



Pathological aspects of experimental infection of *Lactococcus garvieae* in European Sea Bass (*Dicentrarchus labrax* L.): Clinical, hematological, and histopathological parameters

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ABSTRACT

This study, it was aimed to examine the clinical, hematological, and histopathological aspects of lactococcosis induced in European sea bass (*Dicentrarchus labrax*), which was experimentally infected with *Lactococcus garvieae*. For this purpose, the infection was induced intraperitoneally with *L. garvieae* strain (10^8 CFU/mL), and blood samples were collected from the infected fish on different days (6, 18, 26, 31, 36, 44, and 48th) of infection. The morphological structures, erythrocyte and leukocyte count, hematocrit value, sedimentation rate, and coagulation time of the blood cells in the collected samples were calculated. As a result of the infection, while there was a decrease in erythrocyte count, hematocrit value, and coagulation rate, in addition to changes in the morphological structure of blood cells, it was determined that there was a significant increase in the leukocyte count and sedimentation rate. Furthermore, histopathological examination was also performed in the organs of infected fish such as the brain, liver, spleen, kidney, heart, gill, and intestine. Histopathologically, in the infected sea bass, while hemorrhage, diffuse necrosis, and hyaline droplets were detected in the granular brain tissue, hyperemia and hemorrhage were noted in the liver and spleen, and widespread necrosis in the hematopoietic tissue of the kidney, in the gills intensive hyperplasia, an increase in the goblet cell were detected. Although there are studies on lactococcosis in various marine fish species around the world, the infection of *L. garvieae* in farmed sea bass and various parameters and pathological aspects were investigated in detail for the first time in this study. *L. garvieae* was determined to have clinical significance for European sea bass with a high economic value.

Keywords: Lactococcosis, European sea bass, Experimental infection, Blood, Histopathology



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Introduction

Lactococcosis is a bacterial infection caused by *Lactococcus garvieae* and characterized by hemorrhagic septicemia (Vendrell et al., 2006; Austin and Austin, 2012). The infectious agent is zoonotic (Lee et al., 2020), and it has spread to many countries of the world and causes significant economic losses in marine and freshwater farmed fish (Vendrell et al., 2006; Ortega et al., 2020; Duman et al., 2020). The wide range of strains reported in many countries suggests that *L. garvieae* can be an opportunist bacteria cohabitant in different fish farming systems (Austin and Austin, 2012; Shahi et al., 2018). Previous studies indicate that it has been reported that the causative agent of lactococcosis causes disease in marine fish such as yellowtail (*Seriola quinqueradiata*) (Kawanishi et al., 2005), mullet (*Mugil cephalus*) (Chen et al., 2002), black rockfish (*Sebastes schlegeli*) (Kang et al., 2004), olive flounder (*Paralichthys olivaceous*) (Lee et al., 2001) and wild red sea wrasse (*Coris aygula*) (Colorni et al., 2003) and sorubim (*Pseudoplatystoma* sp.) (Rodrigues et al., 2020). However, there is no report stating that it causes disease in European sea bass (*Dicentrarchus labrax*).

It has been reported that lactococcosis develops depending on fish species in experimental infection studies performed on different fish species (Muzquiz et al. 1999; Chen et al., 2002; Urku and Timur, 2014), and trout has been reported to be the most susceptible species to the disease (Vendrell et al., 2006). Regardless of the infected fish species, exophthalmos, hemorrhage in the periorbital and intraocular area, base of the fins, perianal region, operculum, and swollen abdomen are among the typical external clinical findings of the disease. Internally, different researchers (Muzquiz et al., 1999; Chen et al., 2002; Urku and Timur, 2014; Didinen et al., 2014) have reported fluid accumulation in the peritoneal space, diffuse hemorrhage in the internal organs, splenomegaly, focal necrosis in the spleen and liver.

