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Diagnosis of Vibriosis Involving Members of the Splendidus Clade in Cultured European Seabass (*Dicentrarchus labrax*) in Turkey

R. Eda Yardimci*

[*] *Department of Fish Disease, Faculty of Aquatic Sciences, University of Istanbul, 34470, Ordu Cad. No: 200. Laleli-Istanbul/Turkey*

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Abstract

This study aimed to investigate a case of acute vibriosis associated with *Splendidus* clade members in cultured European sea bass (*Dicentrarchus labrax*) from the Aegean Region of Turkey. Moribund fish (150-250 g) had lesions on their skin that ranged from small patches to shallow haemorrhagic ulcers and fin rot. Gram-negative bacteria were isolated from the liver, kidney, and spleen of affected fish and through 16S rRNA gene sequencing these isolates were identified as *Vibrio pomeroyi*, *Vibrio crassostreae*, *Vibrio* sp., and *Vibrio anguillarum. V. anguillarum* was isolated from only one examined fish, the bacterium seems less important to this case of vibriosis than the other identified vibrio species. All isolates were susceptible to flumequine, kanamycin, oxytetracycline, florphenicol and enrofloxacine. Oval shaped *Amyloodinium* spp. on the skin and *Diplectanum aquans* among the secondary lamellae of gills were determined as secondary pathogens. Parasitical asphyxia, epithelial cells hyperplasia in the gills was observed in addition to typical histopathological findings associated with Vibriosis. In this study, *V. crassostreae* and *V. pomeroyi* have been reported for the first time in Turkey as a fish pathogen.

* Corresponding author. e-mail:etepecik@istanbul.edu.tr

Introduction

Vibrio species belonging to the *Vibrionaceae* family are Gram-negative bacteria commonly found in coastal waters and estuaries. Since the first report by Bergman in 1909 in eels *Anguilla anguilla*, *Vibrio anguillarum* has been isolated from much marine fish (Actis et al., 1999; Thompson et al., 2004; Austin and Austin, 2016). Vibriosis, caused by *Vibrio* species, is one of the most important problems that impact fish production in Turkey (Karataş and Candan, 2007; Korun and Timur 2008; Öztürk and Altınok, 2014). Besides vibriosis, motile Aeromonas septisemia, yersiniosis, furunculosis, pasteurellosis, tenacibaculosis, staphylococcosis, and fish disease caused by rickettsia-like organisms and mycobacteria have been also reported in cultured marine fish in Turkey (Karataş and Candan, 2007; *Öztürk and Altınok, 2014).*

Molecular techniques have allowed more precise identification of members from the genus *Vibrio* and they are now classified into 16 monophyletic clades; *Cholerae, Anguillarum, Vulnificus, Harveyi, Halioticoli, Fischeri, Splendidus, Nereis, Orientalis, Coralliilyticus, Scophthalmi, Diazotrophicus, Mediterranei, Pectenicida, Porteresiae* and *Rumoiensis* using multilocus sequence analysis and 16S rRNA gene sequencing according to researchers (Thompson et al., 2005; Thompson et al., 2007; Sawabe et al., 2013). The *Splendidus* clade is the largest and the genetic diversity and polyphyletic nature of the *Splendidus* clade have been demonstrated by previous studies (Le Roux et al., 2004; Thompson et al., 2005; Sawabe et al., 2013). The said clade currently includes 17 species. *Vibrio splendidus, V. atlanticus, V. artabrorum, V. celticus, V. chagasii, V. crassostrea, V. cyclitrophicus, V. gallaecicus, V. gigantis, V. kanaloae, V. lentus, V. pomeroyi, V. tasmaniensis* and *V. toranzoniae* are causing significant losses in the aquaculture industry worldwide (Romalde et al., 2014; Pérez-Cataluña et al., 2016).

Vibrio crassostreae, originally identified as a *V. splendidus*-like isolate, has been described as a species with a pathogenic potential for the oyster *Crassostreae gigas* (Faury et al., 2004). *Vibrio pomeroyi* was originally isolated from healthy bivalve larvae (*Nodipecten nodosus*) in Brazil and from turbot (*Scophthalmus maximus*) in Spain (Thompson et al., 2003). Studies of these two *Vibrio* species by researchers preceded demonstrations of either a virulent or low virulent infections in animals (Gay et al., 2004; Austin et al., 2005). In an experimental study with *V. pomeroyi* injection in the adductor muscle or the pallial cavity, weakness of adductor muscle and mortalities occurred in several days (Gay et al., 2004). These species are known as possible pathogens of oysters, other bivalves, and mollusks but have until now never been associated with fish disease (Thompson et al., 2003; Gay et al., 2004).

