

## Antimicrobial Susceptibility of *Flavobacterium psychrophilum* Isolated from Rainbow Trout (*Oncorhynchus mykiss*)

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Research Article

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### Abstract

This study was conducted to determine antibiotic susceptibility of *Flavobacterium psychrophilum* isolated in diseased rainbow trout (*Oncorhynchus mykiss*) from two hatcheries in the Mediterranean region of Turkey. The samples were taken from the kidney and spleen for bacteriology from rainbow trout juveniles (weight 0.7-1 g) in April and isolates obtained on tryptone yeast extract salts (TYES) agar medium at 15°C for 72 hours. Fourteen isolates were identified as *F. psychrophilum* using biochemical, physiological, and morphological characteristics and API ZYM test. Antibiotic sensitivities of the isolates were determined by the disc diffusion method on TYES agar, and the results were evaluated according to CLSI standards. Only one isolate from each hatchery was used for the antibiogram test. Both isolates were sensitive to amoxicillin/clavulanic acid 2:1, penicillin, nitrofurantoin, florfenicol, amoxicillin, clindamycin, chloramphenicol, ampicillin, doxycycline, streptomycin, and erythromycin; resistant to sulfadiazine, sulfamethoxazole/trimethoprim, oxolinic acid, vancomycin, trimethoprim/sulfadiazine, gentamicin, kanamycin, nalidixic acid, oxacillin, enrofloxacin, flumequine, tobramycin. The results show that isolating *F. psychrophilum* and performing the antimicrobial susceptibility testing is important issue in disease control.

**Keywords:** antibiotics resistance, RTFS, hatcheries, rainbow trout fry.

### Gökkuşuğu Alabalıklarından (*Oncorhynchus mykiss*) İzole Edilen *Flavobacterium psychrophilum* İzolatlarının Antimikrobiyal Duyarlılığı

#### Özet

Bu çalışma, Türkiye'nin Akdeniz bölgesindeki iki kuluçkahaneden temin edilen hasta gökkuşuğu alabalıklarından (*Oncorhynchus mykiss*) izole edilen *Flavobacterium psychrophilum*'un antibiyotik duyarlılığını belirlemek amacıyla yapılmıştır. Nisan ayında hasta gökkuşuğu alabalığı yavrularının (ağırlığı 1-2 g) böbrek ve dalağından alınan örneklerlemelerde tryptone yeast extract salts (TYES) agar besiyerine ekim yapılarak 15°C de 72 saat inkübe edilerek izolatlar elde edilmiştir. Biyokimyasal, fizyolojik ve morfolojik özellikler ve API ZYM testi kullanılarak 14 izolat *F. psychrophilum* olarak tanımlanmıştır. İzolatların antibiyotik duyarlılıkları TYES agarda disk difüzyon yöntemi ile belirlenmiş, sonuçlar CLSI standartlarına göre değerlendirilmiştir. Her kuluçkahaneden sadece bir izolat antibiyogram testi için kullanılmıştır. Her iki izolat da amoksisilin / klavulanik asit 2:1, penisilin, nitrofurantoin, florfenikol, amoksisilin, klindamisin, kloramfenikol, ampisilin, doksisisiklin, streptomisin ve eritromisine duyarlı; sülfadiazin, sülfametoksazol / trimetoprim, oksolinik asit, vankomisin, trimetoprim / sülfadiazin, gentamisin, kanamisin, nalidiksik asit, oksasilin, enrofloksasin, flumequine, tobramisin dirençlidir. Sonuçlar, *F. psychrophilum*'un izole edilerek antimikrobiyal duyarlılık testinin yapılmasının hastalıkla mücadelede de önemli olduğunu göstermektedir.