In recent studies, it has been reported that hematological analyses provide important data on the physiological status and health of cultured fish (Fazio, 2019). It has been stated that changes in hematological values may develop due to stress or environmental factors or in the presence of an infection (Gbore et al., 2006; Fazio et al., 2012). Therefore, hematological examinations should be performed to detect changes in blood parameters that occur in fish blood depending on the source of infection (Chen et al., 2005; Alsaid et al., 2014).

Lactococcosis is an acute systemic disease histopathologically characterized by congestion and hemorrhage (Vendrell et al., 2006; Roberts, 2012). It causes necrosis and diffuses hemorrhage and hyperemia in hemopoietic tissues such as the spleen, kidneys, and liver. It has been reported to cause inflammatory cell infiltration and melanomacrophage centers in the kidney tissue (Chen et al. 2002; Urku and Timur, 2014) as well as pericarditis (Chang et al., 2002; Didinen et al., 2014), panophthalmitis (Chen et al. 2002; Avcı et al. 2010; Didinen et al. 2014) in the fish infected with *L. garvieae*.

European sea bass is extensive and very significant commercial marine fish species in the Mediterranean Sea. According to FAO data (2014), Turkey ranks second among the countries producing sea bass such as Italy (67.00 tons), Greece (42.500 tons), Spain (14.455 tons), and Egypt (13,798 tons) with a production of 65.512 tons. (Di Trapani, et al., 2014). According to TUIK 2020 data, it has been reported that sea bass is the most common fish with a production of 148.907 tons. Due to its high economic value, the sea bass was selected as a model for inducing experimental lactococcosis. The aim of this study is to evaluate the clinical, hematological, and histopathological changes of lactococcosis induced in sea bass experimentally infected with *L. garvieae*.

Material and Methods

Fish Material and Experimental Groups

The experimental study plan was designed according to previous studies (Chang et al., 2002; Urku and Timur, 2014; Rodrigues et al., 2020). 130 European sea bass weighing 50-70 g on average were obtained from a commercial enterprise in the Aegean Sea. The fish were adapted to laboratory conditions for two weeks. TSA and NB media were used in this study. The number of bacteria was calculated according to Plumb and Bowser (1983). For determining the LD₅₀ dose, bacterial concentrations were identified to be 10⁶, 10⁷, and 10⁸ CFU/mL. For every dose, five fish were infected intraperitoneally. The LD₅₀ dose was found to be 10⁸ CFU/mL. After determining the LD₅₀ dose, 100 sea bass were divided into two groups. For this purpose, two fiberglass tanks with a diameter of 1 m and a depth of 70 cm were used in the experiment. The *L. garvieae* strain isolated from diseased fish in an enterprise in Fethiye/Turkey was used in the experimental infection study. Bacteria incubated in Nutrient Broth (NB) medium for 24 hours were washed with PBS at 2500 rpm,

then the optical density was adjusted to 1 (approximately 10^9 CFU/mL) at 540 nm in the spectrophotometer (Barnes et al., 2002) and then it was diluted and the bacterial count was adjusted to 10^8 CFU/mL. The experimental infection was induced by the intraperitoneal injection of 0.1 mL of bacterial culture (10^8 CFU/mL) per fish in the experimental group. 0.1 mL sterile PBS was administered intraperitoneally to 50 fish in the control group. During the experimental period, fish were fed twice a day. The water temperature was maintained at 22-23°C, salinity at 35‰, and oxygen at 5-6, and the fish were monitored for clinical signs and mortality for 50 days.

Hematological Examination

Hematological sampling was performed considering the clinical signs of the disease. For this purpose, blood samples were taken from five fish showing clinical signs on the post-infection (dpi) days (6, 18, 26, 31, 36, 44, and 48th) when mortality were detected in the experimental group. Approximately 0.5 mL of blood sample was taken from each fish. There was a decrease in blood volume due to anemia developing towards the end of the experiment (0.1-0.2 mL/ each fish).

