

DETECTION OF BACTERIAL DISEASES AND DETERMINATION OF ANTIBACTERIAL SUSCEPTIBILITIES OF RAINBOW TROUT (*Oncorhynchus mykiss* Walbaum, 1792) IN TURKEY**Necla Türk¹, Murat Yabanlı², Esin Baba^{2*}, Canan Ontaş², Mehmet Ali Aydın¹**¹Bornova Veterinary Control Institute, Izmir - Turkey²Muğla Sıtkı Koçman University, Faculty of Fisheries, Department of Aquaculture, Kötekli-Muğla, Turkey.

Abstract: In this study, the bacterial pathogen agents of cultivated rainbow trout (*Oncorhynchus mykiss* Walbaum) in Turkey were investigated. Pathogens from 692 samples belonging to 20 rainbow trout populations from different localities of Turkey were isolated between 2005 and 2008. 11 different bacterial pathogens were identified: *Acinetobacter* sp., *Aeromonas hydrophila*, *Aeromonas sobria*, *Citrobacter freundii*, *Flavobacterium psychrophilum*, *Lactococcus garvieae*, *Micrococcus luteus*, *Streptococcus iniae*, *Vibrio alginolyticus*, *Vibrio anguillarum*, and *Yersinia ruckeri*. The most frequently isolated pathogen was *Yersinia ruckeri*. Disc diffusion method was used to determine antimicrobial susceptibility to several antibiotics. The isolates were found to be susceptible to enrofloxacin, oxytetracycline and florfenicol.

Keywords: Bacterial disease, *Oncorhynchus mykiss*, Turkey

Özet: **Türkiye’de Gökkuşluğu Alabalığı (*Oncorhynchus mykiss* Walbaum, 1792)’nda Görülen Bakteriyel Hastalıkların Tespiti ve Antibiyotik Duyarlılıklarının Belirlenmesi**

Bu çalışmada Türkiye iç sularında ve kafeslerde yetiştiriciliği yapılan gökkuşluğu alabalıklarındaki bakteriyel patojen etkenler araştırılmıştır. Bu kapsamda 2005–2008 yılları arasında Türkiye’ nin farklı lokalitelerinden temin edilen ve bakteriyel hastalık şüphesi gösteren 20 alabalık popülasyonuna ait 692 adet materyalden etken izolasyonu yapılmıştır. İdetifikasyon çalışmaları sonucunda 11 farklı bakteriyel patojen etken izole edilmiştir. Bunlar *Acinetobacter* sp., *Aeromonas hydrophila*, *A. sobria*, *Citrobacter freundii*, *Cytophaga psychrophila*, *Lactococcus garvieae*, *Micrococcus* sp., *Streptococcus iniae*, *Vibrio alginolyticus*, *V. anguillarum*, *V. salmonicida* ve *Yersinia ruckeri* türleri olup en sık izole edilen etken *Yersinia ruckeri*’dir. Yapılan antibiyogram testleri sonucunda enroflaksasinin, oksitetrasiklin ve florfenikol elde edilen etkenlere karşı etkili olduğu belirlenmiştir.

Anahtar Kelimeler: Bakteriyel hastalık, *Oncorhynchus mykiss*, Türkiye

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Introduction

In Turkey, fish culture began with rainbow trout farming in concrete and earthen ponds in 1960's. In 1980 the rainbow trout production that was 3.000 tonnes, reached 85.000 tons in 2010 (Celikkale et al., 1998; TUIK, 2010).

Rainbow trout, *Oncorhynchus mykiss*, one of the most important fish species for freshwater aquaculture in Turkey also cultured in sea cages in the Black Sea since the early 1990s. The most significant factor affecting their culture is the incidence of microbial pathology. The major microbial diseases in freshwater farms are: furunculosis, caused by *Aeromonas salmonicida*; enteric redmouth disease by *Yersinia ruckeri*, and infections with *Flavobacterium psychrophilum*. These diseases are also common worldwide and produce considerable economic losses in the fish farming industry (Austin and Austin, 2007).

Economic consequences can be reduced by decreased mortality and treatment with appropriate antibiotics. Antibacterial treatments are most often administered to fish through drug supplemented feeds. Contamination of the surrounding environment by these drugs occurs principally through uneaten feed, and feces (Hirsch et al., 1999). In addition the use of inappropriate antibiotics can in some cases lead to the development of antimicrobial resistance in fish pathogens and environmental bacteria (Alderman and Hastings, 1999).

