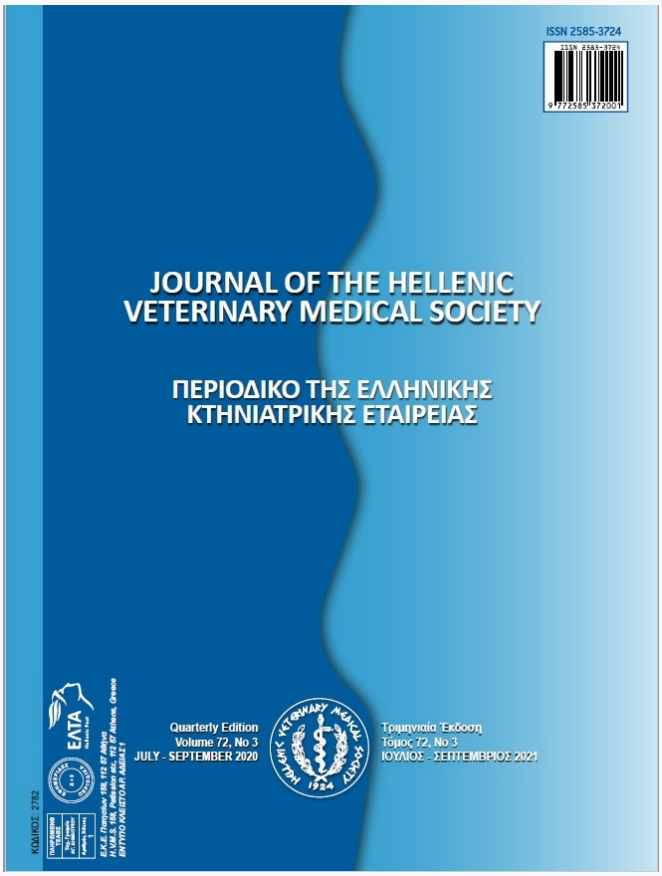
Journal of the Hellenic Veterinary Medical Society

Vol 72, No 3 (2021)



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doi: 10.12681/jhvms.28486

To cite this article:

TANSEL TANRIKUL, T., & DINÇTÜRK, E. (2021). A New Outbreak in Sea Bass Farming in Turkey: Aeromonas veronii. *Journal of the Hellenic Veterinary Medical Society*, *72*(3), 3051–3058. https://doi.org/10.12681/jhvms.28486

J HELLENIC VET MED SOC 2021, 72(3): 3051-3058
ΠΕΚΕ 2021, 72(3): 3051-3058

Research article
Ερευνητικό άρθρο

A New Outbreak in Sea Bass Farming in Turkey: *Aeromonas veronii*

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ABSTRACT: Turkey produces most of the European sea bassin Europe and bacterial diseases are the main cause of economic loss during the production cycle. This research presents the first extended study of the *Aeromonas veronii* infection in sea bass on the Turkish coast of the Aegean Sea. An outbreak was observed in three different sea bass farms and diseased fish with clinical symptoms were sampled. Abdominal distention, hemorrhages on the body and anus, enlarged spleen and liver, and hemorrhages in the internal organs were detected from clinical and pathological examinations. Biochemical and molecular identification confirmed the pathogen to be *A.veronii*.The histopathological observations demonstrated that the pathogen caused bacterial colonies in the fibrous connective tissue, granuloma, and vacuolar degeneration.The primary causes of the disease were proved through an infection experiment. 80% and 90% mortality were calculated in 0.85 x 10^8 CFU ml^{-1} and 1.28 x 10^8 CFU ml^{-1} experimentally infected groups with clinical and pathological signs of the disease respectively. Recently, the pathological symptoms of the disease had been confused with pasteurellosis infection in cage farms but the presence of *A.veronii* has been confirmed in the current study. A detailed study is needed to investigate the overall status of the disease in the Aegean Sea in order to design an appropriate preventive strategy.

