

1-1-2015

## Comparison of in vitro and in vivo antibacterial efficacy for the control of *Flavobacterium psychrophilum* in rainbow trout (*Oncorhynchus mykiss*) fry: the first genotypical evidence in West Aegean region of Turkey

MURAT BOYACIOĞLU

CAVİT KUM


ŞÜKRÜ KIRKAN

SELİM SEKKİN

UĞUR PARIN

*See next page for additional authors*

Follow this and additional works at: <https://journals.tubitak.gov.tr/veterinary>

 Part of the [Animal Sciences Commons](#), and the [Veterinary Medicine Commons](#)

### Recommended Citation

BOYACIOĞLU, MURAT; KUM, CAVİT; KIRKAN, ŞÜKRÜ; SEKKİN, SELİM; PARIN, UĞUR; KARADEMİR, ÜMİT; and AKAR, FERDA (2015) "Comparison of in vitro and in vivo antibacterial efficacy for the control of *Flavobacterium psychrophilum* in rainbow trout (*Oncorhynchus mykiss*) fry: the first genotypical evidence in West Aegean region of Turkey," *Turkish Journal of Veterinary & Animal Sciences*: Vol. 39: No. 3, Article 10. <https://doi.org/10.3906/vet-1502-36>

Available at: <https://journals.tubitak.gov.tr/veterinary/vol39/iss3/10>

This Article is brought to you for free and open access by TÜBİTAK Academic Journals. It has been accepted for inclusion in Turkish Journal of Veterinary & Animal Sciences by an authorized editor of TÜBİTAK Academic Journals. For more information, please contact [academic.publications@tubitak.gov.tr](mailto:academic.publications@tubitak.gov.tr).

---

**Comparison of in vitro and in vivo antibacterial efficacy for the control of *Flavobacterium psychrophilum* in rainbow trout (*Oncorhynchus mykiss*) fry: the first genotypical evidence in West Aegean region of Turkey**

**Authors**

MURAT BOYACIOĞLU, CAVİT KUM, ŞÜKRÜ KIRKAN, SELİM SEKKİN, UĞUR PARIN, ÜMİT KARADEMİR, and FERDA AKAR

## Comparison of in vitro and in vivo antibacterial efficacy for the control of *Flavobacterium psychrophilum* in rainbow trout (*Oncorhynchus mykiss*) fry: the first genotypical evidence in West Aegean region of Turkey

Murat BOYACIOĞLU<sup>1\*</sup>, Cavit KUM<sup>1</sup>, Şükrü KIRKAN<sup>2</sup>, Selim SEKKİN<sup>1</sup>, Uğur PARIN<sup>2</sup>, Ümit KARADEMİR<sup>1</sup>, Ferda AKAR<sup>1</sup>

<sup>1</sup>Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Adnan Menderes University, Aydın, Turkey

<sup>2</sup>Department of Microbiology, Faculty of Veterinary Medicine, Adnan Menderes University, Aydın, Turkey

Received: 11.02.2015

Accepted/Published Online: 26.03.2015

Printed: 10.06.2015

**Abstract:** The aim of this study was to investigate the presence of *Flavobacterium psychrophilum* and to examine the in vitro and in vivo efficacy of enrofloxacin (ENR), doxycycline (DOX), and florfenicol (FFC) treatment in rainbow trout fry (*Oncorhynchus mykiss* Walbaum) against *F. psychrophilum*. A total of 220 naturally infected rainbow trout fry were examined and 26 bacterial isolates were phenotypically and genotypically identified. Minimum inhibitory concentrations (MICs) of ENR, DOX, and FFC were determined. ENR was the most active, with an MIC for 90% of the isolates (MIC<sub>90</sub>) of 4 µg/mL. DOX was less active, with an MIC<sub>90</sub> of 32 µg/mL for isolates. Approximately 25,000 naturally infected fry were divided as the control, ENR, DOX, and FFC group. ENR and FFC groups were given 10 mg kg<sup>-1</sup> day<sup>-1</sup> for 10 days, and DOX was given at 30 mg kg<sup>-1</sup> day<sup>-1</sup> on specified days. Among the isolates, 12% were resistant to ENR, 38% were resistant to DOX, and 19% were resistant to FFC. Despite these in vitro results, ENR and DOX were not effective in rainbow trout fry syndrome treatment. FFC reduced the mortality when compared with the other groups (P < 0.001). This study indicated that in vivo and in vitro results are different from each other.

**Key words:** *Flavobacterium psychrophilum*, rainbow trout fry, polymerase chain reaction, antimicrobial efficacy, in vitro, in vivo

### 1. Introduction

*Flavobacterium psychrophilum* (previously known as *Cytophaga psychrophila* and *Flexibacter psychrophilus*) is the causative agent of bacterial coldwater disease in larger fish, seen primarily in salmonids. Worldwide, the disease in fry is called rainbow trout fry syndrome (RTFS), and for 20 years it has caused serious problems in rainbow trout (*Oncorhynchus mykiss* Walbaum) hatcheries (1,2). Outbreaks of the disease typically occur in water at temperatures below 14 °C.

