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Diagnosis of *Vibrio anguillarum* in Cultured Rainbow Trout (*Oncorhynchus mykiss*) by Different Methods

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ABSTRACT

Objective: In this study, the diagnosis of infection caused by *Vibrio anguillarum* in cultured rainbow trout (*Oncorhynchus mykiss*), in Turkey using bacteriological, histopathological and immunohistochemical methods was attempted.

Materials and Methods: 15 rainbow trout samples showing signs of disease were investigated using bacteriological and histopatological methods.

Results: Because of physiological and biochemical characteristics of bacteria growing on the medium, these bacteria were isolated and identified as *V. anguillarum*. As a result of the histopathologic examination of the tissues of diseased rainbow trout, hyperemia in the liver, degeneration of the epithelium of kidney tubules and necrosis in the interstitial area, enlargement in the gill filaments, cells having picnotic nucleus and several necrotic areas in the spleen were observed. It was also detected in the streptavidin-biotin staining method that the causative agent accumulates in the liver, spleen, kidney and also in the gills especially in the areas near around the blood vessels depending on the development stage of the disease.

Conclusion: As a result, an immunoenzimatic method, streptavidin-biotin staining method was used in this study for the detection of the presence of *V. anguillarum* in the infected rainbow trout tissues.

Keywords: Rainbow trout, *Vibrio anguillarum*, histopathology, streptavidin-biotin

INTRODUCTION

Bacterial pathogens are among the leading causes of economic loss in fish farming (1,2). One of the most important bacterial diseases leading to hemorrhagic septicemia in fish both in the natural and culture environment is vibriosis (1,3). It was reported that *Vibrio anguillarum*, known as the most common pathogen of vibriosis, causes mortality in many cultured fish species such as rainbow trout (4,5), sea bass (*Dicentrarchus labrax*) (6,7), and sea bream (*Sparus aurata*) (8,9). In general, it was reported that the primary clinical findings in fish infected with *V. anguillarum* are erythema around the fins and mouth, as well as lethargy, loss of appetite, change in skin color, ulcers on the body surface, formation of red necrotic lesions in the abdominal muscles and accumulation of bloody liquid in the intestine (1,5). It was reported that histopathological changes such as hemorrhage in the liver, gills, spleen, kidney and muscle focal necrosis in the spleen and skeletal muscles and severe degeneration in the intestinal mucosa epithelium were observed in fish infected with *V. anguillarum* (2,5,10).

The identification and localization of the presence of the pathogen in tissues by different immunohistochemical methods used in studies conducted on *V. anguillarum* by different researchers were used for various purposes such as detecting its density in tissues, understanding how the pathogen infects healthy fish, and investigating how bacteria entering through the portal tracts spread to the fish tissues and organs (7,11). Bacterial methods have frequently been used for the identification of the diease agent. However, since this method is somewhat time-consuming, various alternative methods have recently come into use. Immunohistochemical methods are among these specific methods that are used for the identification of the agent since they have a high specificity and provide more efficient results in a shorter time (1).

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In this study, the diagnosis of infections caused by *V. anguillarum* in cultured rainbow trout in Turkey using bacteriological, histopathological and immunohistochemical methods was attempted.

MATERIALS AND METHODS

Fish Sampling

In this study, the 15 moribund rainbow trout (*Oncorhynchus mykiss*) used as material were obtained from 4 different trout fish farms in the Aegean Region. The three healthy rainbow trout used as the control in immunohistochemical investigations was obtained from Istanbul University, Sapanca Inland Waters Fish Culture Research and Application Unit. Table 1 shows data on the diseased fish examined in this study.

Bacteriological Examination

Bacteriological samples of liver, kidney, spleen and blood were streaked onto Tryptic Soy Agar (TSA) and the plates were incubated at 22-23°C for 48-72 hours. Conventional bacteriological methods were applied to pure cultures of bacteria grown in the media after incubation, and the isolated bacteria were identified (1). In the application of conventional bacteriological methods, the RV22 strain of *V. anguillarum* O2 serotype obtained from Santiago de Compostela University in Spain was used as the control.