In this study, the purpose was to investigate an outbreak of vibriosis in cultured sea bass from soil ponds in the Aegean Region of Turkey and to determine the identity of the isolated bacteria that were suspected to belong to the *Splendidus* clade.

Materials and Methods

Case history and sampling

The reported outbreak occurred during May of 2016 with 5% mortality in a soil pond farm located near Milas in the Aegean Region of Turkey. Fifteen moribund sea bass (150-250 g) were examined. The study was conducted according to the principles of the World Medical Association Declaration of Helsinki "Ethical Principles for Medical Research Involving Human Subjects" amended in October 2013.

Parasitological examination

For parasitological examination, skin scrapings and gill biopsy were performed. Smear from skin and squash preparation from gills were examined under the light microscopy. The squash of small section of the lower intestines and gall bladder were also made on glass slides and observed for the presence or absence of internal parasites under the microscope. The identification protocols used by Oliver (1957) and Timur et al. (2004) were followed.

Bacteriological examination

The samples for bacteriology were taken from liver, spleen and kidney and streaked onto Marine Agar (Difco 2216) and thiosulfate-citrate-bile salts-sucrose (TCBS) agar

(Merck 1.10263) (Bulcok, 1978; Buller, 2004; Austine and Austine, 2016). The plates were incubated at 22ºC for 24-72 h and the morphological, physiological and biochemical characteristics of bacterial colonies of the pure cultures were determined by routine laboratory methods and using API 20 E kits.

PCR Amplification and Sequencing

Bacterial isolates with unique morphological and biochemical characteristics were selected for identification and DNA was extracted from the isolates with the GeneJET Genomic DNA Purification Kit (Thermo). This genomic DNA was used as a template for PCR. In the described PCR, the S-20 (5' AGA GTT TGA TCC TGG CTC AG 3') and A-18 (5' GWA TTA CCG CGG CKG CTG 3') primer set were used to amplify a 540bp long fragment of the 16S rRNA gene.

The PCR protocol included an initial denaturation step at 95 °C for 3 min, then 30 cycles of amplification (denaturation at 95 °C for 30 s, annealing at 56 °C for 1 min, extension at 72 °C for 1 min) and a final extension step at 72 °C for 4 min. PCR products were purified and sequenced in both directions by a commercial firm (Medsantek) and thereafter analyzed. Bioedit v7.00 (Hall, 1999), ClustalX2.1 (Larkin et al., 2007) and the BLASTIN 2.2.20 algorithm (Zhang et al., 2007) were used for this sequence editing and analysis.

Antibiotic Susceptibility Tests

All strains were tested for antimicrobial susceptibility by the disc diffusion method. The Kirby-Bauer disk diffusion method was performed using multi-disk. The isolates were plated onto Mueller-Hinton agar (Oxoid) incubated at 20ºC for 48-96 h and results were interpreted based on available CLSI data (CLSI, 2012).

Histopathological Examination

Tissue samples from gills, heart, liver, kidney, spleen and intestine were processed for histopathology after fixation in 10% buffered formalin and then embedded into paraffin blocks. Histological sections of 5µm were stained with hematoxylin and eosin and examined by light microscopy (Culling, 1963).

Results

Cross and Clinical Findings

Moribund fish (150-250 g) had lesions on their skin which ranged from small patches to shallow hemorrhagic ulcers and fin rot (**Figures 1a, 1b**). Internal examination revealed that all fish had pale livers, melted kidneys, small spleens, thinner and transparent intestinal walls as well as excessive fat between visceral organs (**Figure 1b, 1c**).

Parasitological Finding

The squash preparations from gills and scrapings from the skin were examined under the light microscopy and a large number of oval-shaped *Amyloodinium* spp. but also monogenean *Diplectanum aquens* were found present on the gills. Endoparasites were not observed gall bladder and intestines of moribund fish.

Bacteriological Findings

Twenty bacterial isolates were obtained from the visceral organs of the affected fish. All isolates were identified as Gram-negative rods, showing motility with a cell size of about 0,6-3 x 0.5 µm. These isolates gave positive reaction in cytochrome oxidase and catalase tests and showed sensitivity to pteridine phosphate (O/129 vibriostatic agent) (Fig 2a). None of the Facultative anaerobe strains grows without NaCl. All morphological and biochemical testing results are presented (**Table 1**). These test results suggested that all isolates belonged to genus Vibrio and gathered under four groups (**Figure 2a, 2b, 2c**).

