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Current antibiotic sensitivity of *Lactococcus garvieae* in rainbow trout (*Oncorhynchus mykiss*) farms from Southwestern Turkey

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ABSTRACT

The continuity of antimicrobial resistance development in bacteria changes the type and dose of effective antibiotic treatments and makes routine monitoring studies necessary for successful control of bacterial diseases. This study was aimed to determine the current antibiotic susceptibility of *Lactococcus garvieae*, which causes significant economic losses in rainbow trout (*Oncorhynchus mykiss*) farms. In the study, two consecutive visits were made to three farms operating on the banks of a stream during a disease outbreak in the fall of 2018. At each visit, 10 fish showing the signs of disease from each farm (60 fish in total) were sampled and *L. garvieae* was isolated from 16 fish. All isolates were identified using conventional and molecular methods. Then, they were examined for 5 different antibiotic resistance genes and their antibiotic susceptibility profiles were determined using the Kirby Bauer disc diffusion technique. Results of the disc diffusion test revealed that all 16 isolates had a different antibiotic susceptibility profile and the isolates with different antibiotic susceptibility profile could exist within and between farms, using the same water source. Furthermore, they revealed that highly resistant isolates that showed no susceptibility up to 82 to 100% of the 33 antibiotics at the doses evaluated exist in all farms. All isolates carried either *tetA* or *tetB* genes or both and the majority of isolates carried *tetA* gene. Together with this, 25% of the isolates which carried both or one of the resistance genes showed susceptibility to all 4 tetracycline class antibiotics at the doses evaluated. Results of the study and their comparison with previous studies in the same production area or in different regions of the country revealed the dynamic nature of antibiotic resistance development in *L. garvieae*. Additionally, it showed that monitoring studies with a limited number of isolates may not give an accurate picture of the current status of antibiotic resistance from a production area. These results of the study were also discussed in terms of the treatment strategies that trout farmers should follow when treating lactococcosis.

Keywords: Antimicrobial resistance genes, Antimicrobial susceptibility, Pathogenic bacteria, Fish farm, Fish disease

1. Introduction

Intensive culture conditions, in which a large number of individuals are kept in close contact, adversely affect fish welfare as well as weaken the immune system of fish (Sönmez et al. 2022), leading to a disease by existing pathogens in the environment. On the other hand, excessive use of antibiotics and chemotherapeutics for the treatment of diseases leads to the accumulation of antibiotics in the aquatic environment and fish, suppressing the already weakened immune system and further reducing the resistance against pathogens (Lundén et al. 2020). In addition, excessive antibiotic use may raise an important problem because it causes pathogens (bacteria) to develop resistance against antibiotics (Karayakar & Ay 2006). Once such resistance is established, it can be rapidly transferred within and between bacterial species by means of genetic elements such as plasmid and transposon (Doğancı 2001). For this reason, the ability of bacterial pathogens to develop resistance to antibiotics is a very important issue to be considered in the combat against infectious diseases (Vahaboğlu 2004). In addition, the fact that antibiotic resistance developed in aquatic environments has the potential to be easily transferred to terrestrial environments, in other words, the possible transfer of this resistance to human pathogens increases the importance of studies on the development of antimicrobial resistance in aquatic environments (Carvalho 2012).

Lactococcus garvieae is a bacterial species that can cause significant economic losses in fish farms. It was first reported from Japan in 1974 to cause an infection with high mortality rates in yellowtail (*Seriola dumerili*) (Kusuda et al. 1991; Kusuda & Salati 1991; Austin & Austin 1999). In later years, mortalities related to *L. garvieae* infections (streptococcosis or lactococcosis) were observed in many other marine and freshwater fishes from different geographic regions, and this bacterium was accepted as a common fish pathogen (Kusuda & Salati 1991). Spain, Italy, Portugal, England, South Africa, Australia, France and some Asian countries have reported that *L. garvieae* is a dangerous disease agent for rainbow trout (*Oncorhynchus mykiss*) (Ravelo et al. 2001). This pathogen was first detected in rainbow trout farms located in the western part of Turkey in 2001 and reported to cause high mortality rates (Diler et al. 2002). *L. garvieae*, which induce a systemic and rapidly spreading infection characterized by hemorrhagic septicemia, can cause significant economic losses in trout farms because it usually infects pan-sized fish (Çağırgan & Tanrıkul 1997).

Lactococcus infections generally occur in the summer months when the fish biomass is high, the culture waters get warmer and the amount of incoming fresh water decreases. The infections developing under these difficult conditions may leave trout farmers who want to protect their investments with no choice but to use prophylactic, sometimes high-dose antibiotic cures. In this context, routine monitoring studies that inform the farmers about the effective antibiotic cures against common fish pathogens in their production area carry great importance. In addition to helping farmers to protect their investments, such monitoring studies may help to reduce the unnecessary use of antimicrobials in aquaculture and its negative impacts on the environment.

Muğla province is considered as the capital of the Turkish aquaculture industry. In addition to cage farming of marine fishes in the northern part, considerable amount of pan size and larger (≥1 kg) rainbow trout are produced in the southern part of the province. According to provincial authorities, more than 40 licensed rainbow trout farms with annual production capacities ranging from 3 to 2,500 metric tons currently operate in raceway systems constructed on the banks of Eşen Stream (BSGM 2019). Although several different bacterial infections that cause important economic losses occur in these farms, *L. garvieae* is often isolated as the disease agent (Kubilay et al. 2005; Kav & Erganis 2008; Altun et al. 2013; Kurtoğlu & Korun 2018; Balta & Balta 2019).

Various studies conducted before in order to determine the effective antibiotic cure against different *L. garvieae* strains isolated from trout farms in Turkey (Akçam et al. 2004; Doğancı 2001). However, intensive use of antibiotics against bacterial pathogens leads to the development of antibiotic resistance in the aquatic environment. As this continuum of antimicrobial resistance development changes treatments in terms of the type and dose of effective antibiotics, routine monitoring studies are needed for successful control of bacterial diseases (Kum et al. 2004). The last monitoring study in Muğla province was conducted more than ten years ago (Kubilay et al. 2005). Although samples were taken from the area in a more recent study (Altun et al. 2013), only a very limited number of isolates (n=2) and antibiotics were evaluated. Therefore, this study was conducted to assess the current antibiotic susceptibility status of *L. garvieae* during a disease outbreak in the autumn of 2018, when stream flow slowed down, water temperature raised above 20 °C and fish biomass was high. In this assessment, all isolates were profiled based on their susceptibility to 33 different antibiotics and the presence of five different tetracycline resistance genes. Furthermore, the results were compared with previous studies to evaluate the dynamics of antibiotic susceptibility in *L. garvieae* over the years.