Histopathological Examination

After the necropsy examinations tissue samples such as kidney, spleen, liver and gills were fixed in 10 % formalin, dehydrated in ethanol and embedded in paraffin wax, section at 5 µm and stained by routine methods with haemotoxylin and eosin (H&E) (12,13).

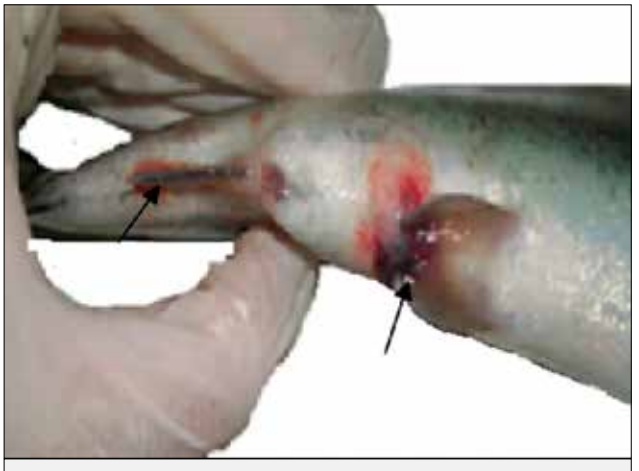

Figure 1. Large hemorrhagic foci and lesions around the pelvic and anal fin of the moribund rainbow trout (arrowed).

Immunohistochemical Staining (Streptavidin-Biotin Method)

The identification of *V. anguillarum* and its localization in the gill, kidney, spleen and liver were determined by Strep-ABC staining method (14). Organs such as gills, kidney, spleen and liver collected from healthy and diseased fishes were fixed in 10% neutral buffered formalin for 24 h. They were subjected to a routine paraffin embedding method. Tissue sections of 4-µm from paraffin-embedded organs were deparaffinized and rehydrated. The sections were treated with 10 mM citrate buffer (pH 6.0) in a microwave oven. The endogenous peroxidase activity in tissues was eliminated by 3% hydrogen peroxide for 30 min. After washing, the sections were treated with a normal goat blocking serum for 15 min at room temperature. And then, the sections were incubated with mouse anti-*Vibrio anguillarum* primary antibody (Ibt FM-040AX-5) at a 1:1000 dilution (the best dilution rate) for 2 h at room temperature. They were labelled by secondary antibodies (goat IgG) according to the manufacturer's instructions for 15 min at room temperature (Invitrogen, Histostain Plus-peroxidase kit 859043). The peroxidase activity was revealed by a 3-amino-9-ethylcarbazole substrate kit (Invitrogen 002007). Sections were counterstained with Mayer's hematoxylin. No immunolabelling was detected when the primary antibody was replaced with either PBS instead of primary antiserum. Sections were investigated under a light microscope.

RESULTS

Clinical and Macroscopical Findings

Externally all moribund rainbow samples showed external signs of disease, hemorrhages on the body surface, in the pectoral, pelvic, and anal fins (Figure 1), darkening in color internally, anemia in the gill, exfoliation at the tip of the gill filaments, petechial hemorrhage on the viscera and in the visceral adipose tissue, splenomegaly and paleness in the liver color (Figure 2).

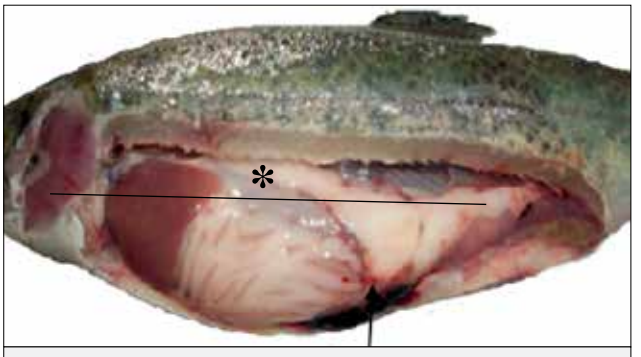

Figure 2. Anemia in the gills of diseased rainbow trout and exfoliation at the ends of gill filaments (*), splenomegaly, hemorrhage in the visceral adipose tissue (arrowed).

