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RESEARCH PAPER

Detection of Haemolysin Genes as Genetic Determinants of Virulence in *Lactococcus garvieae*

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Abstract

Haemolysin genes (*hly1, hly2, hly3*) are functional genetic determinants of virulence in bacterial pathogenesis. In this study, the presence of hly genes was investigated in *Lactococcus garvieae*, previously recovered from infected rainbow trout (*Oncorhynchus mykiss*) in commercial fish farms. Although the bacteriological phenotypic tests failed to correctly identify the six isolates at species level, the amplification products (1100 bp) of partial 16S rRNA gene region and their nucleotide homology confirmed that 21 isolates, including ATCC 43921 control, were *L. garvieae*. All strains carried hly2 (796 bp), while hly1 (522 bp) was carried by 20 strains, and hly3 (549 bp) by 16. The results suggested that hly1 and hly2 could directly participate in pathogenesis whereas hly3 might not be responsible for the strains' virulence. Antibiogram tests indicated that all strains were susceptible to ampicillin while being resistant to clindamycin and streptomycin. Variations in resistance profiles against various antimicrobials were detected among strains. The detection of virulence genes coding for haemolysins and the determination of the antimicrobial resistance profiles might contribute to a better understanding of the mechanisms of virulence and resistance of *L. garvieae*, which may lead to the determination of new targets for therapeutics and vaccines against this pathogen.

Introduction

Lactococcus garvieae (junior syn. *Enterococcus seriolacida*) is a common Gram-positive pathogen of aquatic species (Vendrell *et al*., 2006). A causative agent of haemorrhagic septicaemia in warm-blooded animals, this species is a potential zoonotic pathogen (Vinh, Nichol, Rand, & Embil, 2006; Wang *et al*., 2007; Yiu *et al*., 2007). The pathogen usually causes endemic infections in commercial fish farms, especially during summer (June-September), leading to significant economic losses in aquaculture industry (Vendrell *et al*., 2006; Wang *et al*., 2007). Endemic infections of *L. garvieae* have been reported in different aquatic species from different countries (Eyngor *et al*., 2004; Savvidis, Anatoliotis, Kanaki, & Vafeas, 2007; Evans, Klesius, & Shoemaker, 2009; Sharifiyazdi, Akhlaghi, Tabatabaei, & Mostafavi Zadeh, 2010). *L. garvieae* was

described as a causative agent of lactococcosis in rainbow trout (*Oncorhynchus mykiss*) in Turkey for the first time in 2001 (Diler, Altun, Adiloglu, Kubilay, & Isıklı, 2002).

Symptoms of lactococcosis are anorexia, abnormal (erratic) swimming, single or double-sided exophthalmos, dark pigmentation, internal congestion, meningoencephalitis and haemorrhage of the intestine, spleen, liver and kidney in infected fish (Kusuda, Kawai, Salati, Banner, & Fryer, 1991; Eldar *et al*., 1996; Kang *et al*., 2004; Vendrell *et al*., 2006).

Thirteen biotypes and three serotypes of this pathogen were identified based on phenotypic and serologic characteristics (Vela *et al*., 2000; Vendrell *et al*., 2006). The complete genome sequences of a virulent *L. garvieae* Lg2 and a non-virulent ATCC 49156 strains have been released (Morita *et al*., 2011). The draft genome sequences of some strains, isolated from different sources, have also been reported (NCBI). However, there is limited knowledge about the virulence mechanisms of *L. garvieae*.

Bacterial haemolysins are produced by a variety of Gram-positive and Gram-negative bacteria (Goebel, Chakraborty, & Kreft, 1988; Pandey, Naik, & Dubey, 2010). Their haemolytic capacity causes membrane structure damage in blood cells (Meesters, Brack, Hellmann, & Decker, 2009). Haemolysins and their relationship to virulence and pathogenesis have been studied in several pathogenic bacteria, including *L. garvieae*, at the molecular level. *L. garvieae* had α-haemolytic activity on blood agar (Kusuda *et al*., 1991; Koneman, Allen, Janda, Schreckenbergger, & Winn, 1992), and the following three *hly* genes were identified in the *L. garvieae* genome: *hly*1, *hly*2 and *hly*3. Because those genes code for proteins displaying 56-72 % amino acid identity to known haemolysins, they have been designated as a potential virulence factors (Morita *et al*., 2011; Miyauchi, Toh, Nakano, Tanabe, & Morita, 2012; Ture & Altinok, 2016).