Keywords: *Dicentrarchus labrax, Aeromonas veronii,* Aegean Sea, infectious fish disease

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Date of initial submission: 23-12-2019
Date of revised submission: 31-03-2021
Date of acceptance: 10-04-2021

INTRODUCTION

Turkey is a significant producer of farmed fish in Europe, and sea bass (*Dicentrarchus labrax*) is one of the main species produced. The increase in national aquaculture output in the last 30 years has led to various problems; among these is the prevalence of several bacterial fish pathogens in the Mediterranean area. The number of new pathogens identified has increased over time, and one of the most common causes of the emergence of infectious diseases in marine fish is the *Aeromonas* species.

Aeromonas species are Gram-negative, facultative anaerobic bacteria that have a ubiquitous presence, including in psychrophilic and mesophilic organisms and humans (Nerland, 1996; Austin and Austin, 2007; Janda and Abbott, 2010). These bacteria were previously reported as the causative agent of bacterial hemorrhagic septicemia (BHS), motile aeromonad septicemia (MAS), and epizootic ulcerative syndrome (EUS) in many marine and freshwater fish species (Austin and Austin, 2007; Martinez-Murcia et al., 2008; Liu et al., 2016). In addition, motile aeromonads have been reported with several clinical signs,such as ulceration, fin and tail rot, abdominal distention, and exophthalmia (Sreedharan et al., 2011). Because *Aeromonas* species affect many fish species, interest in understanding the role of the pathogen has been rising in fish farms (Guzman-Murillo et al., 2000). *Aeromonas veronii* is the most virulent of the *Aeromonas* species,which include *Aeromonas caviae*, *Aeromonas hydrophila* and *Aeromonas sobria* (Janda and Kokka, 1991; Sreedharan et al., 2011). It causes pneumonia, soft tissue and wound infection and gastroenteritis in humans (Janda and Abbott, 2010), and hemorrhagic septicemia and epizootic ulcerative syndrome in fish (Cai et al., 2012).

The diseases caused by *A.veronii* have been reported from several locations around the world and from several fish species: cultured channel catfish (*Ictalurus punctatus*) (Liu et al., 2016), cultured snakehead fish (*Ophiocephalus argus*) (Zheng et al., 2012), Chinese longsnout catfish (*Leiocassislongirostris*) (Cai et al., 2012), Nile tilapia (*Oreochromis niloticus*) (Hassan et al., 2017), carp (*Cyprinus carpio*) (Gong et al., 2010; Yu et al., 2010), Siberian sturgeon (*Acipenser baerii*) (Ma et al., 2009) and European sea bass (*D. labrax*) (Smyrli et al., 2017).

To our knowledge, this is the first extended study of infection by *A. veronii*in cultured sea bass in the Aegean coast of Turkey. Sea bass is one of the most produced commercial fish species in the Mediterranean region, and Turkey is the leading producer of sea bass in the Aegean Sea. This infection is thus a significant concern with high mortality and economic loss. The pathological symptoms were initially confused with another major bacterial disease, pasteurellosis; however,it was not previously possible to isolate the agent from chronic cases in fish farms. The study presented here is the first detailed investigation from the Aegean coast of Turkey of the *A. veronii* infection, which is a serious and contagious disease agent in sea bass cage culture, with pure isolation, the biochemical and molecular identification of the agent, and the determination of its histopathological effects and pathogenicity obtained via an experimental infection.

MATERIALS AND METHODS

The outbreak was observed in three different sea bass farms between 2017 and 2019 in the South Aegean region, Turkey. Diseased fish samples that approached commercial size (250-350 g) were sampledover a period of three years (a total of approximately 270 fish) from acute and chronic cases and transferred to the Fish Disease and Biotechnology Laboratory, Faculty of Fisheries, Izmir Katip Celebi University. Clinical, microbiological and pathological examinations were performed, and bacterial isolates from the anterior of the kidneys, spleen and liver were streaked on tryptic soy agar (TSA, Merck) supplemented with 2% NaCl and tryptic soy agar supplemented with sheep blood in order to isolate the causative pathogen. The TSA plates were incubated at 25°C for 24-48 h and colony morphology was observed. The pure colonies were streaked again on TSA for biochemical analyses. Motility, Gram staining was implemented, and catalase and oxidase activity were detected according to standard procedures (Austin and Austin, 2007). The isolates were identified using API 20E tests (BioMerieux S.A., France) by determining the biochemical characteristics of the bacteria.