The aims of this study were to isolate and identify pathogenic fish bacteria from rainbow trout farms in Turkey and determine the most effective antibiotic treatment against them.

Materials and Methods

Fish samples

Diseased rainbow trout were brought from freshwater fish farms in different cities and cultured in sea cages between 2005-2008. Fish were sampled from 20 farms. During the four years, the samples usually consisted of six fish per farm per season. Fish were also sampled during disease outbreaks. In total 692 infected samples from Persembe (Ordu), Yomra (Trabzon), Kozan (Adana), Fethiye (Mugla, 4 different localities), Kemalpaşa (Izmir), Catak (Van), Hassa (Hatay), Banaz (Uşak), Trabzon, Mudurnu (Bolu), Nazilli (Aydın), Akyazı (Adapazarı), Bucak (Burdur, 2 different localities), Dalaman (Mugla), Keles

(Bursa), Almus (Tokat) were received. All fish samples were investigated at Bornova Veterinary Control and Research Institute in Izmir.

Identification of bacteria

The 692 fish (5-300 g) were transported to the laboratory on ice and dissected for bacteriological examination. All were examined externally and internally. Samples from spleen, skin, kidney and liver of all fish from each outbreak were streaked onto the following standard selective media; Tryptic soy agar (TSA), Brain Heart Infusion Agar (BHI), Blood Agar (BA), Thiosulfate-Citrate-Bile Salt Sucrose Agar (TCBS), Shotts-Watman Medium Agar (WS) and Kidney Disease Medium Agar (KDM). All the inoculated media were incubated at 20-22°C for 24±48 h. *F. psychrophilum* colonies that grew on CA at 15 °C for 3-4 days. Bacterial strains were examined by phenotypic tests. Identification was carried out by conventional biochemical tests as described by Buchanan et al., (2001) and Austin and Austin (2007).

Colonies were examined morphologically according to shape, color and bacterial motility. These were characterized biochemically with the following tests: Gram staining, cytochrome-oxidase, oxidative/fermentative test, catalase, O/129, β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H₂S production, urease, indole production, methyl red, voges proskauer, gelatinase, fermentation of glucose, mannitol, inositol, sorbitol, rhamnose, saccharose, amygdalin, arabinose and lactose.

Antimicrobial susceptibility tests

Antibiotic susceptibility test was determined by the disc diffusion method (Dalsgaard, 2001). *Acinetobacter sp.*, *Aeromonas hydrophila*, *Aeromonas sobria*, *Citrobacter freundii*, *Lactococcus garvieae*, *Micrococcus luteus*, *Streptococcus iniae*, *Vibrio alginolyticus*, *Vibrio anguillarum*, and *Yersinia ruckeri* were tested on Mueller-Hinton Agar incubated at 22 °C overnight. *Flavobacterium psychrophilum* was tested on CA, incubated at 15 °C and the zones read 3-4 days. Disks containing the following antibiotics were used; enrofloxacin (5 µg), oxytetracycline (30µg), trimethoprim-sulphamethoxazole (25µg), flumequin (30µg), doxycycline (5µg), oxolinic acid (2 µg) and florfenicol (30µg).



Figure 1. The distribution of isolated pathogens in Turkey (1: *Acinetobacter sp.*, 2: *A. hydrophila*, 3: *A. sobria*, 4: *C. freundii*, 5: *F. psychrophilum*, 6: *L. garvieae*, 7: *M. luteus* 8: *S. iniae*, 9: *V. alginolyticus*, 10: *V. anguillarum*, 11: *Y. ruckeri*)

Results and Discussion

Eleven different species of bacteria were isolated from 692 infected fish. Biochemical characteristics of the isolates are shown in Table 1 and Table 2. Isolated pathogens and regions were shown in Table 3. The geographical distribution of the pathogens has been shown in Figure 1. Comparison between the frequencies of isolation of the different bacterial groups detected in survey of bacteria isolated from isolated farmed rainbow trout in Table 4. From skin and the internal organs of the fish, strains belonging to *Acinetobacter sp.*, *A. hydrophila*, *A. sobria*, *C. freundii*, *F. psychrophilum*, *L. garvieae*, *M. luteus*, *S. iniae*, *V. alginolyticus*, *V. anguillarum* and *Y. ruckeri* were isolated.

