Histopathological Changes of Rainbow Trout After Experimental Infection with Lactococcus Garvieae

Abstract

The purpose of this study is to determine both clinical and histopathological changes in experimentally infected rainbow trout with Lactococcus garvieae. Fourty healthy fish samples (50-60 g in weight) were purchased from a commercial farm near Antalya, Turkey and transferred to the laboratory. Each group of fish were challenged a cell density of $6x10^5$ cfu/ml of L. garvieae isolate. The control group was given with the appropriate dilution of the sterile PBS. Fish showed darkening of skin color, lesion on the upper jaw and prolapsus. Internally, they had pale liver and kidney, enlarged spleen and hemorrhagies on the muscle. The control group did not show clinical signs. Histopathologically, the kidney and liver showed haemosiderin deposits. Lamellar fusion and hyperplasia of primary lamellae in the gills, depletions of red and white pulpas in the spleen and necrosis of the gastro-intestinal tract were detected.

Key Words: Rainbow trout, Lactococcus garvieae, Experimental study

Research Article

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Introduction

Rainbow trout (Oncorhynchus mykiss) is one of the economically important fish species around the world. Production of the species is affected by a variety of factors. These factors include infectious diseases. The infectious diseases are caused by viruses, fungi, bacteria and protozoan parasites. Lactococcosis is a limiting problem for rainbow trout culture in many countries including Turkey and Iran (Sharifiyazdi, et al., 2010; Didinen, et al., 2014). Lactococcus garvieae is the causative agent of Lactococcosis. This pathogen was previously referred as Streptococcus garvieae and then; it was proposed as Enterococcus seriolicida (Sharifiyazdi, et al., 2010). L. garvieae is a non-motile, non-sporulating, facultatively anaerobic, catalase and cytochrome oxidase negative and Gram-positive coccus. It can be isolated from a wide range of animal species including cow, water buffalo, cat, dog and fish. It was also reported in human infections (Vendrell, et al., 2006). Although the evolution of Lactococcosis depends on environmental conditions such as water temperature and water microbiological quality, the disease is described as hemorrhagic septicemia (Vendrell, et al., 2006). The mostly observed signs of the natural disease outbreaks in cultured rainbow trout are uni or bilateral exophthalmia, with periocular hemorrhages, loss of eye or eyes in some cases. Internal clinical signs of the disease include hemorrhagic fluid in the peritoneal cavity, pale liver, enlarged spleen and hemorrhages in the liver and muscle (Pereira, et al., 2004; Savvidis, et al., 2007); however, rainbow trout (80 ± 10 g) experimentally infected with L. garvieae via immersion route showed exophthalmia, paleness of the gills, hemorrhages in the liver, swim bladder peritoneum, muscle and bloody content in the intestine (Avci, et al., 2014). Many infectious agents produce typical tissue responses. These responses may change as the infection progresses from acute to chronic forms. The observed histological changes in different organs such as kidney, brain and liver tissue sections may give enough information to describe the effect of a particular infective agent (Gupta, et al., 2009; Alsaid, et al., 2013). Histopathological examinations of rainbow trout which was infected with L. garvieae in culture conditions showed significantly lesions in the liver, kidney, spleen, gills, heart and stomach (Altun, et al., 2005; Didinen, et al., 2014). Avci, et al. (2014) reported that the most important histopathological finding in kidney and spleen was the presence of macrophage in fish tissue samples which were experimentally infected with L. garvieae. There is a little histopathological information for both natural and experimental Lactococcosis infections in Turkey. For this reason, the purpose of this study is to challenge rainbow trout with L. garvieae and observe both clinical and histopathological changes in experimentally infected fish.