Molecular identification of the bacteria was accomplished with 16SrRNA gene amplification. A EurXGeneMATRIX Tissue Bacteria DNA Isolation Kit (Poland) was used for DNA isolation, and the density and quality of the DNA were determined with a Thermo Scientific Nanodrop 2000 (USA). The PCR amplification reactions were performed by employing universal primers 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3').The amplification was carried out after initial denaturation at 95 °C for 5 minutes, fol-

lowed by 35 cycles at 95 °C for 45 seconds, 57 °C for 45 seconds and 72 °C for 1 minute, then 72 °C for 5 minutes as the final extension. Band screening of the PCR products was observed in the gel electrophoresis. Amplified products of template DNA were sent to the Macrogen direct sequencing service (Macrogen, Holland) for sequence determination. The sequenced DNA data were matched with the GenBank database using the BLASTN 2.6.1. algorithm and a phylogenetic tree was formed by the neighbor-joining method with MEGA7 software.

For histological examination, the liver, spleen, gill, and heart were sampled from diseased fish with clinical signs and preserved in 10% buffered formalin after necropsy. The tissues were then processed routinely and prepared into paraffin blocks. The blocks of tissues were cut to 5 µm thickness and stained with Hematoxylin and Eosin (H-E) and examined under a light microscope (Culling et al. 1985).

The pathogenicity of *A.veronii* was tested on European sea bass which had no infection history. Three duplicated experimental groups were constituted and 10 healthy fish (weighing about 350 g each) were placed in each of 300 L tanks in the recirculated aquaculture system in Faculty of Fisheries Research Center, Izmir Katip Celebi University.

The isolated and identified *A.veronii* strain was grown in Tryptic Soy Broth (TSB, Merck) supplemented with 0.5% NaCl for 20 h at 25°C. The number of colony-forming units (CFU) per ml was determined by plating seven-fold serial dilutions on TSA plates. 0.1 ml bacterial suspension of 0.85 x 10^8 CFU ml^{-1} and 1.28 x 10^8 CFU ml^{-1} *A.veronii* were injected by intraperitoneal injection (IP) into two of the groups, while 0.1 ml PBS were injected IP into the control groups under the same environmental conditions. Moribund and dead fish were observed and clinical and pathological examinations were conducted over a period of 10 days.

RESULTS

The disease caused massive mortality in sea bass farms between May (19 °C) and November (20 °C) in the same region, and cumulative losses were observed over three years from chronic cases. All the sea bass farms are located in the same area and the symptoms of the disease were observed in all of them. Infected fish were observed near the surface of the water and exhibited a loss of appetite. The clinical findings indicated abdominal distention, hemorrhages on the head and around the mouth, especially on the upper and lower jaw,operculum, and ulcerative lesions on the ventral side of the body and around the anus (Figure 1). Necropsy showed enlarged liver, spleen, heart, and kidney with whitish nodules, multiple granulomas, and focal necrosis (Figure 2).