2. Material and Methods

2.1. Sample collection

During a disease outbreak, 60 rainbow trout samples, weighing an average of 165-216 g, were collected from farms operating on the banks of Eşen Stream in Muğla province, in two consecutive visits on 13 September and 15 November 2018. The samples were collected from three different farms at the beginning (farm A), middle (farm B) and end (farm C) of the stream sections where the majority of the trout farms are located. In both visits, ten fish showing disease signs (lethargic and anorexic with darkened skin, bilateral or one-sided exophthalmia or fallen eyeball, hemorrhages in the ocular, perianal area and in the fin basements) were sampled from each farm. After sampling, all fish were euthanized with high doses of MS-222 (100-200 mg/L, Priborsky & Velisek 2018 and references there) and examined for external parasites. Later, their body surfaces were disinfected with 70% ethanol and necropsy was performed. Bacterial isolation was carried out by transplanting tissue samples (anterior kidney, liver and spleen) onto tryptic soy agar (TSA) medium (Biokar-Diagnostics, France) under aseptic conditions and incubating the inoculated plates at 22 °C for 48 h. Afterwards, bacteria from tissue samples (62.5% anterior kidney, 25% spleen and 12.5% liver) that showed the most intense growth in TSA medium were transferred to tryptic soy broth (TSB) medium (Biokar-Diagnostics, France) and incubated at 25 °C for 24 h. Bacterial samples that had been grown in TSB medium were stored at -80 °C until use, after adding 20% sterile glycerin.

2.2. Morphological and biochemical identification of L. garvieae isolates

Identification of isolated bacteria by conventional methods was carried out at Isparta University of Applied Sciences, Eğirdir Fisheries Faculty, Microbiology Laboratory (IUEFF-ML). When the temperatures of the bacteria samples were taken out of the deep freezer equilibrated to room temperature, the samples were planted on TSA medium and incubated at 25 ºC for 48 h. Colony morphology, color and other characteristic features of bacteria were recorded. Afterwards, Gram staining, motility test, cytochrome oxidase, catalase, oxidation/fermentation (O/F) tests were performed (Austin & Austin 1999). For the remaining biochemical analysis, API 20 STREP (Biomerieux, France) test kit was used. API test was carried out under the protocol recommended by the company that produced the API test kit. The reference *L. garvieae* strain (ELG1) was obtained from IUEFF-ML Collection.

2.3. Molecular identification of L. garvieae isolates

Molecular identification of isolates was carried out at Akdeniz University, Faculty of Agriculture, Molecular Genetics Laboratory. For DNA isolation, 5 mL samples were prepared from the cultures incubated overnight in TSB medium. Then, the appropriate number of bacterium cells (approximately $1.5x10^9$ cells equivalent to McFarland 0.5 turbidity) specified in the total genomic DNA isolation kit (ThermoScientific, USA) protocol was taken from the culture. DNA isolation was performed using the protocol specific to Gram-positive bacteria, as suggested by the company that produced the kit. After evaluating the quality and quantity of isolated genomic DNA using a spectrophotometer (ThermoScientific, NanoDrop 1000), the DNA samples were diluted with 10 mM Tris-EDTA buffer up to 200 µl volume and stored at -20 °C until use.

B27F (5 'AGAGTTTGATCCTGGCTCAG 3') and U1492R (5 'GGTTACCTTGTTACGACTT 3') universal primers were used to amplify the targeted 16S rRNA gene sequences from total genomic DNA. The polymerase chain reaction (PCR) reaction mix was prepared by adding 2 ng of genomic DNA to 12.5 µl of 2X master PCR mix (Qiagen, Germany) as recommended in the kit protocol, 1 µl (10 nmol/µl) of each primer, and sterile distilled water to complete the total reaction volume to 25 µl. The PCR conditions applied for the multiplication of 16S rRNA genes consisted of an initial denaturation at 95 °C for 10 min, denaturation at 95 °C for 45 s, primer binding at 60 °C for 45 s, synthesis at 72 °C for 2.5 min and final elongation at 72 °C for 10 min. Denaturation, primer ligation and synthesis steps were repeated for 30 cycles. Afterwards, the PCR products were run in 2% agarose gel in order to check the reaction took place. An electric field of 8 volts/cm^2 was applied during agarose gel electrophoresis (Brody & Kern 2004) and 1 kb DNA ladder was employed for the determination of molecular size of PCR products.

2.4. Antibiotic resistance gene screening of L. garvieae isolates

Whether the *L. garvieae* that were isolated developed resistance against the commonly employed antibiotics in tetracycline class or not were confirmed by using gene-specific primers (Table 1). The PCR reaction mix was prepared as explained in section 2.3 using the same kit. The PCR conditions applied for the amplification of all tetracycline genes consisted of an initial denaturation at 94 °C for 5 min, further denaturation at 94 °C for 45 s, primer binding at 58 °C for 45 s, synthesis at 72 °C for 1 min (90 s for *tetE*) and final elongation at 72 °C for 10 min. Denaturation, primer ligation and synthesis steps were repeated for 35 cycles. Afterwards, the PCR products were run in 2% agarose gel under an electric field of 8 volts/cm^2 in order to check the presence of targeted resistance genes (Brody & Kern 2004). A 100 bp DNA ladder was employed for the determination of molecular size of PCR products. Positive-control bacteria (EAS4: *Aeromonas sobria*, EAH13: *A. hydrophila* and ELG17: *L. garvieae*) bearing targeted resistance genes were obtained from the collection of IUEFF-ML.