Table 1. The rainbow trout used in this study and obtained from different fish farm and its weight

Fish Species	Fish Farm (A)	Fish Farm (B)	Fish Farm (C)	Fish Farm (D)	Control
Rainbow trout	4 fishes (150-200 g)	4 fishes (150-200 g)	4 fishes (170-250 g)	3 fishes (100-150 g)	3 fishes (100-150 g)

Bacteriological Findings

After the incubation of the bacteriological inoculations from the spleen, kidney and liver, bacteria produced round, convex, slightly fluffy, bright, and cream-colored colonies onto TSA (Figure 3). It was determined that gram-negative, motile, bacilli bacteria isolates (15 isolates) isolated from moribund rainbow trout belonged to *Vibrio* genus since they exhibited a fermentative characteristic, were sensitive to O/129 vibriostat test, and positive reaction in cytochrome oxidase and catalase tests. The isolated bacteria were identified as *V. anguillarum* since indole, beta-galactosidase (ONPG), Voges-Proskauer (VP), and arginine dihydrolase tests of the isolates gave a positive reaction, and H_2S, Methyl Red, lysine, and ornithine dihydrolase tests were negative, they did not produce gas from glucose, used citrate in a citrate medium, the urease enzyme production was negative and gelatinase and amylase productions were positive, and also, they were similar to the reference bacteria (RV22).

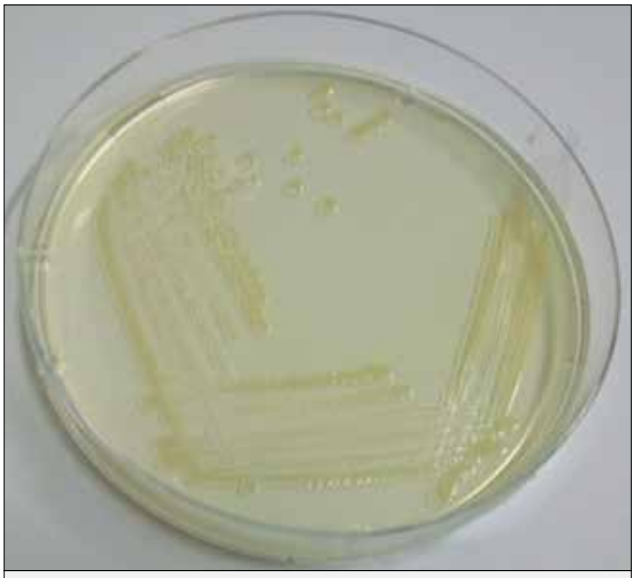

Figure 3. The appearance of Vibrio anguillarum colonies on TSA.

Histopathological Findings

Histopathologically, mild hyperemia in the liver (Figure 4a), degeneration of the epithelium of kidney tubules and very severe necrosis in the interstitial area (Figure 4b), the enlargement in the gill filaments (Figure 4c), cells having picnotic nucleus (Figure 4d) and several necrotic areas in the spleen were observed.

Streptavidin-Biotin Method Findings

As a result of examining the visceral organs (liver, kidney, spleen) and the gills, of moribund rainbow trout infected