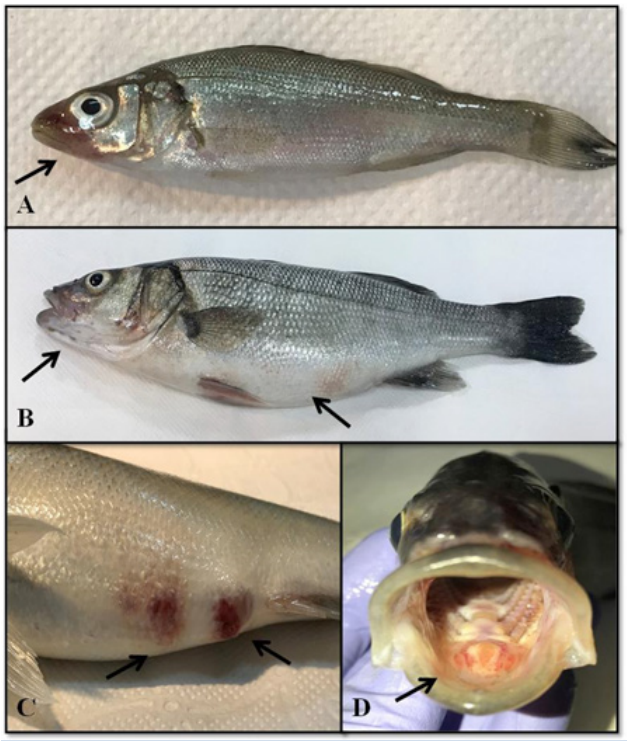

Figure 1. Clinical signs of the disease: A: hemorrhages on head and around mouth; B: hemorrhages around mouth and abdominal distention; C: ulcerative lesions on the ventral side of the body and around anus; D: hemorrhages on the upper and lower jaw

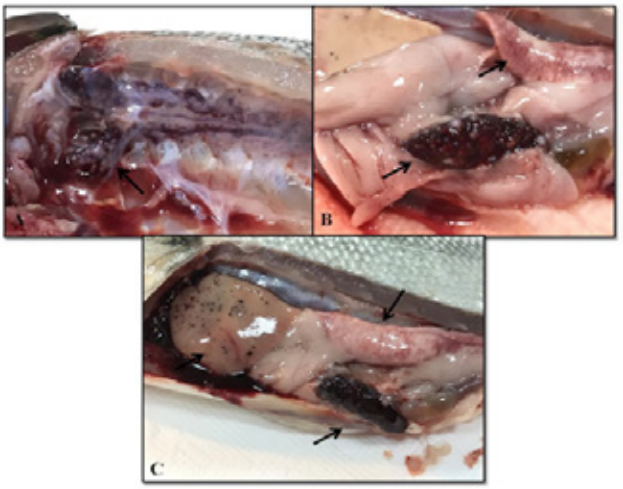

Figure 2. Pathological findings of the disease: A: multiple granulomas on kidney; B: hemorrhages on gonads and internal fat, whitish nodules on spleen; C: petechiae on liver with anemia, hemorrhages on gonads and internal fat, whitish nodules on spleen

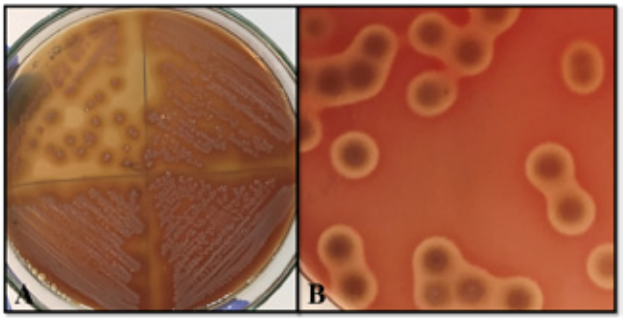

Figure 3. A: Pure *A. veroniic* olonies on TSA; B: Hemolysis of *A. veronii* on BTSA

Smooth and round colonies were detected on the TSA with brown pigmentation on the medium and these caused hemolysis on the BTSA after 48 h incubation (Figure 3). The isolates were found to be motile, Gram-negative, oxidase, and catalase positive rod-shaped bacteria. The morphologic and API 20E biochemical test results are presented in Table 1. ONPG (ortho-nitro-phenyl-galactoside), LDC (lysine decarboxylase), ODC (ornithine decarboxylase), citrate utilization, TDA (tryptophan deaminase), indole, VP (Voges Proskauer test), gelatin, glucose, mannitol and sucrose were found to be positive, while ADH (arginine dihydrolase), H_2S, urease, inositol, sorbitol, rhamnose, melibiose, amygdalin and arabinose were detected to be negative in all isolates (Table 1).