The most common pathogen isolated from rainbow trout freshwater farms were *Y. ruckeri*. *Y. ruckeri* was isolated from the liver, spleen, and kidney. *A. hydrophila*, *L. garvieae*, *V. anguillarum* were isolated from kidney, liver, spleen, and skin lesions. When fish has clinical signs of disease, the following bacteria were isolated; *A. sobria*, *A. hydrophila*, *F. psychrophilum* (dark pigmentation, fried fin), *Y. ruckeri* (hemorrhage on

liver, enlarged spleen, and exophthalmia), *C. freundii* (ulcerative lesions on skin, pale liver, surface haemorrhages), *M. luteus* (haemorrhages, exophthalmia), *S. iniae* (discoloration, bilateral exophthalmia, corneal opacity, haemorrhaging in the eye), *L. garvieae* (exophthalmia, darkening skin, haemorrhagic enteritis), *V. alginolyticus* (bacterial septicaemia, darkening skin) and *V. anguillarum* (haemorrhage in anal and pectoral fins, haemorrhage in liver). Antibiotic susceptibilities of isolated pathogens were given Table 5. The isolates were found susceptible to enrofloxacin, oxytetracycline and florfenicol.

In this study, 692 infected rainbow trout were investigated. As a result of the bacterial samples skin and from internal organs of the fish, *Acinetobacter sp.*, *A. hydrophila*, *A. sobria*, *C. freundii*, *F. psychrophilum*, *L. garvieae*, *M. luteus*, *S. iniae*, *V. alginolyticus*, *V. anguillarum*, and *Y. ruckeri* were isolated. The most frequently isolated strain was identified as *Yersinia ruckeri* (Table 4). The presence of these bacteria both in aquatic environment and the internal fish organs have been often observed (Toranzo et al., 1993; Karataş et al., 2004; Akşit and Kum, 2008).

Table 1. Phenotypic characteristics of the Gram (-) bacteria isolated from rainbow trout.

	<i>V.alginolyticus</i>	<i>V.anguillarum</i>	<i>A.hydrophila</i>	<i>A.sobria</i>	<i>Acinetobactersp.</i>	<i>C.freundii</i>	<i>F.psychrophila</i>	<i>Y.ruckeri</i>
Gram	-	-	-	-	-	-	-	-
Motility	+	+	+	+	-	+	+	+
Oxidase	+	+	+	+	+	-	-	-
Catalase	+	+	+	+	+	+	+	+
O/F test	F	F	F	F	F	F	F	F
ONPG	-	+	+	+	-	-	-	+
citrate	+	+	+	+	ND	+	-	+
H ₂ S	+	-	-	+	-	+	-	-
Indole	+	+	+	+	-	-	-	-
MR	+	-	+	ND	-	+	-	+
VP	+	+	+	-	-	-	-	-
O 129	S	S	R	R	ND	ND	ND	ND
Growth at 37° C	+	+	+	+	ND	+	-	+
0% NaCl, growth	-	-	+	+	ND	+	ND	+
7% NaCl, growth	+	-	-	-	ND	-	ND	-
Arabinose	-	+	+	-	-	+	-	-
Inositol	-	-	-	-	-	-	ND	-
Lactose	-	-	-	+	-	+	ND	-
Maltose	+	+	+	+	+	+	ND	+
D- mannitol	+	+	+	+	-	+	ND	+
Mannose	+	ND	+	+	+	ND	ND	+
Sucrose	+	+	+	+	ND	ND	ND	-
Urease	+	-	-	-	-	-	-	-

+ Positive reaction; - Negative reaction; F: Fermentative; R: resistant; S:Sensitive, ND: not detected

Table 2. Phenotypic characteristics of the Gram (+) bacteria isolated from rainbow trout.

	<i>L. garvieae</i>	<i>S. iniae</i>	<i>M. luteus</i>
Gram stain	+	+	+
Motility	-	-	+
Oxidase	-	-	+
Catalase	-	-	+
O/F test	F	F	O
Nitrate	-	-	-
Hemolysis	α	β	ND
H ₂ S	-	-	-
Indole	-	-	-
Gelatin degradation	-	-	ND
citrate	-	ND	-
Methyl red	+	+	ND
Voges-Proskauer	+	-	-
10° C growth	+	-	+
45° C growth	+	-	+
6,5% NaCl, growth	+	-	+
Arabinose	-	-	-
inositol	-	-	-
Lactose	-	+	ND
Maltose	+	+	ND
D- mannitol	+	+	ND
Mannose	+	+	ND
Sucrose	ND	+	ND
Urease	-	-	ND

+: Positive reaction, -: Negative reaction, F: Fermentative, O: oxidative, ND: not detected.

