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ARAŞTIRMA MAKALESİ **RESEARCH PAPER**

Vibrio Hepatarius Septicemia with Subacute Enteritis in Common Guitarfish (*Rhinobatos rhinobatos*) [*]

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Abstract: Common guitarfish (*Rhinobatos rhinobatos*) are an endangered species and are placed on the red list of "Critically Endangered, CR" by the International Union for Conservation of Nature. Guitarfish are kept in captivity and are protected in public aquaria globally, but there is limited information about the diseases of this species. This research reported mortalities (14%) observed in common guitarfish in a public aquarium. Severe hemorrhagic lesions on the ventral body side and reduced feed intake were observed in the affected animals. Two guitarfish were transferred for necropsy, microbiological and histopathological examination in a cold chain Macroscopic findings included severe ecchymotic hemorrhages in the ventral body, bloating of the gastrointestinal tract due to invasive mass, hyperemia of the colon vessels, and obstruction of the colon lumen. Histopathological examination revealed degenerations and necrosis in the liver, desquamation of intestinal villi, severe mononuclear cell infiltration and fibrosis in distal intestine, severe bacterial aggregates in different organs, pancreatitis, vasculitis, and filamentous bacteria-like structures. *Vibrio hepatarius* was isolated from the samples analysed and the agent was identified with multilocus sequence analysis (MLSA); multilocus sequence typing (MLST) revealed that the agent belonged to a novel sequence type, 247. After the first description of *V. hepatarius* in wild healthy adults of Whiteleg shrimp (*Litopenaeus vannamei*) from Ecuador, this is the first report of deaths due to *V. hepatarius* observed in common guitarfish kept in captivity in a public aquarium.

Keywords: *Vibrio hepatarius*, common guitarfish, ornamental fish, aquarium fish diseases.

Gitar Balıklarında (*Rhinobatos rhinobatos*) *Vibrio hepatarius* Kaynaklı Subakut Enteritik Septisemi

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Öz: Gitar balığı (*Rhinobatos rhinobatos*) Uluslararası Doğayı Koruma Birliği tarafından "Kritik Tehlike Altında, CR" kırmızı listesinde yer alan ve nesli tükenme tehlikesi altında olan bir balık türüdür. Gitar balıkları çoğunlukla şehir akvaryumları gibi global akvaryumlarda tutulur fakat bu türe ait hastalıklar konusunda sınırlı sayıda rapor bulunmaktadır. Bu çalışmada bir şehir akvaryumunda bakılan gitar balıklarında mortalite (%14) vakası araştırılmıştır. Çalışmaya dahil edilen balıkların ventral karın duvarında şiddetli hemorajik lezyonlar ve yem alımında azalma gözlenmiştir. İki gitar balığı soğuk zincir altında laboratuvarımıza nakledilmiş olup Nekropsi sonrasında mikroskobik ve histopatolojik incelemeler yapılmıştır. Makroskobik bulgularda özellikle balığın ventral bölgesinde şiddetli hemoraji, invaziv kitle bulunması ile gastrointestinal kanalda ödem, kolon damarlarında hiperemi ve kolon lümeninde tıkanıklık tespit edilmiştir. Histopatolojik incelemede karaciğerde dejenerasyon ve nekroz, barsak villuslarında deskuamasyon, barsak distalinde şiddetli mononükleer hücre infiltrasyonu ve fibrozis, bazı organlarda şiddetli bakteriyel kümelenmeler, pankreatit, vaskülit ve filamentli bakteri benzeri yapılar belirlenmiştir. Nekropsi yapılan balıklarda izole edilen bakteriyel etken multilocus sekans analizi (MLSA) ile *Vibrio hepatarius* identifiye edilmiştir. Etkenin tiplendirmesinde multilocus sekans tiplendirmesi (MLST) yöntemi kullanılarak izolatların yeni bir sekans tipi olan ST 247 olduğu belirlenmiştir. Ekvator bölgesindeki beyaz bacaklı karideslerden (*Litopenaeus vannamei*) *V. hepatarius* 'un ilk olarak izole edilmesi sonrasında, *V. hepatarius*'a bağlı mortaliteler ilk defa bu çalışmada rapor edilmektedir.