The blood was withdrawn from the caudal vessel into a sterile syringe containing lithium heparin anticoagulant (Svobodova and Vykusova, 1991), and leucocyte (WBC) and erythrocyte counts (RBC) were determined manually by utilizing a Neubauer hemocytometer using Natt-Herrick's stain (Natt and Herrick, 1952). Haematocrits (Hct) were estimated using a microhematocrit machine (Goldenfarb et al., 1971). The micro-ESR method, described by Murachi (1959) was used to determine the erythrocyte sedimentation rate, and the slide coagulation method described by Wolf (1959) was used to determine the coagulation time of the blood taken from infected fish. The results were compared with the control group and the average of all the data obtained was taken according to Office Excel. To determine morphological changes in blood cells and confirm the presence of inoculated bacteria in the blood, thin blood smears were routinely prepared and stained by Giemsa/May-Grunwald (Blaxhall and Daisley, 1973).

Histopathological Examination

Experimental fish were anesthetized with 2-phenoxyethanol. For this purpose, fish samples were exposed to concentrations of 0.15 mL/L 2-Phenoxyethanol dissolved in aerated and dechlorinated tap water. Tissue samples taken from experi-

mentally infected sea bass (heart, liver, kidney, gills, intestines, spleen, eyes, and brain) were fixed in 10% neutral-buffer formalin solution, embedded in paraffin, sectioned in 4-5 μ m and stained routinely with hematoxylin and eosin (H&E) (Roberts, 2012).

Results and Discussion

The detection of hematological and tissue changes caused by pathogens in fish organs by experimental infection studies plays a significant role in controlling the outbreaks of bacterial fish diseases (Alsaid et al., 2014; Fazio et al., 2019). In particular, the evaluation of these data gives detailed information about the pathogenesis, diagnosis, control, and treatment of the disease. Although few studies have been carried out to assess the blood parameters, tissue changes and clinical findings of lactococcosis infected freshwater fish species (Avsever et al., 2014; Khosravi et al., 2018), the present study describes the details of changes in clinical, hematological, and histopathological parameters induced by lactococcosis in European sea bass for the first time.

After the 6th day of the experimental infection, it was observed that deaths also started with the decrease in feed intake and slowing of movements in infected fish. At the end of the experiment, it was observed that four fish remained in the experimental group. Mortality caused by the *L. garvieae* strain is given in Figure 1 in detail. It was observed that an administration dose of 10^8 CFU/mL caused mortality in experimental fish.

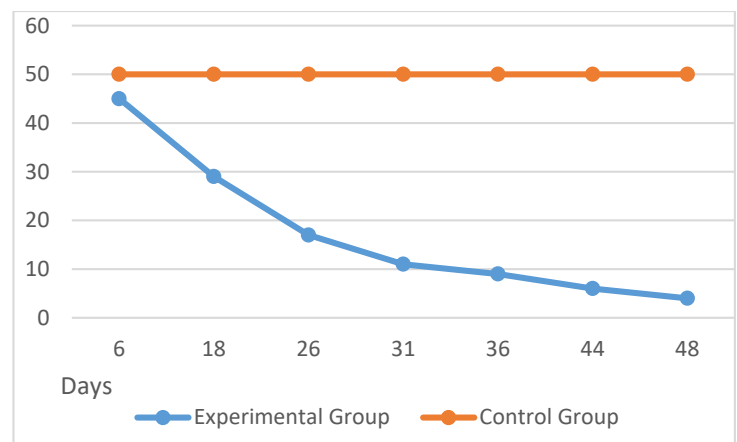


Figure 1. Mortality range of European sea bass infected with *L. garvieae*

Experimental infection studies have reported that the disease-related symptoms develop at different times depending on the fish species and cause mortality. It has been stated that this

time is 2-3 days in yellowtail (Itami et al., 1996), 2 days in mullet (Chen et al., 2002), and 3-4 days in trout (Muzquiz et al., 1999). In a previous study, *L. garvieae* (2×10^6 CFU/mL) did not cause death and any clinical signs in sea bass (Türe et al., 2014). However, in the present study, administration dose of 10^8 CFU fish⁻¹ caused deaths in experimental fish. In this study, it was determined that deaths occurred with a decrease in feed intake and slowing of movements on the 6th day of inoculation in the infected sea bass, as reported by Vendrell et al. (2006). Considering all the studies on experimental infection, the fact that *L. garvieae* has a short incubation period and causes death by the virulence mechanism is also clinically important for other fish species that are economically valuable and farmed.

