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Aeromonas veronii biovar veronii Infection in Cultured European Seabass (*Dicentrarchus labrax*) in Türkiye

Süheyla KARATAŞ[1], Emre TURGAY[2], Remziye Eda YARDIMCI[3], Terje M. STEINUM[4]
[1,2,3]Istanbul University, Faculty of Aquatic Sciences, Dept. of Aquaculture and Fish Diseases, Istanbul, Türkiye, [4]Istanbul University, Faculty of Science, Dept. of Molecular Biology and Genetics, Istanbul, Türkiye
[1]https://orcid.org/0000-0003-2006-7854, [2]https://orcid.org/0000-0001-9964-3919, [3]https://orcid.org/0000-0001-7737-8739
[4]https://orcid.org/0000-0001-5377-9765
✉: skaratas@istanbul.edu.tr

ABSTRACT
Fish in aquaculture systems are susceptible to infectious agents as they are kept in a densely populated and often physically restricted environment. Genus *Aeromonas* include well-known pathogens, and the member *Aeromonas veronii* has been reported to cause diseases in both humans and animals, either as primary infection or as mixed infection with other pathogens. This study describes a low mortality epizootic caused by *Aeromonas veronii* biovar veronii in European seabass (*Dicentrarchus labrax*) farmed in the Aegean Sea. The gills, kidneys and livers of moribund fish were pale. Erythema, haemorrhage and superficial ulcerative lesions were detected on the skin. In addition, petechial haemorrhage was observed on the tongue, maxilla, and operculum. The spleen was enlarged and multiple granulomas were detected in both the kidney and the spleen. Some fish had skin depigmentation, ecchymosis in the liver, and a bloody exudate in the abdominal cavity. The intestinal walls were lined with a clear yellowish fluid. Twenty-nine motile, Gram-negative bacterial isolates were obtained from the internal organs of diseased fish. According to morphology, biochemical properties and 16S rRNA gene sequencing results, all isolates were identified as *Aeromonas veronii* bv. veronii. All isolates were resistant to amoxicillin and ampicillin, and sensitive to oxytetracycline, enrofloxacin, ciprofloxacin, florfenicol, and flumequine.

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Histopathology

Türkiye'de Yetiştiriciliği Yapılan Levrek Balıklarında (*Dicentrarchus labrax*) *Aeromonas veronii* biovar veronii Enfeksiyonu

ÖZET
Yetiştiricilik sistemlerindeki balıklar, stok yoğunluğu yüksek ve genellikle fiziksel olarak kısıtlı bir ortamda tutuldukları için enfeksiyöz ajanlara karşı hassastırlar. *Aeromonas* cinsinin içinde pek çok patojen tür içerdiği bilinmektedir ve bu cins üyelerinden *Aeromonas veronii* bakterisinin hem insanlarda hem de hayvanlarda birincil enfeksiyon şeklinde veya diğer patojenlerle karışık enfeksiyon olarak hastalıklara neden olduğu bildirilmiştir. Bu çalışma, Ege Denizi'nde yetiştirilen levrek balıklarında (*Dicentrarchus labrax*) *Aeromonas veronii* biovar veronii bakterisinin neden olduğu düşük ölüm oranına sahip bir epizootiği tanımlamaktadır. Hasta balıklarda solungaç, böbrek ve karaciğerin solgun olduğu tespit edilmiştir. Deride eritem, hemoraji ve yüzeyel ülseratif lezyonlar görülmüştür. Ayrıca dil, maksilla ve operkulumda peteşiyal hemoraji gözlenmiştir. Dalağın büyüdüğü ve hem böbrek hem de dalakta çoklu granülomalar tespit edilmiştir. Dalak ve böbrekte granülomlar gözlenmiştir. Bazı balıklarda deri depigmentasyonu, karaciğerde ekimoz ve karın boşluğunda kanlı eksüda görülmüştür. Bağırsak duvarları berrak ve sarımsı bir sıvıyla kaplı olarak bulunmuştur. Hasta balıkların iç organlarından 29 adet Gram negatif hareketli bakteri izolatı elde edilmiştir. Morfoloji, biyokimyasal özellikler ve 16S rRNA gen dizileme sonuçlarına göre tüm izolatlar *Aeromonas veronii* bv. veronii olarak tanımlanmıştır. Elde edilen

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Levrek balığı
Histopatoloji

izolatların amoksisilin ve ampisiline dirençli ve oksitetrasiklin, enrofloksasin, siprofloksasin, florfenikol ve flumekine duyarlı olduğu tespit edilmiştir.