Table 1- Primer sequence, product size and T_m information of the screened tetracycline class antibiotic resistance genes

Targeted gene	*Primer*	*Primer nucleotid sequence (5'-3')*	*Product size (bp)*	*T_m (°C)*	*Reference*
tetA	Tet A FW	GCTACATCCTGCTTGCCTTC	210	63	Ng et al. (2001)
	Tet A RV	CATAGATCGCCGTGAAGAGG		64	
tetB	Tet B FW	TTGGTTAGGGGCAAGTTTTG	659	59	
	Tet B RV	GTAATGGGCCAATAACACCG		59	
tetC	Tet C FW	CTTGAGAGCCTTCAACCCAG	418	63	
	Tet C RV	ATGGTCGTCATCTACCTGCC		62	
tetD	Tet D FW	AAACCATTACGGCATTCTGC	787	60	
	Tet D RV	GACCGGATACACCATCCATC		60	

Table 1- Continued

Targeted gene	*Primer*	*Primer nucleotid sequence (5'-3')*	*Product size (bp)*	T_m *(°C)*	*Reference*
tetE	Tet E FW Tet E RV	GTGATGATGGCACTGGTCAT CTCTGCTGTACATCGCTCTT	1180	60 63	Schmidt et al. (2001)

T_m: Primer melting temperature

2.5. Antimicrobial susceptibility test

Kirby Bauer disc diffusion method was used to determine antibiotic resistance of the isolates. Bacterial isolates inoculated in TSB medium were incubated at 25 °C for 24 h. At the end of incubation period, turbidity of the bacterial suspensions was adjusted to 0.5 McFarland turbidity (Biomerieux, France) with physiological saline (0.9%). Then, a 0.1 mL sample taken from each suspension was distributed onto Mueller-Hinton agar (MHA) medium (Merck, Germany), containing 5% sheep blood, using a sterile swab and the plates were dried in a sterile cabinet for 5-10 min. Afterwards, antibiotic discs were placed on the plates and the plates were incubated at 25 °C for 48 h. At the end of incubation period, diameter of no growth zones formed around the antibiotic discs were measured with the aid of a millimetric ruler. Then, the isolates were classified as susceptible (S), moderately susceptible (I) or resistant (R) in accordance with the Clinical and Laboratory Standards Institute (CLSI) criteria (for *Enterococcus* spp., Enterobacterales, *Staphylococcus* spp. or *Streptococcus aureus* in the order of availability) or relevant previous studies (Table 2). Clinical breakpoints for lincomycin 2 µg (L2) and colistin 10 µg (CT10) cannot be found in the available literature. Hence the bacterial isolates were classified as "resistant" for these two antibiotics when the diameter of inhibition zones was "0". Remaining 31 different antibiotic discs were used in sensitivity detection of the isolates were ampicillin 10 µg (AM10), amoxicillin 25 µg (AX25), oxacillin 1 µg (OX1), penicillin G 10 U (P10), cephalothin 30 µg (CF30), cefoperazone 75 µg (CFP75), ceftriaxone 30 µg (CRO30), cefuroxime 30 µg (CXM30), spectinomycin 100 µg (SPT100), gentamicin 10 µg (GM10), kanamycin 30 µg (K30), streptomycin 10 µg (S10), trimethoprim 1.25 µg / sulfamethoxazole/23.75 µg (SXT25), vancomycin 30 µg (VA30), clindamycin 2 µg (CC2), apramycin 15 µg (APR15), erythromycin 15 µg (E15), pristinamycin 15 µg (PT15), tylosin 15 µg (TY15), nitrofurantoin 300 µg (FM300), chloramphenicol 30 µg (C30), florphenicol 30 µg (FFC30), ciprofloxacin 5 µg (CIP5), enrofloxacin 5 µg (ENO5), flumequine 30 µg (FLM30), norfloxacin 10 µg (NOR10), oxolinic acid 2 µg (OA2), ofloxacin 5 µg (OFX5), doxycycline 30 µg (DOX30), oxytetracycline 30 µg (T30) and tetracycline 30 µg (TE30).

Table 2- Antibiotic susceptibility test breakpoints used in the study

Antibiotics	*S*	*I*	*R*	*Reference*
β-Lactams				
Penicilins				
AM10	≥17	14-16	≤13	CLSI M100 2021
AX25	≥18	14-17	≤13	CLSI M100 2021
OX1	≥13	11-12	≤10	CLSI M31 2008
P10	≥15	-	≤14	CLSI M100 2020
Cephems				
CF30	≥18	15-17	≤14	CLSI M31 2008
CFP75	≥21	16-20	≤15	CLSI M31 2008
CRO30	≥27	25-26	≤24	CLSI M31 2008
CXM30	≥18	15-17	≤14	CLSI M31 2008
Non-β-Lactams				
Aminocyclitols				
SPT100	≥14	11-13	≤10	CLSI VET01S 2020
Aminoglycosides				
GM10	≥15	13-14	≤12	CLSI M100 2021
K30	≥18	14-17	≤13	CLSI M100 2021
S10	≥15	12-14	≤11	CLSI M100 2021
APR15	≥25	17-24	≤16	CLSI VET01S 2020
Folate pathway antagonists				
SXT25	≥16	11-15	≤10	CLSI M100 2021

Table 2- Continued

Antibiotics	*S*	*I*	*R*	*Reference*
Glycopeptides				
VA30	≥17	15-16	≤14	CLSI M100 (2020)
Lincosamides				
CC2	≥19	16-18	≤15	CLSI M31 (2008)
L2	NA	NA	NA	NA
Lipopeptides				
CT10	NA	NA	NA	NA
Macrolides				
E15	≥23	14-22	≤13	CLSI M100 (2020)
PT15	≥22	19-21	<19	Perrin-Guyomard et al. (2005)
TY15	≥26	19-25	≤18	CLSI VET01S (2020)
Nitrofurans				
FM300	≥17	15-16	≤14	CLSI M100 (2021)
Phenicols				
C30	≥18	13-17	≤12	CLSI M100 (2021)
FFC30	≥29	23-28	≤22	CLSI VET01S (2020)
Quinolones				
CIP5	≥26	22-25	≤21	CLSI M100 (2021)
ENO5	≥23	17-22	≤16	CLSI VET01S (2020)
FLM30	≥21	16-20	≤15	Korun et al. (2021)
NOR10	≥17	13-16	≤12	CLSI M100 (2021)
OA2	≥13	11-12	≤10	Balta & Balta (2019)
OFX5	≥16	13-15	≤12	CLSI M100 (2021)
Tetracylines				
DOX30	≥14	11-13	≤10	CLSI M100 (2021)
T30	≥19	15-18	≤14	Balta & Balta (2019)
TE30	≥15	12-14	≤11	CLSI M100 (2021)