Indiscriminate use of antibiotics causes microorganisms to develop antibiotic resistance mechanisms, a potential threat to the environment, as well to aquaculture, and to public health (Vendrell *et al*., 2006; Austin & Austin, 2012; Altun, Onuk, Ciftci, Büyükekiz, & Duman, 2013; Meyburgh, Bragg, Boucher, 2017). The acquisition of antibacterial resistance in *L. garvieae* was shown by screening for antibiotic resistance genes (Raissy & Moumeni, 2016; Türe & Alp, 2016). However, association between antibiotic resistance and virulence mechanisms was not previously discussed. The determination of bacterial antibiotic resistance patterns provides important basic data for the treatment of bacterial diseases –which is why the antimicrobial susceptibility profile of *L. garvieae* should be considered for bacterial infection control.

The aims of this study were to investigate and characterise the virulence genes (*hly*1, *hly*2 and *hly*3) associated with haemorrhagic activity of 19 fish (*O. mykiss*) and one human *L. garvieae* isolates together with the type strain ATCC 43921, and also to determine their antibiotic susceptibility profiles.

Materials and Methods

Bacterial Isolates and Phenotypic Characterisation

A total of 21 *L. garvieae* strains was included in this study (Table 1). 19 samples were isolated from different organs (heart, liver, kidney, blood, spleen) of diseased rainbow trout (*O. mykiss*), and one from an infected human (FMB-H). The *L. garvieae* type strain (FMB-R) which was isolated from water buffalos with subclinical mastitis, was obtained from American Type Culture Collection (ATCC 43921). The strains were grown on Todd Hewitt Broth (THB) and incubated aerobically at 25°C for 24 h. In addition, they were incubated at 22°C for 24-48 h on blood agar (with 5 % sheep blood) medium in order to determine their haemolytic activities.

The morphological and physiological characteristics of bacterial colonies were determined together with their biochemical characteristics, using rapid ID 32 Strep (Biomérieux) which allows for the identification of most streptococci and members of related genera. Bacterial suspensions were loaded into each of 32 wells containing various biochemical tests in dehydrated form. Strips were incubated at 37°C for 4 h

Table 1. Isolates and type strain of *L. garvieae* used for analysis

Strain	Location	Strain	Location
FMB-F1	Fethiye/Turkey	FMB-F12	Fethiye/Turkey
FMB-F2	Fethiye/Turkey	FMB-F13	Fethiye/Turkey
FMB-F3	Fethiye/Turkey	FMB-F14	Fethiye/Turkey *
FMB-F4	Fethiye/Turkey	FMB-F15	Fethiye/Turkey *
FMB-F5	Fethiye/Turkey	FMB-F16	Fethiye/Turkey *
FMB-F6	Fethiye/Turkey	FMB-BL1	Bafra Lake/Turkey
FMB-F7	Fethiye/Turkey	FMB-BL2	Bafra Lake/Turkey
FMB-F8	Fethiye/Turkey	FMB-F17	Fethiye/Turkey **
FMB-F9	Fethiye/Turkey	FMB-H	Human isolate/Turkey
FMB-F10	Fethiye/Turkey	ATCC 43921	Culture Collection/United Kingdom
FMB-F11	Fethiye/Turkey	(FMB-R)	

*: obtained from different hatchery, **: first isolate reported from Turkey

(Freney *et al*., 1992). Reactions were evaluated and isolates were identified following Bergey's Manual (Holt, Krieg, Sneath, Staley, & Willians, 1994).

Antibiotic Susceptibility Tests in *L. garvieae* Isolates

The antimicrobial susceptibility was evaluated using the Kirby-Bauer disc diffusion method (Koneman *et al*., 1992). Each bacterial suspension, whose turbidity was adjusted to 0.5 McFarland standard, was spread on Muller Hinton agar plates. The standard antibiotic discs (Oxoid), widely used in aquaculture industry of Turkey, [ampicillin (AMP 10 µg), erythromycin (E 5 µg), florfenicol (FFC 30 µg), clindamycin (DA 2 µg), kanamycin (K 30 µg), enrofloxacin (ENR 5 µg), ciprofloxacin (CIP 1 µg), chloramphenicol (C 30µg), oxytetracycline (OT 30 µg), sulphamethoxazole/trimethoprim (SXT 25 µg), flumequine (UB 30 µg), streptomycin (S 10 µg)] were then placed on the Muller Hinton agar plates. Double distilled water instead of bacterial suspensions was used as a negative control in the tests. ATCC 43921, which is a study material, was also used to validate of the Kirby-Bauer disc diffusion method. Zones of inhibition were measured after 24 hours of incubation at 25ºC. The diameter of each zone was recorded, and the zone size compared to the set of CLSI standards (CLSI, 2011). The antibiogram tests were repeated three times to ensure reliability.