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INTRODUCTION

Aquaculture is arguably the fastest growing industry in the global food animal production and this growth is expected to continue in the near future. According to the latest data published by FAO (the Food and Agriculture Organization), the total aquaculture production worldwide is 85.3 million tons and %66 of this production volume (approximately 56.3 million tons) is obtained from finfish farming. The trade volume from marine fish farming alone has a calculated value of approximately 14 billion US dollars (FAO, 2021).

The intensive culture condition that is required for this rapid growth and expansion cause, on the other hand, some negative effects on fish health. Among these, fish disease and mortality are notable factors that limit the production volume in aquaculture systems. In addition, although the mortality rate may be very low in some diseases, the external lesions caused by these diseases make the fish unmarketable. Many fish disease-causing bacteria have been identified to date. Most of them have been identified in and isolated from farmed species rather than from wild fish. Fish in aquaculture systems, unlike their wild counterparts, are kept in a densely populated and often physically restricted environment, making them much more susceptible to pathogens (Toranzo et al., 2005; Fryer & Rohovec, 2021).

Members of the *Aeromonas* genus are Gram-negative, facultative anaerobic, non-spore forming bacteria that are ubiquitous in both terrestrial and aquatic environments. But the genus also includes pathogens that have are well known to human and veterinary medicine (Brenner et al., 2005; Janda & Abbott, 2010). *Aeromonas veronii* was first isolated as a potential causative agent from a patient by Hickman-Brenner et al. (1987) and has been subsequently reported as a pathogen from many organisms. This bacterium has been isolated from many fish species to date, either as the primary agent or in mixed infections, including cyprinids (Rahman et al., 2002; Yu et al., 2010; Sun et al., 2016; Zhu et al., 2016), cichlids (Dong et al., 2017), members of Siluriformes (Rahman et al., 2002; Nawaz et al, 2010; Cai et al., 2012; Hoai et al., 2019), gilthead seabream (*Sparus aurata*) (Gashgari & Selim, 2015) and European seabass (*Dicentrarchus labrax*) (Smyrli et al, 2017). In Türkiye, *A. veronii* infections were reported in marine cages cultured European seabass in the Black Sea (Uzun & Oğut, 2015) and in the Aegean Sea (Tanrıkul & Dinçtürk, 2021).

The aim of the present study was to determine the likely cause of an epizootic that occurred in June in European seabass (*Dicentrarchus labrax*) cultured in the Aegean Sea.

MATERIALS and METHODS

The recorded mortality during this epizootic that affected European seabass (*Dicentrarchus labrax*) was less than %1. Ten moribund fish (approx. 250 g) were sampled from offshore floating cages and examined by standard procedures (Whitman, 2004). Sample material was taken from liver, spleen and kidney and streaked onto Tryptic Soy Agar (TSA) medium containing 1.5% NaCl and incubated at 22°C for 48 h. Pure bacterial cultures were obtained from the colonies on primary plates by repeated streaking. Tissue samples for histopathology were taken from gills, liver, kidney, spleen and processed after fixation in %10 buffered formalin solution and then embedded into paraffin blocks. Histological sections of 5µm were stained with hematoxylin and eosin, Ziehl-Neelsen staining, and examined by light microscopy (Culling, 1963).

The morphological and biochemical characteristics of the isolated bacteria were determined by routine laboratory methods including API 20E kits (bioMérieux). For bacterial identification, the isolates were inoculated into Marine Broth 2216 (Difco). After incubation overnight at 22°C, genomic DNA was extracted from the isolates by using the GeneJET Genomic DNA Purification Kit (Thermo) according to the manufacturer's instructions. A universal bacteria primer set S-D-Bact-0008-a-S-20 (5' AGAGTTTGATCCTGGCTCAG 3') and S-*-Univ-0536-a-A-18 (5' GWATTACCGCGGCKGCTG 3') were used to amplify a partial fragment of the 16S rRNA gene (Suau et al., 1999).