External clinical findings such as hemorrhage in the abdomen, operculum (Figure 2a) and eyes, darkening of the skin in the back region, and scoliosis were detected in sea bass experimentally infected with *L. garvieae*. Furthermore, internal clinical findings such as fatty degeneration in the liver, hyperemia and hemorrhage in the visceral organs, splenomegaly, and necrosis in the anterior kidney were observed (Figure 2b). No clinical findings were noted in the control group fish.

External clinical findings, such as hemorrhage in the intraocular area, the base of the fins, and operculum, and darkening, reported in the fish infected with *L. garvieae* were also detected in the infected sea bass in this study (Prieta, 1993; Eldar and Ghittino, 1999; Mu'zquiz et al., 1999). Exophthal-

mos, defined as the typical clinical manifestation of the disease, was not observed in the sea bass infected in this study. Scoliosis, which has not been reported until the present day, was detected in the infected sea bass in this study for the first time. Furthermore, as reported by (Eldar and Ghittino, 1999; Mu'zquiz et al., 1999; Urku and Timur, 2014) depending on the course of the disease, diffuse hemorrhage and hyperemia, splenomegaly in the internal organs, focal necrosis in the spleen and liver were also observed in the infected sea bass.

As a result of the 50-day experimental infection study, it was determined that the hematocrit value (Hct) of the fish in the control group remained constant at 30%, while it was observed to decrease to 4% in the blood samples taken from the infected fish (Table 1). While the sedimentation rate of the blood samples taken from the control group fish was determined to be 3 mm during the experiment, this value increased to 34 mm in the experimental group (Table 1). It was noted that the coagulation time in the control group fish was 13 minutes throughout the whole experiment, while it decreased to 1.5 minutes in the infected fish (Table 1). While the erythrocyte count (RBC) in the control group fish was between 3.2×10^6 - 5.1×10^6 /mL, it was found that this value was 4.2×10^6 /mL in the fish infected with *L. garvieae* at the beginning and decreased to an average of 0.8×10^6 /mL at the end of the experiment. While the leukocyte count (WBC) was between 0.8×10^3 - 1.6×10^3 /mL in the control fish, the average leukocyte count increased to 8.3×10^3 /mL in the infected fish (48th day) (Table 1).

