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# The Study of Histopathologic Changes of Experimental Infection with *Listonella (Vibrio) anguillarum* in Rainbow Trout

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## Abstract

The rainbow trout production increased more than 100% in the last decade and total rainbow trout production was shown as 107.013 tons according to 2016 statics. According to Federation of European Aquaculture Producer, Turkey was determined as the biggest rainbow trout producer into the European Countries in 2016. Together with high production capacity, a number of outbreaks were reported causing *L. anguillarum* (Vibriosis). The pathogenesis of *L. anguillarum* which were experimentally infected in rainbow trout were examined during 15 days in our study. Spreading of agent that was injected with intraperitoneally into tissue and organs were studied by using histopathological methods. The mortality rate of agent was determined above 70% and deaths were seen in 2-3th days of experiment. In addition of these, liver, spleen, kidney and gills were determined as the most affected organs and tissues. In the present study was obtain for original pathological findings of *L. anguillarum.*

**Key words**: *L. anguillarum*, Vibriosis, Pathology, Rainbow trout

## Introduction

Vibriosis is an infectious bacterial disease causing Vibrio species which seen aquaculture industry with important economic loss in over the World. The most important agent is *Listonella (Vibrio) anguillarum* in Vibrionaceae family.[1,2] *L. anguillarum* causing by hemorrhagic septicemia both warm water and cold water fishes like Atlantic salmon, rainbow trout, turbot, sea bass, sea bream, striped bass, cod, Japan and Europe eel fishes and ayu fishes.[2-5] There are much of information for disease outbreak in fresh water, brackish-water and marine water fish species.[7,8]

*L. anguillarum* infections can successfully be identified with conventional methods, rapid identification kits and Polymerase chain reaction.[9,10] Histopathologic changes must work for screening disease process and understanding tissue lesions in rainbow trout in case of vibriosis. While there are some research for histopathologic changes in vibriosis[11-14] which subjects were agent colonization and penetration to skin,[15-17] yet there is limited information for disease progression in gill, muscle, liver, spleen, kidney tissues.[18]

We researched pathogenesis of Vibriosis (*L. anguillarum*) in rainbow trout tissues (muscle, kidney, liver, spleen, and gill) by experimental infection for 1, 2, 3, 4, 5, 7, 9, 11, 13 and 15 days. We think that this study provides detailed information study for progression of *L. anguillarum* diseases.

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## Material and Methods

### Create experimental groups, inoculum preparation of *L. anguillarum* and experimental infection

The study was carried out 170 rainbow trout on average weight of 208.7±8.11 g for determining histopathologic changes of *L. anguillarum*. The bacterial strain isolated in rainbow trout and identified before by Ekici et al.,[14] The fishes were taken from Faculty of Egirdir Aquaculture and adapted completely for 10 days in 1 $m^3$ fiber plastic tanks contain 900-liter water are located in Aquatic animal disease unit of Egirdir Aquaculture Research Institute. Experimental fishes were examined for parasitic and bacterial diseases (conventional bacteriologic and parasitic methods) in random selected 20 fishes. 0.2 ml volume of 101 and 102 cfu/ml *L. anguillarum* strain was injected for suitable infective dose (LD50) via intraperitoneal separated in two fish group (25 fish). After determination of infective dose as 2,3x102 cfu/ml, experimental injections were done two groups into two separated tanks (total 50 fishes).[19] For control group, 50 fishes separated two groups in two tanks (each of 25 fishes) injected 0.2 ml PBS buffer (pH 7.2) solution.[20-24] Pathologic examinations were made in 1, 2, 3, 4, 5, 7, 9, 11, 13 and 15 days by euthanized in disease and control groups after experimental injection. Dissolved oxygen, pH and water temperature measured 5.72±0.46 mg/lt, 7.51 and 13.04±0.22°C respectively in experiment tanks.