The PCR mixture included template DNA (approx. 50 ng) 0.4 µM of each primer, PCR master mix (2X) (Thermo Scientific) and DNase/RNase-free distilled water (Thermo Scientific). Amplification was done using a thermal cycler (Biometra, TAdvanced) programmed as follows: 95°C for 3 min (initial denaturation) followed by 30 cycles of amplification

(95°C for 30 s for denaturation, 56°C for 1 min for annealing, 72°C for 1 min for extension) and 72°C for 4 min for a final extension step. PCR products obtained from the amplification were visualized by gel electrophoresis [%1.5 agarose (w/v) in 1X TAE buffer, containing EtBr (0.5 µg ml^{-1})] and running for 45 min at 100 V. All PCR products were purified and sequenced in both directions by a local sequencing company. Editing and analysis of the sequences were performed in Bioedit v7.0.0 (Hall, 1999) using the BLASTN (v2.2.20) (Larkin et al., 2007) and ClustalX (v2.1) (Zhang et al., 2000) algorithms. All sequences obtained in this study have been deposited in the GenBank database under accession numbers OP522255-OP522261.

All bacterial isolates were also tested for antimicrobial susceptibility by the Kirby-Bauer disk diffusion method (Bauer et al., 1966). The isolates were plated onto Mueller-Hinton agar (Oxoid) with eight antimicrobial disks (Oxoid) (amoxicillin, ampicillin, enrofloxacin, ciprofloxacin, oxytetracycline, sulfamethoxazole/trimethoprim, florfenicol, flumequine) and then incubated at 20ºC for 48-96 h and the results were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2010).

RESULTS

Gross Pathology

In the examined fish, the gills, kidneys and livers were pale. Erythema, haemorrhage and superficial ulcerative lesions in the skin, petechial haemorrhage on the tongue, upper jaw and operculum as well as in the visceral fat were observed (Figure 1a-d). The spleen was enlarged and had multiple abscesses (Figure 1e). Some fish had ecchymosis and fatty degeneration in the liver (Figure 1f), bloody exudates in the abdominal cavity, and a transparent intestinal wall. Other observations were yellowish liquid in the intestinal tract, multiple granulomas in the spleen (Figure 1g), and the kidney (Figure 1h).

Bacteriological Findings

A total of twenty-nine Gram-negative motile bacterial isolates were obtained from the visceral organs of ten diseased fish. According to their morphological and biochemical characteristics (Table 1) and 16S rRNA gene sequencing results (%100 similarity), all isolates were identified as *Aeromonas veronii* bv. veronii.