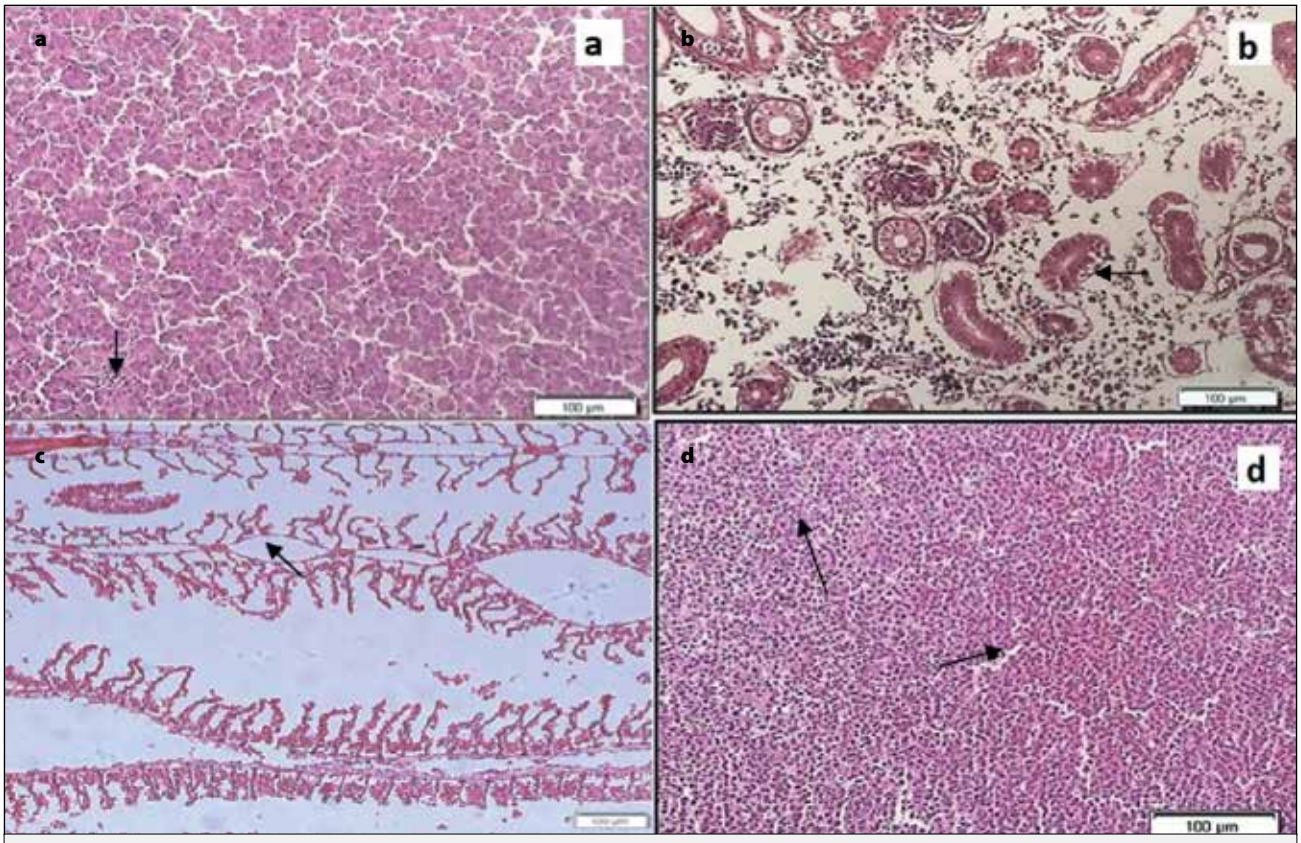

Figure 4. a-d. (a) Moribund rainbow trout infected with *V. anguillarum*. Mild hyperemia (arrowed) in the liver, (b) degeneration of the epithelium of kidney tubules (arrowed) and very severe necrosis in the interstitial area, (c) the enlargement (arrowed) in the gill filaments, (d) cells having picnotic nucleus in the spleen (arrowed) tissue of moribund rainbow trout samples.