Materials and Methods

Forty healthy rainbow trout (50-60 g in weights) were purchased from a commercial fish farm near Antalya, Turkey and the fish were transferred to the AUFF research laboratory. The fish were separated four group. Each group including control group consisted ten fish and the ten fish were placed into 300 L fiberglass tank. Water temperature was 18 °C and it was kept constant during the acclimatization period. All fish were acclimatized to the tanks for 2 weeks before challenge and the fish in each tanks were observed in terms of mortalities and clinical sings for possible disease outbreak. During acclimatization period and after

challenge, all the fish were fed as 3% of their fish weights at a daily. The fish were starved for 24 h. before challenge. The procedures were reviewed and approved by the Akdeniz University Local Committee on Animal Research Ethics. Number. 2011.10.01. The L. garvieae strains used in the challenge studies were isolated from a survey study on rainbow trouts which was naturally infected streptococcal species (Korun, et al., 2015). The strains (L1, L2 and L3) were Gram-positive, non-motile, cytochrome oxidase and catalase negative. They produced α-hemolysis on the blood agar. PLG1 and PLG2 primers (5′-CATAACAATGAGAATCGC-3′) and PLG-2 (5′-GCACCCTCGCGGGTTG-3′) were used for the amplification of 16S rDNA isolated from the strain (Ravelo, et al., 2003) and we got 1100 bp lenght unique and clear PCR product for the strains. Streptococcus agalactieae (ATCC 12401-20), S. pyogenes (ATCC 19615), Lactococcus garvieae (ATCC 43921) and Gram-negative bacterium, Aeromonas hydrophila (ATCC 19570) were also used as referans for bacterial identification. The amplification products for reference strains were not observed. According to the phenotypical tests and PCR assay results, the isolated strain from moribund rainbow trout was identified as L. garvieae. The challenge procedure was followed that described by Sharifiyazdi, et al. (2010). Briefly, after 2 weeks acclimatization period, the fish samples were challenged by immersion in a strain of L. garvieae grown on BHI agar. Each group of fish except control group were immersed in a bacterial suspension of $6x10^5$ colony forming unit/ml of the strain and kept for 30 min into aquariums. The control group was treated with the appropriate dilution of sterile PBS (Phosphate Buffered Saline). Then, the fish were kept in aerated fiberglass tanks until clinical signs of Lactococcosis and/or fish losses were observed. During this period, the water temperature was maintained at 24 °C. When the clinical signs were evident, moribund fish were subjected to bacterial re-isolation and for histopathological examination, tissue specimens including gills and internal organs from samples were fixed in 10% buffered formalin solution. The fixed tissues were washed in tap water and dehydrated in the ascending concentrations of ethanol. After dehydration, tissues were cleared in xylene and sectioned at 5 µm. Then, the sections were stained with hematoxylin and eosin (H+E) (Culling, 1963).

Results and Discussion

L. garvieae is one of the major Gram-positive coccus pathogen for freshwater salmonid and marine culture species (Khamesipour, et al., 2014). In Turkey, Lactococcosis was firstly reported in cultured rainbow trout (Diler, et al., 2002) and since then, the disease has been appeared regularly in trout farms especially spring and summer seasons (Kav & Erganis, 2007; Avci, et al., 2010).

Ürkü & Timur (2014) reported fish which were experimentally injected via intraperitoneal injection of L. garvieae isolates showed loss of orientation, bilateral exophthalmia with periocular hemorrhage, darkening of the skin, enlargement of the spleen, congestion in the liver, intestine and swim bladder. In the present study, three L. garvieae isolates were found to cause same clinical signs on experimentally infected rainbow trout. The all fish started to show lethargy, loss of orientation and swam near to the tank side on the first day of post inoculation (Fig. 1A.). Fish losses from the tank 1 were observed and the fish exhibited darkening of the skin color on the 2nd day of the trial (Fig. 1B.). Fish from the tank 2 and tank 3 had similar clinical findings of Lactococcosis after 3 days post infection. They had dropsy, exophthalmia, lesions on the upper jaw and anal prolapsus. Internally, they showed pale kidney,

splenomegaly, pale and hemorrhagic liver (Fig. 2A.). Hemorrhagies on the muscle and air bladder were also observed. Fish from the tank 1 had bilateral exophthalmia and hemorrhagies on the eyes (Fig. 2B.), ascites and anal prolapsus on the 4th day of the trial. Internally, they showed general hemorrhagic septicaemia. The control group did not show clinical findings and / or mortality and histopathological changes. Avci, et al. (2014) informed rainbow trout experimentally infected with L. garvieae via immersion route showed exophthalmia, paleness of the gills, hemorrhagies in the liver, swim bladder peritoneum and muscle, and bloody content in the intestine. This indicate that immersion or injection route of L. garvieae isolates caused same clinical findings and our experimentally infected fish were similar to the findings of Avci, et al. (2014) and Ürkü & Timur (2014).

