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Epidemiology, genotypic diversity, and antimicrobial resistance of *Lactococcus garvieae* in farmed rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Bacterial agents must be genotypically analyzed for vaccinations, effective control programs, and antimicrobial resistance genes that could transfer from aquaculture settings to terrestrial ecosystems and humans. Therefore, we investigated the prevalence, genotypic characterization, and antimicrobial resistance of *Lactococcus garvieae* for two years at aquaculture sites throughout Turkey. A total of 137 *L. garvieae* isolates were obtained from rainbow trout (*Oncorhynchus mykiss*) farms in different regions of Turkey, and three reference strains were used. The isolates were confirmed genotypically using species specific primer sets. All isolates were genotyped with RAPD-PCR using M13 primers. Five different genogroups were determined, and the reference strains were found to differ from all the isolates. Some isolates were compared with the GeneBank database and most isolates were within the same European, Asian, Australian, and South African genogroups. Isolates showed differing levels of resistance to most of the commonly used antimicrobials. The *erm*B, *erm*A, *tet*M, and *tetS* genes were identified and confirmed, whereas the *flo*R, *sul*I, *sul*II, *sul*III, *tet*A, *tet*B, and *tet*E genes were not detected. The identification of antimicrobial resistance genes in rainbow trout fry (weight 0.5 g) showed that genes for antimicrobial resistance could be spread during any stage of the fishes' life, thereby facilitating transmission of resistance to humans and other animals. The investigation of antimicrobial resistance genes in phenotypically susceptible isolates revealed that it is insufficient to investigate only phenotypic resistance in antimicrobial resistance studies.

Keywords: *Lactococcus garvieae*, Genotyping, RAPD-PCR, Antimicrobial resistance, Antimicrobial resistance genes

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Introduction

Lactococcosis is one of the most important diseases and causes of economic loss in aquaculture. It is also an important zoonotic disease that has been isolated from the urinary, circulatory, skin, and respiratory systems of humans in the USA; from bacterial endocarditis infections in Canada; and in immunosuppressed individuals suffering from liver abscess in France (Elliott *et al*., 1991; Fefer *et al*., 1998; James *et al*., 2000; Mofredj *et al*., 2000; Fihman *et al*., 2006). Among farmed rainbow trout, the mortality rate from *L. garvieae* infection can reach 50–80% (Itami *et al*., 1996).

Rainbow trout farmers make extensive use of antimicrobial agents for treating lactococcosis. Antimicrobial agents are mixed with feed or used in bathing treatments for fish, and therefore, contaminated feed and fish feces can be transmitted to other areas via water (Hektoen *et al*., 1995; Kerry *et al*., 1996; Coyne *et al*., 1997; Markestad and Grave, 1996; Holten *et al*., 1999; Sorum, 1999; Guardabassi *et al*., 2000; Sorum and L'Abée-Lund, 2002; Boxall *et al*., 2004; Sorum, 2006). In particular, antimicrobial residues are an important risk for agricultural land using the same water supply, which results in contamination of agricultural products and can infect human consumers of these products. The most common antimicrobials for the control of *L. garvieae* infections are erythromycin, oxytetracycline, amoxicillin, and doxycycline (Munday, 1994). Kawanishi *et al*. (2005) found resistance against macrolides (erythromycin and lincomycin), oxytetracycline, and some resistance genes (*erm*B and *tetS*) in *L. garvieae* isolated from yellowtail (*Seriola*). In recent years, *L. garvieae* has been found to be sensitive to erythromycin, ofloxacin, ampicillin, and chloramphenicol, whereas a Turkish study reported resistance to penicillin and clindamycin (Ture and Boran, 2015). Bacterial distribution, genetic heterogeneity, and antimicrobial resistance are important issues for effective vaccines. To our knowledge, there is limited research on the antimicrobial resistance profiles of *L. garvieae*, especially with regard to genotyping. The present study therefore investigated genetic heterogeneity and antimicrobial resistance profiles of *L. garvieae* present in farmed rainbow trout obtained from commercial fish farms in Turkey.

Materials and methods

Phenotypic identification of *L. garvieae*

A series of 137 isolates were collected from six different regions of Turkey between 2013 and 2015 (Fig. 1). After bacterial isolation from ten different rainbow trout farms (all with production capacities of at least 1000 tons per year), every isolate and three reference strains were identified by Gram staining, oxidase, catalase, oxidation–fermentation (O/F), growth in MacConkey's agar, motility tests, and polymerase chain reaction analysis (PCR) (Austin and Austin, 2007).

