Lactococcosis in rainbow trout (*Oncorhynchus mykiss*,Walbaum, 1792) in the middle Black Sea Region in Turkey and antimicrobial susceptibility of the aetiological agent, *Lactococcus garvieae*

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Summary: In this research study, we isolated *Lactococcus garvieae* from rainbow trout in the middle Black Sea region in Turkey. The diseases outbreak occurred at average water temperature of 16.7°C during April-May 2009. Clinically stagnation, inapetence, darkening of the skin, exophtalmia, opacification in the cornea, hemorrhages in the eyes and at the base of pectoral and anal fins, swollen abdomen and wounds on the body surface were observed in infected fish. In necropsy; the existence of accumulation of bloody fluid in the body cavity, ascites in the intestines and the stomach characterized with a yellowish-colored liquid, enlarged and darkening in the spleen and kidney, fading in colour, existence of petechial hemorrhages and enlarged in the liver were detected. In order to isolate the aetiological agent, 20 infected rainbow trout weighting between 50-100 gr were used. As the result of conventional tests used in the identification of the bacteria, the aetiological agent was identified as *L.garvieae*. The isolated *L.gavieae* strain was determined as susceptible to amoxicillin, amoxicillin/clavulanic acid, cephalothin, chloramphenicol, doxycycline, enroflaxacin, pristinamycine and tetracycline.

Key words: *Lactococcus garvieae*, phenotypic identification, antimicrobial sensitivity, rainbow trout.

Türkiye'nin orta Karadeniz Bölgesindeki gökkuşağı alabalıkları (*Oncorhynchus mykiss*, Walbaum, 1792)' nda laktokokkozis ve *Lactococcus garvieae* etkeninin antimikrobiyal duyarlılığı

Özet: Bu çalışmada, Türkiye'nin Karadeniz Bölgesindeki gökkuşağı alabalıklarından *Lactococcus garvieae* izole edildi. Hastalık salgını ortalama 16.7°C su sıcaklığında Nisan-Mayıs 2009 aylarında görüldü. Hasta gökkuşağı alabalıklarında klinik olarak durgunluk, iştahsızlık, renkte koyulaşma, tek ya da çift taraflı eksoftalmus, korneada opaklaşma, gözlerde, pektoral ve anal yüzgeç tabanlarında hemoraji, karında şişkinlik, vücut yüzeyinde yaralar görüldü. Nekropside vücut boşluğunda kanlı-renkli bir sıvının varlığı, barsak ve midede sarı berrak renkte sıvı ile karakterize asites, dalak ve böbrekte büyüme, renklerinde koyulaşma, karaciğerde büyüme, renginde açılma ve peteşiyel kanamaların olduğu tespit edildi. Hastalık etkenini izole etmek için yaklaşık 50-100 g ağırlığında hasta 20 adet gökkuşağı alabalığı bakteriyolojik incelemelerde kullanıldı. Bakterilerin identifikasyonunda konvansiyonel testler kullanıldı ve hastalık etkeni *L.garvieae* olarak tanımlandı. İzole edilen *L.garvieae* suşu, amoxicillin, amoxicillin/clavulanic acid, cephalothin, chloramphenicol, doxycycline, enroflaxacin, pristinamycine ve tetracycline duyarlı olduğu tespit edildi.

Anahtar sözcükler: *Lactococcus garvieae*, fenotipik identifikasyon, antimikrobiyal duyarlılık, gökkuşağı alabalığı.

Introduction

Lactococcus garvieae is an etiological agent of the lactococcosis disease which is effecting many different fish species in marine and freshwater aquaculture. It is a pathogen, which often present in rearing waters. It can cause serious economical losses with a death rate of %50-80 in fish which are not yet in the marketable size (50-80 gr) in trout farms (17,20). In addition, *L.garvieae* has been isolated from many of the aquatic and terrestrial animals other than various species of fish (8). The isolation of the disease in human showing its zoonotic character is also important (19, 21, 22). In order to prevent lactococcosis outbreaks, necessary treatment and control methods should be developed. Furthermore, antimicrobial susceptibility test is necessary to initiate favorable therapeutics and prophylactic measures in order to limit the economic losses generated by these infections in aquaculture.