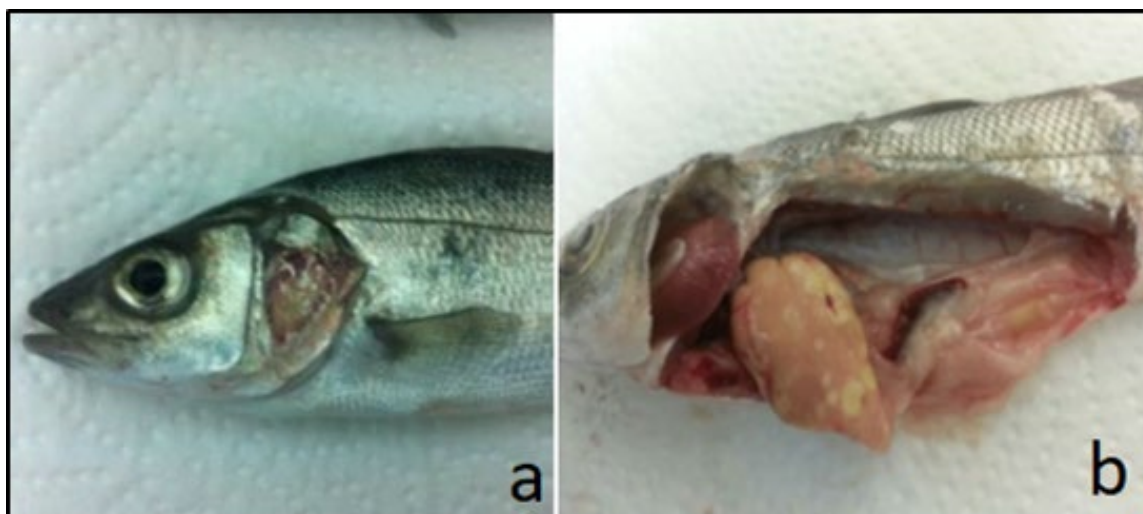


Figure 2. European sea bass infected with *L. garvieae*: (a) Large hemorrhage in the operculum; (b) Fatty degeneration, hyperemia in the liver, necrosis in the anterior kidney

Table 1. Hematological findings in infected and non-infected European sea bass

Experimental groups	Hct (%)	Sedimentation rate (mm)	Coagulation time (min)	RBC ($\times 10^6 \mu\text{L}^{-1}$)	WBC ($\times 10^3 \mu\text{L}^{-1}$)
Post-infection days					
6	21	4	12	3.01	0.9
18	18	8	10	2.66	1.5
26	13	15	9	2.56	2.8
31	10	19	7	2.05	3.6
36	8	24	4	1.45	5.8
44	5	29	2	1.23	7.0
48	4	34	1.5	0.8	8.3
Control	30	3	13	3.2- 5.1	0.8x-1.6

When the blood smears of the diseased fish in the experimental group were compared with the control group (Figure 3a), defects were detected in the morphological structure of erythrocytes from the first week of the inoculation (Figure 3b, c). In the later days of the infection, in addition to lymphocytes, monocyte cells that phagocytized bacterial cells were detected (Figure 3d).

Hematological analysis such as sedimentation quantity, total leukocyte count, and type provides information about the existence of the bacterial fish disease (Fazio, 2019; Alsaid et al., 2014). The erythrocyte count is an important blood parameter used to determine the functions of hemopoietic tissues (Witeska, 2005). Different researchers have reported that bacterial infections in fish usually result in a decrease in the erythrocyte count (Pathiratne and Rajapakshe, 1998; Rehulka, 2002; Chen et al., 2004; Alsaid et al., 2014). However, although it has been stated that there is no change in this value in the Nile tilapia infected with *Enterococcus* sp., which is closely related to the genus of *Lactococcus* (Martins et al., 2008), a decrease in RBC and hematocrit values has been reported in rainbow trout (Barham et al., 1980) and tilapia (Alsaid et al., 2014) infected with *Streptococcus* sp., a closely related species and sorubim infected with *L. garvieae* (Rodrigues et al., 2020). The decrease in the erythrocyte count detected in this study may have developed as a result of the destruction in the hemopoietic tissues of organs such as the spleen and kidney, which we detected histopathologically. Furthermore, it has been reported that the decrease in hematocrit percentage, as well as the erythrocyte count, is associated with anemia (Rajendiran et al., 2008). In this study, it is thought that anemia occurred at the end of the experiment in

relation to hemorrhage and hyperemia that we detected in the spleen and liver tissues of the infected sea bass.

Leukocytes are one of the cellular components associated with the immune system for bacteria and foreign material in the blood (Magnadóttir, 2006; Castro and Tafalla, 2015). An increase in the WBC count may indicate the presence of bacterial pathogens, especially as a response of the non-specific immune system. As reported in trout infected with *L. garvieae* (Avsever et al., 2014; Khosravi et al., 2018) increase in the WBC value was also detected in the infected sea bass in this study.