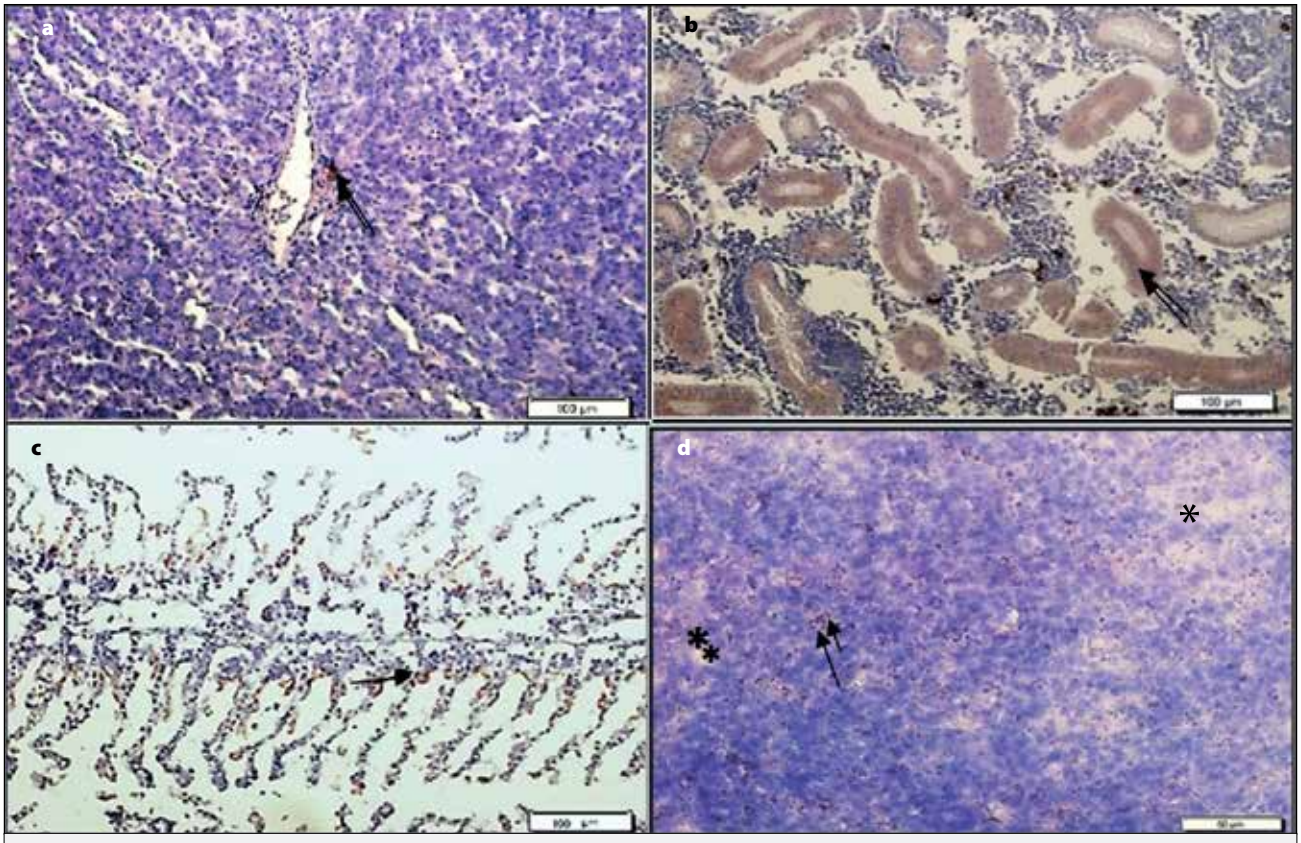

Figure 5. a-d. (a) Immunopositive reactions in the liver sinusoids, necrotic areas and at the vein center (arrowed), (b) in the kidney degenerative tubules (arrowed), (c) in the hyperplasic cells of the gills (arrowed), (d) in the necrotic area cells (arrowed) and several necrotic areas (*) in the spleen.

with *V. anguillarum* by the immunohistochemical staining method, immunopositive reaction was not determined in the cells of the tissue sections such as the gills of the fish in the control group. The immunopositive reaction detected sinusoids in the necrotic areas and at the vein center in the liver (5a), the kidney tubules (Figure 5b), the gill filaments and cells in the hyperplasic areas (Figure 5c) and the necrotic of the spleen tissue (Figure 5d). Upon evaluation of the tissue samples obtained from infected fish according to the result of immunostaining, it was determined that the tissues with the most intense positive staining were in the cells of gills and kidney. Furthermore, this method provided a species-specific identification of *V. anguillarum* in a shorter time when compared with the routine bacteriological methods.