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Lactococcosis epidemics are related with environmental conditions such as stress, overcrowding, sudden water temperature changes, and low water quality. Water temperature is the most important factor in the development of the disease making it seasonal and the appearance of the disease is associated with high water temperature. Most of the acute outbreaks appear when water temperature is over 18 °C, although acute outbreaks have been described at water temperatures of 14-15 °C (20). Normally, the disease follows a peracute route and causes hemorrhagical septicaemia. Among the clinical symptoms of the Lactococcosis; lethargy, anorexia, melanosis, erratic swimming, unilateral or bilateral exophthalmia, hemorrhages in the ocular zone, perianal area, fins, puffiness in the belly, wounds on the body surface and anal prolapsus have been listed (11,20). Outbreaks affecting rainbow trout have been reported in several countries, such as Australia, South Africa, Japan, Taiwan, England and some countries of the Mediterranean area (11). It has been reported that Lactococcosis is regularly seen in *Oncorhynchus mykiss* culture farms since 2001 in Turkey (2,6,10,13,16). Since then, such infections have been reoccurred, especially during the warm summer months. Therefore, *L.garvieae* is now considered one of the most important pathogens in the rainbow trout industry in Turkey (3,10). Therefore, a lot of studies on antimicrobial susceptibility phenotypic, genotypic, biotypic, immunogenic, antigenic profiles determination of *L.garvieae* strains have been conducted (1,4,9,14,18). Despite several studies on *L.garvieae* detected in the western and southern parts of Turkey, there is a few studies on *L.garvieae* isolation from Black Sea region.

The purpose of this study is to elucidate the antimicrobial susceptibility and the phenotypic characersitics of *L.garvieae* isolated from Black Sea region along with its general disease characteristics on rainbow trout.

Materials and Methods

A total of 20 rainbow trout (body weight 50-100 gr) suspected of showing the clinical signs of the disease were collected from a cage farm in Bafra dam lake in the middle Black Sea region of Turkey (at average water temperature of 16.7°C during April-May 2009). Bacteriological samples were collected from spleens, kidney and liver using sterile swabs and streaked onto trypticase-soy-agar (TSA, Merck). Plates were incubated at 22°C for 48 hours. Single colonies were restreaked on the same media to obtain pure isolates. At the end of this period, the morphology and the color of the developed bacterial colonies in the medium were determined. Five isolates obtained from sick fish identified by physiological, biochemical, and enzymatic characterization. The morphology and the color of the developed bacterial colonies in the medium were determined. Gram staining method was applied for the determination of the cell morphology. In order to determine to other phenotypical features of the strain; motility, oxidase reaction (Tetramethyl-p-phenylenediamine dihydrochloride; Sigma), catalase production (3% H_2O_2), nitrate reduction test, oxidation/fermentation test (O/F main medium, Merck with 1% glucose), Methyl Red and Voges-Proskauer tests (MR-VP Broth; Merck), citrate usage (Simmon's Citrate Agar, Merck), gelatinase tests, detection of the hemolysis feature, development at 4 and 45°C and 0%, 2.5%, 6.5% ve 8% NaCl were made (5).

Antimicrobial susceptibility of the isolated *L.garvieae* strain was determined with ATB VET (Bio Mereux). The isolates were inoculated API suspension medium with a turbidity equivalent to 0.5 McFraland. 200 µl from this suspension was inoculated onto ATB S medium. Then, 135 µl from bacterial suspansion in ATB S medium were inoculated into each well in ATB VET strips. The strips were inoculated in at 25oC for 24 hours. After the incubation, clarity in well was evaluated as sensitive, turbidity as resistant. Macroscopic examinations were made in Sinop University, Fisheries and Aquatic Sciences Faculty; and bacteriological examinations were made in Süleyman Demirel University, Eğirdir Fisheries Faculty.