S: Susceptible, I: Moderately susceptible, R: Resistant, AM10: Ampicillin, AX25: Amoxicillin, OX1: Oxacillin, P10: Penicillin, CF30: Cephalothin, CFP75: Cefoperazone, CRO30: Ceftriaxone, CXM30: Cefuroxime, SPT100: Spectinomycin, GM10: Gentamicin, K30: Kanamycin, S10: Streptomycin, SXT25: Trimethoprim/sulfamethoxazole, VA30: Vancomycin, CC2: Clindamycin, L2: Lincomycin, CT10: Colistin, APR15: Apramycin, E15: Erythromycin, PT15: Pristinamycin, TY15: Tylosin, FM300: Nitrofurantoin, C30: Chloramphenicol, FFC30: Florphenicol, CIP5: Ciprofloxacin, ENO5: Enrofloxacin, FLM30: Flumequine, OA2: Oxolinic acid, OFX5: Ofloxacin, NOR10: Norfloxacin, DOX30: Doxycycline, T30: Oxytetracycline, TE30: Tetracycline. Numbers within the abbreviations show antibiotic concentration of the discs in µg, except penicillin G, of which concentration was unit. NA: Clinical inhibition zone breakpoints are not available

2.6. Calculation of multiple antibiotic resistance index values

The multiple antibiotic resistance (MAR) index values were calculated as the ratio of the number of antibiotics to which organisms were resistant in comparison to total number of antibiotics were evaluated. Isolates with a calculated MAR value greater than 0.20 were considered to have MAR (Krumperman 1983; Çapkin et al. 2015).

3. Results

All sampled fish were euthanized with high doses of MS-222 and examined for external parasites. No external parasites were detected on the specimens.

3.1. Morphological and biochemical identification of L. garvieae isolates

Sixteen bacterial isolates (3 each from farm A and B, and 10 from farm C) were obtained from 16 out of 60 fish sampled showed morphological and biochemical characteristics compatible with *L. garvieae*. The API 20 STREP test (Table 3) confirmed that the biochemical properties of all 16 isolates were well matched with *L. garvieae*.

Table 3- API 20 STREP results of *Lactococcus garvieae* isolates

FARM ID	*ISOLATE ID*	*VP*	*HIP*	*ESC*	*PYRA*	*αGAL*	*βGUR*	*βGAL*	*PAL*	*LAP*	*ADH*	*RIB*	*ARA*	*MAN*	*SOR*	*LAC*	*TRE*	*INU*	*RAF*	*AMD*	*GLYG*
	ELG1	+	+	+	+	-	-	-	-	+	+	+	-	+	-	-	+	-	-	+	-
A	LG4, 15, 16	+	+	+	+	-	-	-	-	+	+	+	-	+	-	-	+	-	-	+	-
B	LG3, 13, 14	+	+	+	+	-	-	-	-	+	+	+	-	+	-	-	+	-	-	+	-
C	LG1, 2, 5, 6, 7, 8, 9, 10, 11, 12	+	+	+	+	-	-	-	-	+	+	+	-	+	-	-	+	-	-	+	-

ELG1: Reference *L. garvieae* strain from Isparta University of Applied Sciences, Eğirdir Fisheries Faculty, Microbiology Laboratory Collection, VP: Sodium pyruvate, HIP: Hippuric acid, ESC: Esculin ferric citrate, PYRA: Pyroglutamic acid ß-naphthylamide, αGAL: 6-bromo-2-naphthyl α-D-galactopyranoside, βGUR: Naphthol AS-BI-ß-D-glucuronic acid, βGAL: 2-naphthyl ß-D-galactopyranoside, PAL: 2-naphthyl phosphate, LAP: L-leucine-ß-naphthylamide, ADH: L-arginine, RIB: D-ribose, ARA: L-arabinose, MAN: D-mannitol , SOR: D-sorbitol, LAC: D-lactose, TRE: D-trehalose, INU: Inulin, RAF: D-raffinose, AMD: Starch, GLYG: Glycogen

3.2. Molecular identification of L. garvieae isolates

Concordant with the conventional identification methods, BLASTN algorithm yielded a perfect match (99.86-100%) with *L. garvieae*, when the 16S rDNA sequences of 16 isolates were queried with the bacterial gene sequences in the GenBank database.

3.3. Antibiotic resistance gene profiles of L. garvieae isolates

Our profiling study revealed that 15 out of 16 isolates carried *tetA* gene, 13 isolates carried *tetB* gene, and 12 isolates carried both antibiotic resistance genes (Table 4). Although we detected the presence of *tetC*, *tetD* and *tetE* genes in our reference bacteria evaluated in the same PCR reaction, these three tetracycline resistance genes were not found in any of the 16 *L. garvieae* isolates studied (Table 4).

Table 4- Antibiotic resistance gene profiles of *Lactococcus garvieae* isolates

Farm ID	*Isolate ID*	*Antibiotic resistance genes*				
		tetA	*tetB*	*tetC*	*tetD*	*tetE*
A	LG4	+	+	-	-	-
	LG15	+	+	-	-	-
	LG16	+	-	-	-	-
B	LG3	+	+	-	-	-
	LG13	+	-	-	-	-
	LG14	+	+	-	-	-
C	LG1	+	+	-	-	-
	LG2	+	+	-	-	-
	LG5	+	+	-	-	-
	LG6	-	+	-	-	-
	LG7	+	+	-	-	-
	LG8	+	+	-	-	-
	LG9	+	+	-	-	-
	LG10	+	-	-	-	-
	LG11	+	+	-	-	-
	LG12	+	+	-	-	-
Positive controls	EAS4	+	-	+	-	-
	EAH13	-	-	-	+	-
	ELG17	+	+	-	-	+