**Anahtar Kelimeler:** Antibiyotik direnci, RTFS, kuluçkahane, yavru gökkuşuğu alabalığı.

### INTRODUCTION

The Gram-negative bacterium, *Flavobacterium psychrophilum*, causing bacterial cold water disease (BCWD) and rainbow trout fry syndrome (RTFS) has been seen in mostly freshwater salmonids. During the last twenty years, RTFS has become one of the most serious bacterial diseases

in rainbow trout (*Oncorhynchus mykiss*, Walbaum) hatcheries in many parts of Europe. Outbreaks of diseases caused by *F. psychrophilum* are treated with antibiotics. A limited number of commercial vaccines are currently available in Chile and Norway but they are not suitable for fry fish (Madetoja, 2002; Gómez et al., 2014; Boyacioglu et al., 2015; Hoare et al., 2017). The only course of action which occurs in fry is the antibiotic treatment which has led to increased levels of antibiotic resistance (Henríquez-Núñez et al., 2012, Wahli and Madsen, 2018, Saticioglu et al., 2019).

In recent years, RTFS is causing mortality rates between 10% and 30%. Also, the cumulative mortality rate of fries found to be up to 70% in Turkey (Gultepe and Tanrikul, 2006; Kubilay et al., 2009; Özcan and Sarieyyüpoğlu, 2014; Boyacioglu et al., 2015). Previously, oxytetracycline and chlortetracycline were the only antimicrobial agents licensed for use in aquaculture. Currently, the use of oxytetracycline has dropped about 55% and 75% to treat flavobacteriosis disease in fish farms in the western Aegean region (Kum et al., 2008; Boyacioglu and Akar 2012). Nowadays, oxolinic acid and florfenicol antibiotics are licensed for use in fish. Today, florfenicol is the drug of choice. Until now, the resistance of *F. psychrophilum* to florfenicol has scarcely been reported in Turkey (Kum et al., 2008; Saticioglu et al., 2019).

Several studies carried out in different countries have also shown the differences in resistance profiles among *F. psychrophilum* isolates. Valdebenito and Avendano-Herrera (2009) found that *F. psychrophilum* isolates from Chile were resistant to sulphamethoxazole+trimethoprim but highly sensitive to amoxicillin. In Denmark, *F. psychrophilum* isolates were resistant to amoxicillin (11.6%), oxolinic acid (65.9%), oxytetracycline (67.7%), and sulphamethoxazole+trimethoprim (98.2%) (Bruun et al. 2000). In another study, Del Cerro et al. (2010) found that *F. psychrophilum* isolates originating from Spain were found resistant to oxytetracycline. Özcan and Sarieyyüpoğlu (2013), evaluated the antibacterial susceptibility of resistance profiles strains and found that strains sensitive to oxytetracycline, florfenicol, enrofloxacin, and ciprofloxacin. Among the studies, it is seen that much more variations on the antimicrobial resistance profile of *F. psychrophilum* that is the causative agent of RTFS.

This study aimed to determine the antimicrobial susceptibility of *F. psychrophilum* isolated from sick rainbow trout fries from two hatcheries in the Mediterranean region of Turkey.

## **MATERIALS and METHODS**

### **Samples Bacterial Isolation and Identification**

Fish samples were obtained from two hatcheries (F1 and G1) in the Mediterranean region of Turkey. Tissue samples for bacteriology were taken from rainbow trout fries which showed signs of diseases in April. Fish weighted 0.7-1 g and a total of 14 fish were examined. The samples were collected from the kidney and spleen and streaked onto rypstone yeast extract salts (TYES) agar (Holt et al., 1994). Agar plates were incubated at 15°C for 72 hours. Isolates were identified by using biochemical, physiological, and morphological characteristics by following the guidelines for the diagnosis of fish diseases and the international and national guidelines for animal welfare (OIE, 2003; Buller, 2004; Austin and Austin, 2016). and a rapid identification kit by using API ZYM test (Austin and Austin, 2016).