Anahtar kelimeler: *Vibrio hepatarius*, gitar balığı, akvaryum balığı, akvaryum balığı hastalıkları.

[*] *These students contributed to the study as part of a scholarship program.*

INTRODUCTION

The global capture fisheries production has increased only by 14%, while the total food fish consumption has risen 122% from 1990 to 2018 (FAO, 2020). The high consumer demand for fish causes the overexploitation and irrational usage of nature's resources, leading to the extinction of many animals and some fish species, and threatening many more. Current data show that the number of threatened species increased twice (391 of 1,199) from 2014, when the global assessment was performed for the first time (181 of 1,041), until today (Dulvy et al., 2014). *Rhinobatos rhinobatos*, a cartilaginous teleost in the family *Rhinobatidae*, was included in the Red List of Threatened Species by the International Union for Conservation of Nature (IUCN) on their latest update in 2020 (Jabado et al., 2021).

Public aquaria showcase fishes and play a vital role in conserving endangered species such as sharks and rays. A public aquarium with good environmental conditions mimicking natural habitat is a favorable place for the reproduction of endangered species. However, non-optimal conditions and high interaction with humans place more stress on fish in captivity, and increase their vulnerability to ubiquitous bacteria in the environment (Lieke et al., 2020). Bacterial organisms can cause diseases as primary or secondary invaders. Any stressors would lead bacteria, which are naturally existing in aquatic environments, to become more detrimental toward the host, a phenomenon known as the Rasputin Effect (Hurst, 2016).

Vibrio species are one of the most common bacteria, including heterotrophic species widely distributed in aquatic environments.They are also a common cause of disease and mortality in farmed and aquarium fish (Sampaio et al., 2022). Grimes et al. (1984) were the first to isolate *Vibrio harveyi* from brown sharks (*Carcharhinus plumbeus*) held in captivity. They were also able to experimentally infect lemon sharks (*Negaprion brevirostris*) and spiny dogfish (*Squalus acanthias*) using the same agent. *V. harveyi* was later isolated from moribund Salema (*Sarpa salpa*) in a public aquarium (Turgay et al., 2018). Recently, *V. crassostreae* and *V. cyclotrophicus* were isolated from different organs in lesser-spotted dogfish (*Scyliorhinus canicula*) kept in an Italian public aquarium (Tomasoni et al., 2022). Although there are several reports about *Vibrio* infections in a wide variety of fish species, information about the infectious diseases affecting elasmobranchs and the pathogenic effect of *Vibrio* in common guitarfish is limited (Garner, 2013).

This is the first report of *V. hepatarius* causing septicemia, subacute enteritis, and hemorrhages in guitarfish held in a public aquarium. Clinical and histopathological findings of the affected fish are described, and information about the morphological, phylogenetic, and molecular characteristics of the agent is supplied.

MATERIAL AND METHOD

Two naturally dead common guitarfish (*Rhinobatos rhinobatos*) were transferred from a thematic public aquarium to the Laboratory of Aquatic Animal Diseases. The first guitarfish (g1) died without any macroscopical lesions or disease symptoms, and another one (g2) died five days after the first one after showing reluctance to move, loss of appetite, and severe ecchymotic hemorrhages on the ventral side. Both fish were 1-3 years old with an average weight of 160 g. Fish were maintained in the biggest tank of the company with other sharks, rays, and groupers under regulated temperature (24°C), O_2 (7 ppm, 95-98% saturation), pH (8.1), and salinity (3.0%). Feed was supplied ad libitum for all species with different brands of commercial feeds.

Clinical signs included only slow movement and loss of appetite in two of 14 guitarfish in all aquariums and affected guitarfish showed acute mortality.