+: positive, -: negative, EAS4: *Aeromonas sobria*, EAH13: *A. hydrophila* and ELG17: *L. garvieae* strain from Isparta University of Applied Sciences, Eğirdir Fisheries Faculty, Microbiology Laboratory collection

3.4. Antimicrobial susceptibility of L. garvieae isolates

Disk diffusion tests showed that all 16 isolates had different antibiotic susceptibility profiles (Table 5). While 2 isolates (B-LG14 and C-LG11) were found to be resistant or developing resistance to all antibiotics, 11 out of the remaining 14 isolates showed resistance or have been developing resistance to more than 50% of the 33 antibiotics evaluated. Only 3 isolates (A-LG16, B-LG13 and C-LG1) showed resistance or were developing resistance to less than 50% (39, 33 and 45%, respectively) of the 33 antibiotics evaluated. It was also determined that all isolates were either resistant or developing resistance to 8 antibiotics (OA2, CC2, TY15, N0R10, APR15, FFC30, CIP15 and FLM30), and 50% or more of the isolates were resistant or developing resistance against 20 antibiotics (AM10, VA30, SXT25, E15, ENO5, GM10, K30, FM300, CT10, OFX5, T30, CFP75, CRO30, SPT100, CF30, AX25, PT15, OX1, S10 and L2). As a result, it was seen that only 5 antibiotics (TE30, C30, DOX30, CXM30 and P10) remained to which more than 50% of the isolates still showed susceptibility.

Table 5- Antibiotic susceptibility profiles of *Lactococcus garvieae* isolates according to disc diffusion method (diameter of inhibition zones in mm)

Antibiotics	***FARM A ISOLATE ID***			***FARM B ISOLATE ID***			***FARM C ISOLATE ID***										
	LG4	***LG15***	***LG16***	***LG3***	***LG13***	***LG14***	***LG1***	***LG2***	***LG5***	***LG6***	***LG7***	***LG8***	***LG9***	***LG10***	***LG11***	***LG12***	***Total I+R***
AM10*	R(13)	R(0)	S(33)	I(15)	S(35)	R(0)	R(13)	R(13)	I(14)	R(0)	S(25)	I(14)	R(0)	R(0)	R(0)	R(0)	13
AX25*	S(21)	R(0)	S(35)	I(17)	S(35)	R(0)	S(19)	S(20)	S(20)	R(0)	S(23)	S(23)	R(0)	I(17)	R(0)	R(0)	8
OX1**	R(0)	R(0)	R(0)	I(12)	R(10)	R(0)	S(16)	R(0)	R(0)	R(0)	R(0)	S(14)	R(0)	I(12)	R(0)	S(16)	13
P10**	S(20)	R(0)	S(28)	S(22)	S(30)	R(0)	S(27)	S(20)	S(22)	R(0)	S(22)	R(0)	S(15)	R(12)	R(0)	R(0)	7
CF30**	R(12)	R(0)	S(27)	R(0)	S(30)	R(0)	I(16)	I(15)	S(18)	R(0)	R(0)	S(18)	R(0)	R(0)	R(0)	R(0)	12
CFP75*	R(14)	I(19)	S(30)	R(14)	S(32)	R(0)	R(0)	I(16)	R(0)	S(22)	R(0)	R(14)	R(0)	I(17)	R(0)	R(0)	13
CRO30*	R(13)	R(0)	S(30)	R(14)	S(35)	R(0)	R(21)	R(20)	R(0)	R(23)	S(28)	R(13)	R(14)	R(11)	R(0)	R(0)	13
CXM30**	S(25)	R(0)	S(36)	S(26)	S(40)	R(0)	S(20)	S(21)	S(23)	S(28)	S(27)	R(12)	R(7)	R(8)	R(0)	S(20)	6
SPT100***	I(12)	R(0)	S(20)	S(14)	S(21)	R(0)	S(14)	R(0)	R(0)	R(0)	S(14)	I(12)	R(8)	S(14)	R(0)	S(32)	9
GM10*	S(22)	R(11)	S(17)	S(16)	S(20)	R(0)	S(16)	I(14)	I(13)	R(12)	I(13)	R(0)	S(15)	R(0)	R(0)	R(0)	10
K30*	R(12)	R(0)	S(20)	I(15)	I(15)	R(0)	I(17)	I(15)	R(13)	R(0)	R(0)	R(12)	R(13)	R(11)	R(0)	R(0)	15
S10*	R(0)	R(0)	S(17)	R(0)	S(16)	R(0)	R(0)	R(0)	R(0)	R(0)	R(7)	S(16)	R(10)	I(14)	R(0)	R(0)	13
SXT25**	I(11)	R(0)	R(0)	R(0)	S(20)	R(0)	S(16)	I(13)	I(13)	R(0)	R(0)	I(13)	S(24)	I(15)	R(0)	R(0)	13
VA30*	S(20)	I(15)	S(25)	S(18)	S(25)	R(0)	S(24)	S(20)	I(16)	R(0)	I(15)	S(22)	I(15)	I(10)	R(0)	I(16)	9
CC2**	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(12)	R(12)	R(0)	R(13)	16
L2**	R(0)	R(0)	R(0)	R(0)	-(16)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	-(8)	R(0)	R(0)	R(0)	14
CT10*	-(16)	R(0)	R(0)	-(18)	R(0)	R(0)	-(16)	-(17)	-(15)	R(0)	R(0)	-(10)	R(0)	-(11)	R(0)	-(16)	8
APR15*	R(0)	R(0)	R(16)	R(0)	R(14)	R(0)	R(12)	R(0)	R(10)	R(13)	R(11)	R(13)	R(0)	R(0)	R(0)	R(16)	16
E15*	S(24)	I(15)	S(30)	R(0)	S(30)	R(0)	S(28)	R(0)	S(24)	S(26)	S(26)	R(11)	R(8)	R(0)	R(0)	R(0)	9
PT15**	S(23)	R(0)	S(23)	I(20)	S(23)	R(0)	S(23)	I(19)	I(21)	I (20)	I 20	R(9)	R(13)	R(0)	R(0)	R(0)	12
TY15*	R(13)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(13)	R(11)	R(0)	R(0)	16
FM300***	S(20)	R(11)	S(22)	S(17)	S(21)	R(0)	S(23)	S(20)	S(21)	I(15)	I(15)	R(13)	R(12)	S(22)	R(0)	R(0)	8
C30**	S(22)	S(20)	S(34)	S(20)	S(30)	R(0)	S(30)	S(25)	S(23)	S(23)	R(0)	R(0)	I(14)	I(13)	R(0)	S(28)	6
FFC30**	R(17)	R(22)	R(16)	R(15)	R(0)	R(0)	R(0)	I(24)	R(22)	R(0)	I(23)	R(12)	R(0)	R(21)	I(23)	R(0)	16
CIP5*	R(13)	R(0)	I(24)	R(12)	I(22)	R(0)	R(13)	R(12)	R(14)	R(0)	R(0)	R(15)	R(13)	I(22)	R(0)	R(18)	16
ENO5*	S(23)	R(0)	S(25)	R(16)	S(25)	R(0)	S(23)	I(22)	I(21)	R(13)	I(20)	R(0)	R(12)	R(12)	R(0)	R(8)	12
FLM30*	R(7)	R(0)	R(0)	R(12)	R(12)	R(0)	R(0)	I(17)	R(0)	R(0)	R(0)	R(0)	R(7)	R(12)	R(0)	I(17)	16
OA2*	R(0)	R(0)	R(0)	R(0)	I(12)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(7)	R(9)	R(0)	R(0)	16
OFX5*	I(13)	R(0)	S(20)	R(0)	S(20)	R(0)	S(23)	S(22)	S(22)	R(0)	R(0)	I(13)	I(13)	S(22)	R(0)	R(0)	10
NOR10*	R(8)	R(0)	I(15)	R(0)	R(12)	R(0)	R(12)	R(10)	R(0)	R(0)	R(0)	R(12)	R(0)	R(10)	R(0)	R(0)	16
DOX30**	S(25)	S(16)	S(32)	S(19)	S(31)	R(0)	S(30)	S(25)	S(26)	S(25)	S(25)	I(13)	S(22)	I(12)	R(0)	R(0)	5
T30**	I(16)	R(0)	S(24)	R(0)	S(25)	R(0)	S(21)	I(17)	R(0)	R(0)	S(22)	R(0)	R(7)	I(16)	R(0)	I(16)	12
TE30**	S(22)	I(13)	S(25)	S(22)	S(23)	R(0)	S(25)	S(21)	S(20)	R(0)	R(0)	I(12)	S(19)	I(14)	R(0)	S(20)	7