After 3 days post infection, the fish inoculated with L. garvieae showed disease signs and mortality and the infected fish were used for re-isolation of the isolates. The isolates produced small, ovoid-cocci and white color colonies after incubation at 24 ± 2 °C for 48 hours. The isolates were fermentative, Gram-positive, cytochrome oxidase and catalase negative. Lactococcus garvieae (ATCC 43921) was also included as reference strain in the bacteriological study. The all phenotypic test results which were produced by the isolates are given in Table 1. Positive PCR amplification was obtained with L. garvieae using specific primers (PLG1 and PLG2) for a fragment of the 16S rDNA. The amplification of the target gene for L. garvieae permitted identification of the isolates as L. garvieae with 1100 bp (Fig. 3.). According to the results of bacteriological and molecular studies, L. garvieae was re-isolated from the experimentally diseased fish samples.

In the present study, all experimentally infected fish challenged with L. garvieae had changes in the gill, liver, kidney, spleen and gastro-intestinal tract. The gill showed lamellar fusion, hyperplasia of primary lamellae and epithelial necrosis (Fig. 4.). The liver showed hemorrhagy and increasing of brown haemosiderin deposits (Fig. 5.). Histopathological examination of kidney tissue exhibited heavy haemosiderin deposits and liquefactive necrosis of kidney tubules (Fig. 6.). The spleen tissue showed depletions of red and white pulpas (Fig. 7.). Necrosis of the gastro-intestinal tract was also detected. Ürkü & Timur (2014) found multifocal necrosis and hemorrhages in the liver, kidney and spleen, and talengiectiasis in the gills. Avci, et al. (2013) informed oedema and swelling in the secondary lamellar epithelium in the gills, focal or multifocal coagulation necrosis in the spleen. In this study, our histopathological findings such as hemorrhages in the liver and necrosis of kidney tubules were similar to the findings of Ürkü & Timur (2014). Talengiectiasis and multifocal coagulation necrosis (Avci, et al. (2014) were not observed in this study.

Results obtained in this study indicated that experimentally infected fish showed same clinical signs in other experimental studies; however, histopathological findings of tissue samples in this study were different findings which were reported in the other studies. It was understood that L. garvieae caused different histopathological changes in the tissue samples.

Acknowledgments

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Table 1. Phenotypic properties of reference strain (L. garvieae, ATCC 43921) and isolates (n=8)

Character	**Isolates (n=8)**	***L. garvieae*, reference strain (ATCC 43921)**
Cell morphology	ovoid-cocci	ovoid-cocci
Motility	-	-
Colony colour	w[a]	w
Gram-staining	+	+
O/F (glucose)	F[b]	F
Production of:		
C. oxidase	-	-
Catalase	-	-
Indole	-	-
H_2S	-	-
ADH	+	+
LDC	-	-
ODC	-	-
Nitrate reduction	-	-
Voges-Proskauer	+	+
Metil red	+	+
Degredation of:		
Gelatin	-	+
Starch	-	-
Blood (hemolysis)	α	α
Citrate utilization	-	-
Growth at:		
4 °C	-	-
37 °C	+	+
45 °C	+	+
Growth in:		
0% - 2% NaCl	+	+
4% -6% NaCl	+	+
6.5% NaCl	+	+
ONPGc	-	-
Production acid from:		
Arabinose	-	-
Glucose	+	+
Inositol	+	+
Fructose	+	+