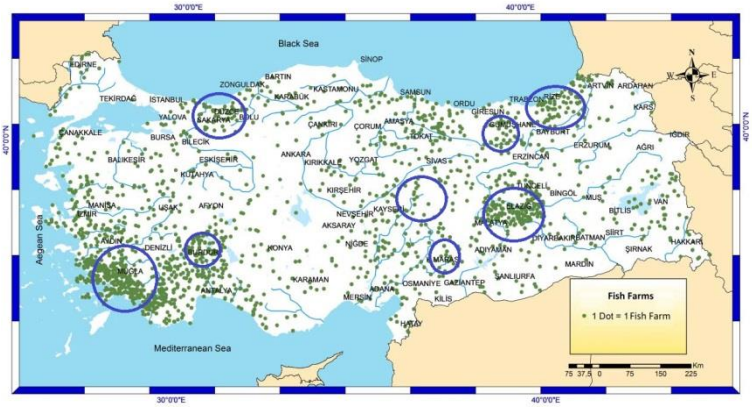

Figure 1: Isolation area in 2013-2015 years, monthly is blue rings.
ºGreen points are distribution of rainbow trout farms in Turkey (Akova, 2015)

Molecular identification

DNA was extracted by spin column filtration kits according to the manufacturer's instructions (QIAamp DNA mini kit, 51306, Hilden, Germany) DNA concentration and purity of isolates were measured at 260 nm and 260/280 nm wavelengths using a spectrophotometer (Multiscan Go, Thermo).

Two different PCR primer pairs, and pLG-1/pLG-2 and ITS Lg 30F/ITS Lg 319R were used for the identification of *L. garvieae* (Zlotkin *et al*., 1998; Dang *et al*., 2012). The PCR primer pairs and conditions are provided in Table 1.

Table 1: PCR primers and conditions for identification and genotyping *Lactococcus garvieae.*

Target gene	Primer name	Primer set	Amplicon size	PCR Condition	Reference
16S-rRNA	pLG-1 (F) pLG-2 (R)	5'-CATAACAATGAGAATCGC–3' 5'-GCACCCTCGCGGGTTG–3'	1.100bp	94°C-3m 94°C-1m 55°C-1m 72°C-1.5m } X30 72°C-10m	Zlotkin *et al*., 1998
16S-23S rRNA	ITS Lg30F ITS LG 319R	5'-ACTTTATTCAGTTTTGAGGGGTCT-3' 5'-TTTAAAAGAATTCGCAGCTTTACA-3'	290 bp	94°C-5 m 94°C-30s 58°C-30s 72°C-40s } X30 72°C-7m	Dang *et al*., 2012
16S-rRNA	M13	5-GAGGGTGGCGGTTCT-3′		94°C-2m 94°C-1m 42°C-40 s 72°C-2 m } X40 72°C-10 m	Rossetti and Giraffa, 2005

Molecular characterization of L. garvieae with random-amplified polymorphic DNA-PCR (RAPD-PCR)

All the isolates were analyzed using RAPD-PCR for determination of genetic diversity with the M13 primer according to Rossetti and Giraffa (2005). The amplification products were screened with a UV transilluminator after loading into agarose gel (1.5%) with added ethidium bromide at 100 V for 100 minutes. To determine the repeatability of RAPD-PCR analysis, every reference strain was analyzed at least three times (Rossetti and Giraffa, 2005; Ferrario *et al*., 2012). A dendrogram was constructed using GelJ software (BMC Bioinformatics, UK) according to the unweighted pair-group method with arithmetic mean (UPGMA) (Heras *et al*., 2015).

Representative isolates of each of the different RAPD-PCR patterns were sequenced with pLG primer pairs and a phylogenetic tree was constructed with MEGA 7 software (Table 1) (Kumar *et al*., 2016). The pLG sequences were deposited in GenBank (accession numbers are given in Fig. 2).

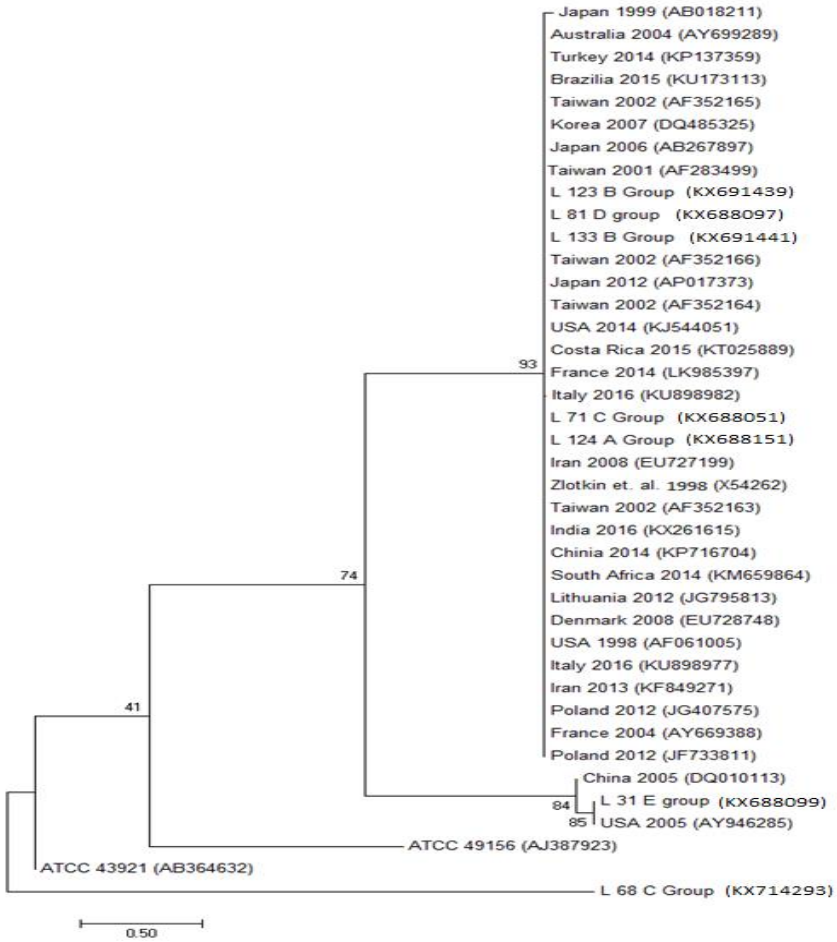

Figure 2: Comparison of isolates with GenBank database (constructed with MEGA 7), accession numbers were given in brackets.