Findings

Throughout the investigation period, a rate of 40-50% mortality was determined in naturally infected *Oncorhynchus mykiss*. Macroscopic findings such as stagnation, inapetence, darkening of the skin (Figure 1a), unilateral or bilateral exophtalmus (Figure 1b), opacification in the cornea (Figure 1c), loss of eye in advanced phases of the disease (Figure 1d), haemorrhagies in the eyes and at the base of pectoral and anal fins, swollen abdomen and

wounds on the body surface were observed in infected fish (Figure 1e-f). In necropsy; the existence of accumulation of bloody fluid in the body cavity, yellowish-colored ascites in the intestine (Figure 1g) and stomach, enlarged and darkening in the spleen and the kidney, fading in colour, existence of petechial hemorrhages and enlarged in the liver were detected.

Gram-positive cocci were seen in the samples prepared from the tissues of the eyes, liver and kidney. As a result of the biochemical and physiological characteristics determined by conventional tests (Table 1) proved that the bacteria isolated from infected fish was *L.garvieae*. Antimicrobial sensitivity of the isolated *L.garvieae* strain was determined with ATB VET (Bio Mereux) and listed in Table 2.

Lactococcus garvieae isolates were examined using 28 different antimicrobials for sensitivity test and results were given in Table 2. *L.garvieae* strain was found to be susceptible to Amoxicillin, amoxicillin/clavulanic acid, cepholothin, cholaramphenicol, doxycycline, enrofloxacin, pristinamycin, tetracycline and to be resistant to apramycin, cefoperazone, colistin, cotrimoxazole, erythromycin, flumequin, fusidic acid, gentamicin, kanamycin, lincomycin, metronidazole, nitrofurantoin, oxacillin, oxolinic acid, penicillin, rifampicin, spectinomycine, sulfamethizole and tylosin.