Necropsy: Two common guitarfish were transferred to the laboratory in a cold chain and necropsies were performed within 4 hours after natural death and morphological lesions were noted. Organs were also sampled for microbiological examination (Suzer et al., 2019;Taskin et al., 2022).

Histopathology: The liver, pancreas, epigonal organ, and intestine were sampled for histopathology. Tissues were fixed in 10% neutral buffered formalin for 48 h, and were embedded in paraffin after routine tissue processing techniques. Several sections were cut at 4 μm and stained with hematoxylin and eosin (H&E) staining.

Bacteriology: Sampling was carried out following the guidelines for diagnosing fish diseases and in consideration of international and national guidelines for animal welfare (OIE, 2015). Samples from the affected intestinal sections, kidney, liver, spleen, testicles, and hemorrhagic part of the colon of common guitarfish (g1 and g2) were inoculated in tryptic soy agar with %1.5 marine salts, marine agar and blood agar, and were incubated in aerobic conditions at 22°C and 28°C for 48-72 h.

Multilocus sequence analysis (MLSA) and sequence typing (MLST): The genomic DNA of the bacterial isolates was extracted using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The amount and purity of

DNA in each sample were measured at 260 nm and 260/280 nm wavelengths with a spectrophotometer (Multiskan Go, Thermo).

Following the previous MLST database, the housekeeping genes used were *atp*A (ATP synthase α subunit), *gyr*B (DNA gyrase β subunit), *pry*H (uridylate kinase) and *rec*A (recombinase A). The PCR was performed as previously described (Rahman et al., 2014). All PCR products were sequenced by Macrogen (Republic of Korea). The DNA fragments used for the analysis were 570 bp for *gyr*B, 501 bp for pyrH, 462 for recA, and 489 for atpA, and the concatenated sequences produced a 2,022-bp fragment.

Housekeeping genes used in MLSA analysis were submitted to the GenBank RefSeq Representative genomes database and 15 *Vibrio* species which have a high similarity to submitted sequences were used to construct a concatenated phylogenetic tree. The evolutionary history was inferred using the maximum likelihood method and the general time-reversible model (Nei & Kumar, 2000). The tree with the highest log likelihood (-8495.42) is shown (Figure 4). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories [+G, parameter = 1.2741]). The rate variation model allowed for some sites to be evolutionarily invariable ((+I), 67.31% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 16 nucleotide sequences. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 1944 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Felsenstein, 1985; Kumar et al., 2018).

RESULTS

Necropsy: External examination revealed a good body condition and no macroscopical lesions in the dorsal side of the head, trunk, and tail in g1 and g2. While the color of the ventral body parts of the g1 guitarfish was white to grey (Figure 1A), diffuse ecchymotic hemorrhages were evident in the ventrodorsal areas (especially the tail, anus region, and pectoral fin) of g2 guitarfish (Figure 1B and 1C). There were no macroscopically lesions on the intestines and testicles of apparently healthy (g1) guitarfish (Figure 1D and 1G). A mass obstructing the lumen and causing hemorrhages was found in the colon (Figure 1E and 1F), and severe hemorrhages were visible on testicles (Figure 1H and 1I) in the diseased fish (g2).

Histopathology: Microscopical examination revealed hepatocellular cytoplasmic vacuoles in the livers. Occasionally, hepatocytes also showed pyknotic nuclei, high nucleus to cytoplasm ratio, and increased intercellular spaces, resulting in disorganization of the hepatic cords (Figure 2A). While the proximal intestine mucosa was normal (Figure 2B), epithelial cells were severely desquamated and villi epithelium was blunted in the distal intestine sections, particularly the colon (Figure 2C). Mononuclear cell infiltrations, fibrosis, and eosinophilic granular cells in the distal intestine were also observed (Figure 2D, 2E).