Genomic DNA Extraction, PCR Amplification and Sequence Analysis

The genomic DNA of all strains was extracted using High Pure PCR Template Preparation Kit (Roche) in accordance with the manufacturer's instructions. Spectrophotometric analysis was performed to evaluate the quality and quantity control of the genomic DNA samples (Maniatis, Fritch, & Sambrook, 2003).

The identification of all strains was confirmed by PCR at species level. Universal primer pair pLG-1 (5'-CATAACAATGAGAATCGC-3') and pLG-2 (5'-GCACCCTCGCGGGTTG-3') described by Zlotkin, Hershko & Eldar (1998) were used to amplify a partial region of *16S rRNA* gene. Specific primer pairs were designed to amplify *hly*1, *hly*2 and *hly*3 genes by using Primer3 program (http://bioinfo.ut.ee/primer3-0.4.0/) (Table 2). Each PCR was performed in a 25µl reaction mix containing 25 ng bacterial DNA, 1x Taq buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTP mix, 15 pmol of each primer, and 1U of *Taq* DNA polymerase (Fermentas). Amplification conditions included a pre-denaturation step of 2 min at 95ºC, 28 cycles of denaturation at 95ºC for 45 sec, annealing at 55-58ºC for 45 sec, extension at 72ºC for 1.5 min, and final extension step at 72ºC for 7 min.

Haemolysin genes were amplified by PCR in a 50 µl reaction mix for DNA sequencing. Each amplicon was purified with the Agarose Gel DNA Extraction Kit (Roche) in accordance to the manufacturer's instructions. Amplicons were sequenced based on Sanger method by RefGen ClustalW (http://www.genome.jp/tools-bin/clustalw) online web tool was used to compare nucleotide and amino acid sequence homologies among *L. garvieae* strains and reference strain Lg2.

Results

Phenotypic Characterisation

The 21 isolates were non-motile, capsulated, Gram-positive cocci, oxidase and catalase negative, α-haemolytic on blood agar, fermentative, and grown at 10-45°C in 0-6.5% sodium chloride and at pH 4.5-9.6. Phenotypic characteristics of all the isolates were found to be homogeneous. However, differences were observed in N-Acetyl-β-glucosaminidase, cyclodextrin, arylamidase and acid production from methyl-β-D glucopyranoside, ribose, lactose, saccharose and mannitol among all isolates (Table 3). According to rapid ID 32 Strep and phenotypic characteristics, while 15 isolates were identified as *L. garvieae*, 6 isolates (FMB-F3, -F7, -F10, F11, F13, F17) were identified as *Enterococcus faecalis*.

Molecular Identification

The *16S rRNA* gene, with amplicon expected length of 1100 bp, was amplified from all isolates by PCR (Figure 1a). Nucleotide sequences of the gene belonging to *L. garvieae* strains were compared to

Table 2. Primers used for PCR

Target gene	Primer	Primer sequence (5'-3')	PS (bp)	Locus	AT (°C)
16S rRNA	pLG1/2	f:CATAACAATGAGAATCGC r: GCACCCTCGCGGGTTG	1100	_	57
*hly*1	p0323	f: TCCTCCGACTAGGAACCAAA r: GCCAGCTTCTCGTGCTTATC	522	LCGL 0323	56
*hly*2	p0374	f: GAGCAAAAAGCGAGTGAAGG r: GCATCTGGAGCATCAAGTCA	796	LCGL 0374	58
*hly*3	p0597	f: CGTGGAGTTATGGCTGGTTT r: CTTGTGGATCTTCGGGTCTT	549	LCGL 0597	55

f: forward primer, r: reverse primer, PS: product size, AT: annealing temperature

reference strain Lg2, registered in the GenBank database, detecting complete sequence identity in the 1018 bp length partial region of the 1100-bp amplification products. Figure 2 shows identical sequences with 50 bp that was randomly selected from 16S rRNA amplicon. All isolates were verified as *L. garvieae*. The nucleotide sequences of FMB-F12, -F11, -F13, -BL1, -F17, -H and -R were registered in the GenBank under the accession numbers MH316756, MH316757, MH316758, MH316759, MH316760, MH316761 and MH316762, respectively. The *hly*1 gene with the size of 522 bp was amplified from all *L. garvieae* strains except FMB-F13 (Figure 1b). In addition, all strains found to be carrying the *hly*2 gene

