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CULTURE- AND POLYMERASE CHAIN REACTION-BASED DETECTION OF *Flavobacterium psychrophilum* IN NATURALLY INFECTED RAINBOW TROUT (*Oncorhynchus mykiss* walbaum, 1792) FROM TROUT FARMS

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Abstract: The present study aimed to detect *Flavobacterium psychrophilum* in fish samples collected from rainbow trout (*Oncorhynchus mykiss* Walbaum 1792) farms in the Southeastern Anatolia region of Türkiye by means of bacteriological culture and polymerase chain reaction and to investigate the antibiotic susceptibility of the causative bacteria. A total of 40 trout farms located in Diyarbakır, Adıyaman, Şanlıurfa, and Batman provinces were visited, and 1200 samples were examined. During January and February 30 fish with an average live weight of 200–250 g were collected from each farm. Samples were obtained from the liver, spleen, kidneys, and tissues following macroscopic laboratory examination of the specimens. Antibiotic treatment is the treatment of choice owing to the lack of an effective vaccine in the control of the disease. Therefore, it is important to rapidly identify the bacterial species and investigate its susceptibility to antibiotics. In this study, the causative bacteria were detected in 5 out of 40 farms. The causative bacteria infected the liver, kidney, and tissues. The sensitivity of Enrofloxacin (5 microgram (µg)), Florfenicol (30µg), Neomycin (5µg), Amoxicillin (25µg), Oxytetracycline (30µg), Erythromycin (10µg), Gentamycin (5µg), Streptomycin (5µg) and Nalidixic acid (10µg) were defined at chancing ratios. In conclusion, these bacteria were detected in regional farms, which should minimize the stress factors by avoiding overstocking and following the required hygiene rules.

Keywords: Antibiotic susceptibility, Bacterial cold-water disease, *Flavobacterium psychrophilum*

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1. Introduction

In recent years, the production quantity, species diversity, and economic value of international aquaculture have increasingly improved. Rainbow trout (*Oncorhynchus mykiss*) is an important salmonidae species farmed worldwide (FAO, 2020). In Türkiye, the rainbow trout production has become a pioneer in the aquaculture sector. Trout is the most important fish species with an output of 127,905 tons (TUİK, 2021). Bacterial-origin fish diseases are one of the factors associated with serious economic losses in aquaculture farms (Özcan, 2022). The severity of the disease varies by the age and species of the fish. Nevertheless, many infections in fish can be successfully treated. However, adverse environmental conditions complicate the efforts or may even make it impossible to protect fish from disease and to take control measures (Buckley et al., 1998). Cold water disease (*Flavobacterium psychrophilum*) is among the primary bacterial diseases in rainbow trout that are associated with economic losses (Austin and Austin 2016). *F. psychrophilum* occurs in the natural flora of the skin, mucous, fin, gill, and operculum of trout (Nematollahi et al., 2003). The causative factor is a Gram negative, thin, long (0.3–0.75 µm × 1.5–7.5 µm) bacillus with a varying size and morphology depending on its reproductive period (Holt et al., 2012). The virulence of the bacteria gradually increases, and a change in environmental factors leads to infection. The bacteria are rapidly transmitted between fish through

water and contact; therefore, they may cause extremely high mortality in the rainbow trout stock in the aquaculture farms, resulting in significant economic losses (Cipriano and Holt, 2005). The mortality rate varies between 30% and 50% when the water temperature drops below 10°C in pools with a high stock ratio, low circulation, low oxygen, and impaired water quality and without hygiene measures in place. Stress-related factors may also play an effective role in the development and spread of the disease. In addition, bacteria can reproduce in water for up to 4 months (FAO, 2020). The causative factor is an infectious and fatal bacterial disease characterized by lesions of various sizes on the dorsal and caudal fins and on the body surface of the fish, bleeding on the skin, exophthalmos, paleness in the gills and liver, swelling of the abdomen, yellow content covering the intestine, and spleen and liver enlargement (Cipriano and Holt, 2005). Antibiotic treatment is the treatment of choice owing to the lack of an effective vaccine for the control of the disease. Therefore, it is important to rapidly identify the bacterial species and investigate its susceptibility to antibiotics. In the present study, samples were collected from separate aquaculture farms in each province to detect *F. psychrophilum* in trout farming facilities located at Diyarbakır, Şanlıurfa, Adıyaman, and Batman provinces of Türkiye. Cold chain transport was used to transfer the samples to the laboratory. After macroscopic examination, culture and polymerase chain reaction (PCR) was used for bacterial identification, followed by the examination of antibiotic susceptibility.