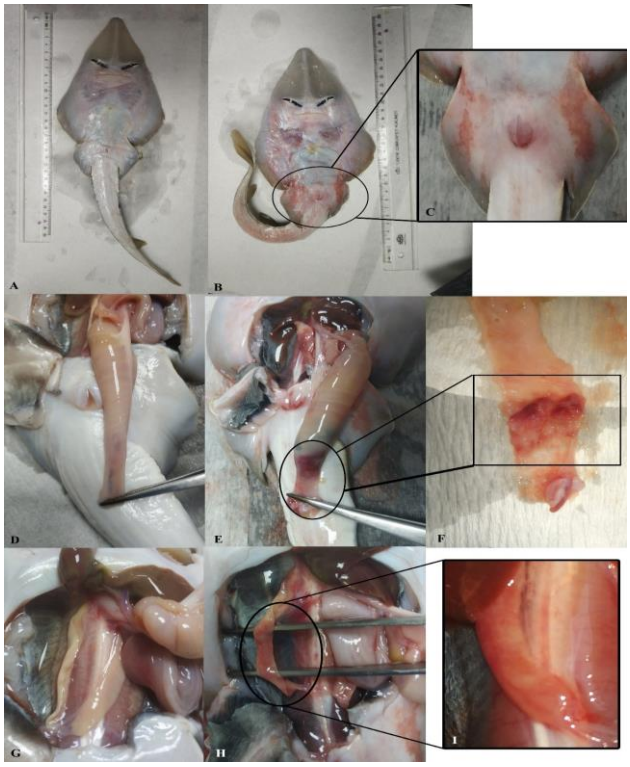

Figure 1. The appearance of the apparently healthy and diseased guitarfish. (A) Clinical presentation of the apparently healthy guitarfish (g1),, (B) Severe ecchymosis on the ventral side, especially in the tail region, of guitarfish (g2), (C) Severe hemorrhage, ulceration and prolapsus of the anus (g2), (D) Macroscopic presentation of healthy intestine sections, spiral valve, and colon (g1), (E) Hemorrhage in the colon (g2), (F) Isolated colon showing invasive mass and severe hemorrhage in the lumen (g2), (G) Observation of the healthy testicles of guitarfish (g1), (H-I) Hemorrhage in the testicles (g2).

Filamentous bacteria-like structure were seen in intestine mucosa in lamina proprialis (Figure 3A, 3B). Severe mononuclear cell infiltrations were observed among the exocrine glands of the pancreas (Figure 3C, 3D). Severe mononuclear cell (lymphocytes and macrophages) infiltrations surrounded the vessels (perivasculitis) in the epigonal organ (Figure 3E, 3F).

Bacteriology and Molecular Phylogeny: Isolated colonies were biochemically characterized by oxidase, catalase, and glucose fermentation in aerobic and anaerobic conditions (O/F basal medium), and also with ram staining and motility test. Strains of Gram-negative, fermentative, motile, oxidase- and catalase-positive comma-like bacteria were purely and dominantly isolated from kidney, liver, testicles, and colon from healthy (g1) and diseased fish (g2).