Methods for prevention of RTFS are limited, and no commercial vaccine is yet available. Several measures (equipment and egg disinfection, hygiene practice, promotion of immunization) can help to reduce the problem, but contamination cannot be excluded (3). *Flavobacterium psychrophilum* infection is still treated with antibiotics. Different pharmacological agents have been evaluated for the prevention and treatment of RTFS in trials (4,5).

In recent years, RTFS is considered to be one of the most serious bacterial fish diseases, and it has reported mortality rates in the range of 10% to 30% and a cumulative

mortality rate of up to 70% in rainbow trout fries in Turkey (6,7). In Turkey, previously, oxytetracycline (OXT) and chlortetracycline were the only antimicrobial agents licensed for use in aquaculture. Currently, the efficacy of OXT has dropped as described in between 55% and 75% of *F. psychrophilum* isolated from Turkish fish farms, especially in the West Aegean region in Turkey (5,8), and oxolinic acid (OXA) and florfenicol (FFC) are licensed for treatment of fish disease. Today, FFC is the drug of choice. Until now, resistance of *F. psychrophilum* to FFC has scarcely been reported in Turkey. Other antimicrobial agents can be requested by a veterinarian obtaining authorization for treatment of a certain disease outbreak in a specific fish farm. Traditionally, OXT incorporated into fish feed has been the drug of choice for treatment of RTFS and columnaris disease (9). Progressively, the efficacy of OXT has dropped in some countries and alternative agents for treating this serious disease have been found. Amoxicillin (AMC) gave faster and more consistent results, i.e. mortality ceased and the infection did not reemerge. For the last decade, fish farms have reported that OXT, AMC, and OXA have become less effective in

\* Correspondence: mboyacioglu@adu.edu.tr

the field, and FFC has become the new agent of choice for the treatment of RTFS (10,11).

To the best of our knowledge, there is no information available on both in vitro and in vivo evaluation of antibacterial activity for the control of RTFS caused by *F. psychrophilum* in rainbow trout fry. Previous investigations of antimicrobial resistance in *F. psychrophilum* only dealt with the in vitro aspect (2,9). Various methods and different media (broth or agar dilutions) have been used to determine antimicrobial susceptibility and classify bacterial isolates as resistant or effective (in some cases intermediate) (12,13). Many antibiotics are effective in vitro. However, the results have not been corrected by in vivo testing (14,15) as depending on early intervention and the aquatic environment, such as water temperature, pH, feed-drug intake amount, use of repeated drugs, or fish density (16). In addition, when the distribution of aquaculture production is examined by types, trout (inland water) has the highest production rate in aquaculture with 52.6% in Turkey (17). RTFS plays an important role in this respect. In the present study, we performed in vitro trials to investigate the sensitivity of *F. psychrophilum* to a wide range of enrofloxacin (ENR), doxycycline (DOX), and FFC agents using an agar dilution method, with the aim of identifying compounds that may be of use in vivo in treating RTFS in this fish.

## 2. Materials and methods

### 2.1. Samples and bacterial isolation

Severe acute infections occurred in four rainbow trout hatcheries of in the West Aegean region of Turkey. Water composition was as follows: temperature =  $9.4 \pm 1.2$  °C; dissolved oxygen =  $10.1 \pm 0.3$  mg/L; pH =  $6.5 \pm 0.2$ . A total of 220 rainbow trout fries (weight 2–3 g, length 2–3.5 cm) with doubtful symptoms of *F. psychrophilum* infection such as failure to feed, spiral swimming, dark skin, spinal column deformities, and dorsal fin white spots or lysis were used as diagnostic samples. The living animals were carried in containers containing fresh water, and the dead were stored on ice in cold boxes.

For isolation of bacteria, samples were taken from the kidneys, liver, spleen, and brain of all fish and from observed pathological lesions in the sick fish fries. *Cytophaga* agar was used for bacterial isolation, and plates were incubated aerobically at 15–20 °C for 48–96 h. After incubation, the yellow-pigmented colonies were stained using the Gram staining technique, and gram-negative isolates were observed under light microscope (18). The experimental protocol was approved by the Animal Ethics Committee of Adnan Menderes University (2013/113).

### 2.2. Identification of *F. psychrophilum*

Pure cultures of the isolated bacteria were identified based on colony morphology, Gram staining, and some

biochemical and fermentation tests (Table 1) according to the methods of Koneman et al. (18). The API ZYM (bioMérieux, Marcy l'Etoile, France) test was performed and the strips were incubated at 15–20 °C for 16–20 h. The *F. psychrophilum* NCIMB (National Collection of Industrial, Marine, and Food Bacteria) 1947 strain was used as the control bacterium.

### 2.3. Isolation of bacterial DNAs

The bacterial chromosomal DNA used in PCR assays was extracted using the Genomic DNA Extraction Kit (MBI Fermentas) according to the manufacturer's instructions. Purified DNA was dissolved in 100 µL of distilled water and then stored at –20 °C until use.