PCR Amplification and 16S rRNA Sequencing Findings

PCR amplification products were shown in **Figure 3**. All obtained 16S rRNA sequences were deposited in the Gen Bank database. According to 16S rRNA gene sequencing results, the isolates were identified at the species level as *V. pomeroyi* (isolates no. 1-8) (acc. no. MF502892), *V. crassostreae* (isolates no 9-16) (acc. no. MF502893, MF502894) and *V. anguillarum* (isolate no 20) (acc. no. MF502896). However, three isolates could only be identified to genus level as *Vibrio* sp. (isolates no 17-19) (acc. no. MF502895).

Antibiotic Susceptibility Tests Findings

All isolates were also determined as resistant to ampicillin but susceptible to flumequine (30 µg), kanamycin (30 µg), oxytetracycline (30 µg), florphenicol (30 µg) and enrofloxacine (5 µg) antibiotics.

Histopathological Findings

Histopathologically, extensive vacuolation of hepatocytes and liquefactive necrosis in the liver were observed (**Figure 4a**). Furthermore, we observed lysis in the heart muscle (**Figure 4b**) and myopathy, multifocal hemosiderin deposits and decreased erythrocytes in the spleen tissue (**Figure 4c**). Other observations included sloughed epithelial cells of intestine into the lumen (**Figure 4d**) and tubular necrosis accompanied by interstitial necrosis such as glomerular oedema in the kidney (**Figures 4e, 4f**) in the tissue sections of affected fish. Because of parasitical asphyxia, hyperplasia of the epithelial cells was observed in the gill sections (**Figure 4g**). Besides, parasitic *Amyloodinium* spp. and *Diplectanum aquens* were found among the secondary lamellae in the histological gill sections (**Figure 4h**).

Table 1. Morphological and biochemical test results of bacterial isolates

	1-8	9-16	17-19	20
Morphology	R	R	R	R
Motility	+	+	+	+
Gram Staining	-	-	-	-
Cytochrome Oxidase	+	+	+	+
MR test	-	-	-	-
VP reaction	+	-	+	+
O/F	F	F	F	F
0/129 (150µu) Resistance	S	S	S	S
Esculin	+	-	+	-
Nitrate Reduction	+	+	+	+
API 20E profiles	304614556	304614556	304616556	324752657
Catalase	+	+	+	+
Production of H_2S	-	-	-	-
Indole	-	-	+	-
B-Galactosidase	+	-	+	+
Arginine dihydrolase	V	+	-	+
Lysine decarboxylase	-	-	+	-
Ornithine decarboxylase	-	-	+	+
Citrate	-	-	+	+
Degradation of Starch	+	-	+	+
Degradation of Gelatin	+	+	-	+
Growth on				
4°C	+	+	+	-
0% NaCl	-	-	-	-
8% NaCl	-	-	-	-
TCBS	+	+	+	+
Acid production from				
Glucose	+	+	+	+
Arabinose	+	+	+	+
Sorbitol	-	-	-	+
Xylose	-	+	+	-

R: rods; +: positive, -: negative; F: fermentative; NF: Non-fermentative; V: variable; S: sensitive;