### Histopathology

Fishes euthanatized by Quinidine were necropsied and liver, kidney, spleen, gill and muscle tissue samples were collected and fixed in 10% buffered formalin solution and further processed following standard techniques. The tissue samples were embedded in paraffin wax, cut into 5 µm thick tissue sections, mounted on slides and stained with hematoxylin-eosin (H&E) and Brown & Brenn stains for bacteria. Tissue sections were then examined under light microscope.

## Results

### Macroscopic Findings

In diseased group; 36 fishes were died injected with $10^2$ live bacteria. Clinical signs, inappetence and motionless were observed in experimental groups. In addition, hemorrhagic lesions were located on anal, ventral and lateral region of body, eyes and fin root were seen in the progressive days of infection (Fig 1, 2). Petechial hemorrhage was especially remarkable on liver in experimental group as necropsy findings (Fig 3). There was no clinical finding in control group injected with sterile PBS before necropsy.


Figure 1. A: Erosion in lateral line
B: Hemorrhage on eye,
C: Hemorrhage on fin

Figure 2. Hemorrhage on eye

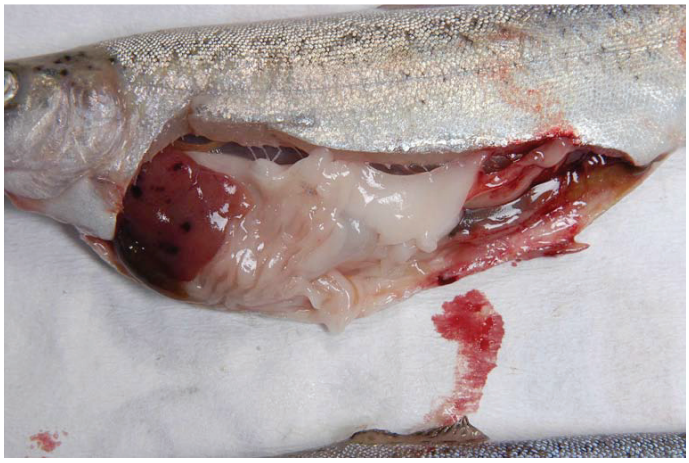

Figure 3. Petechial hemorrhage on liver

Table 1. Lesions of fish in experimental group

| Days of Infection | Gills | | | | | | | | Skeletal muscle | | | | | | Spleen | | | | Liver | | | | | | | Kidney | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Desquamation of seconder | Mononuclear cell | Atrophy Seconder lamella | Hyperemia | Hemorrhage | Necrosis | Telangiectasia of Seconder | Exudation of Seconder | Exudation | Mononuclear Cell | Hemorrhage | Zenger's Degeneration | Necrosis | Calcification | Hemorrhage | Hyperemia | Siderosis | Necrosis | Hemorrhage | Necrosis | Dissociation | Vacuolar Degeneration | Bile stasis | Mononuclear Cell | Siderosis | Siderosis | Hyperemia | Necrosis | Hemorrhage | Mononuclear Cell | Vacuolar Degeneration |
| 1a | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 1b | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - |
| 1c | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 1d | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 1e | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 2a | - | - | - | - | - | - | - | - | + | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 2b | - | - | + | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 2c | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 2d | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 3a | + | + | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| 3b | + | + | - | + | + | + | - | ++ | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| 3c | + | - | - | + | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 3d | + | + | - | - | - | - | - | - | - | - | - | + | - | - | ++ | - | ++ | - | - | - | - | + | - | - | ++ | - | - | - | - | - |
| 4a | - | - | - | - | - | - | - | - | + | + | - | - | - | - | - | - | - | - | + | - | - | - | + | - | - | + | - | - | - | - |
| 4b | - | - | - | + | - | - | - | - | + | - | - | - | - | - | - | - | - | - | + | - | - | - | | ++ | - | - | - | - | - | - |
| 4c | - | - | - | - | - | - | - | - | + | + | - | - | + | - | - | - | - | - | - | + | - | + | + | ++ | - | ++ | - | - | - | - |
| 4d | - | - | + | - | - | - | - | - | + | + | - | + | - | - | + | - | - | - | - | + | - | + | - | - | - | ++ | - | - | - | - |
| 4e | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | + | - | - | - | - | + | - | - | - | + | - | - | - | - |
| 5a | - | ++ | - | - | - | - | - | - | + | - | - | + | - | - | - | - | - | + | - | + | - | - | - | + | - | - | - | - | - | - |
| 5b | + | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | + | - | - |
| 5c | - | ++ | | - | - | - | - | - | - | - | - | + | - | - | - | - | + | + | - | - | - | - | - | ++ | - | - | - | - | - | + |
| 5d | - | + | + | - | - | - | - | - | - | - | - | + | - | - | ++ | + | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| 5e | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | ++ | - | - | - | - | - | |
| 7a | - | - | - | - | - | - | - | - | - | + | - | - | - | - | + | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - |
| 7b | + | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | + | - | - | + | - | - | - | ++ | - | - | - | - |
| 7c | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | ++ | - | ++ | + | ++ | - | - | - | - | - | - |
| 7d | - | - | ++ | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| 7e | - | - | ++ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| 9a | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - |
| 9b | + | + | - | - | - | - | - | - | - | ++ | - | - | - | - | - | - | - | - | + | - | + | - | + | | - | - | - | - | - | - |
| 9c | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | + | - | - | - | - | ++ | + | - |
| 9d | - | - | - | - | - | - | - | - | - | + | + | - | - | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - |
| 9e | - | + | + | + | - | - | - | - | - | - | - | + | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 11 | + | - | - | - | + | + | - | + | - | + | - | - | + | - | - | - | - | - | + | ++ | - | - | ++ | - | - | - | - | - | - | - | - |
| 11 | - | + | - | - | - | - | + | + | - | + | - | - | - | - | + | - | ++ | - | + | - | + | + | ++ | - | - | - | - | - | - | - | - |
| 11 | + | + | - | - | - | + | + | - | - | - | - | + | - | - | + | - | - | - | - | - | + | ++ | - | - | - | - | + | - | - | + | - |
| 11 | - | + | + | + | - | - | - | - | - | - | - | + | - | - | - | - | + | - | - | - | + | + | - | - | - | - | - | - | - | - | - |
| 11 | + | + | - | - | + | - | - | - | - | - | - | ++ | + | - | - | - | + | - | - | - | + | + | - | - | - | - | - | - | + | - | - |
| 13 | - | - | - | - | - | - | - | - | - | - | - | ++ | - | - | - | - | - | - | - | - | - | | - | - | - | - | - | - | - | - | - |
| 13 | ++ | + | - | - | - | - | - | - | - | - | - | ++ | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - |
| 13 | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - |
| 13 | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - |
| 15 | + | + | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | ++ | - | + | - | - | - | - | - | - | ++ |
| 15 | - | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 15 | - | ++ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | ++ | | - | + | + | ++ | - | - | - | - | - | - | - | + | ++ |
| 15 | - | ++ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - |
| 15 | - | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + |

-: no lesion, +: mild lesion, ++: severe lesion; a, b, c, d, e: refer to each of sampled fish

**Microscopic findings**

Control group; Desquamation of gill seconder lamella, hyperemia of primer lamella, and cell infiltration of the gill lamella were examined in control groups during 2, 4, 5, 7, 11, 13, 15th days after experimental PBS injection. These fairly mild lesions were seen on histopathological examination in several fishes.

Hyaline degeneration, necrosis, mononuclear cell infiltration and hemorrhagic exudation were observed in muscle tissue samples in 2, 3, 4, 11, 15th days. Slightly hemorrhage and erythrocyte loaded macrophages only were recorded only on 4th day in spleen tissues. Necrosis, mononuclear cell infiltration, hyperemia and bile stasis were seen in 2, 4, 7, 9, 11th days in liver tissues. Hyperemia, necrosis, mononuclear cell infiltration and melanin pigmentation were observed on 9, 11, 15th days in kidney tissues of control and infected fish groups.