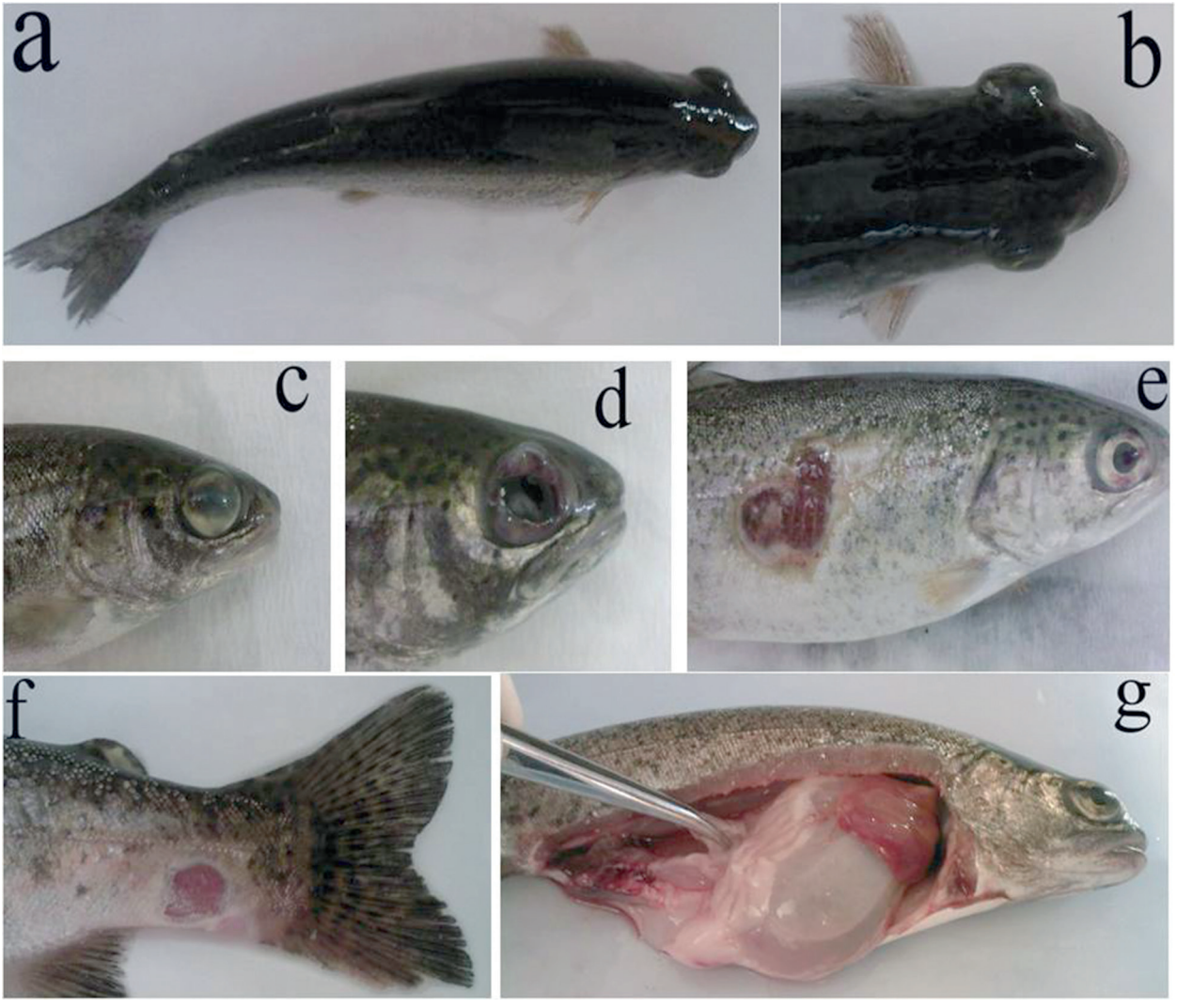

Figure 1. The macroscopic appearances observed in infected fishes. **a:** darkening of the skin, **b:** bilateral exophtalmus, **c:** opacification in the cornea, **d:** loss of eye in advanced phase, **e-f:** wounds on the body surface, **g:** ascites in the intestines.

Table 1. Phenotypic characteristic of *L.garvieae* strain with conventionally tests.

Phenotypic characteristics	
Gram staining	+
Motility	-
Oxidase	-
Catalase	-
O/F	+/+
H_2S production	-
Hydrolysis of gelatine	-
Hydrolysis of starch	-
Hydrolysis of indole	-
MR	+
VP	+
Utilization of citrate	-
Hemolysis (sheep blood)	α
Growth in TSA with 0% NaCl	+
Growth in TSA with 2.5% NaCl	+
Growth in TSA with 6.5% NaCl	+
Growth in TSA with 8% NaCl	-
Growth at 4°C	+
Growth at 25°C	+
Growth at 37°C	+
Growth at 45°C	+

Table 2. Antimicrobial sensitivities of *L.garvieae* strain (**S:** Susceptible, **R:** Resistant).