2. Materials and Methods

In the present study, samples were collected from 40 different commercial rainbow trout farms located in Diyarbakır, Adıyaman, Şanlıurfa, and Batman provinces in southeastern Türkiye, between January and February 2021. Thirty fish with an average live weight of 200–250 g, which were insensitive to feed, immobile, floating on the water surface, and showing signs of disease, including a darkened color, were collected from each farm. The samples were transferred on ice cubes to the laboratory of the Department of Aquaculture and Fish Diseases, Faculty of Veterinary Medicine, Dicle University. The 1200 samples were first macroscopically examined in the laboratory, followed by dissection and examination of the internal organs. Liver, kidney, and tissue samples were collected from the dissected samples and cultivated on a medium to detect bacteria. Anacker-Ordal agar (AO) was employed for bacterial isolation for 5–7 days at 18°C, and the dominant uniform bacterial colonies were purified by streaking onto the AO plates 3 times. The methods used in the identification were slow and time-consuming owing to the difficulties associated with *F. psychrophilum* culture and the need for a prolonged incubation period (3–4 days) (WIklund et al., 2000). Fluorescence polyclonal antibodies and enzyme linked immunosorbent assay techniques are also used to identify the bacteria in the host organism (Rangdale and Way 1995). However, there is a requirement for faster and more sensitive genetic techniques to identify a lower count of *F. psychrophilum* in fish and environmental samples and to determine the epidemiology of the causative factor. PCR, a molecular genetic method, is a recommended technique and has found widespread use for diagnostic purposes (Rocha et al., 2017). The disease usually has a poor prognosis and is associated with high mortality rates. Therefore, there is a requirement for accurate and rapid diagnostic methods to fight the disease. PCR gives fast and accurate results. Nyztech *Flavobacterium psychrophilum* Real Time PCR Kit (Catalog No: MD01331) was used for identification. The Kirby–Bauer Disc Diffusion method, Mueller–Hinton medium, and Bauer et al., 1966 was used for testing antibiotic susceptibility, and the assessment was made by the procedures suggested by the CLSI (2004), Ruangpan and Tendencia (2004), and BDC (2011).

3. Results

The present study used culturing to isolate and identify *F. psychrophilum* in suspected trout samples collected from 40 different rainbow trout (*Oncorhynchus mykiss*) aquaculture farms in Diyarbakır, Batman, Şanlıurfa, and Adıyaman provinces in the southeastern Türkiye. The general clinical picture of the cultivated rainbow trout samples included darkening of their color, paleness of the liver, abdominal swelling, splenic enlargement, bleeding in the fins, wear on the dorsal and adipose fins, bleeding in the jaw, exophthalmos, bleeding in the kidney, and congestion in the liver (Figure 1 and 2).

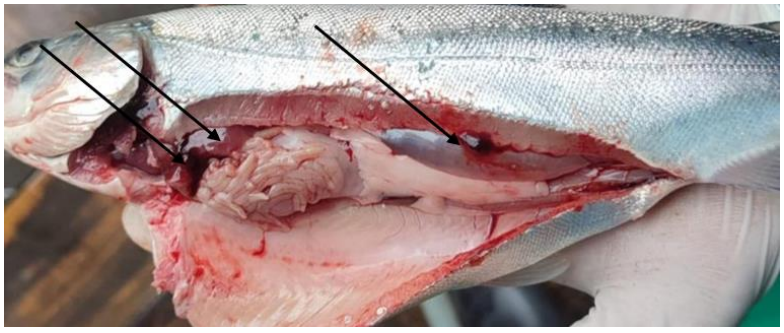

Figure 1. Examination upon autopsy indicated bleeding in the internal organs, paleness of the liver and splenic enlargement.