a

b

c

d

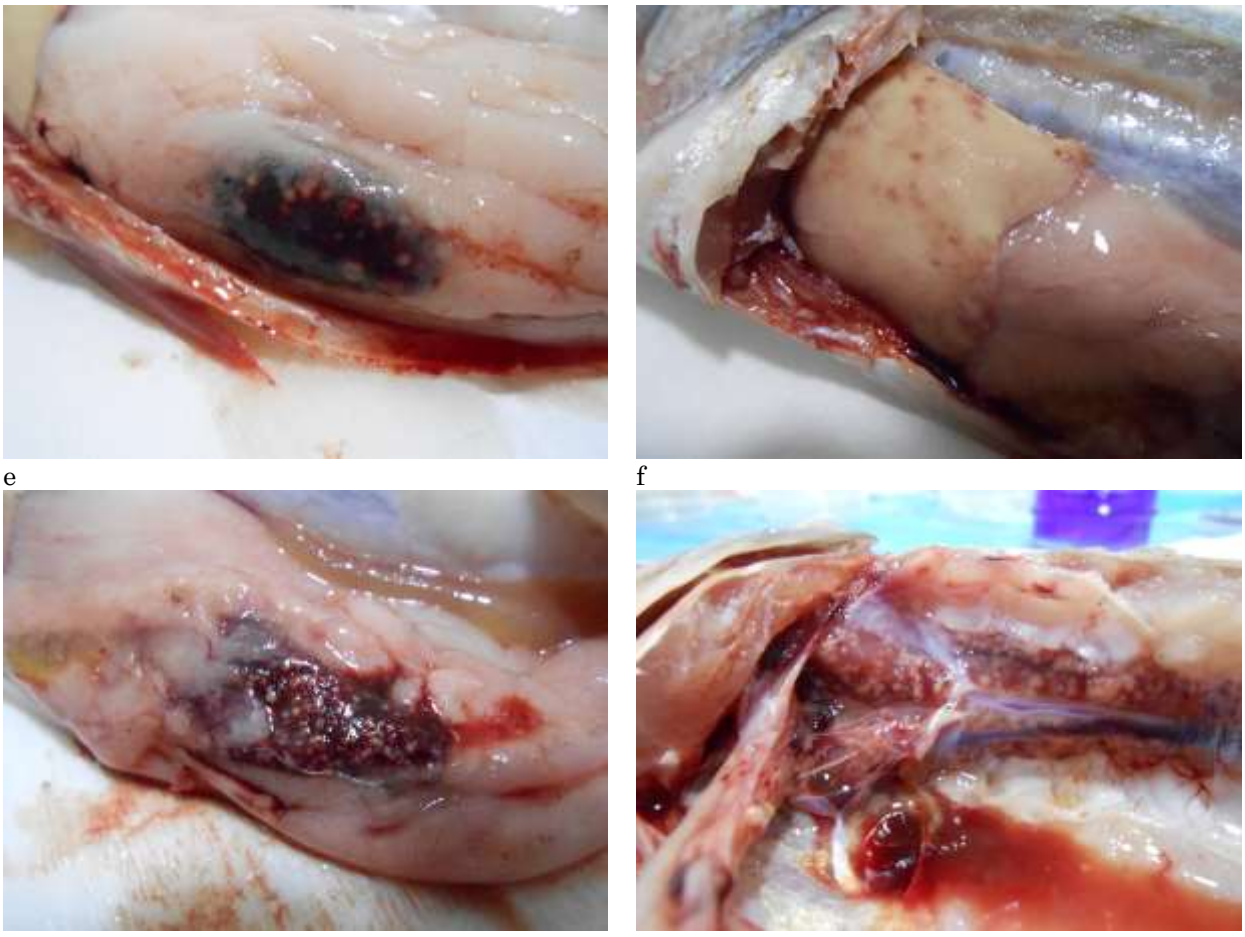


Figure 1. Moribund fish exhibited (a) corneal opacity, haemorrhage and superficial ulcerative lesions in the skin, (b) petechial haemorrhage on the tongue, (c) haemorrhage, haemorrhagic ulcers on the ventral side of the body and between the pelvic fins, mild prolapse, (d) haemorrhage on the upper jaw and operculum, (e) multiple abscesses in the enlarged spleen, (f) pale liver with ecchymosis, (g) transparent intestinal wall and granulomas in the spleen and (h) granulomas in the kidney.

Şekil 1. Hasta balıklarda balıklarda (a) deride korneal opaklık, hemoraji ve yüzeysel ülseratif lezyonlar, (b) dilde peteşiyal kanama, (c) kanama, vücudun ventral tarafında ve pelvik yüzgeçler arasında hemorajik ülserler, hafif prolapsus, (d) üst çene ve kapakçıkta kanama, (e) genişlemiş dalakta çoklu nodüller, (f) ekimoz ile soluk karaciğer, (g) şeffaf bağırsak duvarı ve dalakta granülomlar ve (h) böbrekteki granülomlar.

According to antimicrobial susceptibility testing results, all isolates were determined to be sensitive to oxytetracycline, enrofloxacin, ciprofloxacin, florfenicol and flumequine and to be resistant to amoxicillin and ampicillin as well as intermediate resistant against sulfamethoxazole/trimethoprim.