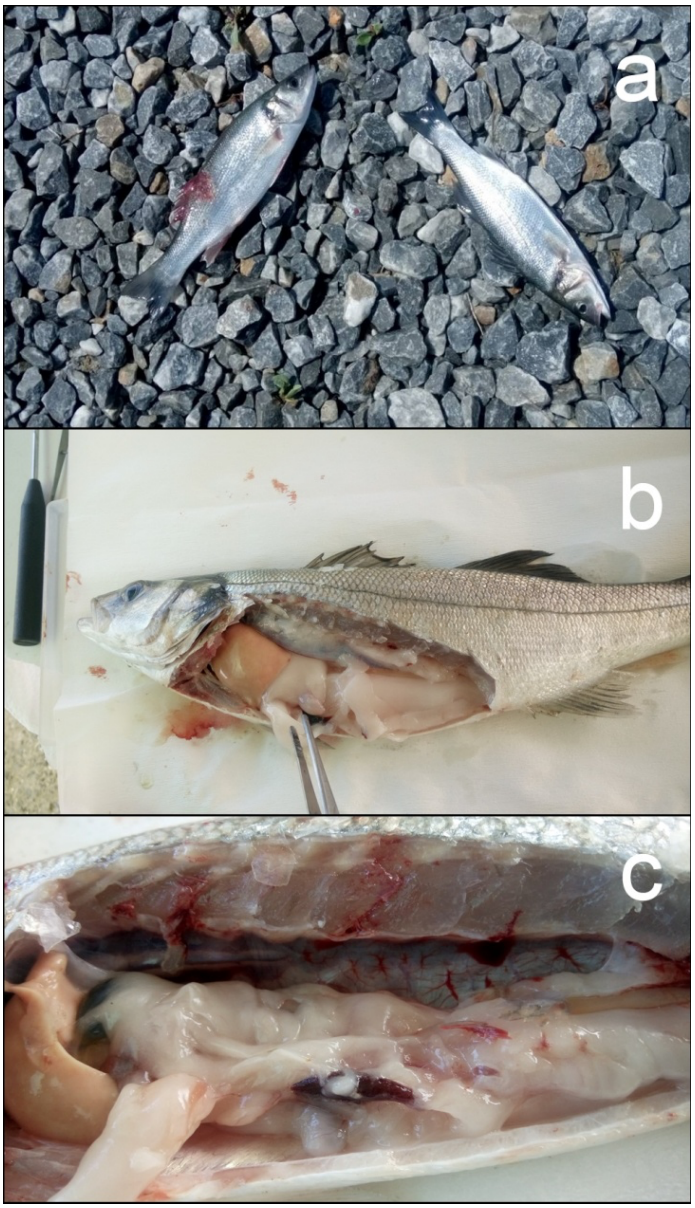

Figure 1 Moribund sea bass (150-250g) external and internal clinical findings (a) haemorrhages and shallow haemorrhagic ulcers, (b) pale liver and fin rot, (c) lysis in kidney and visceral fats.

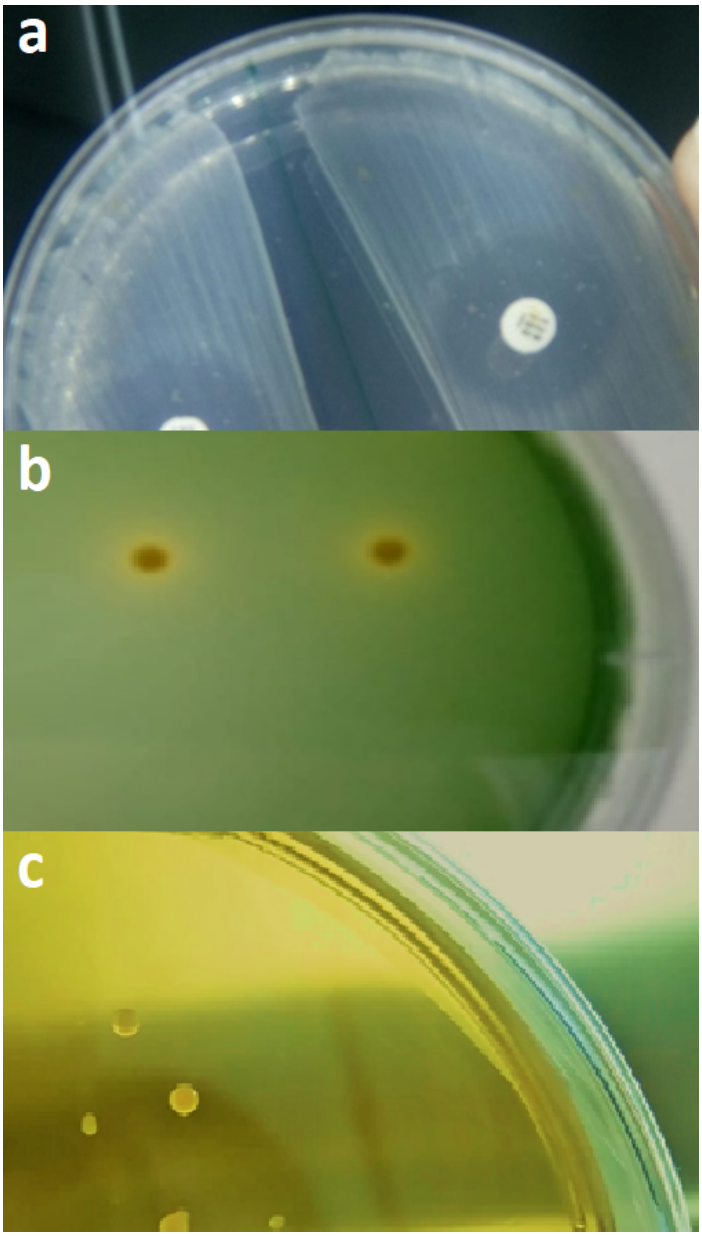

Figure 2 Bacterial isolates were obtained from the visceral organs of the affected fish (a) pteridine phosphate sensitivity on MA (isolates no. 19 and 20), (b) approximately 3 mm in diameter translucent yellowish colonies with entire margins growth on TCBS (isolate no. 7), (c) yellowish colonies growth on TCBS agar (isolate no. 12).