Experimental group; mononuclear cell infiltration and intensively desquamation of gill tissues were seen (Table 1, Fig 5, 6) in the infected group. In some cases, atrophy and desquamation were noticed (Fig. 5). Hyperemia and necrosis were seen in afferent and efferent arteriols of primer and seconder lamella (Fig. 6). Hemorrhages were observed sporadically in some fishes gill tissue. There was enlargement of primer lamella because the mononuclear cell infiltration was observed (Table 1, Fig. 9). Zenker's degeneration, mononuclear cell infiltration hemorrhage and calcification between muscle bundles were observed (Fig 12, 15), (Table 1).

Hemorrhage, siderosis and necrosis were major findings from 3th day of experimental infection in spleen tissue (Table 1, Fig. 4).

Vacuolar degenerations were observed from 3th day of infection depicting beginning of the degeneration of hepatocytes (Fig. 14). From 4th day of infection, vacuolar degeneration, necrosis and bile stasis, depending on inflammation on liver tissue, were determined (Fig. 8, 11, 14). There were increasing multifocal necrosis area, invading mononuclear cell infiltrations and progressive bile stasis showing severely progressive infection (Fig. 8, 10).

Exudation and mononuclear cell infiltration of some livers capsule demonstrating peritonitis were noticed (Table 1).

In kidney, siderosis were increased from 1 day to 4, 5, 7, 9, 11 and 15th days. Besides siderosis, hydropic degenerations of tubules, hyperemia, mononuclear cell infiltration hemorrhage and necrosis in kidney were observed (Fig. 7, 13), (Table 1).

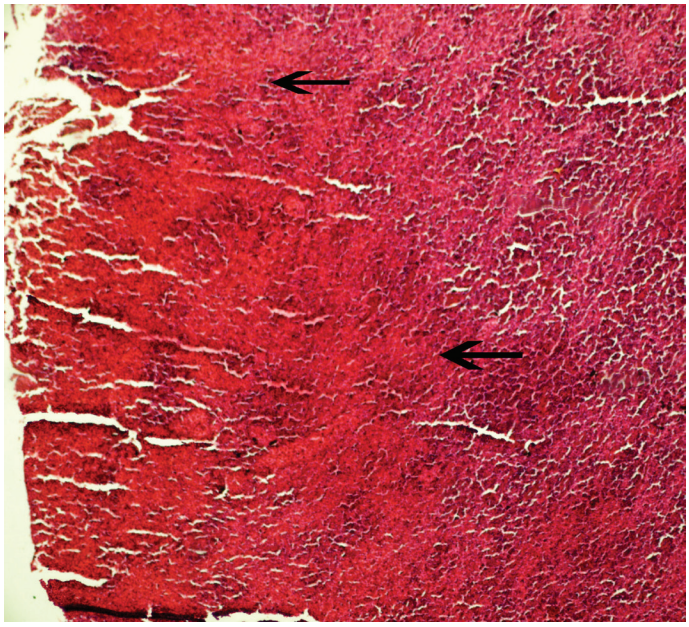

Fig. 4. Hemorrhage on spleen (arrows) H&E x100

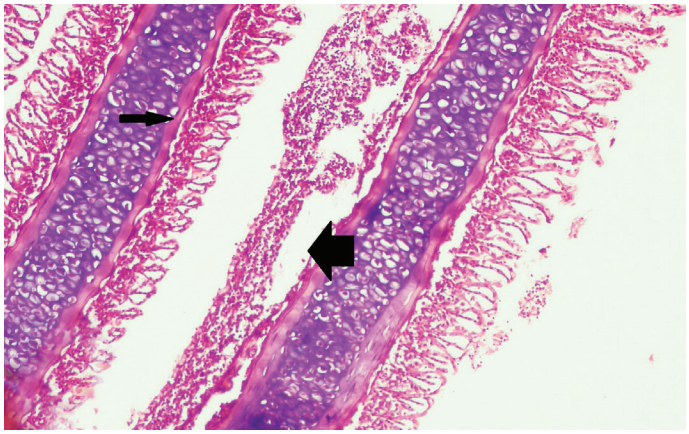

Fig. 5. Desquamations on seconder lamella (thick arrows), Normal seconder lamella (thin arrows) H&E x100