Susceptibility testing

The isolates were analyzed for erythromycin (ERY) (Sigma, 46256), florfenicol (FFC) (Sigma, F1427), tetracycline (TET) (Sigma, 31741) and sulfamethoxazole (SUL) (Sigma, S7507) resistance using broth dilution methods according to Miller *et al*. (2014) and Kawanishi *et al*. (2005). Minimum inhibitory concentration (MIC) values were determined with a 0.008–256 mg mL^{-1} dilution according to Miller *et al*. (2014) and CLSI (2014). After incubation at 22°C for 24–48 hours, the bacterial growth in each of the plates was screened and measured at 595 nm wavelength in a microplate reader (Multiscan Go, Thermo) (CLSI, 2014).

PCR amplification and the sequence of antimicrobial resistance genes

To determine the antimicrobial resistance genes, we analyzed the *ermA*

and *erm*B genes for erythromycin, the *flo*R gene for florfenicol, the *tet*M, *tetS*, *tet*A, and *tet*B genes for tetracycline, and *sul*1, *sul*2, and *sul*3 for sulfamethoxazole resistance using specific primer pairs (Table 2) and PCR conditions (Table 2) with some modifications (Ng *et al*., 2001; Schmidt *et al*., 2001; Chen *et al*., 2007; Van *et al*., 2008; Nawaz *et al*., 2011; Wang *et al*., 2014). The PCR analysis was conducted in our laboratory with positive control genes. The amplification products were screened with a UV transilluminator after loading into agarose gel (1–2%) with ethidium bromide and 100 V for 100 minutes. Following PCR analysis, the antimicrobial-resistant genes were sequenced and deposited in GenBank.

Table 2: PCR primers and conditions of antimicrobial resistance genes.

Primer name	Primer set	Amplicon size	PCR Condition	Reference
flo(R)	F. 5'-TATCTCCCTGTCGTTCCAG R. AGAACTCGCCGATCAATG	399bp	94°C-4 m 94°C-30s 55°C-30s 72°C-1m X30 72°C-7m	Van *et al*., 2008
erm(B)	F. 5'-GATACCGTTTACGAAATTGG R. GAATCGAGACTTGAGTGTGC	364bp	94°C-4m 94°C-30s 58°C-30s 72°C-1m X30 72°C-7m	Chen *et al*., 2007
erm(A)	F. 5'-GAAATYGGRTCAGGAAAAGG R. AAYAGYAAACCYAAAGCTC	332bp	94°C-4m 94°C-30s 55°C-30s 72°C-1m X30 72°C-7dk	Chen *et al*., 2007
tet(A)	F. 5'-GCTACATCCTGCTTGCCTTC 3' R. 5'-CATAGATCGCCGTGAAGAGG 3'	210bp	94°C-5m 94°C-1m 55°C-1m 72°C-1,5m X30 72°C-10m	Ng *et al*., 2001
tet(B)	F. 5'-CTCAGTATTCCAAGCCTTTG 3' R. 5'-CTAAGCACTTGTCTCCTGTT 3'	416bp	95°C-3m 95°C-30s 59°C-30s 72°C-3m X25 72°C-5m	Schmidt *et al*., 2001
tet(M)	F. 5'-GTTAAATAGTGTTCTTGGAG-3' R. 5'-CTAAGATATGGCTCTAACAA-3'	657bp	95°C-5m 95°C-45s 55°C-45s 72°C-1m X30 72°C-7m	Nawaz *et al*., 2011
tet(S)	F. 5'-ATCAAGATATTAAGGAC-3' R. 5' -TTCTCTATGTGGTAATC-3'	573bp	94°C-4m 94°C-30s 55°C-30s 72°C-1m X30 72°C-7m	Nawaz *et al*., 2011
tet(E)	F. 5'-GTGATGATGGCACTGGTCAT-3' R 5'-CTCTGCTGTACATCGCTCTT-3'	1180	95°C-4m 95°C-30s 62°C-30s 72°C-45s X25 72°C-7m	Schmidt *et al*., 2001
sul(1)	F. 5'-CGGCGTGGGCTACCTGAACG-3' R 5'-GCCGATCGCGTGAAGTTCCG-3'	433bp	94°C-4m 94°C-30s 60°C-30s 72°C-1m X30 72°C-7m	Wang *et al*., 2014
sul(2)	F. 5'-GCGCTCAAGGCAGATGGCATT-3' R 5'-GCGTTTGATACCGGCACCCGT-3'	293bp	94 °C-4m 94°C-30s 55°C-30s 72°C-1m X30 72°C-7m	Wang *et al*., 2014
sul(3)	F. 5'-TCAAAGCAAAATGATATGAGC-3' R 5'-TTTCAAGGCATCTGATAAAGAC-3'	787bp	94°C-4m 94°C-30s 55°C-30s 72°C-1m X30 72°C-7m	Wang *et al*., 2014