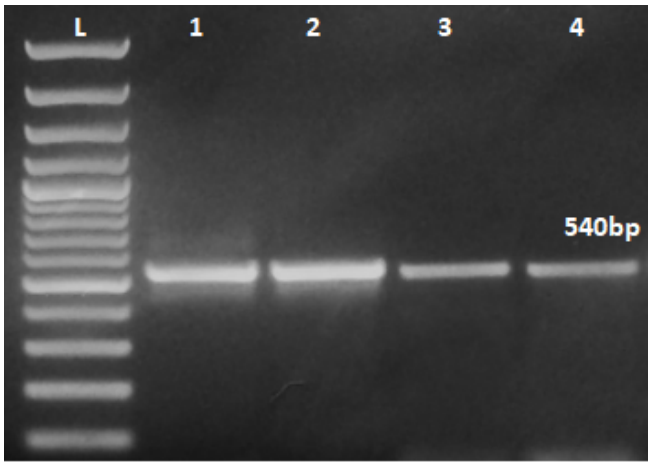

Figure 3 540bp long fragments of the 16S rRNA gene. L: Ladder (GeneRuler 100 bp Plus DNA Ladder - Thermo Scientific), 1: *V. pomeroyi*, 2: *V. crassostreae*, 3: *Vibrio* sp., 4: *V. anguillarum.*