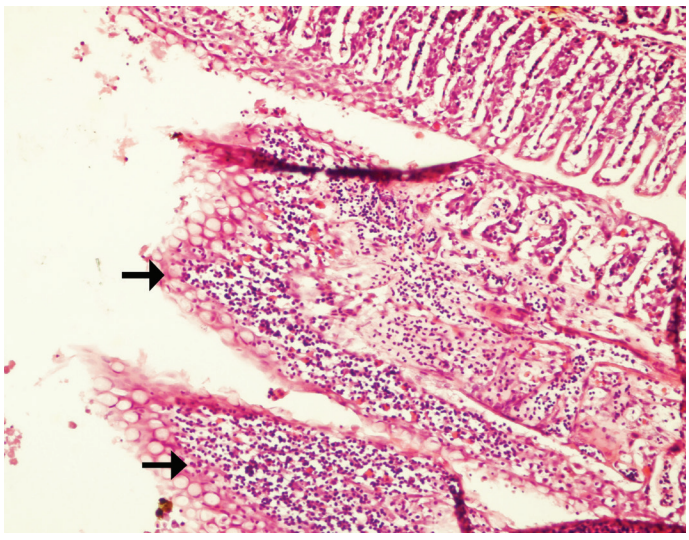

Fig. 6. Severe mononuclear cell infiltration on primer lamella, (arrows) H&E x200

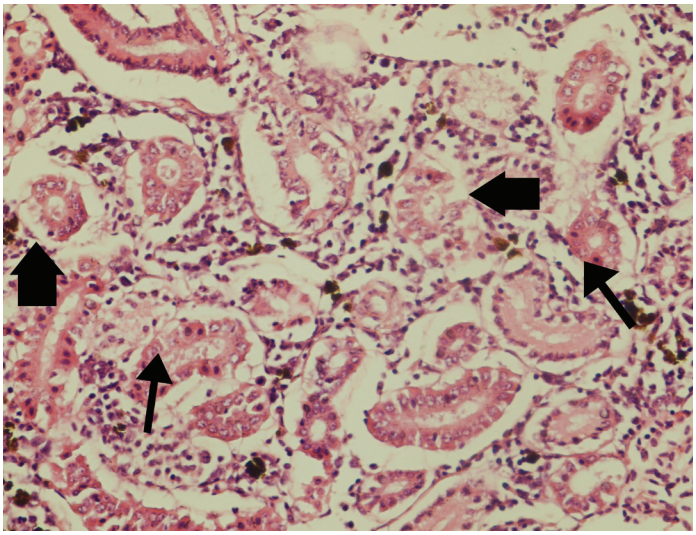

Fig. 7. Vacuolar degeneration on kidney tubules (thin arrows), necrosis (thick arrows) H&E x400

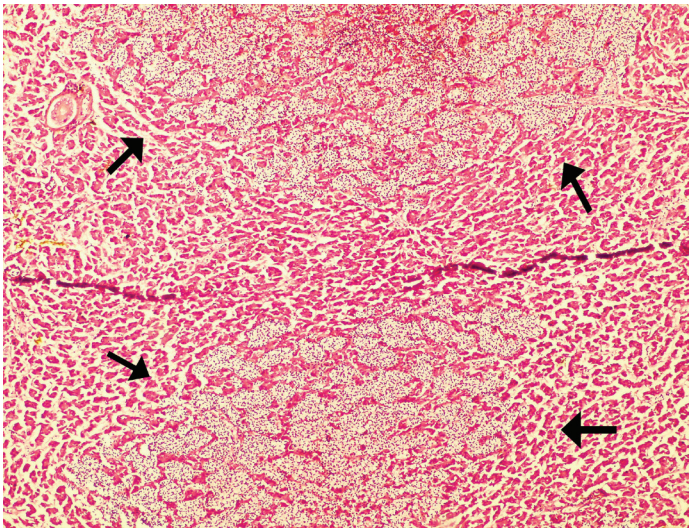

Fig. 10. Multifocal necrosis area in liver that contain inflamma tion cells on center (arrows) H&E x100