Table 3. Phenotypic characteristics of 21 *L. garvieae* isolates

Characteristics	Isolates	ATCC 43921	FMB-H	Characteristics	Isolates	ATCC 43921	FMB-H
Gram	+	+	+	Raffinose	-	-	-
O/F	F	F	F	Saccharose	V	+	+
Oxidase	-	-	-	Arabinose	-	-	-
Catalase	-	-	-	Arabitol	-	-	-
Motility	-	-	-	Cyclodextrin	V	+	+
%5 Blood Agar	α	α	α	Voges-Proskauer	+	+	+
1.5% NaCl	+	+	+	Alanyl-phenylalanyl-proline arylamidase	+	+	+
6.5% NaCl growth	+	+	+	β-galactosidase	-	-	-
10 C° growth	+	+	+	Acide pyroglutamique	+	+	+
45 C° growth	+	+	+	N-acetyl-β-glucosaminidase	V	+	-
Indian ink capsule staining	+	+	+	Glycyl-tryptophane Arylamidase	V	-	-
Arginine	+	+	+	Hippurate hydrolysis	+	+	+
β-glucosidase	+	+	+	Glycogene	-	-	-
β-galactosidase	-	-	-	Pullulan	-	-	-
β-glucuronidase	-	-	-	Maltose	+	+	+
α-galactosidase	-	-	-	Melibiose	-	-	-
Phosphatase alkaline	-	-	-	Melezitose	-	-	-
Ribose	V	-	-	Methyl-β-D-glucopyranoside	V	+	+
Mannitol	V	+	-	Tagotose	+	-	+
Sorbitol	-	-	-	β-mannosidase	-	-	-
Lactose	V	-	-	Urease	-	-	-
Trehalose	+	+	+				

+: positive, -: negative, V: variable result, F: fermentative, α: α haemolytic activity

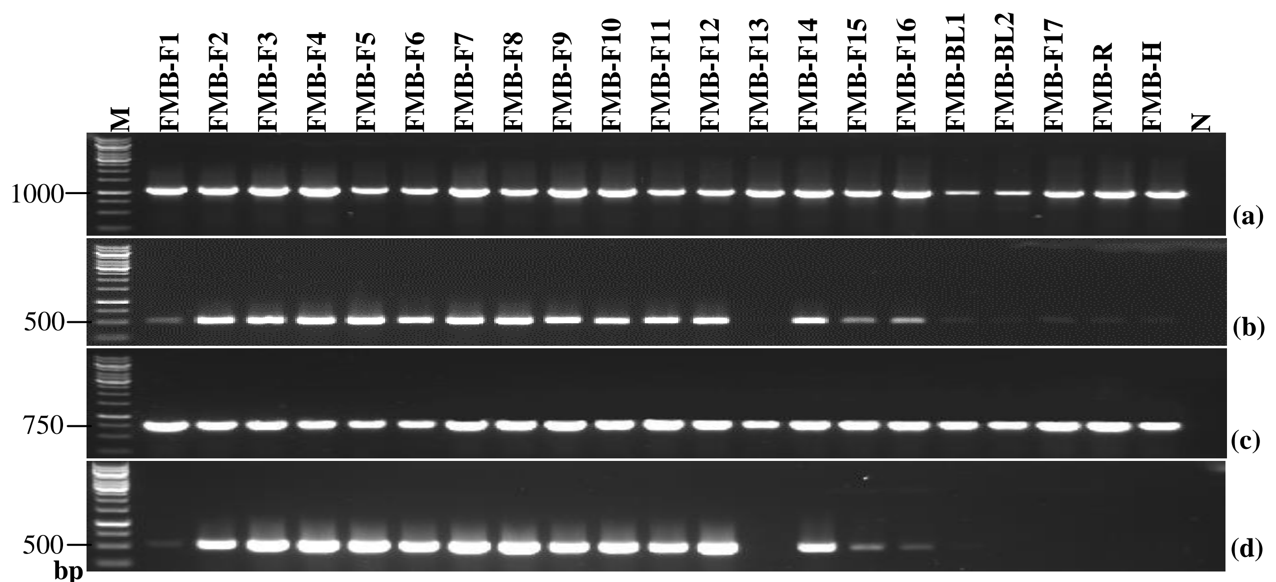

Figure 1. *16S rRNA* gene products with 1100 bp-length amplified from all *L. garvieae* isolates using specific primer pLG-1 and pLG-2 (a). Identification of three hemolysin genes (*hly*1, *hly*2, *hly*3) in *L. garvieae* strains. 522 bp length *hly*1 amplicons (b), amplification products with 796 bp length belongs to *hly*2 (c) and 549 bp fragments of *hly*3 gene (d). M: 1 kb DNA ladder; N: Negative control.