Histopathological Findings

Vacuolar degeneration, haemorrhage and necrosis in parenchyma cells of the liver, hemosiderin deposits and depletion of white blood cells in the spleen and hyperaemia and depletion of haemopoietic tissue in the anterior kidney were observed in histological sections. Other findings were melting lamellae, necrosis in the secondary gill lamellae and epithelial cell hyperplasia in the gills. Granulomas were observed in spleen tissue of some of the examined fish (Figure 2 a-f). These granulomas were stained with Ziehl-Neelsen acid-fast staining to determine if they contained any acid-resistant bacilli, but no bacilli were found (Figure 2g). In addition, a blood parasite, *Trypanosoma* sp., was detected in the histopathological spleen section in one fish.

DISCUSSION

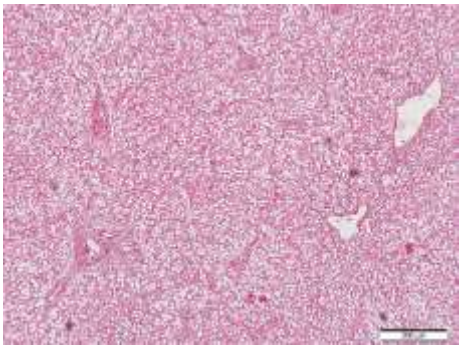
Motile Aeromonads (MAS) are commonly found in terrestrial and aquatic environments as natural members of both the environment and the microbiota of animals, including fish. In the aquaculture industry, this group of bacteria is regarded as opportunistic and often causes diseases under stressful conditions. Besides that, based on disease reports, *Aeromonas veronii* appears to have a wider host range than other fish pathogenic motile *Aeromonas* species (Smyrli & Katharios, 2020).

Table 1. Morphological and biochemical characteristics of the examined *Aeromonas veronii* isolates (n=29)
Çizelge 1. İncelenen Aeromonas veronii izolatlarının morfolojik ve biyokimyasal özellikleri (n=29)

Morphology	Rod	Growth on	
Motility	+	4°C	-
Gram staining	-	37°C	+
Cytochrome oxidase	+	44°C	-
Catalase	+	%0 NaCl	+
O/F	F	%1.5 NaCl	+
Indole	+	%3 NaCl	+
MR test	-	%5 NaCl	-
VP reaction	+	TCBS	+
B-Galactosidase	-	McConkey agar	+
0/129 (10µg)	R	Acid Production from	
0/129 (150µg)	R	Glucose	+
Arginine dihydrolase	+	Fructose	+
Lysine decarboxylase	+	Lactose	+
Ornithine decarboxylase	+	Sucrose	+
Nitrate reduction	+	Mannose	+
Esculin hydrolysis	+	Maltose	+
Citrate utilization	-	Inositol	-
Urease	+	Sorbitol	-
Production of H_2S	-	Arabinose	-
API 20E profile	716712757	Xylose	-

F, Fermentative; +, Positive; -, Negative; R, Resistant.

It is known that *A. veronii* isolates vary phenotypically and the species has been divided into two different biovars: *A. veronii* bv. veronii and *A. veronii* bv. sobria. Studies have been reported that *A. veronii* bv. sobria is negative for esculin hydrolysis and ornithine decarboxylase tests, whereas *A. veronii* bv. veronii is positive. The latter biovar is arginine dihydrolase negative but, produces acid from salicin and utilizes tartrate (Abbott et al., 2003). While degradation of urea, Voges-Proskauer reaction, esculin hydrolysis and



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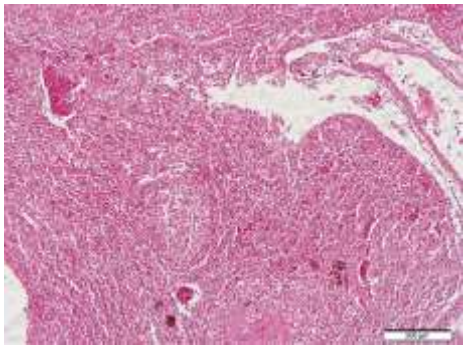