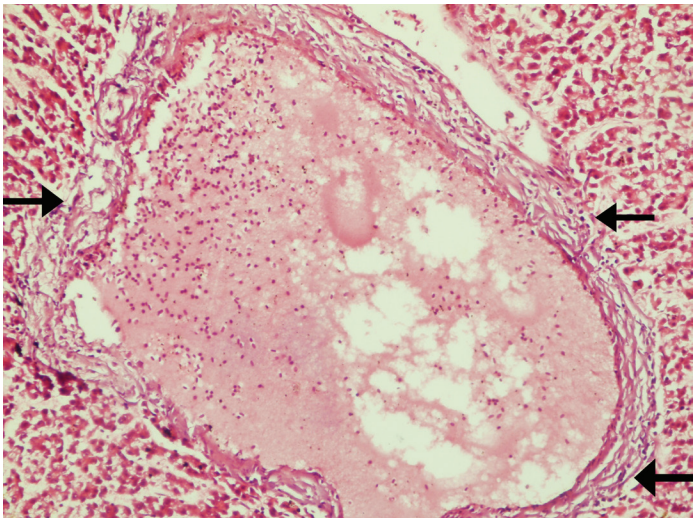

Fig. 8. Mononuclear cell infiltration on portal region of liver (arrows) H&E x200