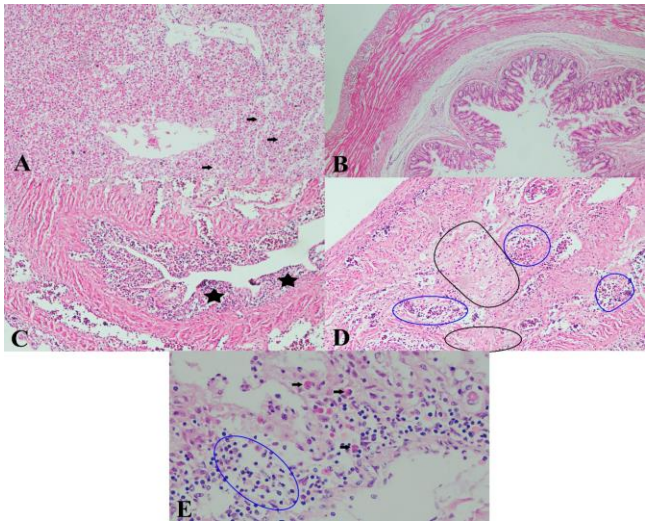

Figure 2. (A-E) Pathological observation of affected organs of diseased common guitarfish (g2), hematoxylin and eosin (H&E) staining. (A) Severe degeneration (arrows) and necrotic changes in liver, x100, (B) A normal villi epithelium and layers in distal intestine x40, (C) Desquamation of intestinal villi and blunted villi epithelium (stars) x100, (D) Mononuclear cells infiltration (blue circles) and fibrosis (black circles) in distal intestine x100, (E) Mononuclear cells infiltration (blue circle) and eosinophilic granular cells (arrows) in distal intestine x400.

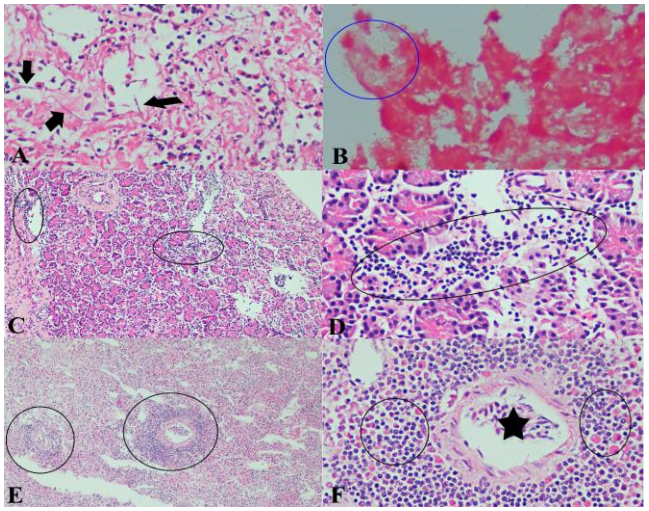

Figure 3. (A-F) (A) basophilic staining filamentous bacteria-like structure (arrows and blue circled area) in intestine mucosa x40 in liver, H&E, (B) filamentous bacteria-like structure (circled area) in gram-staining x100 in liver, (C) mononuclear cell infiltration (circle) in pancreas, x10, (D) Mononuclear cell infiltration (circle) among exocrine gland in pancreas, x40, (E and F) Vasculitis (circle) in around vessel (star) epigonal organ, x10 and x40.

Since nine *Vibrio* isolates recovered from sampling tissues have 100% sequence similarity for *atp*A, *gyr*B, *pyr*H and *rec*A genes, sequences obtained for *atp*A, *gyr*B, *pyr*H and *rec*A of two representative *Vibrio* isolates were deposited in the PubMLST Database (https://pubmlst.org/organisms/*Vibrio*-spp), allele numbers and sequence types (STs) were generated by PubMLST database (Jolley et al., 2018; Rahman et al., 2014). Moreover, the sequences of all strains were deposited in PubMLST (https://pubmlst.org/organisms/*Vibrio*-spp).

Isolated bacteria were found to be closely related (above 99%) with *Vibrio hepatarius* (DSM 19134) by each gene region in the GenBank RefSeq Representative genomes database. By MLSA analysis, the concatenated tree has resulted in a close relationship with *V. hepatarius* strain DSM 19134 but far from *V. nereis*, *V. aquaticus*, *V. brasiliensis*, and other low similar species shown in Figure 4.