### **Antimicrobial Susceptibility Testing**

Antimicrobial sensitivities of the isolates were determined by the disc diffusion method on TYES agar. Only one isolate from each hatchery (F1, G1) was used for the antibiogram test. Antibiotic discs (Oxoid, England) (Total 24) were used for determining the resistance profiles. The results were evaluated according to guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2014). Briefly, TYES broth was used to prepare bacterial suspensions. The turbidity of suspensions was adjusted to McFarland 0.5 and 100 µl aliquots were spread over TYES agar surface. Antibiotic discs were placed on the surface of the inoculated agar plates and the plates were incubated at 15°C for 5 days. After the incubation period, the inhibition zone diameters were measured and the results were evaluated according to the CLSI, 2014 and Van Vliet et al., 2017.

## **RESULT and DISCUSSION**

In this study, sick fish samples (14 fish) were obtained from two hatcheries (F1 and G1) in April from farms where the water temperature at (F1) was 8 °C and another hatchery (G1) had 13 °C and the fries infected with RTFS showed signs of diseases such as dorsal fin erosion, darkening body, and

splenomegaly (Figure 1). The result of the rapid identification test API ZYM on the isolates (F1 and G1) showed the pathogen as *F. psychrophilum* (Figure 2).

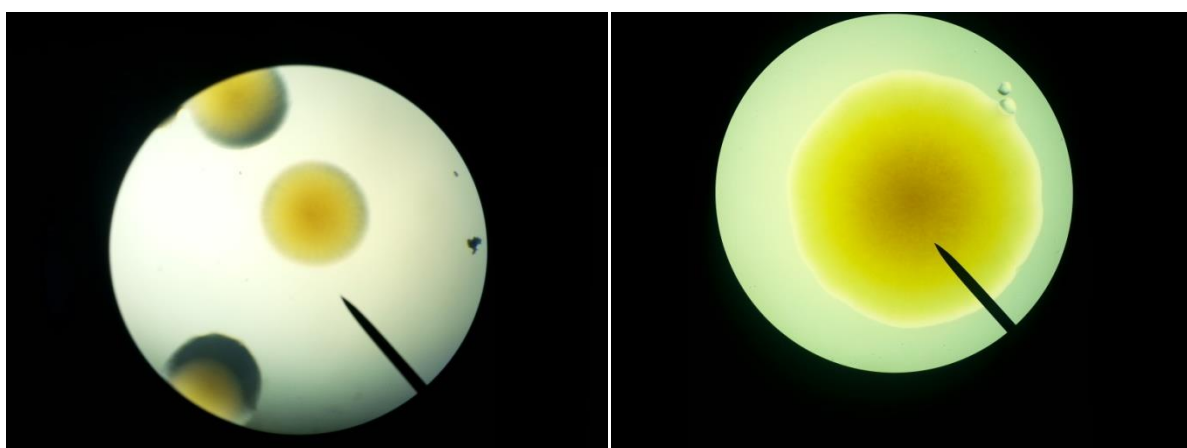


**Figure 1.** Dorsal fin deep erosion (left) and splenomegaly (right) in rainbow trout fries with RTFS



**Figure 2.** Result of API ZYM test for *F. psychrophilum*

The improvement of antimicrobial resistance by *F. psychrophilum* could be a concern since outbreaks regularly require the utilize of antimicrobials. During outbreaks, *F. psychrophilum* strains (Figure 3) were identified in fries of rainbow trout in the hatcheries. Both isolates from rainbow trout were sensitive to amoxicillin/clavulanic acid 2:1, penicillin, nitrofurantoin, florfenicol, amoxicillin, clindamycin, chloramphenicol, ampicillin, doxycycline, streptomycin, erythromycin, and tetracycline. The isolates were resistant to sulfadiazine, oxolinic acid, vancomycin, nalidixic acid, oxacillin, and tobramycin. While the examined isolates were sensitive to florfenicol, they were resistant to sulfonamides. The results indicate that antimicrobial susceptibility testing of *F. psychrophilum* is an important issue to be able to fight RTFS disease outbreaks.