#### 2.3.1. Primer and PCR amplification conditions

The extracted DNA was amplified using an oligonucleotide primer set specific for *F. psychrophilum*. The sequence of the two primers was designed as FLV-1 (5'-CTT AGT TGG CAT CAA CAC-3') and FLV-2 (5'-ACA CTG GCA GTC TTG CTA-3'). The controls consisted of a polymerase chain reaction (PCR) mixture without DNA template (negative control), and with DNA extracted from *F. psychrophilum* NCIMB 1947 (positive control). PCR was performed in an Eppendorf Master Cycler (Eppendorf AG, Hamburg, Germany) with a thermal cycling capacity of 25 samples. PCR conditions were set according to del Cerro et al. (19).

The PCR products were electrophoresed in 2% agarose gel (Bio-Rad Laboratories), stained with ethidium bromide, viewed with ultraviolet light, and photographed using the Vilber Lourmat Gel Documentation System (Vilber Lourmat, Germany). Samples were considered positive when a 971-bp PCR product specific for *F. psychrophilum* was detected.

The specificity of the primer set was tested using the cell lysates of the *F. psychrophilum* NCIMB 1947 strain. An amplification product of the expected size (971 bp) was observed for *F. psychrophilum* NCIMB 1947. To determine the sensitivity of the PCR, a suspension of *F. psychrophilum* NCIMB 1947 containing  $6 \times 10^3$  CFU/mL was serially diluted two-fold to 6 CFU/mL, and then 10 µL of each dilution was boiled for 10 min and added directly to the PCR mixture. The bacterial concentration was verified by plating 20 µL of each dilution onto *Cytophaga* agar containing 1.5% agar.

### 2.4. Antimicrobial susceptibility testing

The minimal inhibitory concentration (MIC) values were obtained using an agar dilution method as suggested by the Clinical and Laboratory Standards Institute (20). Mueller Hinton broth (Oxoid) was used for testing of *F. psychrophilum*. The density of the culture was adjusted to a turbidity equivalent to that of a 0.5 McFarland standard (approximately  $10^8$  CFU/mL). Doubling dilutions of antimicrobial stock solutions were incorporated (10 µL)

**Table 1.** Phenotypic characteristics and API ZYM profile of *F. psychrophilum* isolates (n = 26).

| Character                           | Reactions     | Character                       | Reactions |
|-------------------------------------|---------------|---------------------------------|-----------|
| Cell morphology                     | Slender rod   | Acid from (as aerobic)          |           |
| Gram                                | 0/26          | Glucose                         | 0/26      |
| Motility - Gliding movement         | 0/26 - 26*/26 | Fructose                        | 0/26      |
| Catalase                            | 26/26         | Lactose                         | 0/26      |
| Congo red                           | 0/26          | Maltose                         | 0/26      |
| Flexirubin pigment                  | 26/26         | Mannitol                        | 0/26      |
| Hydrogen sulfide (H <sub>2</sub> S) | 0/26          | Saccharose                      | 0/26      |
| Indole                              | 0/26          | API ZYM profile                 |           |
| Methyl red (MR)                     | 0/26          | Acid phosphatase                | 12*/26    |
| Nitrate reduction                   | 0/26          | Alkaline phosphatase            | 26/26     |
| Oxidase                             | 26/26         | Cystine arylamidase             | 0/26      |
| Oxidation-fermentation (O/F)        | 3*/26         | Esterase (C4)                   | 0/26      |
| Simmons' citrate                    | 0/26          | Esterase lipase (C8)            | 26*/26    |
| Urease                              | 0/26          | Lipase (C14)                    | 26*/26    |
| Voges Proskauer (VP)                | 0/26          | Leucine arylamidase             | 26/26     |
| Growth at                           |               | N-Acetyl-β-glucosaminidase      | 0/26      |
| 15 °C                               | 26/26         | Naphthol-AS-BI-phosphohydrolase | 19*/26    |
| 37 °C                               | 0/26          | Trypsin                         | 0/26      |
| Growth in                           |               | Valine arylamidase              | 24*/26    |
| 0% NaCl                             | 26/26         | α-Chymotrypsin                  | 15/26     |
| 0.5% NaCl                           | 26/26         | α-Galactosidase                 | 12*/26    |
| 1.5% NaCl                           | 26/26         | α-Glucosidase                   | 12/26     |
| 3% NaCl                             | 0/26          | α-Mannosidase                   | 0/26      |
| Hydrolysis                          |               | α-Fucosidase                    | 0/26      |
| Casein                              | 26/26         | β-Galactosidase                 | 0/26      |
| Gelatin                             | 26/26         | β-Glucuronidase                 | 0/26      |
| Starch                              | 0/26          | β-Glucosidase                   | 0/26      |

\*: Weak positives included.

into the plates, with final concentrations ranging from 0.0312 to 256 µg/mL, and incubated at 15–20 °C for 48–96 h. ENR (Fluka, 17849), DOX (as doxycycline hyclate; Sigma, D9891), and FFC (Sigma, F1427), which are used in aquaculture, were selected in this study. The antimicrobial agents were dissolved in distilled water (ENR), methanol (DOX), or dimethyl sulfoxide (FFC), and stock solutions were used within 1 h of preparation.