Table 1. Morphologic and biochemical test results of the isolated *Aeromonas veronii* strain

Aeromonas veronii			
Gram stain	-	TDA	+
Motility	+	Indole	+
Oxidase	+	VP	+
Catalase	+	Gelatin	+
O/129	-	Glucose	+
OF	+	Mannitol	+
ONPG	+	Inositol	-
ADH	-	Sorbitol	-
LDC	+	Rhamnose	-
ODC	+	Sucrose	+
Citrate utilization	+	Melibiose	-
H_2S	-	Amygdalin	-
Urease	-	Arabinose	-

The 16S rRNA sequence results of the isolated strains showed 99% sequence similarity with *A.veronii* in the BLASTN 2.6.1 database and were registered in NCBI GenBank with accession number MT126417. The phylogenetic tree of the isolated strain and other homologous sequences which were isolated from different fish species is presented in Figure 4.

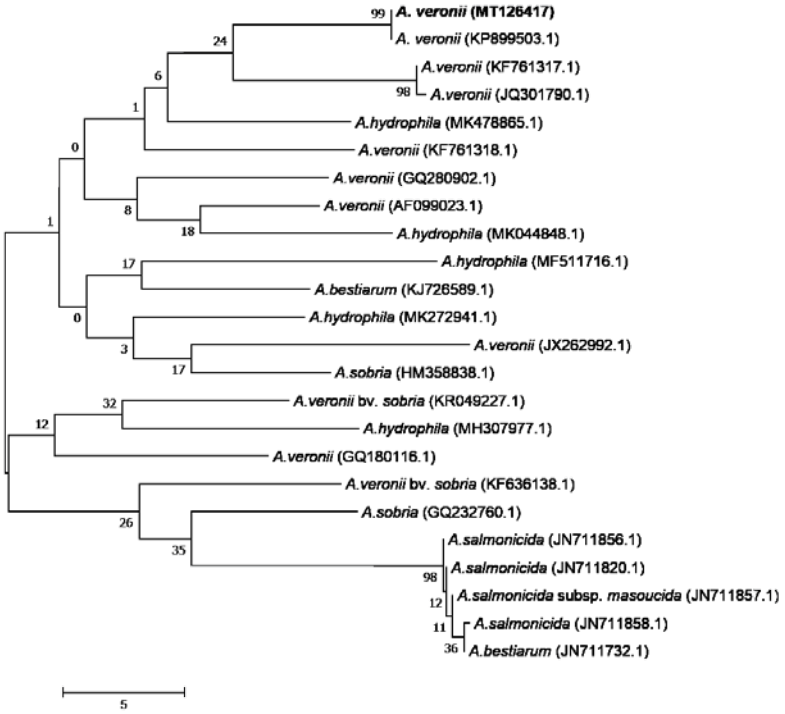

Figure 4. Phylogenetic tree of *Aeromonas veronii* MT126417 (in bold) and related matches isolated from different fish species in GenBank (NCBI) based on 16S rRNA sequences using MEGA7 with Maximum Composite Likelihood Method, 1000 bootstrap replicates. The bootstrap values are shown next to the branches and the scale bars present the distance values.

The histopathological results for the spleen, liver, gill and heart tissues are presented in Figure 5. Degeneration in cells such as lymphocytic cell infiltration was noticed in the spleen tissue. In the liver,granuloma, intravenous hyperemia, hemorrhages between cells, vacuolar degeneration, necrotic tissue were observed, as well asbacterial colonies in the fibrous connective tissue. Lamellar epithelial hypertrophy and hyperplasia with degenerative changes were found in the gill epithelium. In addition, degeneration and lymphocyte cell infiltration were determined, especially in subendocardial cells (Figure 5).