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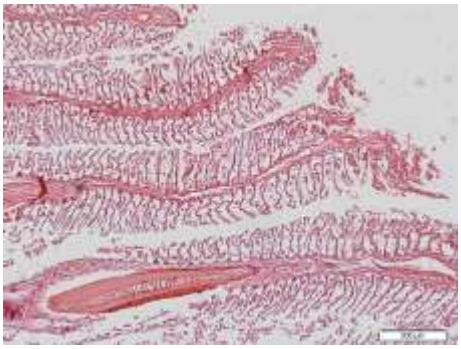

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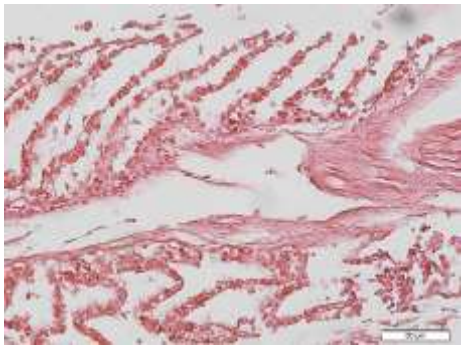

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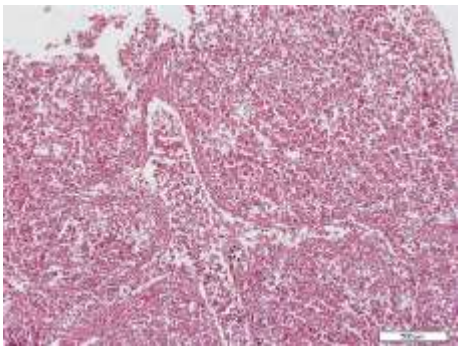

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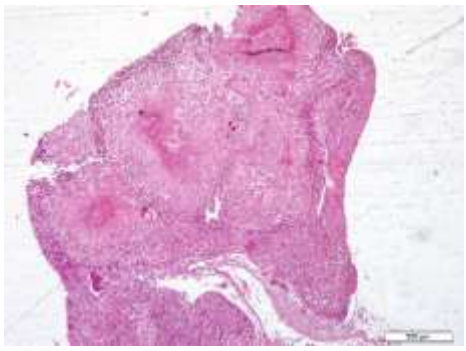

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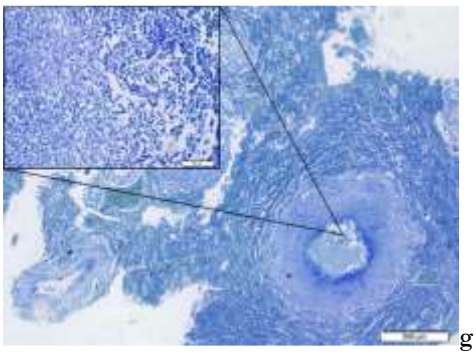

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Figure 2. Findings observed in photomicrographs of tissue sections: (a) vacuolation and necrosis in the parenchyma cells and haemorrhage in the liver, (b) hemosiderin deposits and depletion of white blood cells in the spleen, (c) melting lamella and epithelial cells hyperplasia in the gills (arrows), (d) fusion of the gill lamella (arrows), (e) hyperaemia and depletion of hematopoietic tissue in the anterior kidney, (f) granulomas (stars) in the spleen, (g) Ziehl-Neelsen negative staining result of granuloma in the spleen.

Şekil 2. Doku kesitlerinin fotomikrograflarında gözlenen bulgular (a) karaciğerde hemoraji ve parankim hücrelerinde vakuolasyon ve nekroz, (b) dalakta hemosiderin birikintileri ve beyaz kan hücrelerinde boşalma, (c) solungaç lamelalarında erime ve epitel hücre hiperplazisi (okla gösterildi), (d) lamellar füzyonu (okla gösterildi), (e) ön böbrekte hiperemi ve hematopoetik dokuda boşalma, (f) dalakta granülomlar (yıldızla gösterildi) ve (g) dalakta granülomanın Ziehl-Neelsen negatif boyama sonucunu.

ornithine decarboxylase were positive in agreement with previous reports, our arginine dihydrolase tests for the isolates were positive. Taken together with the 16S rRNA gene sequencing results, these isolates were identified as *A. veronii* bv. veronii (Janda & Abbott, 2010; Shameena et. al, 2020).