**Figure 3.** Typical yellow colonies of *F. psychrophilum* on TYES agar

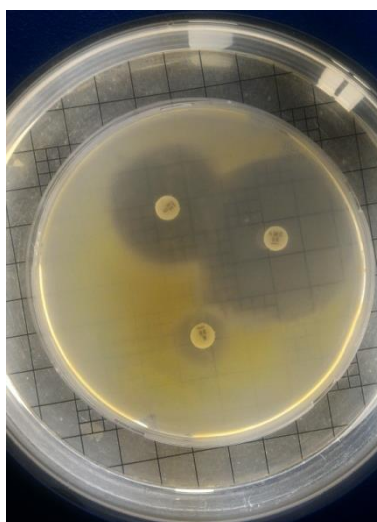
The results of the antibiotic susceptibility test in both *F. psychrophilum* isolates were found to be susceptible to 12, intermediate to 3, and resistant to 5 out of 24 examined antibiotics (Table 1). While, isolate F1 showed resistance against trimethoprim/sulfadiazine and kanamycin, isolate G1 was

susceptible to these antibiotics (Figure 4). The reason for it might be the developing resistance of the bacteria to these antibiotics in the hatchery F1. Saticioglu et al. (2019) reported that all isolates of *F. psychrophilum* were susceptible to amoxicillin and florfenicol.

**Table 1.** Antibiotic susceptibility profiles of *F. psychrophilum* isolates

Antibiotic Disc	Isolate, F1	Isolate, G1
Sulfadiazine (25µg)	R (0 mm)	R (0 mm)
Florfenicol (30µg)	S (53mm)	S (57mm)
Sulphamethoxazole/Trimethoprim (25µg)	I (10mm)	I (14mm)
Chloramphenicol (30µg)	S (47mm)	S (43mm)
Ampicillin (10µg)	S (38mm)	S (39mm)
Erythromycin (15µg)	S (22mm)	S (24mm)
Oxolinic acid (2µg)	R (0 mm)	R (0 mm)
Streptomycin (10µg)	S (28mm)	S (35mm)
Vancomycin (30µg)	R (0 mm)	R (13mm)
Trimethoprim/ Sulfadiazine (30µg)	R (0 mm)	S (25mm)
Gentamycin (10µg)	I (13mm)	S (15mm)
Nitrofurantoin (300µg)	S (57mm)	S (53mm)
Doxycycline (30µg)	S (41mm)	S (34mm)
Kanamycin (30µg)	R (0 mm)	S (20mm)
Nalidixic Acid (30µg)	R (0 mm)	R (0 mm)
Amoxicillin (25µg)	S (53mm)	S (55mm)
Penicillin (10µg)	S (57mm)	S (60mm)
Clindamycin (2µg)	S (47mm)	S (44mm)
Oxacillin (1µg)	R (0 mm)	R (0 mm)
Enrofloxacin (5µg)	I (15mm)	I (20mm)
Flumequin (30µg)	I (20mm)	I (10mm)
Tetracycline (30µg)	S (38mm)	S (30mm)
Tobramycin (30µg)	R (0 mm)	R (0 mm)
Amoxicillin/Clavulanic acid 2:1 (30µg)	S (60mm)	S (60mm)

R, Resistance; S, Susceptible; I, Intermediate



**Figure 4.** Susceptibility test of *F. psychrophilum* against Amoxicillin/Clavulanic acid 2:1 (S), Chloramphenicol (S) and Oxacillin (R)