Results

Phenotypic and molecular identification

A total of 137 strains were isolated from carrier or diseased rainbow trout from 10 farms located in four different regions of Turkey except for the Black Sea and Marmara regions (Table 3). The isolates (Gram-positive, oxidase-catalase negative, with non-growth in MacConkey's agar, O/F fermentative, and non-motile) and the three reference strains were identified with pLG and ITS primer pairs using PCR (Figs. 3 and 4). *L. garvieae* were isolated in almost every month during every season from rainbow trout (body weight 0.5–3000 g) located in the Central Anatolian (7 isolates), Aegean (124 isolates), Mediterranean (1 isolate), and Eastern Anatolian (5 isolates) regions of Turkey (Table 3, Fig. 5).

Table 3: Isolation information of *Lactococcus garvieae.*

Genogroup	Isolate no	Fish species-weight	Isolation Date	Region
A	L69	RT-150g	2013-March	Aegean
B	L6, L7, L8	RT-0,5g	2013-March	Aegean
C	L68	RT-150g	2013-March	Aegean
C	L70	RT-0,5g	2013-March	Aegean
C	L71	RT-130g	2013-March	Aegean
B	L67	RT-250g	2013-March	Central Anatolia
B	L87, L88	RT-150g	2013-April	Aegean
B	L72, L73, L77	RT-150g	2013-May	Aegean
A	L83	RT-65g	2013-July	Central Anatolia
A	L86	RT-250g	2013-July	Central Anatolia
D	L81	RT-250g	2013-July	Central Anatolia
A	L78	RT-250g	2013-July	Central Anatolia
A	L79	RT-200g	2013-July	Central Anatolia
A	L80	RT-3000g	2013-July	Central Anatolia
A	L20	RT-30g	2013-August	Aegean
A	L21	RT-30g	2013-August	Aegean
A	L22, L25	RT-300g	2013-August	Aegean
A	L124	RT-5g	2013-August	Aegean
B	L24	RT-8g	2013-August	Aegean
B	L26	RT-3g	2013-August	Aegean
B	L74, L75, L76	RT-10g	2013-August	Aegean
A	L27	RT-250g	2013-September	Aegean
A	L29, L32	RT-0,5g	2013-September	Aegean
A	L33, L34	RT-2g	2013-September	Aegean
A	L36-L51 (16 isolates)	RT-250g	2013-September	Aegean
A	L52	RT-100g	2013-September	Aegean
B	L28, L30, L35	RT-150g	2013-September	Aegean
E	L31	RT-150g	2013-September	Aegean
A	L17, L18	RT-150g	2013-September	Aegean
A	L11	RT-15g	2013-December	Aegean
A	L116	RT-30g	2013-December	Aegean
A	L130	RT-15g	2013-December	Aegean
A	L19, L118	RT-200g	2014-March	Aegean
A	L1	RT-200g	2014-April	Aegean
A	L4	RT-40g	2014-April	Aegean
A	L128	RT-40g	2014-April	Aegean
B	L5, L125	RT-200g	2014-April	Aegean
A	L2, L3, L16, L115, L63	RT-200g	2014-April	Aegean
A	L66	RT-300g	2014-May	Aegean
B	L65	RT-60g	2014-May	Aegean
A	L62, L64	RT-200g	2014-May	Aegean

Table 3 continued:

A	L53	RT-8g	2014-June	Aegean
A	L59	RT-150g	2014-June	Aegean
A	L55	RT-200g	2014-June	Aegean
A	L56	RT-100g	2014-June	Aegean
A	L54	RT-8g	2014-June	Aegean
A	L57, L58	RT-250g	2014-June	Aegean
A	L60, L82, L84	RT-200g	2014-June	Aegean
B	L85	RT-200g	2014-June	Aegean
A	L90, L94	RT-12g	2014-July	Aegean
B	L89	RT-300g	2014-July	Aegean
B	L91	RT-150g	2014-July	Aegean
A	L92	RT-200g	2014-July	Aegean
B	L93	RT-200g	2014-July	Aegean
A	L97	RT-10g	2014-August	Aegean
B	L99	RT-200g	2014-August	Aegean
A	L96	RT-100g	2014-August	Aegean
A	L98	RT-7g	2014-September	Aegean
B	L100, L103	RT-250g	2014-September	Aegean
A	L101	RT-60g	2014-September	Aegean
A	L102	RT-250g	2014-September	Aegean
A	L104	RT-60g	2014-October	Aegean
B	L105	RT-40g	2014-October	Aegean
A	L108	RT-14g	2014-October	Aegean
B	L107	RT-250g	2014-October	Aegean
A	L106, L111	RT-30g	2014-October	Aegean
A	L110	RT-300g	2014-October	Aegean
B	L109	RT-300g	2014-October	Aegean
A	L12, L131	RT-250g	2014-December	Aegean
A	L13, L14, L122	RT-200g	2014-December	Aegean
B	L123	RT-200g	2014-December	Aegean
A	L15, L132	RT-400g	2014-December	Aegean
ND	L140	RT-250g	2014-December	East Anatolia
A	L 9, L135	RT-200g	2015-January	Aegean
A	L10, L114	RT-150g	2015-January	Aegean
A	L129	RT-250g	2015-February	Mediterranean
A	L119	RT-100g	2015-February	Aegean
B	L133	RT-200g	2015-February	Aegean
A	L112, L120, L126, L127	RT-250g	2015-March	Aegean
A	L117	RT-12g	2015-May	Aegean
A	L121	RT-200g	2015-May	Aegean
A	L113	RT-350g	2015-May	Aegean
A	L134	RT-25g	2015-May	Aegean
A	L136-L139 (4 isolates)	RT-250g	2015-May	East Anatolia
	ATCC 49156	Yellowtail	1974	Japan
	ATCC 49157	Yellowtail	1974	Japan
	ATCC 43921	Cow	1984	Japan