Table 3. Regions isolated pathogens

<i>Isolates</i>	<i>Regions</i>
<i>Acinetobacter sp.</i>	Akyazı (Adapazarı)
<i>A. hydrophila</i>	Fethiye (Mugla), Dalaman (Mugla) Çatak (Van), Bucak (Burdur), Almus (Tokat)
<i>A. sobria</i>	Bucak (Burdur), Almus (Tokat)
<i>C. freundii</i>	Fethiye (Mugla)
<i>F. psychrophilum</i>	Akyazı (Adapazarı), Keles (Bursa)
<i>L. garvieae</i>	Nazilli (Aydın), Fethiye (Mugla)
<i>M. luteus</i>	Trabzon, Fethiye (Mugla)
<i>S. iniae</i>	Fethiye (Mugla)
<i>V. alginolyticus</i>	Yomra (Trabzon) (marine water)
<i>V. anguillarum</i>	Kozan (Adana), Kemalpaşa (İzmir), Banaz (Usak), Trabzon, Mudurnu (Bolu), Keles (Bursa)
<i>Y. ruckeri</i>	

Table 4. Comparison between the frequency of isolation of the different bacterial groups detected in survey of bacteria isolated from isolated farmed rainbow trout (%).

Strains	Frequency of isolation				
	2005 (n=150)	2006 (n=102)	2007 (n=190)	2008 (n=250)	Total (n=692)
<i>V.anguillarum</i>	6.66	9.80	5.23	8	29.69
<i>V. alginolyticus</i>	20	14.70	2.61	6	43.31
<i>Y. ruckeri</i>	13.33	49.01	47.12	12	121.46
<i>M. luteus</i>	20	4.90	5.23	8	38.13
<i>L. garvieae</i>	26.66	11.76	2.61	6	47.03
<i>F. psychrophilum</i>	0	0	3.141	6	9.14
<i>A. hydrophila</i>	13.33	9.80	15.70	24	62.83
<i>A. sobria</i>	0	0	15.70	20	35.70
<i>Acinetobacter sp.</i>	0	0	2.61	4	6.61
<i>S. iniae</i>	0	0	0	4	4
<i>C. freundii</i>	0	0	0	2	2

Table 5. Susceptibility/Resistance profiles of bacterial isolated from rainbow trout

	ENR	OTC	SxT	UB	DO	OA	FFC
<i>Acinetobacter sp.</i>	S	S	S	R	R	S	R
<i>Aeromonas hydrophila</i>	S	S	S	R	R	S	R
<i>Aeromonas sobria</i>	R	S	S	R	R	R	S
<i>Citrobacter freundii</i>	R	R	R	R	S	R	S
<i>F. psychrophilum</i>	S	S	S	R	R	S	S
<i>Lactococcus garvieae</i>	S	R	R	R	S	R	S
<i>Micrococcus luteus</i>	S	S	R	R	S	R	S
<i>Streptococcus iniae</i>	R	R	R	R	S	R	S
<i>Vibrio alginolyticus</i>	S	S	R	S	R	S	R
<i>Vibrio anguillarum</i>	S	S	S	S	R	R	R
<i>Yersinia ruckeri</i>	S	R	S	R	R	R	R

S: sensitive, R: resistant; ENR: Enrofloxacin (5µg), OTC: Oxytetracycline (30µg), SxT: Trimethoprim-sulphamethoxazole (25µg), UB: flumequin (30µg), DO: doxycycline (5µg), OA: Oxolinic acid (2µg), FFC: Florfenicol (3µg).

Previous investigations on bacterial infections in Turkish marine and freshwater rainbow trout hatcheries have focused on disease outbreaks from a single pathogen *Y. ruckeri* (Timur and Timur, 1991; Karataş et al., 2004; Akşit and Kum, 2008; Kayış et al., 2009). To date, other than *Y. ruckeri* reported bacterial pathogens of rainbow trout in Turkey are *Acinetobacter sp.* (Yonar et al., 2010), *A. hydrophila* (Kirkan, et al., 2003; Kayış et al., 2009), *A. sobria* (Kayış et al., 2009), *C. freundii* (Aydın et al., 1997), *F. psychrophilum* (Gültepe et al., 2006; Kayış et al., 2009), *L. garvieae* (Akşit and Kum, 2008; Diler et al., 2002), *M. luteus* (Aydın et al., 2005), *S. iniae* (Aydın et al., 2005), *V. anguillarum* (Tanrıkul, 2007; Akşit and Kum, 2008). All these agents were isolated in this study too. *V. algi-*

lyticus was isolated from rainbow trout cultured in the sea cages, although it was not reported in previous investigations Akşit and Kum, 2008; Kayış et al., 2009.