It has been reported that the hematocrit value of healthy fish living in seawater varies between 15.3-52.5% according to different species (Satheeshkumar et al., 2012), while this value is 51.18% in sea bass (Fazio, 2019). As reported in the farmed Korean rockfish (*Sebastes schlegeli*) and rainbow trout (*O. mykiss*) infected with *L. garvieae* (Kobayashi et al., 2004; Khosravi et al., 2018), the decrease in the hematocrit value due to the disease was also detected in the infected sea bass in this study, and this value was found to be 4%.

Cells located in the inner wall of the vessel activate many cells and systems involved in the coagulation mechanism. Fish need very effective hemostatic mechanisms to respond to vascular damage and other general injuries in the case of infection (Tavares-Dias and Oliveira, 2009). However, the knowledge of the coagulation system in fish is rather limited. It has been reported that *L. garvieae* causes lesions in the vascular endothelium and damages these cells (Prieta, 1993; Vendrell et al., 2006). In this study, we predict that the gradual decrease in the coagulation level originates from the

pathomorphological changes caused by the agent in the infected fish's vascular systems, as reported by Prieta (1993).

The sedimentation rate is a parameter related to erythrocytes and can change in situations such as disease, stress, and exposure to pollutants (Kori-Siakpere and Ubogu, 2008). In these cases, it is reported that the platelet counts and coagulation time, which are coagulation-related parameters, may change (Tavares-Dias and Oliveira, 2009). In the presence of an infection, protein structures such as fibrinogen and globulin in the plasma combine with red blood cells, causing erythrocytes to precipitate faster, and the sedimentation rate of the blood with anemia due to infection increases (Fazio, 2019). Likewise, in this study, a significant increase in sedimentation rate was detected due to the decrease in the erythrocyte count in infected fish, in addition to the picture of anemia formed as a result of severe hyperemia and hemorrhage in the last days of the infection.

Histopathologically diffuse necrotic areas (Figure 4a), hemorrhage, hyperemia, and melanomacrophage foci in the spleen (Figure 4b); vacuolar degeneration, hyperemia, and hemorrhage in the liver (Figure 4c); necrosis, hyperemia, and tubular edema in the inter-renal hematopoietic tissue of the kidney (Figure 4d); hyperemia, large necrotic areas, and hyaline droplets in the brain tissue (Figure 4e) were detected in the infected sea bass. Furthermore, diffuse necrosis and hemorrhages were observed in the cardiac muscle cells of heart,

and as a result of necrosis in the enterocyte cells covering the intestinal lumen, spillage into the lumen of the necrotic enterocyte cells (Figure 4g) and severe hyperplasia in the gills and an increase in the goblet cell count (Figure 4i) were observed.

Pathomorphological changes in the fish infected with *L. garvieae* indicate acute systemic disease characterized by congestion and hemorrhages in internal organs (Prieta, 1993; Aizpurua et al., 1999). Likewise, diffuse hemorrhage and hyperemia were detected in the spleen and liver tissue samples in this study, when sections obtained from spleen and liver compared with control fish group. It was determined that this picture transformed into anemia with the change in blood values in the last days of the infection. Furthermore, other researchers (Eldar and Ghittino, 1999; Vendrell et al., 2006; Avci et al., 2010; Urku and Timur, 2014) have already described hemorrhages among the myocardial muscles of the heart and necrosis, hemorrhage and hyperemia in the liver, spleen, and kidney, observed in this study. It has been reported that some histopathological findings we detected in the infected sea bass are also similar to the pathological findings observed in diseases with hemorrhagic septicemia (Vendrell et al., 2006; Roberts, 2012). Furthermore, although hyaline droplets have been reported in the tubular lumens of the kidney tissue of infected trout (Chang et al., 2002; Didinen et al., 2014), the presence of hyaline droplets in the brain tissue of infected sea bass was detected for the first time in this study.