in the length of 796 bp (Figure 1c). The 549-bp length *hly*3 gene fragment could not be produced from FMB-F13, -BL2, -F17, -R, -H strains (Figure 1d). Amplification products belonging to three *hly* genes were given in Table 4. The subregion of the *hly* genes containing SNPs and deletion amplified from FMB-F11 and -12 isolates were given in Figure 3 Nucleotide sequence comparison of the three *hly* genes indicated that the similarity among strains and Lg2 varied from 94 to 99%. The highest sequence homology was detected between FMB-F11 strain and Lg2 reference for the *hly*1 gene sequence. The sequences of *hly*1, *hly*2 and *hly*3 genes belonging to FMB-F11 were deposited in the GenBank under the accession numbers MG999526, MH316613 and MH316614, respectively. The most common mutations were found to be single nucleotide polymorphisms (SNPs). Deletions have also been shown to be present in all *hly* gene sequences. Also, high degree of similarity ranged from 98 to100% was found between amino acid sequences of *L. garvieae* strains.

Antibiotic Susceptibility Patterns

Antibiogram test results indicated that all *L. garvieae* isolates (100%) are susceptible to ampicillin; resistant to clindamycin and streptomycin. Moreover, variations among the antibiotic susceptibility profiles of *L. garvieae* isolates were determined in regard to their sensitivities against erythromycin, florfenicol, kanamycin, enrofloxacin, ciprofloxacin, chloramphenicol, oxytetracycline, sulfamethoxazole/trimethoprim and flumequine (Table 5). 80% of the isolated bacteria acquired resistance to kanamycin, enrofloxacin (38%), ciprofloxacin (71%), and flumequine (83%). Also, 62% and 81% of the bacteria were classified as intermediately sensitive to sulphamethoxazole/trimethoprim and erythromycin, respectively. The bacteria were sensitive to florfenicol and chloramphenicol (81%), oxytetracycline (95%). Antibiotic sensitivity percentage of all isolates was shown in Table 6.

Discussion

Developments in genetic studies have led to an increase in data about the biochemical profiles and virulence mechanisms of fish pathogens (Naka & Crosa, 2011). Many research groups have attempted to understand the virulence mechanisms of *L. garvieae* in view of more efficient lactococcosis treatment and pre-exposure prophylaxis (Kimura & Kusuda, 1982; Schmidtke & Carson, 2003; Kawanishi *et al*., 2007; Miyauchi *et al*., 2012; Ture & Altinok, 2016; Castro *et al*., 2017). In this study, *L. garvieae* isolates were identified through bacteriological and molecular approaches, and the *hly* gene (*hly*1, *hly*2 and *hly*3) variations, contributing to the isolates' virulence, were screened.

Various microbiological methods are used in the detection and subsequent identification of bacterial species causing fish diseases. The most frequently used are diagnostic kits based on phenotypic and biochemical properties such as Gram staining, enzyme production and oxidation / fermentation (Austin & Austin, 2012). The commercial API test systems (API 20 STREP, Rapid ID 32 STREP, API 50CH etc.) are the most widely used conventional assays for rapid detection (Appelbaum, Chaurushiya, Jacobs, & Duffett, 1984; Bosshard, Abels, Altwegg, Böttger, & Zbinden, 2004).

Figure 2. Multiple alignment of partial nucleotide sequences belonging to 16S rRNA gene amplified from L. garvieae isolates by PCR. ' type strain found in GenBank, * identical bases.