Galactose	+	+
Mannitol	+	+
Mannose	+	+
Sorbitol	+	+
Sucrose	+	-
Xylose	+	+

+: positive, -: negative, a: white, b: fermentative

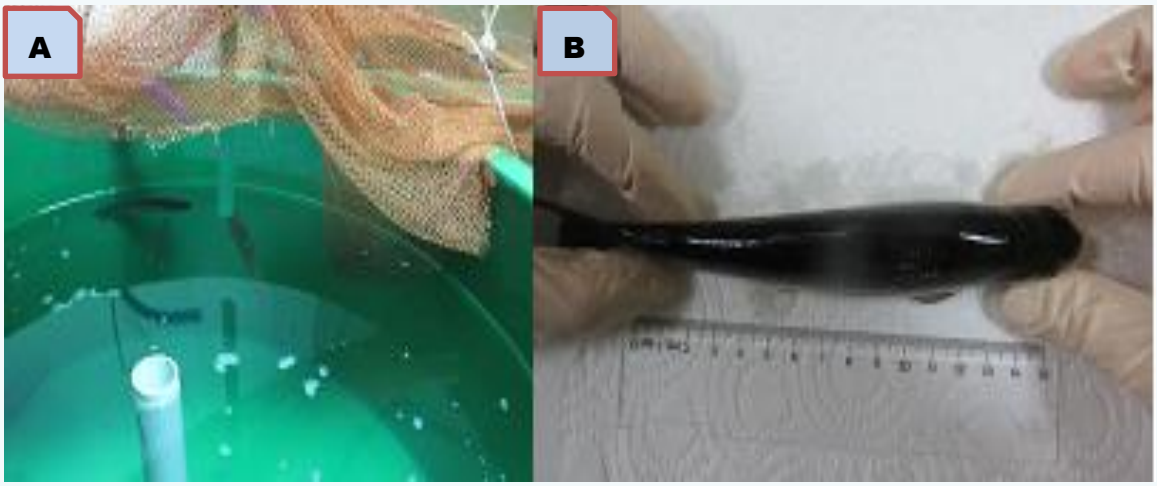

Figure 1A: Infected fish showed loss of orientation on the 1st day of post oculation
Figure 1B: Darkening of the skin color on the 2nd day of the trial

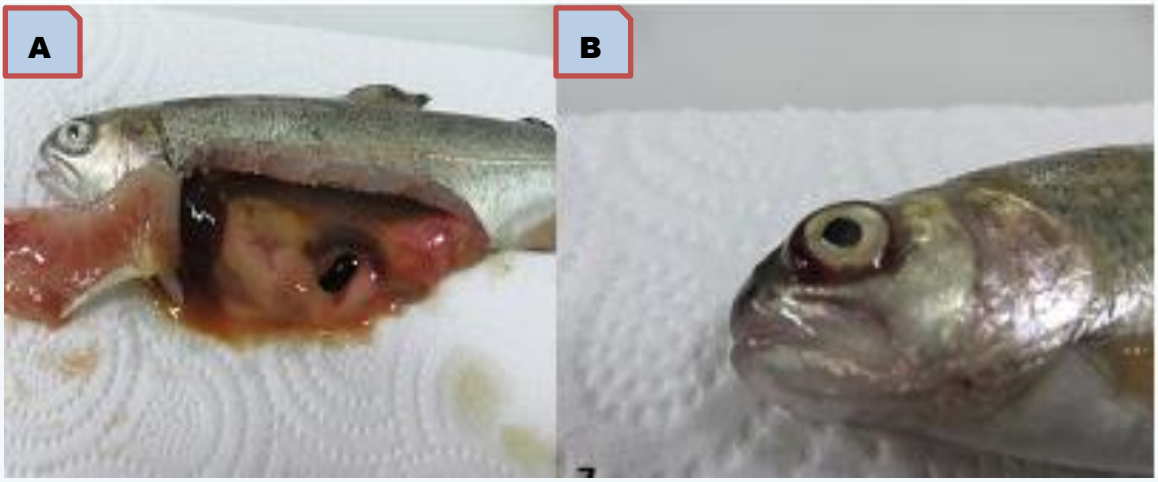

Figure 2A: Splenomegaly and hemorrhagic liver on the 3rd day of the trial
Figure 2B: Bilateral exophthalmia and hemorrhagies on the eyes on the 4th day of trial