RT: Rainbow Trout; ATCC: American type culture collection; A, B, C, D and E: represent Genogroups of *L. garvieae*, ND: not determined

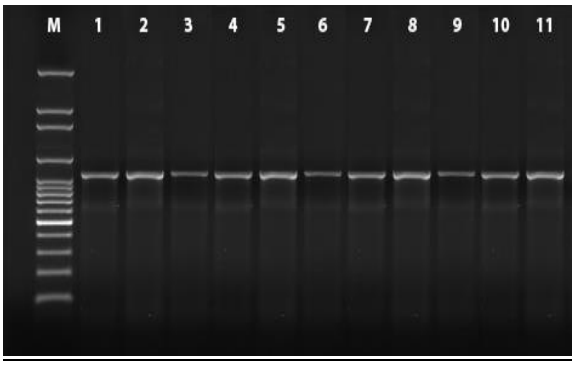

Figure 3: PCR image of *Lactococcus garvieae* with pLG primer. 1-11 are positive isolates at 1100bp. M: Marker 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000bp.

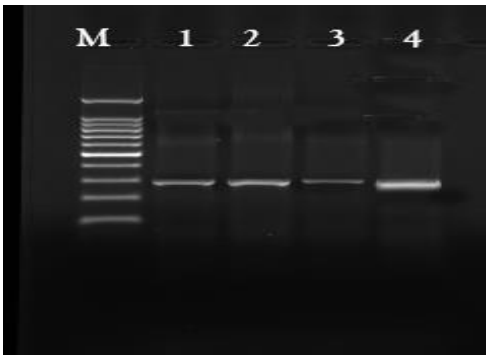

Figure 4: PCR images of *Lactococcus garvieae* with ITS primer at 290bp. M: Marker 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500.

Figure 5: Geographic distribution of *Lactococcus garvieae* isolated in 2013-2015 years (n= isolate number).

Molecular characterization of L. garvieae

Eight different genogroups were determined by RAPD-PCR analyses of 140 *L. garvieae* strains include the reference strains (see Fig. 6). RAPD-PCR analysis of the 137 isolates (excluding reference strains) showed 99 *L. garvieae* isolates (72.2%) in group A (Fig. 7), 32 (23.3%) in group B, three (2.1%) in group C, and one isolate was in groups D and E and genogroup of one isolate (isolate L140) wasn't determined because of band confusion (Table 3). RAPD-PCR showed no strong similarity between the 137 *L. garvieae* isolates and the three reference strains (Fig. 6). Similarities were 82% between ATCC 49156 and 49157, 86% for group D and A isolates, 72% for ATCC 43921 and the group E isolates, 77% for group C and B isolates, and all isolates shared at least 61% similarity among each other (Fig. 6). Sequence of representative isolates from different RAPD-PCR patterns based on pLG primers was deposited in the GenBank database under the accession numbers KX688051, KX688097, KX688099, KX688151, KX691439, KX691441, and KX714293. Almost all our isolates were in the same phylogenetic genogroup with Japan, Australia, Turkey, Brasilia, Taiwan, Korea, USA, Costa Rica, France, Italy, Iran, China,

South Africa, Lithuania, Denmark, Poland except for L31 and L68. Unlike all sequenced isolates, L68 was in genogroup C in RAPD-PCR analysis. This isolate showed the highest distance from our isolates and GenBank database isolates.