## 2.5. Definition of MIC resistance

Endpoint determinations were performed after 96 h, and the MIC for each strain was determined as the last well or last dilution for which absence of growth of the microorganism was detected with the unaided eye, as well as when compared to the negative control well. The MIC<sub>50</sub>, MIC<sub>90</sub> (minimum concentration of antimicrobial compound required to inhibit 50% and 90%, respectively),

and resistance percentage to each drug was then calculated in relation to the total number of *F. psychrophilum* strains isolated from fish fries.

## 2.6. In vivo treatment

The naturally infected fish fries in four different hatcheries were randomly divided into four main groups, each containing approximately 25,000 fries. These main groups were examined as divided into four pools, each containing 6250 fries for better feed intake and following of antimicrobial efficacy for control of *F. psychrophilum*: Group 1 (control or untreated), Group 2 (10 mg kg bw<sup>-1</sup> day<sup>-1</sup> ENR; Baytril 10% oral solution, Bayer, Turkey), Group 3 (30 mg kg bw<sup>-1</sup> day<sup>-1</sup> DOX; Hipradoxi-S, Gürtav İlaç, Turkey), and Group 4 (10 mg kg bw<sup>-1</sup> day<sup>-1</sup> FFC; Florocol 50% Premix, Schering-Plough Animal Health Corp., UK). These fries, prior to the start of the study, were transferred to the test pools where mean water temperature, dissolved oxygen, and pH levels were determined as 9.2 ± 1.4 °C, 10.4 ± 0.4 mg/L, and 6.6 ± 0.1, respectively. They were fed a commercial pelleted fish feed (control group) or medicated feed (treatment groups) (Bagci Aqua Feed, Turkey) ad libitum (mean total 2% feed) twice a day during the treatment period (10 days). Fries of control and treatment groups were fasted for 12 h. The medicated feeds (ENR, DOX, and FFC) for oral administration were mixed into ordinary fish feed by Bagci Aqua Feed, utilizing the double coating technique at concentrations of 0.66 g of ENR, 2 g of DOX, and 0.66 g of FFC per kilogram of feed. The deaths were recorded daily and dead fish were examined. Compared to the control group, 10 fish from each of the treatment groups underwent gross external and internal examination and microbiological confirmation of RTFS during the treatment period.

## 2.7. Statistics

Pearson chi-square ( $\chi^2$ ) tests were used to compare the groups. All analyses were performed using the SPSS 11.5. Differences were considered statistically significant if  $P < 0.05$ ,  $P < 0.01$ , or  $P < 0.001$ .

## 3. Results

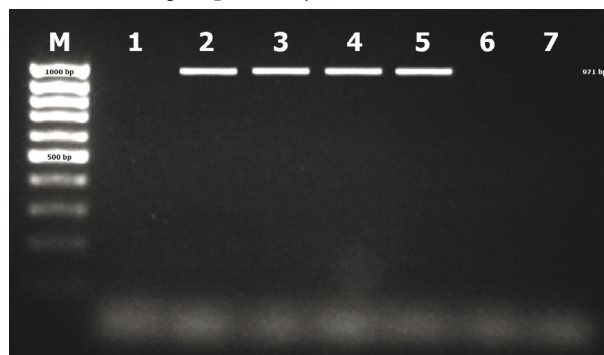
Under laboratory conditions, 26 *F. psychrophilum* strains were identified from 220 fries in outbreaks detected in rainbow trout (*Oncorhynchus mykiss* Walbaum) hatcheries. The number of strains obtained according to the hatcheries are 6, 7, 8, and 5, respectively. These strains were isolated from the kidneys (2.72%,  $n = 6$ ), liver (1.81%,  $n = 4$ ), spleen (2.27%,  $n = 5$ ), and brain (1.81%,  $n = 4$ ) and from observed pathological lesions of the caudal peduncle (5%,  $n = 11$ ). The cultural, biochemical, and physiological characteristics and API ZYM profile tests were used in the identification of *F. psychrophilum* strains (Table 1).

Molecular confirmation of the 26 isolates was performed using an *F. psychrophilum* species-specific

primer set targeting the 16S rRNA genes. The amplification of PCR product of the expected size (971 bp) confirmed the identity of the isolated bacteria as *F. psychrophilum* isolates. All isolates were confirmed as *F. psychrophilum* with PCR (Figure 1). The PCR test was specific for *F. psychrophilum* strains. The amplification of *F. psychrophilum* yielded the expected 971-bp amplicon. The PCR assay had a detection limit of 60–65 cells per milliliter of PCR mixture, assuming that the lysate procedure was completed, since no viable cells were detected after the boiling treatment. This level equals  $6 \times 10^1$  CFU/mL.

