of silver eel

After 72 hours of incubation period, 10 bacterial strains were isolated. 6 strains produced orange colored colonies on BHIA-S and 4 strains produced cream colored colonies were motile, Gram-negative, cytochrome oxidase and catalase positive. The strains reduced nitrate to nitrite and they showed resistance against vibriostatic agent (O/129) (10 µg/disk and 150 µg/disk). Most *Aeromonas* species are motile and facultatively anaerobic. They give positive reactions to the cytochrome oxidase and catalase tests and produce acid from many carbohydrates. They show resistance against 150 µg of vibriostatic agent (Martin Carnahan and Joseph, 2005). The four strains from cream colored colonies depending on the bacteriological

study were tentatively identified as motile *Aeromonas* species according to Martin Carnahan and Joseph (2005). Six strains from silver eel samples produced orange colonies on BHIA-S. The strains were motile, Gram-negative, cytochrome and catalase positive and non-fermentative. The strains produced H_2S. *Shewanella putrefaciens* is motile, Gram-negative, cytochrome oxidase and catalase positive, pschrothropic and non-fermentative. It typically produces H_2S on Triple Sugar Iron (TSI) agar (Pekala et al., 2015). The six strains in this study were tentatively identified as *Shewanella putrefaciens* according to the study results of Pekala et al. (2015). Phenotypical characteristics of *Aeromonas* spp. and *Shewanella putrefaciens* from silver eel samples were given in Table 1.

Table 1. Phenotypical characteristics of *Aeromonas* spp. and *Shewanella putrefaciens*

Characteristic	*Aeromonas* spp.	*S. putrefaciens*
Motility	+	+
Gram staining	-	-
Cytochrome oxidase	+	+
Catalase	+	+
O/F	F	NF-NO
Indol production	-	-
VP	+	+
H_2S production	V	+
Gelatinase	-	-
Amylase	+	V
Citrate utilization	-	-
NO_2 production	+	+
ONPG	V	-
Acid from		
Arabinose	+	+
Fructose	+	+
Galactose	+	+
Glucose	+	+
Mannitol	+	V
Sorbitol	V	V
Xylose	+	V
Growth in		
0% NaCl	+	-
2-4% NaCl	+	+
6% NaCl	+	V
8%NaCl	-	-
Growth at		
4 °C	-	+
37	+	+
O/129 (10 µg/disk)	R	R
O/129 (150 µg/disk)	R	R

+: positive (100%), -: negative (100%), V: variable, R: resistance

The PCR product of each strain was extracted from the agarose gel and sequenced. 16S rDNA sequencing of the strains were compared with the GenBank database using the BLASTN. The closest matches were obtained with *Aeromonas sobria* (2 strains) (GenBank accession number KC573782.1; maximal score 2636, E value 0.0, and maximal identity 99% (1430/1431), *A. veronii* (2 strains) (GenBank accession number KC166864.1; maximal score 2632, E value 0.0; and maximal identity 100% (1425/1425) and *Shewanella putrefaciens* (6 strains) (GenBank accession number DQ307731.1; maximal score 2627, E value 0.0, and maximal identity 100% (1422/1422).

DISCUSSION

Esteve et al. (1993) reported disease outbreaks from an eel farm that was located in Spain in 1987 and 1988. They noticed that the etiology of the disease was complex because of isolations of *A. hydrophila*, *A. jandaei*, *Pseudomonas fluorescens* and *S. putrefaciens* from sick eel samples during epizootics and also routine survey studies. In Turkey, *A. hydrophila* was firstly reported from the farmed European eel in 1983 (Timur, 1983). In this study, we isolated *S. putrefaciens* with *A. sobria* and *A. veronii* and this finding was similar to the reports of Timur (1983) and Esteve et al. (1993); however, *A. veronii* from the eel samples in the study was first isolated. The freshly dead silver eel samples showed gross haemorrhages on the body surface, enlarged spleen and pale liver in this study. These findings were similar to those of *S. putrefaciens* and the motile *Aeromonas* species (Koziñska and Pekala, 2004; Altun et al. 2014; Stratev and Odeyemi, 2017).

A variety of phenotypical charateristics of the bacterial species, genetic hybridization, reverse transcriptase sequencing and 16S rRNA sequence analysis of the species have been useful tools to identify microorganisms (Bascomb and Manafi, 1998; Austin, 2011). But, some bacterial species show some phenotypical characteristics which are similar to those of species from the same genus and this could be difficult to differentiate the bacterial species from another species (Shewan and McMeekin, 1983; Austin, 2011; Kumar et al., 2014). 16S rDNA sequencing has been accepted for the identification of pathogenic and also opportunistic bacterial species (Austin, 2011). For this reason, the bacterial strains in this study were identified by PCR technique.

As conclusion, the eel stocks in natural conditions are decreasing and this problem may be multifactoral which include disease, overhunting and migration barriers. *Shewanella putrefaciens* and the motile *Aeromonas* species are members of intestinal bacterial flora of fish and they are also opportunistic fish pathogens. When the fish are under stress conditions, these bacterial species cause disease outbreaks and affect fish population. *Aeromonas veronii* was first isolated and identified in this study.

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