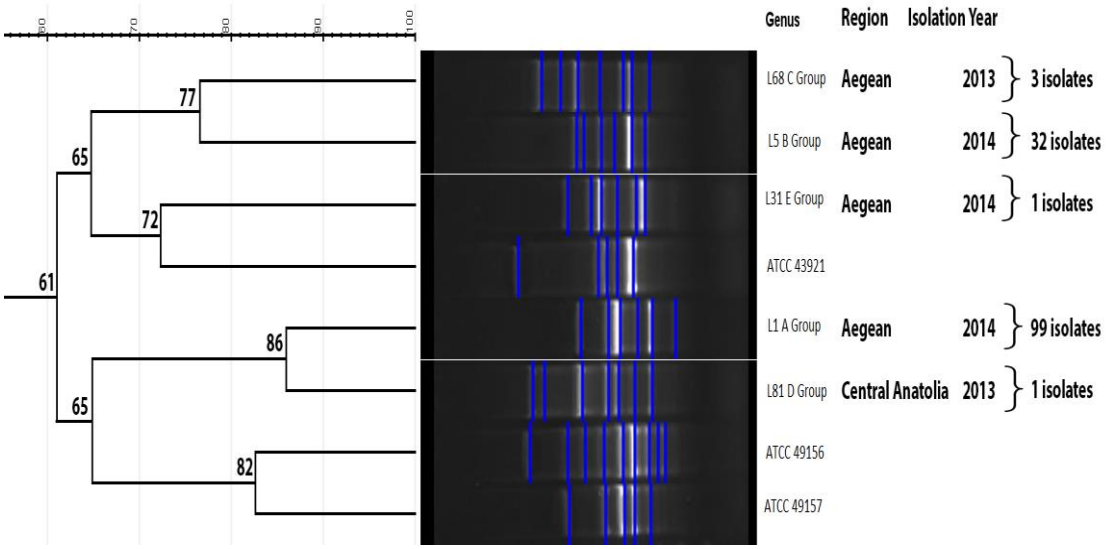

Figure 6: Dendrogram of *Lactococcus garvieae* isolates with RAPD-PCR (49157, 49156, 43921: ATCC strains; 81, 1, 5, 31, 68: each Genogroups of *Lactococcus garvieae*).

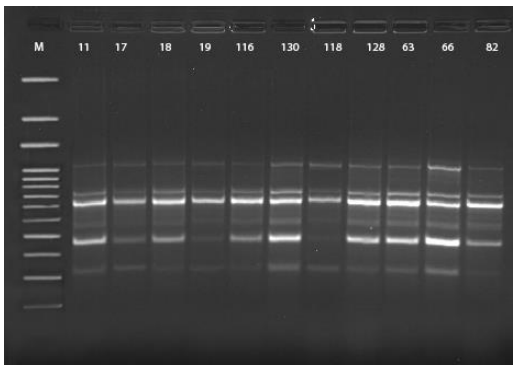

Figure 7: PCR images of A Genogroup with RAPD-PCR (the most common profile). 11, 17, 18, 19, 116, 130, 118, 128, 63, 66 and 82 representative isolates of this group (M: Marker: 100, 200, 300, 400, 500, 600, 700, 800, 900, 1.000, 1.500, 2.000, 3.000bp).

MIC, resistance genes, and sequencing

MIC values and resistance genes of 140 *L. garvieae* isolates are provided in Tables 4 and 5, respectively. MIC ranges for ERY and FFC were appreciably lower than those of the other two antimicrobials. Among all the isolates 25 were phenotypically resistant to FFC, eight to TET, four to ERY, and 132 to SUL. Two phenotypically TET-resistant *L. garvieae* isolates and one phenotypically TET-susceptible isolate had both *tet*M and *tetS* genes, whereas two phenotypically resistant isolates and three non-resistant isolates had *tet*M or *tetS*. *ermA* is the most widely detected gene among the 137 isolates,

and in all cases these genes were detected in phenotypically ERY-susceptible isolates. Only two ERY-resistant isolates carry the *erm*B gene, despite two *erm*B genes being detected as phenotypically sensitive isolates. The *sul*1, *sul*2, *sul*3, *tet*A, *tet*B, and *flo*R genes were not detected in any of the 137 isolates. The detected antimicrobial resistance genes were sequenced and deposited in the GenBank database (accession numbers KX722454–KX722466). The *ermA* and *tet*M genes isolated in *L. garvieae* were deposited for the first time in GenBank.

Table 4: Antimicrobial concentration of *Lactococcus garvieae* isolates for Florfenicol, Tetracycline, Sulfamethoxazole and Erythromycin.

Antimicrobials	(n)	MIC concentration (mg L^{-1})															Resistant isolate (%)
		0,008	0,016	0,032	0,064	0,128	0,256	0,512	1	2	4	8	16	32	64	128	256	
Florfenicol	140	6					2	13	32	26	36	13	9	3				17,8
Tetracycline	140	1			1		10	29	46	38	6	1		2	1	5			10,7
Sulfamethoxazole	140	3					2								3	1	131		94,2
Erythromycin	140	13	2	7	48	33	27	6	4										2,8

n: Isolate number

Table 5: MIC concentration and resistance genes of *Lactococcus garvieae* isolates*.