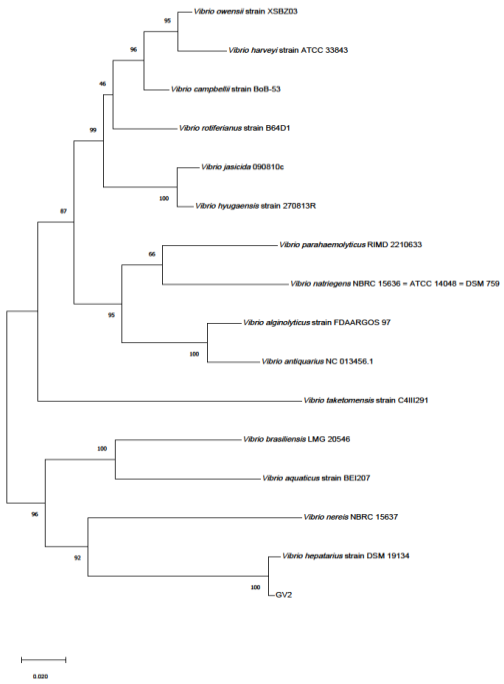

Figure 4. Concatenated sequence by using *gyr*B, *pyr*H, *rec*A, and *atp*A in maximum composite likelihood (MCL) analysis model.

Sequences of *gyr*B, *pyr*H, *rec*A, and *atp*A gene regions were submitted to the PubMLST database (https://pubmlst.org/organisms/*Vibrio*-spp) and allelic numbers of each gene were determined as 147, 111, 140 and 105, respectively (Table 1). Strains were uploaded to the PubMLST database with the names V2 and V3, and a novel sequence type, ST 247, was described for the study isolates. While our isolate had a close relationship with *V. hepatarius* DSM 19134, our isolates had allelic differences in nine positions at *gyr*B, four positions at *rec*A, and one at *atp*A genes.

Table 1. Allelic profiles and ST information of the study isolates.

ID	ST	*gyr*B	*pyr*H	*rec*A	*atp*A	Species
518	247	147	111	140	105	*Vibrio hepatarius*
519	247	147	111	140	105	*Vibrio hepatarius*

DISCUSSION

Although some fish species are not ornamental or pet fish, such as shark and ray species, they are collected from the western North Atlantic Ocean or the Mediterranean Sea and kept in captivity in public aquaria for the showcase. After transferring the marine fish to the aquariums, a lack of optimal environmental and nutritional conditions triggers the stress factors that lead to the outbreak of infectious diseases (Fioravanti & Florio, 2017). In the present study, we sampled common guitarfish, *Rhinobatos rhinobatos* kept in a public aquarium after a disease outbreak.

The isolation of *Vibrio* species is made easily on marine agar or agar media prepared with marine salts and the agent can be pre-identified as comma type bacteria under the microscope (Ina-Salwany et al., 2019). In our study, observation of Gram-negative, motile, oxidase- and catalase-positive comma type bacteria directed us to the *Vibrio* genus. No other bacterial organism except for *V. hepatarius* was isolated from the guitarfish (g1 and g2) which supported that *V. hepatarius* was the main etiological agent of the presented mortalities. The isolation of the same agent from both the healthy (g1) and the lesioned fish (g2) suggested different phases of bacterial disease, peracute and acute, respectively. The causative agent was determined as *V. hepatarius* in the MLSA analysis using four different gene loci. *V. hepatarius* was originally isolated from the hepatopancreas of wild healthy adults of Whiteleg shrimp (*Litopenaeus vannamei*) from Ecuador (Thompson et al., 2003). Although the reports about *V. hepatarius* are limited since its first description, the database of the National Center for Biotechnology Information (NCBI) reveals that the agent was isolated from a wide variety of sources such as shellfish hatchery, surface seawater, coastal sea water, sediment, giant tiger prawn, symptomatic fish, and crustaceans from 2003 to 2021 (unpublished data, NCBI). The MLSA analysis revealed that our isolate showed a close relationship to *V. hepatarius* isolated from Ecuador, with few nucleotide differences. In addition, *V. hepatarius* isolated in common guitarfish had a unique allelic profile and was assigned a novel ST 247. In this study, we revealed *V. hepatarius* septicemia in common guitarfish after macroscopic, microscopic, and bacteriological examinations.