MIC values ( $\mu\text{g/mL}$ ) for ENR, DOX, and FFC are presented in Table 2. The MIC values determined by agar dilution method indicated that they were susceptible to 88% ENR, 62% DOX, and 81% FFC in this study. For these antimicrobial agents, the MIC ranges were 0.5 to 64, 0.25 to 64, and 0.25 to 32  $\mu\text{g/mL}$ , respectively, and MIC<sub>50</sub> and MIC<sub>90</sub> values were 2, 2, and 1  $\mu\text{g/mL}$  and 4, 32, and 16  $\mu\text{g/mL}$ , respectively.

The total number of dead fish of the control, ENR, DOX, and FFC groups were 17,240, 10,293, 7701, and 4873, respectively, during the treatment period. Death numbers of experimental groups were significantly different ( $P < 0.001$ ). Death numbers as a function of time in the antimicrobial treatment period are shown in Figure 2. Cumulative mortality rates (%) of the control, ENR, DOX, and FFC groups were 57.47, 34.31, 25.67, and 16.24, respectively, during 10 days. The groups were significantly different when compared according to cumulative mortality ( $P < 0.001$ ). When compared to the treatment groups, the mortality rate was much higher in the control group ( $P < 0.001$ ), although there was no significant difference between control and ENR groups on day 1 ( $P > 0.05$ ). However, there was a significant difference between control and ENR groups on day 2 ( $P < 0.01$ ). FFC reduced the mortality as compared to the other groups after day 5 ( $P < 0.001$ ). There was no significant difference between DOX and FFC groups on days 1, 3, or 6 ( $P > 0.05$ ).

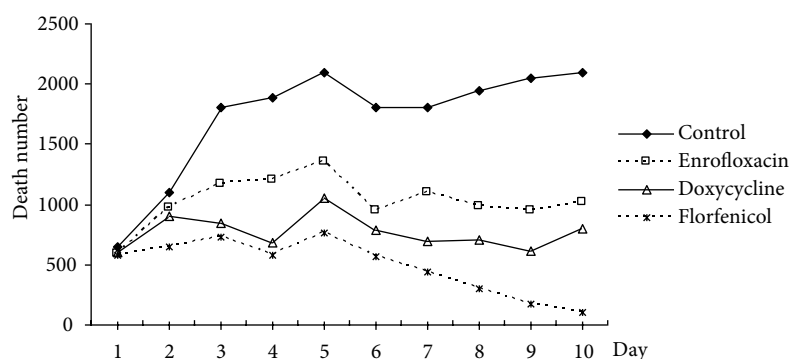


**Figure 1.** *F. psychrophilum* PCR results. M: 100-bp DNA ladder, 1: negative control, 2: *F. psychrophilum* positive control (*F. psychrophilum* NCIMB 1947 strain), 3–5: *F. psychrophilum* PCR positive samples, 6–7: *F. psychrophilum* PCR negative samples.

**Table 2.** Antimicrobial MICs, MIC ranges, MIC<sub>50</sub>, MIC<sub>90</sub>, and resistance rates by agar dilution method for *F. psychrophilum* isolates (n = 26).

| Antimicrobial agents | Number of isolates with MIC ranges (µg/mL) |        |       |      |     |   |   |   |   |    |    |    |     |     |         | MIC range (µg/mL) | MIC <sub>50</sub> (µg/mL) | MIC <sub>90</sub> (µg/mL) | Resistance (%) |
|----------------------|--|--------|-------|------|-----|---|---|---|---|----|----|----|-----|-----|---------|-------------------|---------------------------|---------------------------|----------------|
|                      | 0.0312                                     | 0.0625 | 0.125 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512     |                   |                           |                           |                |
| Enrofloxacin         |  |        |       |      | 1   | 5 | 9 | 8 | 1 |    | 1  | 1  |     |     |         | 0.5–64            | 2                         | 4                         | 12             |
| Doxycycline          |  |        | 1     | 2    | 6   | 4 | 3 |   |   | 5  | 2  | 3  |     |     |         | 0.25–64           | 2                         | 32                        | 38             |
| Florfenicol          |  |        | 5     | 5    | 4   | 4 | 3 | 1 | 3 | 1  |    |    |     |     | 0.25–32 | 1                 | 16                        | 19                        |                |

MIC: Minimum inhibitory concentration; doxycycline was used as doxycycline hyclate.

**Figure 2.** Death numbers as a function of time in the antimicrobial treatment period (10 days).

Compared to the control group, 10 fish from each of the treatment groups underwent gross external and internal examination and microbiological confirmation of RTFS during the treatment period.

There were no specific findings about RTFS in the FFC group after the treatment. Between 4 to 8 fish died every day in this group. Bacteriological examination yielded no *F. psychrophilum* or other fish pathogens. Deaths continued to increase in the control group and the other treatment groups (ENR and DOX) after the treatment. For this purpose, 10 surviving fish in each raceway were bacteriological examined for 5 days. Sixteen isolates that were phenotypically identified as *F. psychrophilum* were cultured from the control group (4 isolates from the caudal peduncle, 2 isolates from the spleen, 1 each from the brain and liver), ENR group (1 each from the spleen, kidney, and brain), and DOX group (2 isolates from spleen, 2 isolates from brain, 1 from kidney).