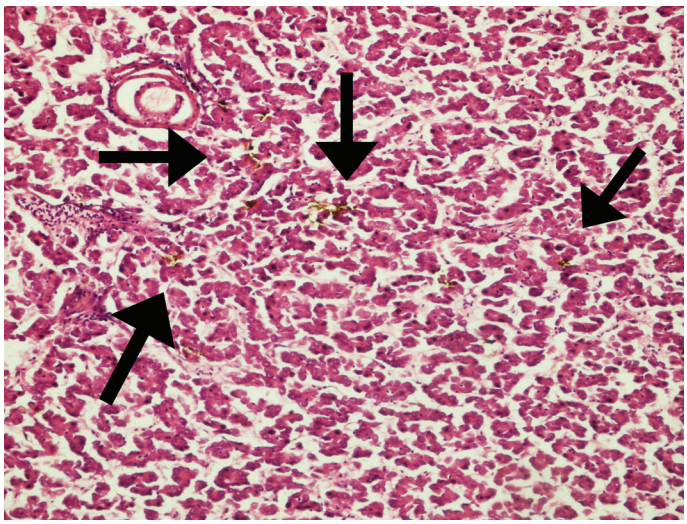

Fig. 11. Gall stasis on liver (arrows) H&E x200

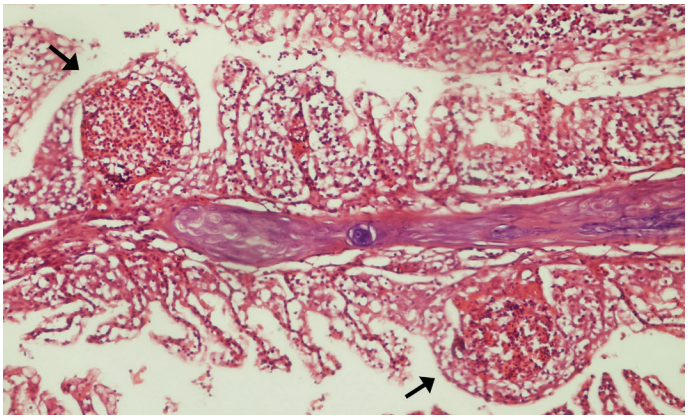

Fig. 9. Telangiectasia on seconder lamella (arrows) H&E x200

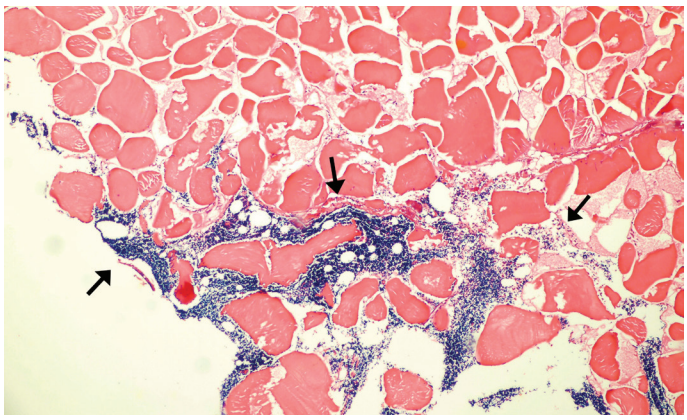

Fig. 12. Severe mononuclear cell infiltrations on muscle (arrows) H&E x100

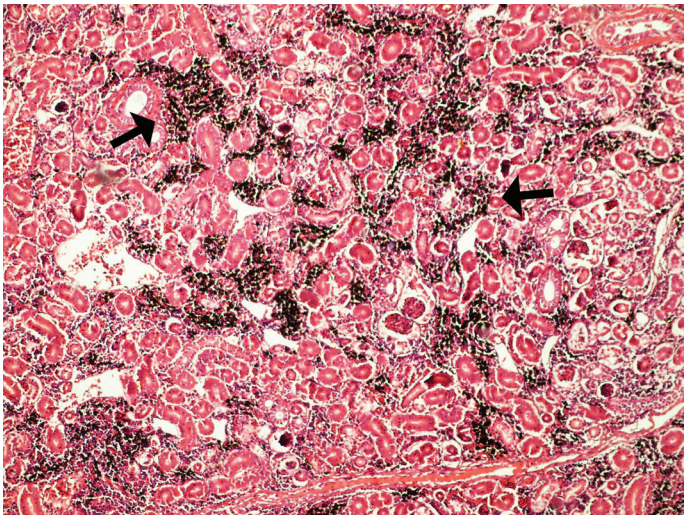

Fig. 13. Severe melanin pigmentation on kidney tubules H&Ex100

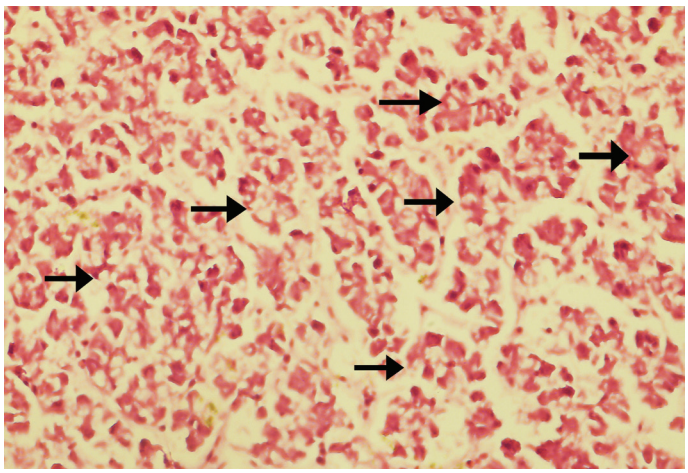

Fig. 14. Vacuolar degenerations on liver epithelials (arrows) and dissociation H&E x400