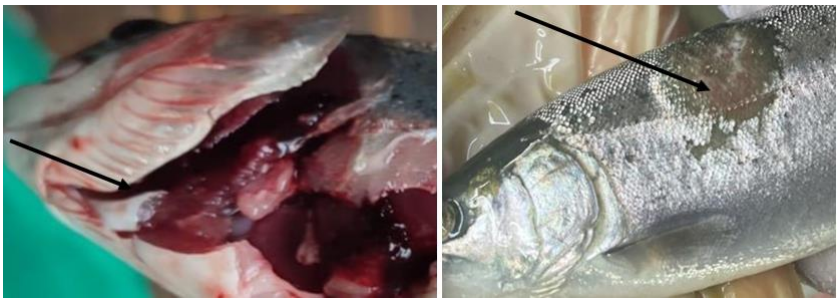

Figure 2. Melting and petechial hemorrhages in the gills, ulcer formation in the dorsal region.

In the present study, the causative bacteria were identified in 5 out of 40 farms upon examination of 1200 samples. The causative bacteria infected the liver, kidney, and tissues. The trout farms in the southeastern Anatolia region were contaminated by *F. psychrophilum* at a rate that ranged from 0% to 100%, and the causative bacteria developed different levels of resistance to antimicrobials. In the present study, the bacteria were isolated at a higher rate in farms with excessive water and overstocking. The antibiogram found that the causative bacteria were susceptible to enrofloxacin, florfenicol amoxicillin, and oxytetracycline (Table 1).

Table 1. Antimicrobial susceptibility of *Flavobacterium psychrophilum*

Antimicrobial agent	Isolate 1	Isolate 2	Isolate 3
Enrofloxacine (5µ)	S	S	S
Florfenicol (30µ)	S	S	S
Neomycine (5µ)	R	R	R
Amoxicillin (25µ)	S	S	S
Oxytetracycline (30µ)	S	S	S
Erythromycin (10µ)	R	R	R
Gentamycine (5µ)	R	S	R
Streptomycine (5µ)	R	R	R
Nalidixic acid (10µ)	R	R	R

S= susceptible, R= resistant

4. Discussion

The morbidity rates were considerably high in the intensive aquaculture farms owing to the increased output. High stocking density and changes in water quality might result in a higher prevalence of bacterial diseases. Today, cold water disease is an enzootic disease in many countries, is associated with severe fish mortality in rainbow trout farms worldwide (Ersoy *et al.,* 2018). *Flavobacterium psychrophilum* grows in the natural flora of the skin, mucous, fin, gill, and operculum of trout (Nematollahi et al., 2003). The virulence of the causative factor may increase and changes in environmental factors, including lower water temperatures, poor quality of water, high stock density, and poor maintenance and feeding may lead to infection. The bacteria are rapidly transmitted between fish through water and contact horizontally, leading to extremely high mortality in rainbow trout stock in the aquaculture farms, resulting in significant economic losses (Cipriano and Holt 2005). The clinical manifestations of *F. psychrophilum* infections in rainbow trout included anorexia, lethargy, darkening of the skin, ascites, bilateral exophthalmos, and periocular bleeding (Yıldırım and Özer 2010). The clinical findings in the present study included the fish immobility, decreased feed consumption, and irregular floating and were consistent with those of previous reports. Many fish had an eroded epidermis. The erosions were transformed into ulcers penetrating deep muscle layers in certain locations (Figure 2). The presence and distribution of bacteria in those ulcers were indicative of the fact that the skin acted as an entry port. Previous studies reported signs of anemia-related paleness, hyperemia, and petechial bleeding in the gills of infected fish (Nematollahi et al., 2003). As a matter of fact, degeneration and eruption were observed in the gills (Figure 2) in the present study. In addition, the spread of infection from these gill lesions might have been a factor that increased the severity of the disease. Furthermore, there was hyperemia in the primary lamella and edema in the secondary lamella in the gills along with degeneration and eruption in the epithelium. The abundant foreign particles in the gills indicated water pollution, which was interpreted as a factor that increased the severity of the disease and disease-associated mortality rate. A 2018, study by Ersoy et al. on the identification of *F. psychrophilum* in rainbow trout samples collected from 14 different farms in the Mediterranean region reported a general clinical picture, including darkening of color, paleness of the liver,