#### 4. Discussion

The aim of this study was to demonstrate the presence of *F. psychrophilum* and the antibacterial susceptibility and effective antibacterial treatment in RTFS. FFC was the most active among the antimicrobial agents used in this study.

The improvement of the environment and the use of recommended doses of antibacterials have shown benefits in controlling an outbreak of RTFS (21). In vitro antibiotic resistances vary according to geographic region in Turkey. Didinen et al. (6) reported that 13 *F. psychrophilum* isolates were determined from the Mediterranean region of Turkey, and the isolates were susceptible to DOX (100%) and ENR (76.9%). Five *F. psychrophilum* isolates, which were isolated from the Middle and Eastern Black Sea Regions of Turkey, were found to be susceptible (100%) to OTC and ENR (22). In our study, 26 isolates of *F. psychrophilum* were susceptible to 88% ENR, 62% DOX, and 81% FFC. There are limited data on antimicrobial efficacy investigations in vivo against many species of marine bacteria in Turkey. In this study, antimicrobial agents currently used in veterinary and aquaculture therapy were tested, and they included ENR, DOX, and FFC. No similar in vivo studies have apparently been performed on fish infected with *F. psychrophilum*.

In the FFC group, 4–8 fish died after the treatment. This situation may be related to a decrease in food intake. The fact that the fishes receiving ENR and DOX died could be related to RTFS, which is the etiological agent of *F. psychrophilum*. The most significant symptoms in fries were dark skin, spiral swimming, spinal column deformities, dorsal fin white spots or lysis, and failure to

feed after the treatment in these groups. The results of the present study are in agreement with those of other researchers (11).

Authors reported successful use of ENR in field outbreaks of RTFS in in vitro investigations (8,14), but no correlation between MIC values and in vivo efficacy has been done. This study shows that this theoretical approach can be verified in vivo, at least in this treatment setting. Although susceptibility rates of ENR were higher than those of other antimicrobial agents, ENR did not reduce mortality in the current study. DNA gyrase (*gyrA*) is an important target for quinolones in *F. psychrophilum* infection and is transferred vertically in bacterial strains (23). Because of development of bacterial resistance (quinolone), ENR is used infrequently in the aquaculture industry.

Tetracyclines, especially OTC, are among the therapeutic agents most commonly used in aquaculture and veterinary treatment. Because of the widespread use of tetracyclines, resistance to it has disseminated to many species of marine bacteria, as noted in the present study for DOX. This drug also has greater plasma protein binding than the other tetracyclines, which produces a prolonged half-life of the drug in humans and animals. Nevertheless, and despite its higher lipophilicity and general recognition as the most potent agent (especially true for DOX and minocycline) in this class, and thus strong capacity for penetrating tissues (24,25), it may be of interest to assay the in vivo efficacy of this compound administered orally.

Although DOX treatment reduced mortality on the second day, the therapeutic activity remained at low levels. This activity was decreased after the fifth day, while providing a significant initial therapeutic efficacy of FFC. The reason for this might be chelation or resistance. Burka et al. (26) reported that tetracyclines may be inactivated by exposure to  $Ca^{2+}$  and  $Mg^{2+}$  in the water and the intestine of the fish. Resistance mechanisms have been examined in recent studies as potential causes of antimicrobial aquaculture treatment failures (27). Resistance to OTC in *F. psychrophilum* is caused by uptake of transposons carrying tetracycline resistance determinants or the resistance is caused by other unspecific changes in membrane permeability (9). *tetQ* was demonstrated to function in *F. psychrophilum*, conferring resistance to tetracycline (28).

FFC is used in aquaculture to control susceptible bacterial diseases. The Food and Drug Administration's Center for Veterinary Medicine has approved FFC to control mortality in enteric septicemia, coldwater disease, furunculosis, streptococcal septicemia, and columnaris disease. In the present study, the protective effect of FFC against *F. psychrophilum* has been evaluated in rainbow trout fry. In our previous study (5), we found that FFC administration for 10 days caused reduced death numbers and mortality rates. In this study, in vivo results

demonstrated that FFC was effective in RTFS treatment. Our findings are in agreement with the above-cited reports. Additionally, FFC's better effectiveness may be dependent on high plasma levels in the current study. Popovic et al. (29) reported that decreased cytochrome P450 levels (e.g., 7-ethoxyresorufin O-deethylase and aldrin epoxidase) under higher fenicol antibiotic concentrations resulted in high plasma drug levels and long drug half-life in rainbow trout. FFC resistance has been detected in a wide variety of bacterial species with increasing frequency (30). This situation may depend on residues from medicated feed (15). However, resistance to FFC could be overestimated by the occurrence of innately resistant bacteria with multiple nonspecific resistances, and use of FFC is not a necessary causal condition for the development of elevated frequencies of FFC resistance (31). Additionally, biochemical homogeneity of *F. psychrophilum* is relative (10). Therefore, antibiotic resistance may vary according to geographic region. Del Cerro et al. (32) suggested that the population of *F. psychrophilum* in Spain is quite heterogeneous, and resistance to OTC was much higher, while all isolates remained susceptible to FFC. MIC ranges for OTC and FFC were 2.4–9.7  $\mu\text{g/mL}$  and 1.2–2.4  $\mu\text{g/mL}$ , respectively. In our study, MIC ranges for DOX and FFC were 0.5–64  $\mu\text{g/mL}$  and 0.25–32  $\mu\text{g/mL}$ , respectively. The resistance of pathogenic bacteria to antibacterials is a growing problem in aquaculture (33). The consequences of increasing resistance in bacteria and the diminishing impact of therapeutic drugs reach far beyond the geographic origins of antibacterial compounds (34). Studies of antibiotic resistance in fish are needed for aquaculture management of microorganisms (23).