In our study, the vets working in the aquarium stated that the common guitarfish started to die despite no changes in the environmental conditions of the aquarium, and later the cownose rays in the same aquarium showed signs of disease and were immediately treated with antimicrobials. Vibriosis is known as “red pest” because it causes severe hemorrhage in fish (McCarthy, 1976). We observed severe hemorrhages in the common guitarfish similar to previous reports related to vibriosis. Most often, the disease vibriosis in fish starts with external pathological changes including lethargy, anorexia, abnormal swimming, hemorrhagic or ulcerative lesions on the skin, abdominal distension, necrosis in various organs, skin discoloration, and later the infection may become systemic, contributing to mortality (Ina-Salwany et al., 2019; Ransangan & Mustafa, 2009; Sonia & Lipton, 2012). In our study, the histopathological lesions were particularly prominent in the skeletal muscle, liver, kidney, spleen, and heart (Emam et al., 2019; Ina-Salwany et al., 2019; Won et al., 2009). Geng et al., (2014) previously observed vasculitis in the kidney, spleen, and liver, and severe degeneration and necrosis of the liver. We macroscopically observed severe hemorrhage on the ventral side of common guitarfish and testicles, and additionally, a major invasive mass obstructing the colon and causing hemorrhage. A delay in the initiation of therapy causes vibriosis to become a systemic infection (Sonia and Lipton, 2012). We detected the bacteria in the liver, intestines, and epigonal organ similar to those previously described for fish infected with *Vibrio* (Geng et al., 2014; Kang et al., 2022; Li et al., 2006; Marudhupandi et al., 2017; Pramila & Lipton, 2018; Sumithra et al., 2019; Won et al., 2009; Zhang et al., 2020).

It was interestingly reported that *Vibrio*-predatory filamentous bacteria has a potential predator effect for several *Vibrio* species (Yeoh et al., 2021). *Vibrio*-predatory filamentous bacteria (VPFB) have been potentially used in the control of *Vibrio* infections and they were assumed to be a biocontrol agent for *Vibrio* infections (Yeoh et al., 2021). Contrastingly, filamentous bacteria have also been reported in the gastroenteritis in rainbow trout, and pathological lesions were detected caused by filamentous bacteria (Del-Pozo et al., 2010). In the bacteriological examinations of our study, we have not detected bacteria other than *Vibrio* agents. But, we detected a structure similar to filamentous like-bacteria which has a similar structure to the previous reports. The presented data revealed that *Vibrio*-predatory filamentous bacteria may play a role in the infection caused by *V. hepatarius* in common guitarfish. *Vibrio*-predatory filamentous bacteria or filamentous bacteria role in fish disease could be studied in the future research to illuminate their pathological roles in fish disease. The pansystemic dissemination of the bacteria suggested a septicemic course for the infection.

As a conclusion, the presented data revealed that *V. hepatarius* causes a serious septicemic disease in common guitarfish. We suggest that the endangered species should be protected not only in their natural habitats, but also in the aquarium environment.

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ETHICAL STATEMENT

After a natural death, the fish were obtained from a public aquarium, transferred our laboratory in cold chain, and necropsy was performed. The care and use of animals complied with Local Ethics Commission of Bursa Uludag University animal welfare laws, guidelines and policies as approved by 2021-07/07 commission report

DATA AVAILABILITY STATEMENT

All data were presented in the manuscript.

AUTHOR CONTRIBUTION

Burak Ozdemir, Ebrucan Bulut, Ece Yurddas, Fatih Hira Aytekin, Kemal Bagci, Kubra Balci, Merve Tasgin, Neslihan Kostebekci: Transfer of the fish to the laboratory and necropsy and bacteriology; Nihed Ajmi: Necropsy, bacteriology, and manuscript writing; Ozkan Yavas: Pathological examinations, manuscript writing and editing; Muhammed Duman: Study planning, necropsy, bacteriology, molecular analysis, bioinformatics, photograph editing, writing and editing manuscript, and supervision. These students contributed to the study as part of a scholarship program

DECLARATION OF COMPETING INTEREST

The authors declare no competing or financial interests.

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