Antimicrobial drugs	Concentration (mg/mL)	*L.garvieae* strain
Amoxicillin (AMO)	4	S
Am oxicillin/ clavulanic acid (AMC)	4/2	S
Apramycin (APR)	16	R
Cefoperazone (CFP)	4	R
Cephalothin (CFT)	8	S
Chloramphenicol (CMP)	8	S
Colistin (COL)	4	R
Cotrimoxazole (TSU) (Trimethoprim/ sulphamethoxazole, TSU)	2/38	R
Doxycycline (DOT)	4	S
Enrofloxacin (ENR)	0.5	S
Erythromycin (ERY)	1	R
Flumequin (FLU)	4	R
Fusidic Acid (FUC)	2	R
Gentamicin (GEN)	4	R
Kanamycin (KAN)	8	R
Lincomycin (LYN)	2	R
Metronidazole (MTR)	4	R
Nitrofurantoin (FUR)	25	R
Oxacillin (OXA)	2	R
Oxolinic Acid (OXO)	2	R
Penicillin (PEN)	0.25	R
Pristinamycin (PRI)	2	S
Rifampicin (RFA)	4	R
Spectinomycine (SPE)	64	R
Streptomycine (STR)	8	R
Sulfamethizole (SUL)	100	R
Tetracycline (TET)	4	S
Tylosin (TYL)	2	R

Discussion and Conclusion

The typical symptoms such as changes in the skin pigmentation, opacification in the cornea, exophtalmia, swelling in the belly and hemorrhage in various body parts have been reported in the infections caused by gram-positive cocci. In the present study, *L.garvieae* produced clinical findings such as darkness in colour, opacification in the cornea, exophtalmia, swelling in the belly, the existence of a bloody-colored liquid in the body cavity, yellowish-colored ascites in the intestine and the stomach, growth and darkness in the spleen and the kidney, fading in color, existence of petechial hemorrhages and growth in the liver in infected *O.mykiss*. The similar clinical findings have been reported by Bragg and Broere (7), Muzquiz et al., (15), Eldar and Ghittino (11), Diler et al., (10), Altun et al., (2), Vendrell et al., (20). Here in the present study, open wounds were also observed on the body surfaces of the infected fish in addition to the previous findings as a result of possible subacute route of the disease.

Phenotypic characteristic of *L.garvieae* strain in our study are similar to those reported by Akşit and Kum (1), Altun et al., (4), Çağırgan, (9), Diler et al., (10), Kubilay et al., (14) and Türe, (18).

The antibiotics most often used to control Lactococcosis in rainbow trout outbreaks have been erythromycin, oxytetracycline, amoxicillin

and low-level doxycycline. The study of *L.garvieae* strains from different geographic origin showed that all of them were sensitive to enrofloxacyn and nitrofurantoin, and were resistant to oxolinic acid and sulphametoxazol-trimethoprim. However, the results differed with regard to erythromycin, chloramphenicol, oxytetracycline and ampicillin (20). The reference strains of *L.garvieae* were found sensitive to erithromycin, with a minimum inhibitory concentration (MIC) of 0.12 mg/ml (12). Recent outbreaks in Turkey have demonstrated that the strains of *L.garvieae* were sensitive to erythromycin, tetracycline, ofloxacin, ampicillin, chloramphenicol (10); penicilin, ampicillin, amoxycillin, amoxicillin/clavulanic acid, erythromycin, oxytetracycline (13); gentamycin, oxitetracycline, neomycin, enrofloxacin, eritromycin and streptomycin (16). On the other hand, some strains of *L.garvieae* were found resistant to penicilin, clyndamycin and cefriaxon (10); clyndamycin, lincomycin, spiramycin, cloxacillin, oxacillin, methicillin, gentamicin, neomycin, bacitracin and sulphamethoxazole/trimethoprim (13); sulphamethoxazole–trimetoprim (16). When the antimicrobial sensitivity test results obtained in this research are examined (Table 2), bacterial resistance seems to be quite higher than the above-mentioned studies. Moreover eritromycine and streptomycine were also found resistant to *L.garvieae* in the present study. Considering the high antibacterial resistance of the streptococci, this resistance could be the result of the insensible antibacterial drug usage in aquaculture.

In conclusion, this paper makes some valuable contribution to our knowledge about known Lactococcosis from the Black Sea region in Turkey by providing data on *L.garvieae* isolation and phenotypic identification. Moreover, the results obtained from this investigation are also important to eliminate resistant and sensitive therapeutics on the etiological agent *L.garvieae*.

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