Table 5- Continued

Antibiotics	*FARM A ISOLATE ID*			*FARM B ISOLATE ID*			*FARM C ISOLATE ID*										
	LG4	*LG15*	*LG16*	*LG3*	*LG13*	*LG14*	*LG1*	*LG2*	*LG5*	*LG6*	*LG7*	*LG8*	*LG9*	*LG10*	*LG11*	*LG12*	*Total I+R*
Total I+R	21	31	13	24	11	33	15	23	22	28	24	27	27	29	33	27	
Total R	16	27	10	18	8	33	13	13	16	26	18	21	24	18	32	24	
MAR	0.48	0.82	0.30	0.55	0.24	1.00	0.39	0.39	0.48	0.79	0.55	0.64	0.73	0.55	0.97	0.73	

S: Susceptible, I: Moderately susceptible, R: Resistant, -: not classified, *Critically important antimicrobials, **Highly important antimicrobials, ***Important antimicrobials, AM10: Ampicillin, AX25: Amoxicillin, OX1: Oxacillin, P10: Penicillin G, CF30: Cephalothin, CFP75: Cefoperazone, CRO30: Ceftriaxone, CXM30: Cefuroxime, SPT100: Spectinomycin, GM10: Gentamicin, K30: Kanamycin, S10: Streptomycin, SXT25: Trimethoprim/sulfamethoxazole, VA30: Vancomycin, CC2: Clindamycin, L2: Lincomycin, CT10: Colistin, APR15: Apramycin, E15: Erythromycin, PT15: Pristinamycin, TY15: Tylosin, FM300: Nitrofurantoin, C30: Chloramphenicol, FFC30: Florphenicol, CIP5: Ciprofloxacin, ENO5 Enrofloxacin, FLM30: Flumequine, OA2: Oxolinic acid, OFX5: Ofloxacin, NOR10: Norfloxacin, DOX30: Doxycycline, T30: Oxytetracycline, TE30: Tetracycline. The numbers in the abbreviated names show antibiotic concentration of the discs in µg, except penicillin G, of which concentration was unit. MAR: Multiple Antibiotic Resistance index value

3.5. Multiple antibiotic resistance index values

The MAR index values of the 16 isolates ranged from 0.24 (B-LG13) to 1.00 (B-LG14) (Table 5). Based on these high (>0.20) MAR index values, we can say that all 16 isolates evaluated in this study carried MAR.

4. Discussion

Since the first record of *L. garvieae* from rainbow trout farms located in the western part of Turkey in 2001 (Diler et al. 2002), different antibiotics have been used to control lactococcal infections (Kubilay et al. 2005; Balta & Balta 2019). Studies have been conducted since then and the results of present study clearly reveal the dynamic nature of antibiotic resistance development in *L. garvieae*. Our results showed that all 16 *L. garvieae* isolates studied had unique antibiotic susceptibility profile. In addition, it was detected that 2 of these isolates (B-LG14 and C-LG11) were either resistant or developing resistance to all 33 antibiotics at the doses evaluated. Fifty percent or more of the remaining 14 isolates also showed resistance to or were developing resistance against 28 antibiotics, indicating that only 5 antibiotics remained (penicillin G, cefuroxime, chloramphenicol, doxycycline and tetracycline) to which more than 50% of the isolates were still susceptible (Table 5). In contrast to these, previous studies reported resistance against 3 of these 5 remaining antibiotics (for penicillin G in Diler et al. 2002 and Kubilay et al. 2005, for cefuroxime in Kubilay et al. 2005, and for doxycycline in Altun et al. 2013).