In conclusion, despite in vitro results, this study supports the claim that FFC has attenuating effects in RTFS. These effects of FFC seem to be closely involved with the suppressing of *F. psychrophilum* isolates' activities. Eradication of *F. psychrophilum* from the hatchery is unlikely, and using an antibiotic unnecessarily to treat RTFS can lead to resistance to antibiotics. Furthermore, residues from medicated feed may enhance further development of antimicrobial resistance in *F. psychrophilum*. Due to the growing prevalence and incidence of infection and increasing antibiotic resistance, investigations on alternative antibiotics or vaccination should be given high priority. It should be noted that the antibiotic resistance profiles vary according to geographic area in *F. psychrophilum* infection.

#### Acknowledgment

The authors would like to acknowledge Dr Behire Işıl Didinen, Department of Aquaculture, Faculty of Fisheries, Süleyman Demirel University, for providing the *F. psychrophilum* NCIMB 1947 strain.



## References

- Nilsen H, Olsen AB, Vaagnes Ø, Hellberg H, Bottolfsen K, Skjelstad H, Colquhoun DJ. Systemic *Flavobacterium psychrophilum* infection in rainbow trout *Oncorhynchus mykiss* (Walbaum) farmed in fresh and brackish water in Norway. *J Fish Dis* 2011; 34: 403–408.
- Nunez HH, Evrard O, Kronvall G, Herrera RA. Antimicrobial susceptibility and plasmid profiles of *Flavobacterium psychrophilum* strains isolated in Chile. *Aquaculture* 2012; 354–355: 38–44.
- Starliper CE. Bacterial coldwater disease of fishes caused by *Flavobacterium psychrophilum*. *Journal of Advanced Research* 2011; 2: 97–108.
- Wagner EJ, Oplinger RW, Bartley M. Effect of single or double exposures to hydrogen peroxide or iodine on salmonid egg survival and bacterial growth. *N Am J Aquacult* 2012; 74: 84–91.
- Boyacioglu M, Akar F. Isolation of *Flavobacterium psychrophilum* causing rainbow trout fry syndrome and determination of an effective antibacterial treatment in rainbow trout (*Oncorhynchus mykiss*) fry. *Kafkas Univ Vet Fak* 2012; 18: 197–203.
- Didinen BI, Diler Ö, Ekici S, Altun S. Identification of *Flavobacterium psychrophilum* isolates using API ZYM and determination of antimicrobial susceptibilities with ATB VET. *Süleyman Demirel Üniversitesi Egirdir Su Urunleri Fakültesi Dergisi* 2005; 1: 64–70 (in Turkish with English abstract).
- Kubilay A, Altun S, Didinen BI, Ekici S, Diler Ö. Isolation of *Flavobacterium psychrophilum* in farmed rainbow trout (*Oncorhynchus mykiss*). *Kafkas Univ Vet Fak* 2009; 15: 709–715 (in Turkish with English abstract).
- Kum C, Kirkan S, Sekkin S, Akar F, Boyacioglu M. Comparison of in vitro antimicrobial susceptibility in *Flavobacterium psychrophilum* isolated from rainbow trout fry. *J Aquat Anim Health* 2008; 20: 245–251.
- Bruun MS, Madsen L, Dalsgaard I. Efficiency of oxytetracycline treatment in rainbow trout experimentally infected with *Flavobacterium psychrophilum* strains having different in vitro antibiotic susceptibilities. *Aquaculture* 2003; 215: 11–20.
- Lumsden JS, Young K, Welsh K, MacInnes J, Russell S, Hesami S. Management approaches for coldwater disease caused by *Flavobacterium psychrophilum*. In: *Proceedings of the Canadian Freshwater Aquaculture Symposium*. Saint Andrews, Canada: AAC Special Publication No. 11; 2004. pp. 111–117.
- Barnes ME, Brown ML. A review of *Flavobacterium Psychrophilum* biology, clinical signs, and bacterial cold water disease prevention and treatment. *Open Fish Science Journal* 2011; 4: 1–9.
- Dalsgaard I. Selection of media for antimicrobial susceptibility testing of fish pathogenic bacteria. *Aquaculture* 2001; 196: 267–275.
- Schmidt AS, Bruun MS, Dalsgaard I, Pederson K, Larsen JL. Occurrence of antimicrobial resistance in fish-pathogenic and environmental bacteria associated with four Danish rainbow trout farms. *Appl Environ Microb* 2000; 66: 4908–4915.
- Hesami S, Parkman J, MacInnes JI, Gray JT, Gyles CL, Lumsden JS. Antimicrobial susceptibility of *Flavobacterium psychrophilum* isolates from Ontario. *J Aquat Anim Health* 2010; 22: 39–49.
- Sundell K, Wiklund T. Effect of biofilm formation on antimicrobial tolerance of *Flavobacterium psychrophilum*. *J Fish Dis* 2011; 34: 373–383.
- Shotts EB, Starliper CE. Flavobacterial diseases: columnaris disease, cold-water disease and bacterial gill disease. In: Woo PTK, Bruno DW, editors. *Fish Diseases and Disorders*, Volume 3: Viral, Bacterial and Fungal Infections. 1st ed. Wallingford, UK: CABI Publishing; 1999. pp. 559–573.
- Turkish Statistical Institute. *Fishery Statistics*. Ankara, Turkey: Turkish Statistical Institute Printing Division; 2013.
- Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn WC. *Color Atlas and Textbook of Diagnostic Microbiology*. 5th ed. Philadelphia, PA, USA: Lippincott; 1997.
- del Cerro A, Marquez I, Guijaro A. Simultaneous detection of *Aeromonas salmonicida*, *Flavobacterium psychrophilum*, and *Yersinia ruckeri*, three major fish pathogens, by multiplex PCR. *Appl Environ Microb* 2002; 68: 5177–5180.
- Clinical and Laboratory Standards Institute. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically*; Approved Standard. 7th ed. Wayne, PA, USA: CLSI; 2006.
- Bebak JA, Welch TJ, Starliper CE, Baya AM, Garner MM. Improved husbandry to control an outbreak of rainbow trout fry syndrome caused by infection with *Flavobacterium psychrophilum*. *J Am Vet Med Assoc* 2007; 231: 114–116.
- Durmaz Y, Onuk EE, Çiftçi A. Investigation of the presence and antibiotic susceptibilities of *Flavobacterium psychrophilum* in rainbow trout farms (*Oncorhynchus mykiss* Walbaum, 1792) in The Middle and Eastern Black Sea Regions of Turkey. *Ankara Univ Vet Fak Derg* 2012; 59: 141–146.
- Izumi S, Aranishi F. Relationship between *gyrA* mutations and quinolone resistance in *Flavobacterium psychrophilum* isolates. *Appl Environ Microb* 2004; 70: 3968–3972.
- Chopra I, Roberts M. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol R* 2001; 65: 232–260.
- Riviere JE, Spoo JW. Tetracycline antibiotics. In: Adams HR, editor. *Veterinary Pharmacology and Therapeutics*. 8th ed. Ames, IA, USA: Iowa State University Press; 2001. pp. 828–840.
- Burka JF, Hammel KL, Horsberg TE, Johnson GR, Rainnie DJ, Speare DJ. Drugs in salmonid aquaculture. *J Vet Pharmacol Ther* 1997; 20: 333–349.