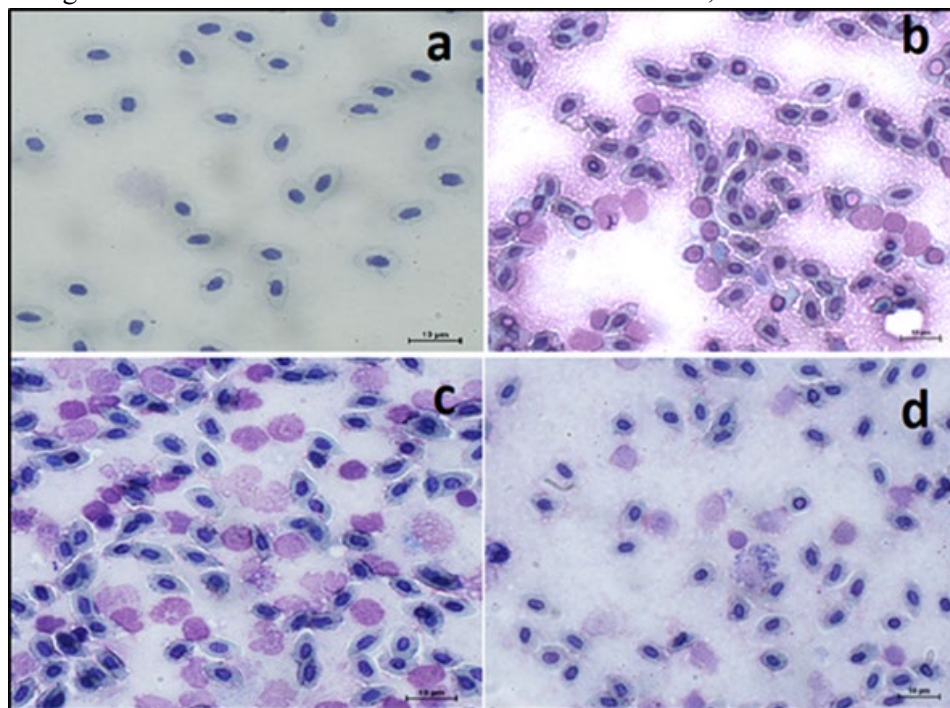


Figure 3. Morphological changes seen in blood cells of infected sea bass: (a) Control group; (b) Erythrocyte cells with altered morphological structures adhered to each other (18th day); (c) Lymphocyte cells on day 26th of infection; (d) Monocyte cells phagocytosed bacterial cells.