Unlike previous studies that were evaluated, only a limited number of isolates from a production area (Kubilay et al. 2005; Altun et al. 2013; Kurtoğlu & Korun 2018; Balta & Balta 2019) or all 30 isolates obtained had the same antibiogram test results (Kav & Erganis 2008), our study evaluated 16 different isolates showed that *L. garvieae* with different antibiotic susceptibility profiles can be found in a farm or in different farms within the same production area. Under these circumstances, trout farmers would be unlikely to cure lactococcosis without having a prior antibiogram testing or by simply administering one type of antibiotic. Farmers who cannot treat their sick fish may contribute more to the development of resistance if they unconsciously increase the dose of antibiotics or exploit with various antibiotics. Although, it is not possible to establish a clear link without precise data on the type and intensity of antibiotic treatments applied by trout farmers over the years, our study showed that *L. garvieae* isolated from the rainbow trout raised in Muğla province either had already developed resistance or had been developing resistance to ampicillin, cephalothin, spectinomycin, vancomycin, erythromycin, pristinamycin, nitrofurantoin, chloramphenicol, enrofloxacin, ofloxacin and tetracycline (Table 5). Whereas previous studies including isolates from the region reported that *L. garvieae* isolates were susceptible to these 11 antibiotics (Table 6). Studies involving samples from other parts of the country also reported isolates of *L. garvieae* were still susceptible to cefoperazone, florphenicol, ciprofloxacin and oxolinic acid (Kav & Erganis 2008; Altun et al. 2013; Balta & Balta 2019), but 81% of the 16 isolates in this study were resistant to cefoperazone and all 16 isolates were resistant to the same doses of the last 3 antibiotics (Table 6). High MAR values ranging between 0.24-1.00 also supported these results and suggested that all 16 isolates developed MAR (Krumperman 1983). Thus, we can say that results of the present study indicate that the antibiotic resistance of *L. garvieae* has increased over the years in Muğla province. On the other hand, we should point out that the procedure used in antibiotic susceptibility tests might have contributed to outcomes of the study. Unlike previous studies, we followed the latest CLSI guidelines (CLSI M100 2021) for antibiotic susceptibility determination, thus used 5% sheep blood supplemented MHA and a 48 h incubation period. However, all previous studies presented in Table 6 used MHA only, and some used shorter (20 and 24 h) incubation periods (Kav & Erganis 2008; Balta & Balta 2019). Both procedural differences have potential to induce smaller inhibition zones, as sheep blood providing additional nutrients promotes faster and more efficient bacterial growth and a longer incubation period allow more colony formation (CLSI M100 2021).

Thus, it is possible that these procedural differences had increased our rate of classifying isolates as resistant or intermediate resistant. However, this does not mean that the latest CLSI procedure has resulted in a misclassification of the antibiotic susceptibility of bacteria. Instead, it demonstrates that the use of a standard procedure for antibiotic susceptibility testing on fish pathogens is important to obtain accurate and comparable results. Especially, considering that CLSI test breakpoints are commonly employed when classifying antibiotic susceptibility of fish pathogens.

Table 6- Antibiotic resistance profiles of *Lactococcus garvieae* in previous studies from Turkey

Antibiotics	*Location*					
	Muğla province				*Konya province*	*Eastern Blacksea region*
	Diler et al. (2002)	*Kubilay et al. (2005)*	*Altun et al. (2013)*	*Kurtoğlu & Korun (2018)*	*Kav & Erganis (2008)*	*Balta & Balta (2019)*
AM10 (81)	S	S	-	S	S	I+R
AX25 (50)	-	-	R+I	-	S	S+I+R(10 µg)
OX1 (81)	-	R	-	-	R (5µg)	-
P10 (44)	R	R	-	-	S	-
CF30 (75)	-	S+R	-	-	-	-
CFP75 (81)	-	-	-	-	S (30 µg)	-
CRO30 (81)	R	R	-	-	-	-
CXM30 (38)	-	R	-	-	-	-
SPT100 (56)	-	S*	-	-	-	-
GM10 (63)	-	-	R (120 µg)	-	R	-
K30 (94)	-	R	-	R	-	-
S10 (81)	-	R	-	R	-	R
SXT25 (81)	-	R	R	-	R	R
VA30 (56)	-	S	-	-	S (5 µg)	-
CC2 (100)	R	R	-	-	R	-
L2 (88)	-	R	R	-	R(10 µg)	-
CT10 (50)	-	R*	-	-	-	-
APR15 (100)	-	R	-	-	-	-
E15 (56)	S	S	R+I	S+I	S	S+I+R
PT15 (75)	-	S*	-	-	-	-
TY15 (100)	-	R*	-	-	-	-
FM300 (50)	-	S	-	-	-	-
C30 (38)	S	S	-	S	S	-
FFC30 (100)	-	-	R+I		S	S+I+R
CIP5 (100)	-	R	-	-	S	-
ENO5 (75)	-	S	-	-	I	S+I+R
FLM30 (100)	-	-	-	R	-	-
OA2 (100)	-	-	-	R	-	S+I+R

Table 6- Continued

Antibiotics	*Location*					
	Muğla province				*Konya province*	*Eastern Blacksea region*
	Diler et al. (2002)	*Kubilay et al. (2005)*	*Altun et al. (2013)*	*Kurtoğlu & Korun (2018)*	*Kav & Erganis (2008)*	*Balta & Balta (2019)*
OFX5 (63)	S	S+I+R	-	-	-	-
NOR10 (100)	-	R	-	-	-	-
DOX30 (31)	-	S	R+I	-	-	S+I+R
T30 (75)	-	-	R+S+I	-	S	S+I+R
TE30 (44)	S	S	-	S	-	-