27. Dalsgaard I, Madsen L. Bacterial pathogens in rainbow trout, *Oncorhynchus mykiss* (Walbaum), reared at Danish freshwater farms. *J Fish Dis* 2000; 23: 199–209.
28. Alvarez B, Secades P, McBride MJ, Guijarro JA. Development of genetic techniques for the psychrotrophic fish pathogen *Flavobacterium psychrophilum*. *Appl Environ Microb* 2004; 70: 581–587.
29. Popovic NT, Klobucar RS, Strunjak-Perovic I, Jadan M, Barisic J, Coz-Rakovac R. Piscine cytochromes P450 (CYP) and their response to antimicrobial drugs. *Aquac Res* 2015; 46: 257–271.
30. Schwarz S, White DG. Phenicol resistance. In: White DG, Alekshun MN, McDermott PE, editors. *Frontiers in Antimicrobial Resistance: A Tribute to Stuart B Levy*. Washington, DC, USA: ASM Press; 2005. pp. 124–147.
31. Miranda CD, Rojas R. Occurrence of florfenicol resistance in bacteria associated with two Chilean salmon farms with different history of antibacterial usage. *Aquaculture* 2007; 266: 39–46.
32. del Cero A, Marquez I, Prieto JM. Genetic diversity and antimicrobial resistance of *Flavobacterium psychrophilum* isolated from cultured rainbow trout, *Oncorhynchus mykiss* (Walbaum), in Spain. *J Fish Dis* 2010; 33: 285–291.
33. Oliveira J, Catilho F, Cunha A, Pereire MJ. Bacteriophage therapy as a bacterial control strategy in aquaculture. *Aquacult Int* 2012; 20: 879–910.
34. Kemper N. Veterinary antibiotics in the aquatic and terrestrial environment. *Ecol Indic* 2008; 8: 1–13.