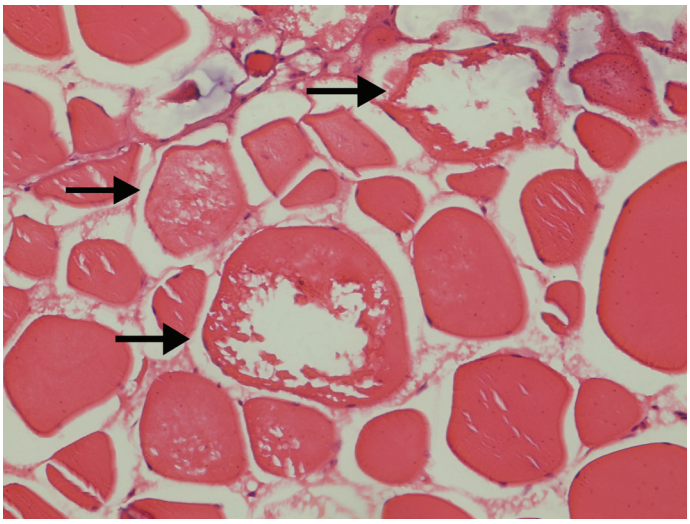

Fig. 15. Piecemeal necrosis on muscle fibrils H&E x400

## Discussion

The detailed pathogenesis of *L. anguillarum* which is one of the most deadly agent of Vibriosis for rainbow trout was examined that was injected via intraperitoneally. Austin and Austin[33] were reported that there is limited information about pathogenesis of *L. anguillarum*, that so; we would like to give macroscopic clinical signs and microscopic findings in different tissue and organs during experimental infection from one to 15 days.

Generally, *L. anguillarum* causes about 40-70% mortality in natural infection for fish[6,25,26], mortality could be reach 100% in experimental infections.15,27-30 Avci et al.,[11] were reported that L. anguillarum caused 40% mortality in experimental infection by immersion and 55% mortality by intraperitoneal injection. As similar these findings, our results showed that mortality reached above 70% by intraperitoneally.

Experimental infections with *L. anguillarum* showed that mortality generally observed between one and 15th day,[31,32] similarly, we observed mortality was shown after injection up till 15th day of experiment. The results showed that *L. anguillarum* caused acute mortality when agent enter the fish body and has short time for incubation and spread all of the body. *L. anguillarum* is also named as red pest because of agent causing hemorrhage in different region of body.[33] We observed motionless, loss of appetite, swimming abnormality, hemorrhages on anal, ventral, lateral region, eyes and fin rots as seen previously disease outbreaks.[6,7,11,34-36] Prolapsus of anus and adhesion in abdominal cavity also reported clinical symptoms[35-38] while we could not detected them, inflammation in liver's capsula was detected which is caused peritonitis. We argued that if infection would progressive, peritonitis could cause adhesion on abdominal cavity and may exudate should be seen. Primarily there is some report showed that agent enter the body via gill tissue, while some of them reported that anus and skin mainly route of entry.[15,16,27,37] As similar pathological signs of desquamation of seconder lamella, atrophy in lamella epithelia, exudation, hyperemia on seconder lamella and submucosa vessel showed that *L. anguillarum* has high affinity of gill tissue regardless of route of entry in the fish body.

One of the most effected tissue is skeletal muscle due to *L. anguillarum* infection was reported[5,11,13,30,40], zenker's degeneration and mononuclear cell infiltration were the one of the most observed findings on skeletal muscle with the experimental infection of our study. But in addition of these findings on skeletal muscle, there are severe lesions were determined on gill, liver and spleen tissue distinct from previous report. There is limited information about pathogenesis of *L. anguillarum* infection on liver tissue,[6,37,40] so we presented dissociation, bile stasis and severe accumulation of siderocyte in liver tissue in the first time. Generally hyaline degeneration and mononuclear cell infiltration were mostly occurred pathological findings in kidney,[6,30] we determined accumulation of siderocyte, hydrobic and vacuolar degeneration and after 11th day mononuclear cell infiltration in our experiment.

In the control group of experiment, the showing microscopic findings demonstrated that fish could easily affected any stressor factor like as PBS injection. There is no macroscopic findings and clinical signs in fish of control group. As a consequence of our study, the pathogenesis of *L. anguillarum* by experimental infection were presented from day to day in the first time. After entry of agent into the fish body, agent reached all body regions via blood stream from 2. and 3th days and especially skeletal muscle, liver and spleen are the most effected tissue were observed. These findings showed that *L. anguillarum* is an acute infection that can affect all tissue severely in short time.

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