S: Susceptible, I: Moderately susceptible, R: Resistant, -: not classified, AM10: Ampicillin, AX25: Amoxicillin, OX1: Oxacillin, P10: Penicillin G, CF30: Cephalothin, CFP75: Cefoperazone, CRO30: Ceftriaxone, CXM30: Cefuroxime, SPT100: Spectinomycin, GM10: Gentamicin, K30: Kanamycin, S10: Streptomycin, SXT25: Trimethoprim / sulfamethoxazole, VA30: Vancomycin, CC2: Clindamycin, L2: Lincomycin, CT10: Colistin, APR15: Apramycin, E15: Erythromycin, PT15: Pristinamycin, TY15: Tylosin, FM300: Nitrofurantoin, C30: Chloramphenicol, FFC30: Florphenicol, CIP5: Ciprofloxacin, ENO5: Enrofloxacin, FLM30: Flumequine, OA2: Oxolinic acid, OFX5: Ofloxacin, NOR10: Norfloxacin, DOX30: Doxycycline, T30: Oxytetracycline, TE30: Tetracycline. The numbers in the abbreviated names show the antibiotic concentration of the discs in µg, except penicillin G, of which concentration was unit. Numbers in parentheses after abbreviated names indicate the percentage of isolates classified as I+R in the present study. Classifications written in bold are for isolates obtained from rainbow trout raised in Muğla province. *Classified using ATB VET stript. Doses different from those used in the present study are given in parentheses

In addition to determining their susceptibility to 3 different antibiotics (doxycycline, oxytetracycline and tetracycline) from the tetracyclines class (Chopra & Roberts 2001), we investigated the presence of 5 different tetracycline resistance genes in all isolates. Among the 5 genes, *tetA* was the most common one in the studied *L. garvieae* isolates. This finding of our study is compatible with the reported results of Raissy and Shahrani (2015). Additionally, all isolates had either *tetA* or *tetB* gene and the majority of isolates (75%) carried both resistance genes. Together with this, 4 isolates (C-LG1, C-LG2, B-LG13, and A-LG16) showed susceptibility to all tetracycline class antibiotics, 4 isolates (B-LG3, A-LG4 and C-LG5 and C-LG9) showed susceptibility to doxycycline and oxytetracycline, one isolate (C-LG7) showed susceptibility to doxycycline and tetracycline, and one isolate (C-LG12) was susceptible to oxytetracycline. These results of the study suggest that the antibiotic resistance genes carried by *L. garvieae* are not sufficiently expressed to provide resistance against the antibiotic doses used in the study or antibiotic resistance genes may be silent as reported in another study (Randall et al. 2004). A similar contrast has also been reported in other studies involving *Pantoea agglomerans* (Saticioglu et al. 2018) and *L. garvieae* (Duman et al. 2020).

In addition to genes, molecules such as AmpC β-lactamases can lead to the development of MAR in bacteria (Noor ul Ain et al. 2014). This means that acquired resistance to one antibiotic can lead to the development of resistance against many other antibiotics, depending on the origin of encounter. Therefore, it is possible for existing or acquired antibiotic resistance to cause bacteria to develop resistance to many other antibiotics with the same mode of action. In the present study, we used large numbers of antibiotics, the majority of which are not used by trout farmers to treat lactococcosis or other bacterial fish diseases, to see the extent of antibiotic resistance development in *L. garvieae*. Unfortunately, we detected isolates showing resistance to all or almost all antibiotics at the doses evaluated. Determining the true causes of such a wide range of antibiotic resistance requires more extensive studies. Together with this, we should mention that there is no settlement that will cause sewage or other types of pollution before the stream section occupied by trout farms in the production area. Additionally, one of the sampled farms and some other farms in the area buy eyed eggs and fingerlings from the hatcheries located in various different regions of the country and also abroad. These two facts lead us to think fish inflows from contaminated areas could be one reason for this wide range of resistance. Nevertheless, as we stated above, further extensive research is necessary for a proper addressing of the issue.

Besides being one of the most important fish pathogens with devastating effects in aquaculture (Algöet et al. 2009), *L. garviae* is also defined as an opportunistic bacterium that can infect humans, especially the elderly (Gibello et al. 2016). Thus, it is possible that bare hand handling and raw or undercooked consumption of infected fish may have humans exposed to this zoonotic pathogen (Chan et al. 2011). Within the 33 antibiotics evaluated in this study, 18 were listed as critically and 13 were listed as highly important antimicrobials for human medicine (Table 5) by the World Health Organization (WHO 2019). And, we determined that 31-100% of the *L. garviae* strains isolated have already developed or have been developing resistant to these two groups of antibiotics. This result of the study

also suggests that antibiotic resistance developed by *L. garviae* may have reached a level that may pose significant health risks for farm workers and consumers as well.

5. Conclusions

In conclusion, no antibiotic to which all *L. garvieae* isolates are susceptible could be determined in this study. Antibiotics to which ≥50% of the strains are still susceptible (penicillin, tetracycline, pristinamycin, chloramphenicol, amoxicillin, nitrofurantoin, cefuroxime, doxycycline) should be used very carefully without forgetting that the remaining strains had already acquired or are developing resistance against them. As demonstrated in this study, several isolates of *L. garvieae* with different antibiotic susceptibility profiles can exist within and between farms using the same water source. In such a case, treatments with a single antibiotic may not be effective or the antibiotic treatment that works in one farm may not work in another. Therefore, we strongly recommend that each farm determine the effective antibiotic cure in terms of type and dose according to the antibiogram test performed specifically for each bacterial disease case. In addition, it should be kept in mind that antibiotic resistance already developed in the studied *L. garvieae* isolates may spread over time between the same or other pathogenic bacteria species in the production area. Thus, we also recommend to screen fish before all transfers between different water bodies to prevent the spreading of pathogenic bacteria. Monitoring the current antibiotic susceptibility of fish pathogens with periodic studies and preventing the spread of new or resistant fish pathogens throughout the country by establishing national monitoring programs will contribute to healthier development of the aquaculture industry. However, it should be noted that monitoring studies with a limited number of isolates may not give the full picture. Therefore, to make a more accurate assessment of current antimicrobial susceptibility in a production area, we recommend evaluating as many bacterial isolates as possible in the future studies. Finally, we would like to emphasize that handling infected fish with bare hands and consuming them raw or undercooked carry risk of exposing humans to zoonotic pathogens that are resistant to many of the antibiotics used in human medicine.

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Data availability: Data are available on request due to privacy or other restrictions.

Ethics Committee Approval: The procedures applied in this study were evaluated by the Akdeniz University Animal Experiments Local Ethics Committee and their ethical compliance was approved with the protocol number 2018.03.001 (date:26.02.2018).

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