Table 4. Amplification products belonging to *hly* genes obtained from *L. garvieae* strains

	FMB-	FMB-	FMB-	FMB-	FMB-	FMB-	FMB-	FMB-	FMB-	FMB-	FMB-	FMB-	FMB-	FMB-	FMB-	FMB-	FMB-	FMB-	FMB-	FMB-R	FMB-
*hly*1	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
*hly*2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
*hly*3	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	-	-

+: presence of amplification products

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NC_017490.1_c332295-331642'    GGTTCCGATGCCATGTGTGATGGCATTAAAAACCTGGTCGACAATTTGAT
p0323-FMB-F11                  GGTTCCGATGCCGTGTGTGATGGCATTAAAAACCTGGTCGACAATTTGAT
p0323-FMB-F12                  GGTTCCGATGCCGTGTGTGATGGCATTAAAAACCTGGTCGACAATTTAAG
                               ************ ********************************* *
                                                                            (a)
NC_017490.1_382251-383579'     ATTTCCGTGAAATGTTGCAAGAACCTCTCTTTGTTCCTGAAACAGTCTTT
p0374-FMB-F11                  ATTTCCGTGAGATGTTGCAAGAACCTCTCTTTGTTCCTGAAACAGTCTTT
p0374-FMB-F12                  ATTTCCGTGAGATGATGCAAGAACCTCTCTTTGTTCCTGAAACAGTCTTT
                               ********** *** ***********************************
                                                                        (b)
NC_017490.1_615478-616287'     TAGTGGATGCCAGTTTTGACAAACCTGGACAAAAGATAGAAGAAGCCACA
p0597-FBM-F11                  TAGTGGATGCCAGTTTTGACAAACCGGGACAAAAGATAGAAGAAGCCACA
p0597-FMB-F12                  TGGTGGA-GCCAGTTTTGACAAACCTGGACAAAGGATAGAAGAAGCCACA
                               * ***** **************** ******* ***************
                                                                        (c)
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Figure 3. ClusterW analysis of partial regions of *hly*1 (a), *hly*2 (b) and *hly*3 (c) genes in FMB-F11 and FMB-F12 isolates. SNPs were highlighted. ' type strain *L. garvieae* Lg2 found in GenBank, * identical bases, - deletion.

Table 5. Assessment of 21 isolates according to antibiotic resistance according to CLSI standards

	Antibiotic											
Isolates	AMP 10	E 5	FFC 30	DA 2	K 30	ENR 5	CIP 1	C 30	OT 30	SXT 25	UB 30	S 10
FMB-F1	S	I	S	R	R	R	R	S	S	I	R	R
FMB-F2	S	S	S	R	I	S	R	S	S	S	R	R
FMB-F3	S	I	R	R	R	R	I	I	S	I	R	R
FMB-F4	S	I	S	R	R	I	R	S	S	I	R	R
FMB-F5	S	I	S	R	R	S	I	S	S	I	R	R
FMB-F6	S	I	S	R	R	S	R	S	S	S	R	R
FMB-F7	S	S	S	R	R	S	I	S	S	I	R	R
FMB-F8	S	I	S	R	R	I	R	S	S	I	R	R
FMB-F9	S	I	S	R	I	S	I	S	S	S	S	R
FMB-F10	S	I	S	R	R	I	R	I	S	I	R	R
FMB-F11	S	I	S	R	I	S	I	S	S	S	I	R
FMB-F12	S	I	S	R	I	S	I	S	S	S	R	R
FMB-F13	S	R	S	R	R	R	R	S	S	R	R	R
FMB-F14	S	I	I	R	R	I	R	S	S	I	R	R
FMB-F15	S	I	S	R	R	I	R	S	S	I	R	R
FMB-F16	S	I	S	R	R	R	R	S	S	I	R	R
FMB-BL1	S	I	I	R	R	R	R	I	S	I	S	R
FMB-BL2	S	I	I	R	R	R	R	S	S	R	R	R
FMB-F17	S	I	S	R	R	R	R	S	S	I	R	R
FMB-R	S	I	S	R	R	I	R	I	S	I	R	R
FMB-H	S	S	S	R	R	R	R	S	R	R	R	R

Previous studies have revealed that *L. garvieae* strains may have variations in utilisation of hippurate, β-glucuronidase, citrate, Voges-Proskauer (VP), pyrrolidonyl arylamidase, urease, and also in acid production from D-mannose, D-lactose, D-ribose, sorbitol and raffinose (Çağırgan, 2004; Soltani, Nikbakht, Ebrahimzadeh Moussavi, & Ahmadzadeh, 2008; Altun *et al*., 2013; Didinen, Yardimci, Onuk, Metin, & Yildirim, 2014). Similarly, it has been observed in the present study that isolates were found to have different phenotypic profiles in acid production from ribose and lactose (Table 3). Furthermore, phenotypic heterogeneity was detected among bacterial strains in their ability to: utilise N-acetyl-β-glucosaminidase, arylamidase, methyl-β-D-glucopyranoside; the acidification of mannitol, saccharose and cyclodextrin (Table 3). Rapid ID 32 Strep test results showed that 15 isolates used in the study belonged to *L. garvieae* and 6 isolates may be *E. faecalis*. However, amplification products obtained from highly conserved *16S rRNA* gene as well as their nucleotide sequence homologies with the reference strain Lg2 confirmed that all isolates were *L. garvieae.* It is known that API test systems enable the identification of many species with high accuracy (Türe & Alp, 2016). However, they still remain insufficient to distinguish between closely related species. Therefore, *L. garvieae* included within the family *Streptococcaceae* was formerly identified as *E.*