In this study antibiotic susceptibility tests revealed that *Acinetobacter sp.* strains were sensitive to enrofloxacin, oxytetracycline, trimethoprim-sulphamethoxazole and oxolinic acid. On the other hand, some of strains were found to be resistant to flumequin and doxycycline. Previous reports were indicated *Acinetobacter sp.* strains were sensitivity oxytetracycline (Austin and Austin, 2007). *Aeromonas spp.* is adapted for antimicrobial resistance monitoring because of its natural susceptibility to main antimicrobial drugs used in aquaculture (Spanggard et al., 1993), par-

ticularly to trimethoprim-sulphamethoxazole, oxytetracycline, quinolones (oxolinic acid and flumequine) and florfenicol. In this study, *A. hydrophila* strains were sensitivity to enrofloxacin, oxytetracycline, trimethoprim-sulphamethoxazole and oxolinic acid as predicted. *A. sobria* strains were sensitivity to oxytetracycline, trimethoprim-sulphamethoxazole and florfenicol as predicted. On the other hand, some of strains were found to be resistant to enrofloxacin and doxycycline.

According to Baya et al. (1990), *C. freundii* strains were resistant to chloramphenicol, potentiated sulphonamides and tetracycline. In this study, the strains were sensitive to doxycycline and florfenicol. Oxytetracycline and oxolinic acid are considered to be the antibiotic of choice for control of infection by *F. psychrophilum* (Holt et al., 1993). Our antibiotic susceptibility tests revealed that all of the pathogens isolates were susceptible to enrofloxacin, oxytetracycline, trimethoprim-sulphamethoxazole, oxolinic acid and florfenicol. According to the results of this study, *L. garvieae* isolates were resistant to oxytetracycline, trimethoprim-sulphamethoxazole, flumequin and oxolinic acid and susceptible to enrofloxacin, doxycycline and florfenicol. When the other studies were evaluated, it was determined that *L. garvieae* was susceptible to amphenicoles, oxytetracycline and novobiocin. Tetracycline, and chloramphenicol susceptibilities were also reported (Diler et al., 2002). These different results may be due to the differences of *L. garvieae* strains and antibiotics usage in the area.

M. luteus was resistant to potentiated sulphanamides and oxytetracycline which contrast to the findings of Austin and Stobie (1992). According to the results of present study enrofloxacin, oxytetracycline, doxycycline and florfenicol have been observed to effective to treat fish infected *M. luteus*. *S.iniae* isolates was susceptible to oxytetracycline (Darwish and Ismail, 2003) for the control of the infection. *S.iniae* was sensitive to doxycycline and florfenicol in the present study. *V. alginolyticus* strains were sensitive to enrofloxacin, oxytetracycline, flumequin and oxolinic acid as predicted. On the other hand, some of strains were found to be resistant to trimethoprim-sulphamethoxazol, doxycycline and florfenicol. Previous reports indicated that *V. alginolyticus* was sensitive to chloramphenicol and nitrofurantoin (Report, 1969). According to antimicrobial susceptibility test in this study, *V. anguillarum* isolates were found to be sensitive to

enrofloxacin, oxytetracycline, trimethoprim-sulphamethoxazole and florfenicol. *Y. ruckeri* strains were sensitive to enrofloxacin and trimethoprim-sulphamethoxazole in present study. These susceptibility results were similar to Karatas et al., (1990) and Savvidis (2004).

Conclusion

In conclusion, the present study Gram negative and Gram positive bacteria were identified at different areas in Turkey. The results in the present study show that it is necessary to carry out extensive monitoring of fish pathogenic bacteria, in order to obtain more knowledge of the most important diseases which occur in different rainbow trout farm in Turkey. The knowledge of the distribution of bacterial pathogens and a subsequent investigation of the characteristics of the bacteria is necessary to evaluate which factors are important in establishing an infection. Also, antibacterial susceptibility test should be performed in consideration of antibacterial drugs which are used in aquaculture medicine in Turkey.

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