Many studies have been performed to determine the antibiotic resistance profiles of *F. psychrophilum* in various regions of Turkey and quite variable profiles have been observed. Balta (1997) recorded that *F. psychrophilum* was sensitive to nitrofurans but resistant to flumequine, sulphonamides, and oxolinic acid. Diler et al. (2003) have reported that two *F. psychrophilum* isolates were sensitive to amoxicillin-clavulanic acid, oxytetracycline, and gentamicin but resistant to trimethoprim. In another study, five *F. psychrophilum* isolates from rainbow trout farms in eastern Anatolia were reported to be sensitive to oxytetracycline, erythromycin, gentamicin, nitrofurantoin, and amoxicillin-clavulanic acid, but resistant to chloramphenicol and penicillin (İspir et al., 2004). Boyacıoğlu (2007) reported that a total of 20 *F. psychrophilum* strains isolated from an outbreak in Muğla province were resistant to ampicillin (95%), sulphamethoxazole (95%), erythromycin (45%), and oxytetracycline (20%) but sensitive to enrofloxacin (100%). In these studies, different techniques were used to evaluate antibiotic resistance profiles. In the Mediterranean region, 13 *F. psychrophilum* strains isolated from different outbreaks were resistant to enrofloxacin (23%), amoxicillin (46.2%), erythromycin (61.5%), kanamycin (30.8%), cefoperazone (46.2%), and oxolinic acid (53.8%) (Didinen et al., 2005). Durmaz et al. (2012), showed that *F. psychrophilum* isolates were resistant to neomycin, ampicillin, amoxicillin, and kanamycin. Furthermore, 80%, 40%, 20%, and 20% of these isolates were resistant to erythromycin, cefoperazone, oxolinic acid, and sulphamethoxazole+trimethoprim, respectively. However, all the strains were 100% sensitive to oxytetracycline and enrofloxacin. It was considered that these variable resistance profiles may be due to the widespread use of different antibiotics or to differences among the strains. Several studies carried out in different countries have also shown the differences in resistance profiles among *F. psychrophilum* isolates. Valdebenito and Avendano-Herrera (2009) found that *F. psychrophilum* isolates from Chile were resistant to sulphamethoxazole+trimethoprim but highly sensitive to amoxicillin. 387 *F. psychrophilum* isolates were resistant to amoxicillin (11.6%), oxolinic acid (65.9%), oxytetracycline (67.7%), and sulphamethoxazole+trimethoprim (98.2%) in Denmark (Bruun et al., 2000). In another study, Del Cerro et al. (2010) found that 25 *F. psychrophilum* isolates originating from Spain were found resistant to oxytetracycline (>80%). The findings of the present study were not in agreement with those mentioned above. However, Rangdale et al. (1997), reported identical results to the current study concerning sensitivity to enrofloxacin. These differences in antibiotic resistance profiles of *F. psychrophilum* might be due to the use of different techniques or isolated strain variations. Ngo et al. (2018) determined the antimicrobial susceptibilities of 133 *F. psychrophilum* strains. They found a high categorical agreement between the classifications of the isolates for florfenicol (100%), oxytetracycline (93%), and oxolinic acid (99%). Mwega et al. (2020), reported that assessing the antimicrobial susceptibility of Flavobacteriaceae family members isolated from Nile tilapia (*Oreochromis niloticus*). The antimicrobial susceptibility of 67 Flavobacteriaceae isolates originating mainly from ponds and Lake Victoria against 19 antimicrobial agents was determined. Overall, most isolates were found to be susceptible to enrofloxacin (97%). Özcan and Sarıeyüpoğlu (2013), found *F. psychrophilum* strains were sensitive to oxytetracycline, florfenicol, enrofloxacin, and ciprofloxacin. These results are similar to our results for florfenicol (sensitive) but different for enrofloxacin (intermediate).

In conclusion, *F. psychrophilum* was isolated from commercial rainbow trout hatcheries in the Mediterranean region of Turkey. To control *F. psychrophilum* infections and to ensure the rational use of antibiotics, it is important to run the antibiotic susceptibility tests by using standard methods (CLSI, 2014). It is concluded that the most critical point is *F. psychrophilum* antimicrobial resistance patterns should be determined to treat the disease in farms in each region and country.

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