Table 6. *L. garvieae* isolates' sensitivities to antibiotics

Antibiotic	% (n=21)		
	R	I	S
AMP10	0	0	100
E5	5	81	14
FFC30	5	14	81
DA2	100	0	0
K30	80	19	1
ENR5	38	29	33
CIP1	71	29	0
C30	0	19	81
OT30	5	0	95
SXT25	14	62	24
UB30	83	0	17
S10	100	0	0

R: resistant, I: intermediate, S: sensitive

seriolicida by using conventional tests (Prieta *et al*., 1993; Morita *et al*., 2011). Furthermore, it was reported that some bacterial species were not identified through these commercial bacterial diagnostic systems (Roach, Levett, & Lavoie, 2006). As a result, it is a requirement that bacterial isolates, which are identified based on their cultural, physiological and biochemical properties, should be verified at species level by molecular taxonomic approaches. It has been experienced in this work that molecular taxonomy is a powerful and reliable approach in identifying *L. garvieae* isolates at species level.

Mechanisms responsible for the pathogenesis of *L. garvieae* have been poorly understood, yet (Vendrell *et al*., 2006; Austin & Austin, 2012). Various virulence factors of *L. garvieae* have been investigated in order to explain the pathogenesis of bacterial infection. Haemolysins are the extracellular toxic proteins, produced by many Gram-positive and Gram-negative bacteria. Haemolysins play a role as virulence factors in pathogenesis due to their lytic activities. Their enzymatic effects cause lysis of red blood cells and phagocytes both by forming pores and by disrupting phospholipid structures in the membrane. Therefore, at the same time they are also known as cytolysins (Goebel *et al*., 1988). Morita *et al*. (2011) compared the amino acid sequences of *L. garvieae* with the sequences of evolutionarily related taxa and they detected amino acid sequence homology (56-72%) among them. They predicted for the first time that haemolysins could be potential virulence factors for this species with the alignment and validation analysis. After that, these suggested haemolysin proteins, encoded by three *hly* genes, have also been shown by different research groups, in α-haemolytic bacteria.

L. garvieae is an α-haemolytic bacterium, and has three genes (*hly*1, *hly*2, *hly*3) which are responsible for haemolytic activity (Gibello *et al*., 2016). For this reason, these *hly* genes are used as genetic determinants in the detection of the haemolytic effect. Miyauchi *et al*. (2012) and Ture and Altinok (2016) have previously showed that these three *hly* genes were carried by the *L. garvieae* genome. In this study, amplification of the full length (796 bp) *hly*2 gene from all strains revealed that the gene encoding the product with haemolytic activity may directly participate in pathogenesis, as reported by other researchers. Also the *hly*1 gene has been amplified from all strains except FMB-F13. It is possible that *hly*1 in FMB-F13 could not be amplified due to mutation in priming site, resulting in a null-allele, and the product encoded by the gene may also play a role as a possible virulence factor in pathogenesis for all strains. In contrast to findings obtained from previous studies (Miyauchi *et al*., 2012; Ture & Altinok, 2016), as shown in the Figure 1d, the 549 bp-length products of *hly*3 gene associated with virulence, could not be yielded from five strains including FMB-F17 and FMB-R (Table 4). However, it has been reported that the FMB-F17 and FMB-R strains caused mortality in experimentally infected rainbow trout (Ürkü & Timur, 2014) and sea bass (*Dicentrarcus labrax*) (Göken, 2016). The fact that both FMB-F17 and -R strains carry *hly*1 and *hly*2 genes, but do not contain *hly*3, indicates that the *hly*1 and *hly*2 are effective in bacterial virulence of the strains. For this reason, this experimental result suggests that *hly*3 gene may not directly participate in the pathogenesis. Data obtained from the characterisation of these haemolytic effective genes shall contribute to understanding the pathogenesis mechanisms of α-haemolytic *L. garvieae*.