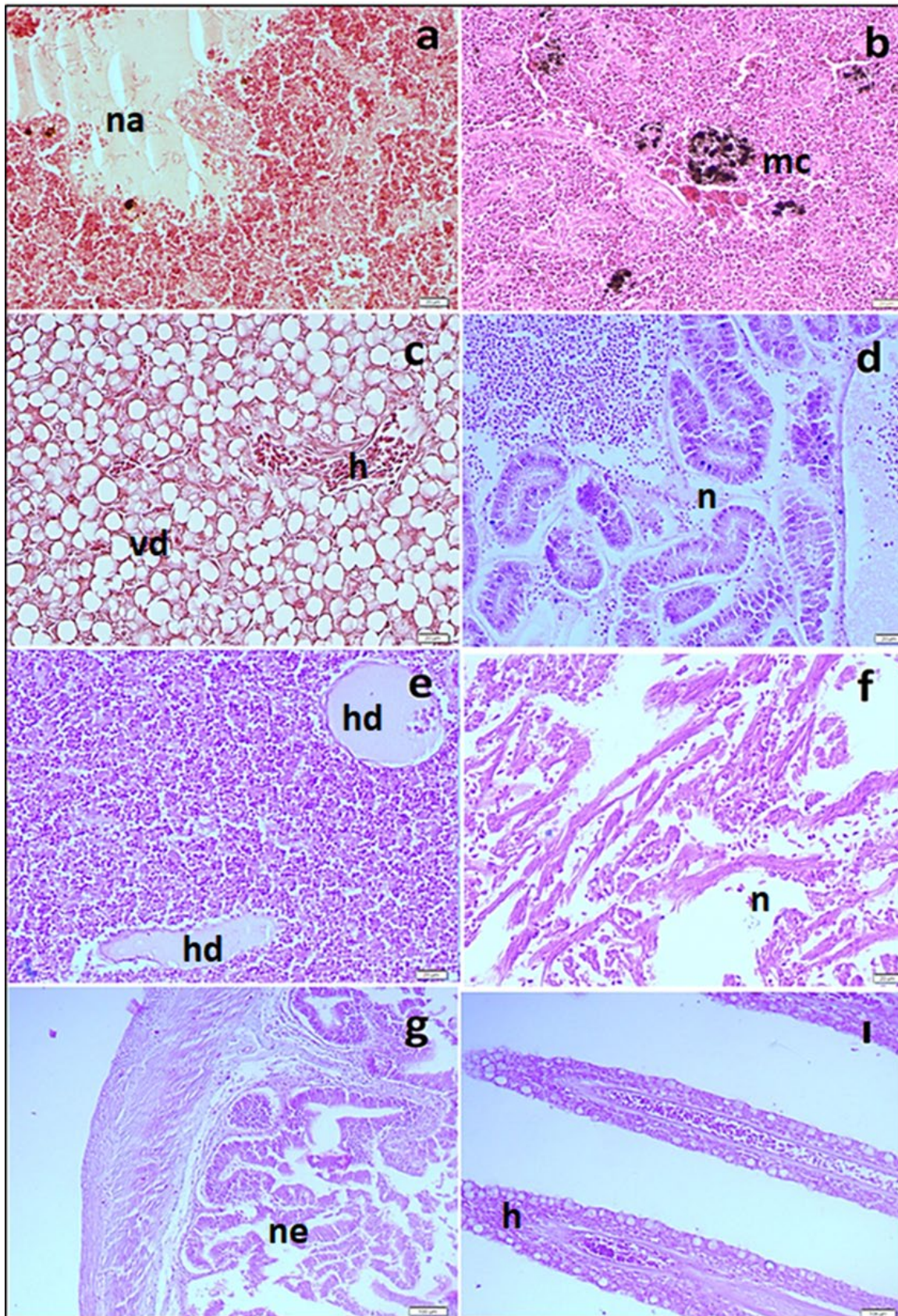


Figure 4. European sea bass infected with *L. garvieae*: (a) Diffuse necrotic areas (na); (b) hemorrhage, hyperemia, and melanomacrophage centers (mc) in the spleen; (c) vacuolar degeneration (vd), hemorrhage and hyperemia (h) in the liver; (d) necrosis (n) in the inter-renal hematopoietic tissue, tubular edema in the kidney; (e) hyaline droplets (hd) in the brain tissue; (f) necrosis (n) in the heart muscles and hemorrhage; (g) spillage into the lumen of the necrotic enterocyte (ne) cells; (i) intensive hyperplasia (h) in the gills and an increase in the goblet cell

Conclusion

In this study, clinical, hematological, and histopathological changes caused by the infection were revealed in sea bass experimentally infected with *L. garvieae*. It was determined that this pathogen bacterium caused mortality in sea bass by creating a pathomorphological structure. Furthermore, it was revealed that *L. garvieae* caused acute systemic disease characterized by congestion and hemorrhage in sea bass histopathologically, and this clinical finding was confirmed by hematological data. Clinically, it was observed that the structure of anemia took shape in infected fish in the later stages of the disease. As a result, *L. garvieae* was found to have clinical significance, especially for sea bass with high economic value.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential, or perceived conflict of interests.

Ethics committee approval: Experimental protocol was approved by the Ethics Committee for Animal Experiments of the University of Istanbul (2014/47).

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Disclosure: -

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