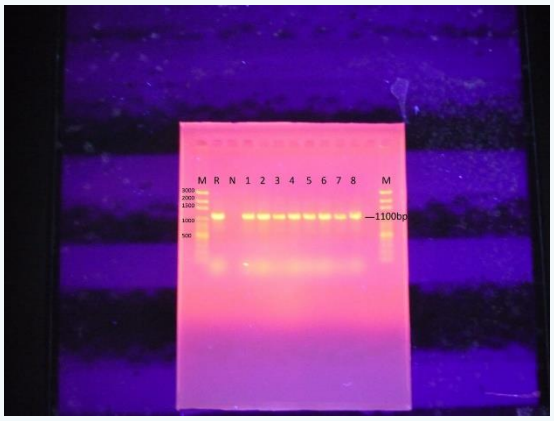

Figure 3: Amplification products of the isolates from the experimentally infected rainbow trout in the study. M: Marker, Solip BioDyne 100 bp DNA ladder; R: positive control, *L. garvieae*, (ATCC 43921); N: negative control, double distilled water

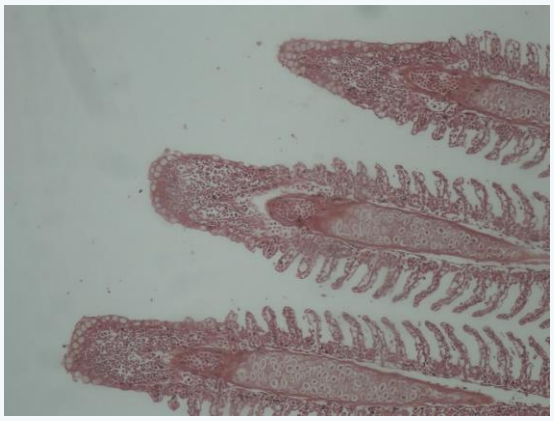

Figure 4: Gill of rainbow trout infected with *L. garvieae*. The gill showed lamellar fusion, hyperplasia (arrow) and epithelial necrosis (H+E x 20)

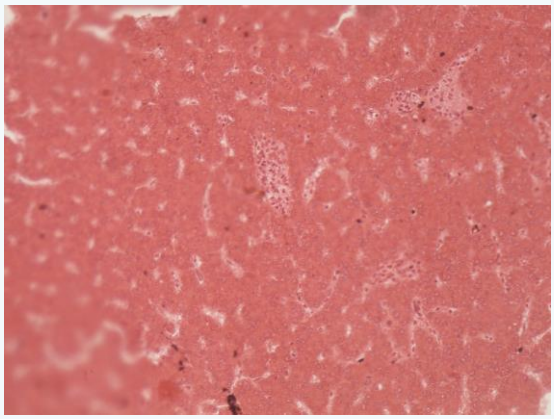

Figure 5: Hemorrhagies (arrow) in the liver (H+E x 20)

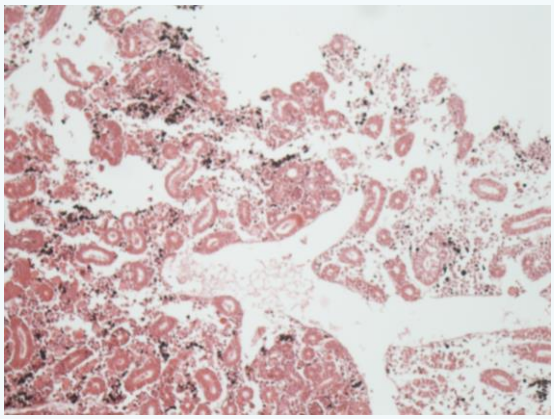

Figure 6: Massive haemosiderin deposits (arrow) and liquefactive necrosis in kidney (H+E x 10)

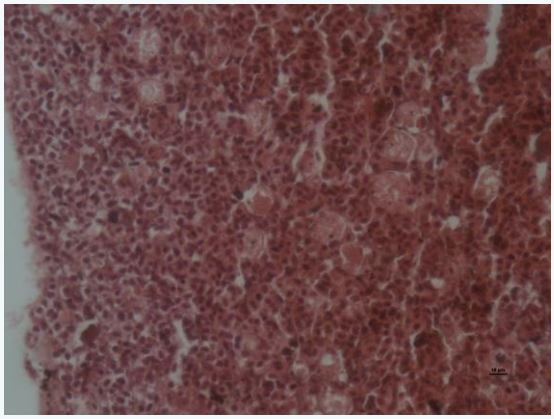

Figure 7. Depletions of red and white pulpas in the spleen (H+E x 40)

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