Sequence similarity searching is a strategy used for the characterisation of the genes (Pearson, 2013). Nucleotide sequencing of the three *hly* genes and alignment with Lg2 sequence revealed the presence of high nucleotide homology. However, the sequence comparison of the three genes indicated that many mutations (such as SNPs and deletions) were present in all *hly* gene sequences. It has been demonstrated that functional haemolysins might have been produced by these variant genes (especially *hly*1 and *hly*2) present in FMB-F17 and FMB-R strains, infecting the rainbow trout and sea bass. The alignment of the potential hly

amino acid sequences showed that there were consensus sequences at high level (98-100%) between strains and Lg2. This amino acid homology data supports that SNPs and deletions detected in the *hly* genes do not alter the code carried in the open reading frame, which is responsible for amino acid profiles. Although amino acid identity ranged from 56 to 72% in different pathogenic species, different researchers demonstrated that these proteins encoded by *hly*1, *hly*2 and *hly*3 genes were responsible for haemolysis by comparing to consensus amino acid sequences (Miyauchi *et al*., 2012; Ture & Altinok, 2016). Hence, high level of amino acid homology, found in the present study, supported that the variations in *hly* genes might not have an effect on phenotypic traits of *L. garvieae* strains.

Turkey is one of the biggest fish producers among European countries according to the Turkish Statistical Institute data (TUIK, 2015). Like in the other large-scale producers, bacterial infectious diseases are spreading among fish farms, and antimicrobial treatments are inadequate for the health management (Türe & Alp, 2016). An excessive and incorrect usage of antibiotics for the control of lactococcosis in fish farms has led to an increase in acquired antibiotic resistance which has negative effects on animal and human health (Cabello, 2006). Therefore, the detection of current antibiotic resistance of bacterial pathogens is crucial for treatment and pre-exposure prophylaxis. This study revealed that all *L. garvieae* strains were resistant to clindamycin and streptomycin, while being susceptible to ampicillin. 80% of the isolated bacteria acquired resistance to kanamycin, enrofloxacin (38%), ciprofloxacin (71%), and flumequine (83%) (Table 6). Diler *et al*. (2002) reported that *L. garvieae* isolates were susceptible to ampicillin and chloramphenicol, but resistant to clindamycin. Clindamycin resistance is a common identification feature which is utilised to discriminate *L. garvieae* from *L. lactis* (Elliot & Facklam, 1996). Other previous studies pointed that *L. garvieae* strains displayed sensitivity to florfenicol and oxytetracycline (Kav & Erganiş, 2007; Raissy & Ansari, 2011; Didinen *et al*., 2014). This research also indicated isolates previously recorded as resistant to sulphamethoxazole/trimethoprim and susceptible to erythromycin (Diler *et al.*, 2002; Kav & Erganiş, 2007; Raissy & Ansari, 2011; Didinen *et al.*, 2014), to be of intermediate profile. To prevent the emergence of antibiotic resistance and the failure in disease treatment, it should be determined of antibiotic susceptibility profiles of bacteria and applied of suitable antimicrobial agents in sufficient dosage and time (Altun *et al*., 2013). Pathogenic bacteria which acquire antibiotic resistance continue to survive and maintain the capacity to cause disease. Therefore the antibiotic resistances may contribute to spread of virulent bacteria. Consequently, the determination of antimicrobial susceptibility of causative agents in fish farms has a great importance in the monitoring of pathogenic bacteria and diseases on aqua-cultural areas.

The determination and characterisation of virulence genes of *L. garvieae* will make a significant contribution to the understanding of the pathogenesis of this pathogen. Because our data confirms that there is genotypic variability in *hly*3 gene, the present study revealed that *hly*1 and *hly*2 genes are effective in the virulence mechanism of *L. garvieae* one of the important fish pathogens. These virulence genes can be used as genetic determinants of virulence and at the same time as suitable targets in the development of new therapeutics and vaccines which are used to treat lactococcosis. Antibiotic resistance helps the survival of the bacterial pathogen, and the maintenance of its virulence. It should be considered that there is a relationship between antibiotic resistance and bacterial virulence since the acquisition of antibiotic resistance contributes to the spread of pathogenic bacteria. The current study has provided valuable genotypic and phenotypic data to be used to control the lactococcosis infection caused by *L. garvieae* in rainbow trout.

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