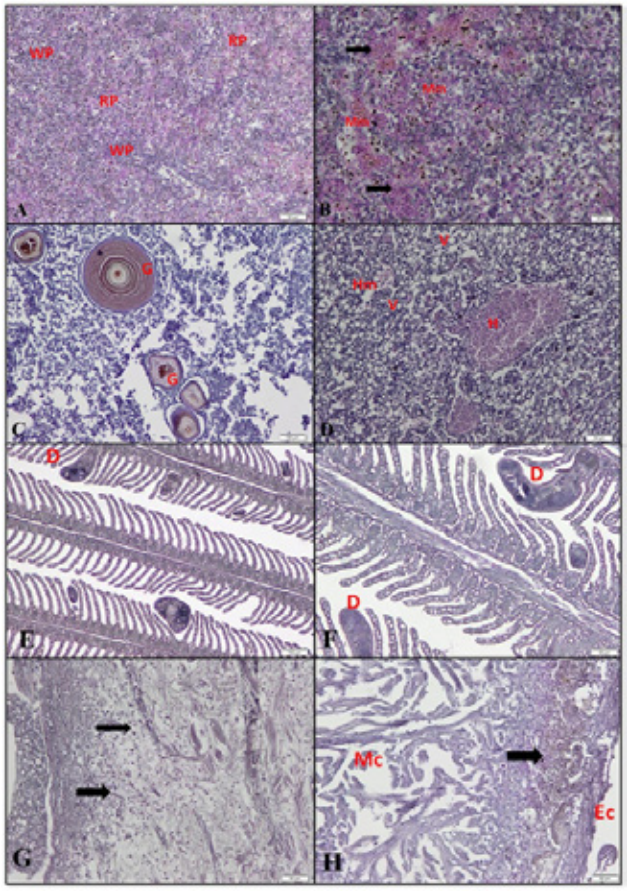

Figure 5. Hematoxylin and Eosin (H-E) stain of *Aeromonas veronii* infected tissues; A (X 200) WP: white pulp; RP: red pulp -B (X 400): spleen tissue with degeneration in cells;Mm: melanomacrophage and lymphocytic cell infiltration; C (X 100) G: granuloma - D (X 200): V: vacuolar degeneration;H: hyperemia;Hm: hemorrhages between cells and bacterial colonies in liver tissue, E (X 100) - F (X 200) D: degeneration - G (X 200): lymphocytic cell infiltration and lamellar epithelial hypertrophy, hyperplasia with degenerative changes in the gill epithelium; H (x 200): Mc: myocardium; Ec: endocardium, degeneration and lymphocyte cell infiltration in subendocardial cells.

In the infection experiment, the mortality of the groups after 36 hours of injection was determined and found to be 80 % and 90 % in the 0.85 x 10^8 CFU ml^{-1} and 1.28 x 10^8 CFU ml^{-1}groups respectively. The symptoms of the disease were observed to be ascites, and hemorrhages on the body surface and internal organs,and the causative pathogen was reisolated from the kidneys, spleens and livers of the IP-injected fish. Death stopped in the sixth day of the experiment, and no clinical or pathological signs of the disease were detected in the control groups.

DISCUSSION

This study investigated the disease caused by *A.veronii* in farmed sea bass in the Aegean Region of Turkey and examined it in detail through biochemical and molecular identification, histopathological study, and the determination of pathogenicity. European sea bass production reached 116,915 tonnes in 2018 in Turkey (TUIK, 2019), and it thus became the primary species in the marine aquaculture industry. This paper highlights the significance of the presence of a new bacterial disease in this area. *A.veronii* was isolated from the commercial-sized diseased fish showing clinical signs and mortality. Determining the impacts of *A.veronii* infection on seabass is important for the future of aquaculture production in the Mediterranean region in order to prevent further outbreaks that could cause several pathological disorders and mass mortality.

Uzun and Ogut (2015) reported *A.veronii* biovar-*sobria* from European sea bass in their study of the occurrenceand frequency of bacterial pathogens in sea bass in the Black Sea Region of Turkey, which sampled two different fish farms monthly between 2009 and 2011. Overall, *A. veronii* bv *sobria* was identified as the most common pathogen (65.2%), although it was isolated from only 13 fish with signs of disease. The study was the only statement of the presence *A.veronii* in Turkey and there was no significant evidence that *A.veronii* was a pathogenic disease agent in that area that could cause an epidemic causing mass mortalities and pathologic disorders in cages. Previous reports have shown the existence of disease caused by *A.veronii* on the Greek coast of the Aegean Sea (Smyrli et al., 2017). The present study verifies the presence and the effects of the pathogen on the Turkish side of the Aegean Sea as a primary causative disease agent.