Genotype	DNA	Florfenicol	Tetracycline	*tet*M	tetS	Erythromycin	*erm*B	*erm*A	Sulfamethoxazole
B	L6	1	1	-	-	0,08<	-	+	256<
B	L7	0,008	0,256	-	-	0,08<	-	+	256<
B	L8	1	1	-	-	0,016	-	+	256<
C	L68	2	1	+	+	0,128	-	-	256<
C	L70	4	64	+	-	0,128	-	-	256<
C	L71	2	64	+	+	0,128	-	-	256<
B	L87	2	0,512	-	+	0,064	-	-	256<
B	L77	2	64	-	-	0,064	-	-	256<
A	L78	4	0,256	+	-	0,256	-	-	256<
A	L79	1	0,512	+	-	0,256	-	-	256<
A	L20	0,512	2	-	-	0,512	-	-	64
A	L21	2	16	-	-	0,032	-	-	256<
A	L25	2	0,512	-	-	0,032	-	+	256<
B	L26	1	0,128	-	-	0,064	-	+	256<
A	L27	0,512	0,512	-	-	0,256	-	+	256<
A	L29	2	0,512	-	-	0,064	-	+	256<
A	L34	0,512	64	+	+	0,256	-	-	256<
B	L28	1	0,512	-	-	0,256	-	+	256<
B	L5	0,008	0,512	-	-	0,08<	-	+	0,128
B	L99	2	1	-	-	1	+	-	256<
B	L100	2	0,256	-	-	1	+	-	256<
A	L101	4	0,256	-	-	0,064	+	-	256<
A	L104	2	0,512	-	-	0,064	+	-	256<
A	L119	4	2	+	-	0,256	-	-	256<

*Some phenotypic resistant and all of genotypic resistance isolates were given, grey cells demonstrate resistant values, A, B and C are Genogroup of *L. garvieae*

Discussion

This study provides further details on the genetic diversity, prevalence, and mortality rates associated with lactococcosis in rainbow trout. Establishing the genotypic characteristics of *L. garvieae* isolates enables comparison with GenBank isolates from different countries. Antimicrobial resistance studies show

which antimicrobial agents should be used for control of lactococcosis, and reveal the prevalence of antimicrobial resistance genes isolated from fish of various weights in different countries.

Rainbow trout production in cage systems plays an important role in the high aquaculture production in Turkey. We observed a mass mortality associated with high water temperatures (above 15°C) due to lactococcosis in cage systems (field experiment). In addition, water temperature fluctuations contributed to this mortality. When many rainbow trout farms are established within the same water source, and the same water is used for agriculture land and other animals, this can lead to cross-contamination between aquaculture ponds and the terrestrial ecosystem (Boxall *et al*., 2004). As far as we know, lactococcosis was previously reported in rainbow trout weighing 5 g and 1 kg, and is a cause of mortality in fish fry (Chang *et al*., 2002; Pereira *et al*., 2004; Vendrell *et al*., 2006; Avci *et al*., 2014). We observed lactococcosis in 0.5 g rainbow trout fry due to the practice of transferring small fish to high-temperature water sources to ensure production continuity. In addition, all sizes of fish (fry, fingerling, portion, and broodstock) could be infected with *L. garvieae* when kept in water warmer than 15°C. Additionally, *L. garvieae* were isolated from some rainbow trout farms that had water sources colder than 15°C, but clinical symptoms were not observed. pLG and ITS primer pairs are mostly used for identification of *L. garvieae* (Zlotkin *et al*., 1998; Dang *et al*., 2012); we therefore used both primer pairs, which successfully identified *L. garvieae*. Ferrario *et al*. (2012) used three different RAPD-PCR primers and reported high discriminatory power of the M13 primer that was used in the present study. Foschino *et al*. (2008) reported similar results for RAPD-PCR, *sau*-PCR, and AFLP methods for genotyping *L. garvieae*. We identified five different genogroups of our *L. garvieae* isolates with the M13 primer. These results showed that the M13 primer has high discriminatory power, which is in agreement with the findings of other studies. Ravelo *et al*. (2003) found seven different RAPD profiles that separated three genogroups and reported Turkish isolates that were similar to groups with Spanish, English, Portuguese, and Italian isolates. Altun *et al*. (2013) identified three different *L. garvieae* genotypes with RAPD-PCR, and these three were the predominant isolates (66.6%). In addition, some were grouped with English and Spanish isolates. We identified *L. garvieae* isolates divided between five different genogroups, and found that reference strains showed differing similarities to those of our isolates. Most of the isolates were in genogroup A, which was predominant (72.2%) in Turkey and showed similar results to those of Altun *et al*. (2013). The present study is novel in that we used 137 *L. garvieae* isolates in five different genogroups, which represented different regions of Turkey. RAPD pattern similarities showed that transport of infected fish (especially asymptomatic) could spread

lactococcosis infection to different regions. After *L. garvieae* was first reported in the Aegean region (Diler *et al*., 2002), the agent was isolated from different regions in Turkey and its rapid spread was reported by Altun *et al*. (2004). Our isolates had similar genetic profiles to those of Aegean region isolates, showing that infections could spread from the Aegean region to different regions of Turkey. When the sequences were compared with isolates from the GenBank database, the isolates from Turkey were in the same genogroups as European (France, Italy, Denmark, and Poland), Asian, Australian, USA, and South African isolates, which were similar to the results of Altun *et al*. (2004). These results showed that most of the *L. garvieae* isolates were homogenous but that heterogeneity was not too low to be ignored.