abdominal swelling, splenic enlargement, bleeding in the fins, bleeding in the jaw, exophthalmos, bleeding in the kidney, and hepatic congestion. The macroscopic findings of another study by Epikmen et al. dated 2020 are consistent with the results of the present study. Dead fish were collected from the farms and included in the study. They demonstrated abdominal bloating owing to fluid accumulation, exophthalmos, darkening of the skin color, and few fish demonstrated the presence of white-colored lesions on the back and caudal fins, with the caudal fin completely destroyed along with the appearance of radiuses. The liver and kidneys of the fish were pale, and the fish demonstrated splenic enlargement in cases of advanced disease.

As a matter of fact, these results are consistent with those previously reported in the relevant literature (Wiklund et al., 2000). The antibiogram found that the *F. psychrophilum* strains were susceptible to enrofloxacin, florfenicol amoxicillin, and oxytetracycline and were resistant to neomycin, erythromycin, gentamicin, nalidixic acid, and streptomycin (Table 1). Durmaz et al. (2012) reported that all the strains were susceptible to oxytetracycline and enrofloxacin, and that the antibiotic susceptibility profiles of the strains were varied. Another study by Ersoy et al. reported in 2018 that the strains were susceptible to trimethoprim/sulfametoxazol, clindamycin, ampicillin, tetracycline, enrofloxacin, chloramphenicol, oxytetracycline, fluorophenicol, and tobramycin. These results are consistent with those of the present study.

5. Conclusion

In conclusion, the present study isolated and identified *F. psychrophilum*, the psychrophillic causative bacteria associated with morbidity and heavy economic losses in rainbow trout farms in Türkiye and in many countries across the world, using culturing. Pathogen positive was found in 150 of the samples examined. Among the tested antibiotics, Enrofloxacin (5µ), Florfenicol (30µ), Amoxicillin (25µ) and Oxytetracycline (30µ) were found to be sensitive to the pathogen. However, the isolation of the bacteria is difficult and time consuming. Therefore, PCR, a molecular technique that provides rapid results in the diagnosis of bacterial diseases, was used for the identification of *F. psychrophilum.* Thus, major economic losses suffered by aquaculture farms can be prevented by timely treatment as cultured *F. psychrophilum* can be diagnosed over a relatively short period of time using PCR. Further, the bacteria were identified in trout farms across the southeastern Anatolia region, and it was concluded that more attention should be paid to hygiene and that high stock density should be avoided.

Author Contributions

The percentage of the author(s) contributions is presented below. All authors reviewed and approved the final version of the manuscript.

	F.Ö.	N.B	M.K	K.A	N.K	N.Ö.	B.Ç.
C	40	10	10	10	10	10	10
D	40	10	10	10	10	10	10
S	40	10	10	10	10	10	10
DCP	40	10	10	10	10	10	10
DAI	40	10	10	10	10	10	10
L	40	10	10	10	10	10	10
W	40	10	10	10	10	10	10
CR	40	10	10	10	10	10	10
SR	40	10	10	10	10	10	10
PM	40	10	10	10	10	10	10
FA	40	10	10	10	10	10	10

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval is not required because of this study used rainbow trout obtained from commercial farms as experimental material.

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