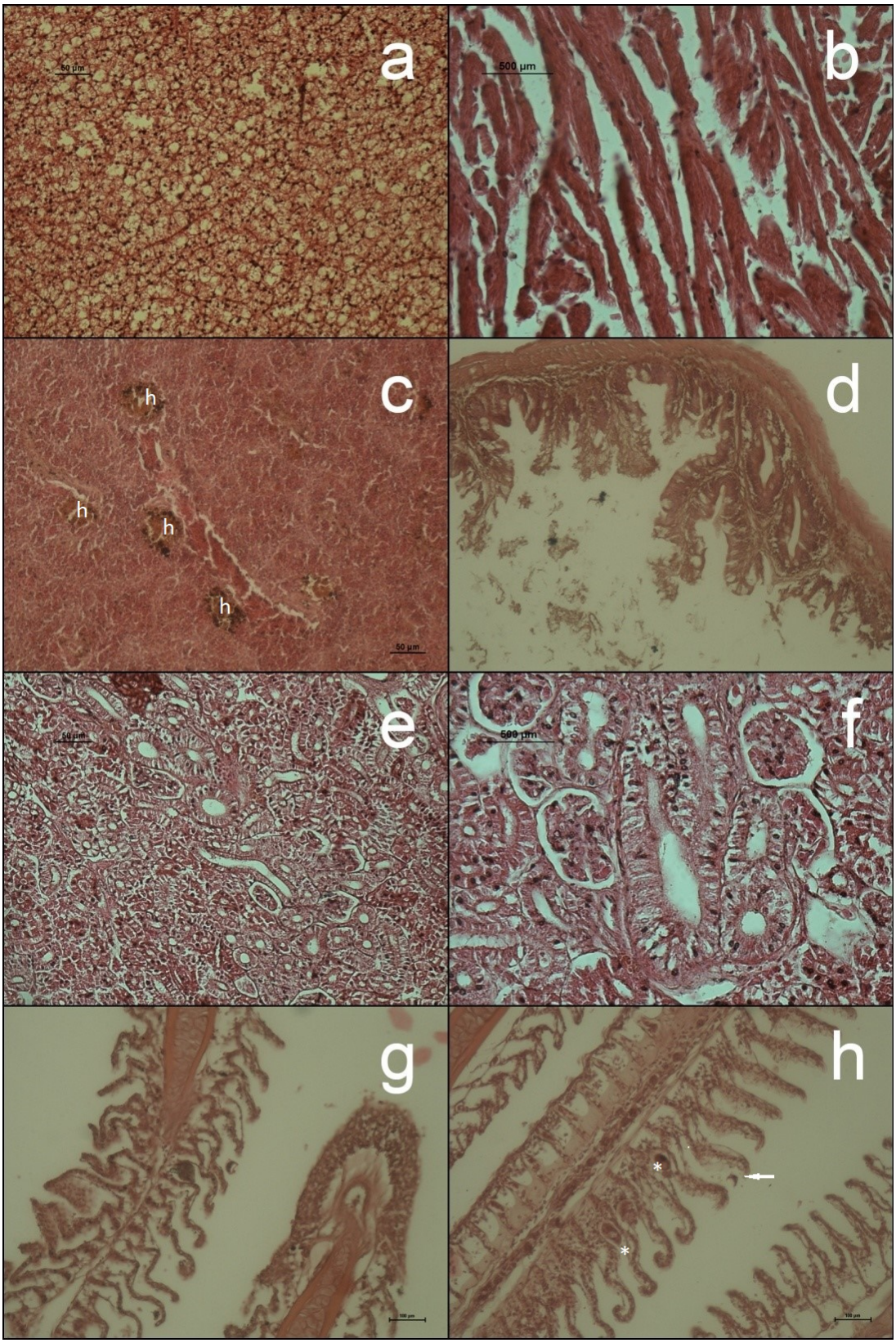

Figure 4 A photomicrograph of moribund fish tissue sections (a) vacuolation and liquefactive necrosis in the liver, (b) lysis in the heart muscle, (c) multifocal hemosiderin deposits (h) and depletion of red pulp in the spleen, (d) epithelial cell of intestine sloughed into the lumen, (e) and (f) tubular necrosis and glomerular oedema in the kidney, (g) haemorrhagic, erosive gill filaments and distal hyperplasia of secondary lamella, (h) *Amyloodinium* spp. (arrowed) and *Diplectanum aquens* (*) among the secondary lamellae (H&E).

Discussion

Vibriosis is a common bacterial disease in wild and cultured marine fish and causes acutely or chronically haemorrhagic septicaemia. Many studies address the influence of environmental parameters on the diversity of *Vibrio* species. In this study, acute vibriosis was observed in diseased sea bass. All clinical findings were similar to previously reported vibriosis observations as described (Actis et al., 1999; Thompson et al., 2004; Karataş and Candan, 2007; Korun and Timur, 2008; Öztürk and Altınok, 2014; Austin and Austin, 2016). These histopathological findings are compliant with the acute form of the disease, which corresponded with excessive stress levels in affected fish.

A high phenotypic variability has been described within the *Splendidus* clade which makes it difficult to discriminate among several species (Thompson et al., 2003; Le Roux et al., 2004; Thompson et al., 2005). In several studies, species of *Vibrio* have been defined as clusters of isolates with both high phenotypic and genotypic similarity. It was reported by Sawabe et al. (2013) that based on 16S rRNA gene sequence phylogeny, there is no robust monophyletic lineage formed within the genus Vibrio. In this study, most isolates could be identified as *V. pomeroyi, V. crassostreae*, and *V. anguillarum* based on 16S rRNA gene sequencing results. However, even with additional gyrB gene sequencing, three isolates (no 17-19) could only be reliably identified to genus *Vibrio* level.

Oval shaped *Amyloodinium* spp. on the skin and *Diplectanum aquens* among the secondary lamellae of gills were assessed as secondary pathogens.

Vibrio anguillarum is naturally resistant to amoxicillin and ampicillin (Manfrin, 2020). A previous study reported that ampicillin, flumequin, furazolidone, oxolinic acid and sulfamethoxazole/ trimethoprim are the most effective chemical substances in the treatment of vibriosis and resistant to erythromycin and ampicillin in Turkey (Korun and Timur, 2008). In another study for *Vibrio pomeroyi* sp. nov., polymyxin B (300 U), tetracycline (30 mg) and chloramphenicol (30 mg) sensitivity and ampicillin (25 mg) resistance were obtained by Thompson et al. (2003). In this study, all strains were found resistant to erythromycin and ampicillin (10 mg).

V. anguillarum was isolated from only one examined fish in this study, most likely as a result of the development of effective commercial vaccines and the widespread use of them. In conclusion, *V. crassostreae* and *V. pomeroyi* have been reported for the first time as fish pathogens of cultured sea bass in Turkey. Pathogenicity tests of the obtained isolates should be performed in the future.

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