Due to this genetic heterogeneity, there are no effective immunizations or preventions for all fish production periods (Austin and Austin, 2007). Therefore, farmers must use different antimicrobial agents such as erythromycin, florfenicol, and oxytetracycline to control lactococcosis. There are differing reports that some isolates are susceptible to enrofloxacin and nitrofurantoin, whereas others are resistant to oxolinic acid and sulfamethoxazole, and differing susceptibilities to erythromycin, chloramphenicol, oxytetracycline, and ampicillin have been found (Ravelo *et al*., 2001; Soltani *et al*., 2008; Raissy and Ansari, 2011; Raissy and Shahrani, 2015; Raissy and Moumeni, 2016). Some authors found that *L. garvieae* was phenotypically susceptible to erythromycin and chloramphenicol, but was resistant to penicillin and clindamycin (Diler *et al*., 2002; Altun *et al*., 2013). In the present study MIC tests showed that, of our 140 *L. garvieae* isolates, four (2.8%) were phenotypically resistant to ERY, 25 (17.8%) were phenotypically resistant to FFC, 15 (10.7%) to TET, and 132 (94.2%) to SUL. Our isolates were mostly susceptible to ERY and TET but highly resistant to SUL. Antimicrobial resistance is more important in aquaculture than terrestrial ecosystems because resistant bacteria can be easily transferred within the aquatic environment and between other farms and humans via water (Itami *et al*., 1996). Some researchers found *tetS*, the integrase gene, *erm*B, *gyr*A, and *par*C genes in *L. garvieae* (Hirono and Aoki 2001; Kawanishi *et al*., 2005; Maki *et al*., 2008; Morandi *et al*., 2015). Ture and Boran (2015) found *ere*A but not *ere*B in 29 *L. garvieae* isolates. In contrast to other studies, we found that only four isolates were phenotypically resistant to ERY and two of these four isolates carried the *erm*B gene. A total of nine *L. garvieae* isolates carried the *ermA* gene, 13 different *L. garvieae* isolates carried the *erm*B gene, and interestingly 11 genotypically resistant isolates showed susceptibility to ERY, with an average MIC of 0.064 mg L^{-1}. Ture and Boran (2015) found that *tet*B was the most common gene in *L. garvieae* isolated from rainbow trout, whereas Raissy and Shahrani (2015) identified *tet*A gene in 94% of

phenotypically TET-resistant *L. garvieae* isolates. We found that only 15 isolates were phenotypically resistant to TET, and only four isolates carried the TET resistance genes, which is in contrast to other studies. Also in contrast to other studies, we did not find the *tet*B, *tet*E, and *tet*A genes. While the most common TET genes were found to be *tet*B and *tet*A in other studies, we found that the most common TET resistant genes were seven *tet*M (5%) and four *tetS* genes. One isolate carried the *tet*M and *tetS* genes (multiple antimicrobial resistance), and was phenotypically susceptible to TET. In addition, an important finding was that the four *L. garvieae* isolates carried the *tet*M or *tetS* gene even though they were not phenotypically resistant to TET. However, Maki *et al*. (2008) worked with 146 *L. garvieae* isolates and did not detect the *flo*R gene, which is phenotypically susceptible or moderately resistant to FFC. Ture and Boran (2015) found that 14 isolates (with ATCC 49156) carried the *flo*R gene, all of which were susceptible to FFC according to the disc diffusion test. Similar to Maki *et al*. (2008), we did not detect the *flo*R gene, and we found that 25 isolates were resistant to FFC. Previous studies showed that the resistance of sulphonamides varies from 53% to 86.6% for *L. garvieae* isolates, and there is no detailed research on the sulphonamide resistance of *L. garvieae* (Raissy and Ansari, 2011; Ture and Boran, 2015). Similar to other studies, we found that our *L. garvieae* isolates were phenotypically highly resistant (91.4%) to SUL, whereas no sulphonamide combinations are commonly used for treating lactococcosis. These discrepancies could be based on transferring mobile genetic elements and differences of strains.

In our study, RAPD-PCR showed that four phenotypically TET-resistant isolates carried *tet*M or *tetS*, one TET-susceptible isolate carried both *tet*M and *tetS*, and three *L. garvieae* isolates belonged to the genogroup C. Among the 32 *L. garvieae* isolates within genogroup B, six have the *ermA* gene, two have *erm*B, and one has the *tetS* gene. *ermA* is the most common gene within the B genogroup, whereas the *tet*M and *tetS* genes are the most common in genogroups A and C. Some isolates in genogroups D and E do not show either phenotypic or genotypic antimicrobial resistance.

In conclusion, lactococcosis was the most common infection among farmed rainbow trout from the Aegean region of Turkey, and all isolates are divided into five different genogroups. Knowledge of genetic similarity with isolates from other countries, genetic heterogeneity and homogeneity are recommended for effective aquaculture vaccination programs. We identified high phenotypic SUL resistance, whereas there was low resistance to erythromycin for all the isolates in our study. Consequently, ERY at appropriate dose and time can be used for treating outbreaks of lactococcosis at aquaculture farms in Turkey. The most important finding is that many of the isolates carry resistance genes while being phenotypically susceptible. For

effective control programs against lactococcosis, antimicrobial resistance genes must be evaluated through susceptibility tests and disease prevalence because resistance genes can be transferred from aquaculture settings to terrestrial ecosystems and humans.

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