DISCUSSION

V. anguillarum has been isolated and identified from diseased rainbow trout, in previous studies conducted in Turkey (5,15). In this study, the diagnosis of this bacterium in cultured fish species was made and the presence of this organism was detected in the tissues of rainbow trout samples showing clinical symptoms of vibriosis using bacteriological, histopathological and immunohistochemical methods.

The bacteria isolated from moribund fish samples were identified as *V. anguillarum* due to the fact that 15 isolates isolated from diseased trout formed yellow-colored colonies in the TCBS media, exhibited a fermentative characteristic and were sensitive to O/129 vibriostat test. Moreover, their physiological and biochemical characteristics were similar to the characteristics reported by other researchers (1,15,16). Our clinical findings about moribund rainbow trout are also similar to the clinical findings reported in fish infected with *V. anguillarum* (1,5,15).

As indicated by different researchers conducting studies on rainbow trout infected with *V. anguillarum*, hyperemia observed in the liver and degeneration of the epithelium of kidney tubules were observed in this study (5). Unlike this research, melanomacro activity and hemosiderosis in the center of the melanomacrophages were not observed in the present study.

Scientists working on fish health have determined the effect of *V. anguillarum* bacterium on the tissues of different fish species using mostly peroxidase-antiperoxidase and avidine-biotin-alkaline phosphatase methods among immunochemical methods (17,18). The streptavidin-biotin technique used in this study was used by Planas et al. (19) in the

treatment of the disease caused by *Roseobacter* sp. with a probiotic characteristic in the larvae of turbot (*Scophthalmus maximus* L.). The Strep-ABC method is known to be around 5 to 10 times more sensitive than the avidin-biotin complex method when compared to the peroxidase-antiperoxidase and avidin-biotin-peroxidase complex methods (20). Thus, in contrast to other research, an immunochemical method, streptavidin-biotin staining was used for the determination of the presence and identification of the fish pathogen *V. anguillarum* in the moribund rainbow trout samples with this study.

In spite of the fact that the observation of immunopositive reactions in the liver tissues of all infected rainbow trout in sinusoids and necrotic areas in this study is similar to the findings obtained by other researchers in moribund rainbow trout and turbot infected with *V. anguillarum*, widespread immunopositive reaction was observed in liver tissues, especially in the cells around the artery (19,21). Furthermore, it was noted that immunopositive reactions in the tissues of trout infected with *V. anguillarum* was intense only in kidney tubules (19), similar to the results of previous studies, and that melanomacrophage centers received this stain as indicated by Mutoloki et al. (22). Immunopositive reactions in the examined spleen tissue sections was determined in sinus and necrotic areas, similar to the result of the study conducted by Avcı et al. (17). However, immunopositive reactions were also found in areas near the vein in spleen tissues, in contrast to previous studies.

In this study, the presence of immunopositive reactions in cells near the veins in the spleen and liver tissues is an indication that, as in the turbot, these bacteria are transported from the lamina propria of the intestine to the liver and other viscera by the blood (21), it may be similar in the rainbow trout. However, the intensive presence of the factor in the gill cells confirms that *V. anguillarum* infects the fish not only through the intestinal tract but also through the gills, as indicated by Laurencin and Germon (21).

As a conclusion, in this study, *V. anguillarum* was isolated from and identified in vibriosis-suspected moribund rainbow trout obtained from different fish farms. By using the streptavidin-biotin staining method, which is among immunohistochemical methods, the presence of this pathogen in the moribund rainbow trout was determined for the first time. It was found out that the factor concentrates in gills, especially in the area near the blood vessels, as well as in the liver, spleen, kidney, and heart, depending on the development of the disease. Due to the high immunosensitivity of this method, it was observed that this method can be used in the diagnosis of the bacterium and in the determination of the entry routes of the pathogen in infected tissues. This method provided a species-specific identification of the agent in a shorter time when compared with the routine microbiological methods. Thus, it is thought that this method can be used in fish-health laboratories as a routine method.

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Conflict of Interest: The authors have no conflict of interest to declare.

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