Abdominal distention, hemorrhages on different parts of the head, and ulcerative lesions were all observed in the diseased fish, while multiple granulomas in the major organs were noted as the main pathologic

findings of the disease. Smyrli et al. (2017) observed epidermal lesions that became ulcerated and reddened fins in European sea bass as the clinical signs of the disease. Furthermore, hemorrhages, lesions, necrotic foci, and granulomas were detected on spleen, kidney, and liver tissue in infected fish. Hassan et al. (2017) reported hemorrhages on the surface of the body, detached scales, ulcerations, and abdominal dropsy in Nile tilapia (*O.niloticus*). Moreover, hemorrhagic spots in the liver, bloody exudates in the abdominal cavity, hepatosplenomegaly, and the gall bladder filled with bile were documented as the post-mortem examination results of *A.veronii* infection. The most obvious clinical and pathological symptoms were stated to be hemorrhages in the mouth and fins, enlarged spleen, hemorrhages, and necrosis in the spleen, kidney, and liver in cultured channel catfish (*I. punctatus*) (Liu et al., 2016). In addition, Yu et al. (2010) found ulcerative lesions on the skin, abdominal dropsy, and enlarged spleen and kidney from *A.veronii* infection in farmed carp (*C. carpio*). These research findings are indicated that the pathogen causes similar clinical and pathological symptoms in several fish species.

Isolated *A.veronii* strains were observed with brown pigment-producing colonies on TSA. Smyrli et al.(2017) isolated two different *A.veronii* strains; pigment-producing isolates and non-pigment producing isolates. However, Uzun and Ogut (2015), Yu et al. (2010) and Cai et al. (2012) did not provide any information about the pigment-production of strains. The biochemical test results of the isolated *A.veronii* strains are compared with related research in Table 2. Some indicators, such as arginine dihydrolase (ADH), ornithine decarboxylase (ODC) and tryptophan deaminase (TDA) vary among different isolates, but the parameters are not essential criteria for biochemical identification of *A.veronii*(Abbott et al., 2003).

Table 2. Morphologic and API 20E biochemical test results

	Present isolate	Yu et al. 2010[a]	Zheng et al. 2012[b]	Liu et al. 2016[c]	Smyrli et al. 2017[d]
Gram stain	-	-	.	-	-
Motility	+	+	.	+	+
Oxidase	+	+	.	+	+
Catalase	+	+	.	.	+
O/129	-	-	.	.	.
OF	+	+	.	+	.
ONPG	+	+	.	+	+
ADH	-	+	+	-	-
LDC	+	+	+	+	+
ODC	+	-	.	+	+
Citrate utilization	+	+	.	.	+
H_2S	-	-	.	-	-
Urease	-	-	-	-	-
TDA	+	-	-	.	+
Indole	+	+	.	+	+
VP	+	+	.	+	+
Gelatin	+	+	.	+	+
Glucose	+	+	.	.	+
Mannitol	+	+	+	+	+
Inositol	-	-	-	-	-
Sorbitol	-	-	-	-	-
Rhamnose	-	-	.	-	-
Sucrose	+	+	+	+	+
Melibiose	-	-	.	-	-
Amygdalin	-	-	.	.	-
Arabinose	-	-	-	-	-

a: Isolated from Israeli carp (*Cyprinus carpio*); b: Isolated from snakehead fish (*Ophiocephalus argus*); c: Isolated from channel catfish (*Ictalurus punctatus)*; d: Isolated from European sea bass (*Dicentrarchus labrax*)