The studied disease outbreak occurred during summer (June), with elevated water temperature, similar to other reports in European seabass so far (Smyrli et al., 2019; Tanrıkul & Dinçtürk, 2021). Ulcers, abdominal distention, hemorrhage and in some cases exophthalmia and fin rot/tail rot are reported as a general clinical finding in disease (both in marine and freshwater species) caused by *A. veronii* (Smyrli & Katharios, 2020; Tanrıkul & Dinçtürk, 2021). However, clinical findings vary between biovar types and is dependent on isolate virulence, co-infections and fish host species (Smyrli et al., 2019). In fact, some studies suggest that *Aeromonas veronii* bv. sobria is more virulent than *Aeromonas veronii* bv. veronii (Shameena et al., 2020).

Studies on the histopathology of diseases caused by *Aeromonas veronii* in fish tissues are very limited. Chen et al. (2019) reported in crucian carp (*Carassius auratus gibelio*) that multiple organs and tissues of the diseased fish displayed intense hemorrhaging, infiltrating inflammatory cells and necrosis. Severe intravascular congestion, swelling of liver cells, cell necrosis and karyolysis, as well as hyperplasia of the gill lamellae have been reported. Another study, found that the liver of largemouth bass (*Micropterus salmoides*) was hemorrhagic and had necrotic lesions Pei et al. (2021). Numerous inflammatory cells infiltrated the kidney, as well as necrosis in the glomeruli. The aforementioned study also found that the spleens of diseased fish had large amounts of hemosiderin granules, and secondary gill lamellae were an important sign of necrosis. Similar to these findings; we observed hemorrhages in the liver, hemosiderin deposits in the spleen and necrosis in the gill lamellae of diseased fish. In contrast to previous studies, depletion and hyperemia in the hematopoietic tissue in the anterior kidney were detected in examined fish. Our study also revealed intravascular hyperemia in many organ tissue sections from diseased fish. Some other histopathological findings

were similar to previously reported *A. veronii* epizootics in European seabass described by Tanrıkul and Dinçtürk (2021). These researchers reported lymphocytic cell infiltration in spleen tissue and granuloma, hyperemia, hemorrhages and necrotic tissue in the liver. They also found that the gill epithelium had lamellar epithelial hypertrophy and hyperplasia with degenerative changes as those observed in the present study.

In previous disease reports, the susceptibility of *A. veronii* isolates to antibiotics varies according to the fish host and the environment. Researchers have generally reported that *A. veronii* isolates are resistant to many antibiotics including ampicillin, amoxicillin, carbenicillin, chloramphenicol, clindamycin, enrofloxacin, kanamycin, lincosamide, nalidixic acid, pipemidic acid, teicoplanin and vancomycin (Vila et al., 2002; Cai et al., 2012; Liu et al., 2016). Uzun and Öğüt (2015) reported that biovar *A. veronii* bv. sobria isolates were resistant to ampicillin, sulfadiazine, tilmicosin, trimethoprim, penicillin-G, streptomycin and vancomycin antibiotics. Herein, all *A. veroni* bv. veronii isolates from European seabass were found to be sensitive to oxytetracycline, enrofloxacin, ciprofloxacin, florfenicol and flumequin, but, resistant to ampicillin and amoxicillin.

CONCLUSION

In this work, we identified *Aeromonas veronii* biovar veronii as the causative agent behind an epizootic that affected European seabass (*Dicentrarchus labrax*) cultured in Türkiye. Chronic disease characteristics observed in histopathology are similar to previously reported findings. Accumulated mortality below %1 support that this is a low virulence biovar. Out of the many antibiotics tested, the examined isolates of *Aeromonas veronii* biovar veronii were only resistant to ampicillin and amoxicillin, and sensitive to oxytetracycline, enrofloxacin, ciprofloxacin, florfenicol and flumequine.

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Author's Contributions

The authors declare that they contributed equally to this article.

Conflict of Interest

Authors declare that there is no conflict of interest.

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