Smyrli et al. (2017) reported a systemic infection from European sea bass characterized by chronic granulomatous inflammation; necrotic lesions with granulomas contained the foci of rod-shaped bacteria. Yu et al. (2010) observed hepatocellular vacuolar degeneration and congestion in sinusoids, and pulps in the spleen, which had numerous bacterial invasions, in *A. veronii*-infected *Cyprinus carpio*. In naturally-infected Nile tilapia (*O.niloticus*) vacuolar degeneration in the liver, depletion of hemopoietic tissue in the spleen, and degenerative changes in the parenchymatous organs including the liver and spleen were noted by Hassan et al. (2017). Similarly, in this study,degenerations in the spleen tissue, vacuolar degeneration, hemorrhages between cells, and bacterial colonies in the liver tissue were found among the histopathological results for the European sea bass.

Previous studies on the mortality rates of *A.veronii* infection experiments have demonstrated acute mortalities at different concentrations. Yu et al, (2010) tested the effects of *A.veronii* on carp (*Cyprinus carpio*) and reported that a 10^5 CFU fish^{-1} dose caused 16.7% mortality within 10 day, while a 10^6 CFU fish^{-1} dose raised this percentage to 65% within nine days, and 10^7 CFU fish^{-1} injected fish all died within seven days. All the dead fish indicated typical external and internal signs of the disease. Smyrli et al. (2017) tested *A.veronii* infection in sea bass with two different challenge tests: 10^4 CFU fish^{-1} by injection intraperitoneally and 10^5 CFU fish^{-1} bacterial suspension by immersion. All the fish in the bath-challenge group were reported dead within 10 days, and displayed lethargic swimming, reddening on the skin and fins, diffused hemorrhages in the peritoneum cavity and on the surfaces of internal organs, as well as lesions on the spleen and liver. The IP-injected fish group died within four days with diffused hemorrhages in the peritoneum cavity and internal organs. Sreedharan et al. (2011) tested the pathogenicity of the *A.veronii* strain on goldfish (*Carassius carassius*). They calculated the LD_{50} values to be $10^{5.071}$ CFU/ml and observed loss of scales with hemorrhagic scale pockets in the fish tested. Hassan et al. (2017) established the LD_{50} dose of *A.veronii* strain for the Nile tilapia (*O. niloticus*) 5.2 x 10^6 CFU/ml and determined 78% mortality in the challeng egroups,observing hemorrhage and redness at scale pockets, enteritis and the filling of the intestine with transparent content. Many recent studies have shown that *A.veronii* causes similar clinical and pathological symptoms in different fish species and the present study provides similar results in sea bass.

Photobacterium damselae subsp. *piscicida* infection was described as "pseudotuberculosis" by Egusa (1993), due to its having similar symptoms and histopathological characteristics as tuberculosis, including numerous tubercles in the internal organs, especially in the kidney and spleen. The clinical and pathological symptoms of the disease caused by *A.veronii* are very similar to those of pasteurellosis, including the appearance of severe granulomas in the internal organs, and the disease in this current case could be considered as "pseudopasteurellosis".The pathological symptoms were generally confused with pasteurellosis infection in cage farms, especially in Turkey but this study verifies the presence and pathological effects of *A.veronii* in sea bass.

In conclusion, this is the first study to our knowledge to report *A.veronii* infection in European sea bass from the Turkish coast of the Aegean Sea. The study has determined the presence of the pathogen as a disease agent in seabass in this area through clinical, pathological, biochemical, molecular, and histopathological findings, and has also confirmed the results with an infection experiment. Overall, these findings highlight the potential for anout break in a region that is one of the main seabass producers in the world, which could lead to massive economic loss. More research is required to fully understand the prevalence of *A.veronii* in the Aegean Sea and to explore its effects on other fish species and the longer-term economic implications.

ACKNOWLEDGEMENTS

We wish to thank Dr. Fatma Şimşek of the Department of Histology and Embryology, Faculty of Medicine, Izmir Katip Celebi University for her support with the histopathological analyses in this study.

CONFLICT OF INTEREST STATEMENT

None declared by the authors.

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