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Detection of putative virulence genes of *Lactococcus garvieae*

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ABSTRACT: *Lactococcus garvieae* is the causative agent of lactococcosis and has been isolated from a wide variety of animals. In the present study, 34 strains of *L. garvieae* isolated from fish from different sources and locations were tested for the presence or absence of the following putative virulence genes: a capsule gene cluster (CGC), hemolysins 1, 2, and 3 (*hly*1, -2, -3), NADH oxidase, superoxide dismutase (*sod*), phosphoglucomutase (*pgm*), adhesin Pav (*adh*Pav), adhesin PsaA (*adh*PsaA), enolase (*eno*), LPxTG-containing surface proteins 1, 2, 3, and 4 (LPxTG-1, LPxTG-2, LPxTG-3, LPxTG-4; where LPxTG means Leu-Pro-any-Thr-Gly), adhesin clusters 1 and 2 (*adh*CI, *adh*CII), and adhesin (*adh*). To determine the presence of the CGC, we developed a multiplex PCR. All strains of *L. garvieae* had the *hly*1, -2, -3, NADH oxidase, *pgm*, *adh*Pav, LPxTG-2, LPxTG-3, *sod*, *eno*, *adh*PsaA, *adh*CII, and *adh*CII genes, while only the Lg2 strain contained the CGC. The virulent Lg2 strain contained all 17 virulent genes. All Turkish, Spanish, Italian, and French strains did not contain the CGC. The multiplex PCR assay was useful for the detection of the CGC genes. In conclusion, the CGC is not the only virulent factor in *L. garvieae* because strains that lack the CGC are virulent to rainbow trout. Single genes also might not be responsible for the virulence of *L. garvieae*.

KEY WORDS: Capsule gene cluster · Hemolysin · LPxTG-containing surface protein · Enolase

INTRODUCTION

Lactococcus garvieae is the causative agent of lactococcosis and belongs to the lactic acid bacteria. It has been isolated from various sources such as fish, food products, terrestrial animals, and humans. Although *L. garvieae* currently can be isolated from many different sources, it is a major pathogen of fish and causes fatal hemorrhagic septicemia in fish (Vendrell et al. 2006). It was first isolated from yellowtail in Japan (Kusuda et al. 1991) and has caused great economic losses in the aquaculture industry worldwide (Eldar et al. 1996), including Turkey (Ozturk & Altinok 2014).

Although it has been considered to be of increasing clinical significance in both veterinary and human medicine, to our knowledge, limited studies have reported on the pathogenic mechanisms of *L. garvieae*. Several studies have proved that capsule formation is one of the virulence factors for fish (Yoshida et al. 1997, Kawanishi et al. 2007, Miyauchi et al. 2012). *L. garvieae* isolated from yellowtail *Seriola quinqueradiata* has been divided into agglutinating (KG^+) and nonagglutinating (KG^-) phenotypes. Morita et al. (2011) claimed that the capsulated serotype (KG^-) Lg2 strain of *L. garvieae* is more virulent in yellowtail than the noncapsulated (serotype KG^+) ATCC 49156 strains, and these capsules are thought to play an important role in the pathogenicity of *L. garvieae* infection, by increasing the bacterium's resistance to fish phagocytosis (Ooyama et al. 2002). On the other

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hand, Ture et al. (2014) found that uncapsulated *L. garvieae* Lgper and the ATCC 49156 strain were pathogenic to rainbow trout *Oncorhynchus mykiss* and caused more than 89% mortality. It seems that the pathogenicity of *L. garvieae* cannot directly be related to the presence of a capsule. Therefore, it is important to investigate the putative virulence genes of *L. garvieae* strains. The main objective in this study was to screen for a wide array of putative virulence genes for 30 *L. garvieae* strains isolated from fish farms throughout Turkey. All isolates were tested for the presence of putative virulence genes including a capsule gene cluster (CGC), hemolysins, NADH oxidase, phosphoglucomutase, adhesin, adhesin clusters, adhesin Pav, adhesin PsaA, enolase, superoxide dismutase, and LPxTG-containing surface proteins (where LPxTG means Leu-Pro-any-Thr-Gly). All apart from the CGC were analyzed using uniplex PCR. To determine the presence of the CGC, we also developed a multiplex PCR.

MATERIALS AND METHODS

Bacterial isolates and culture conditions

A total of 34 isolates of *Lactococcus garvieae* and 1 isolate of *L. lactis* were studied. Seventeen *L. garvieae* strains were isolated from local rainbow trout farms in Turkey between 2002 and 2011 (Table 1). Six Spanish and 1 French isolates were kindly provided by J. F. Fernandez-Garayzabal (Dpto. Sanidad Animal Facultad de Veterinaria, Universidad Complutense, Madrid, Spain). Four Italian strains were provided by A. Manfrin (Istituto Zooprofilattico Sperimentale delle Venezie, Adria, Italy). The Lg2 strain was provided by H. Morita (Azabu University, Japan). Some of the Turkish strains were also kindly provided by F. Balta (Recep Tayyip Erdogan University, Rize, Turkey). Iranian strains were provided by M. Soltani (University of Tehran, Tehran, Iran). A strain of *L. lactis* was isolated in another study in Turkey. All isolates were stored in 15–20% glycerol containing tryptic soy broth (TSB, Merck) at –80°C. For analyses, they were inoculated on tryptic soy agar (TSA, Merck) and incubated at 30°C for 18 h.

Capsule staining

The method was performed as described by Anthony (1931), with a slight modification. Briefly, a smear from each strain was prepared on a slide after overnight incubation at 30°C in 10 ml of skim milk broth. Air-dried smears were covered with 1% crystal violet for 2 min and then rinsed gently with a 20% solution of copper sulfate. Slides were air-dried and examined under an oil immersion lens.

DNA preparation

Genomic DNA from all isolates was extracted using the QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions. To increase the DNA

Table 1. *Lactococcus garvieae* isolates and a *L. lactis* isolate, with source, country, and isolation year

Isolate (strain)	Source	Origin	Year of isolation
Lg2	Yellowtail	Japan	2002
225-1	Rainbow trout	Artvin, Turkey	2008
399-18	Rainbow trout	Artvin, Turkey	2008
673-5	Rainbow trout	Gumushane, Turkey	2008
671-14	Rainbow trout	Gumushane, Turkey	2008
K9	Rainbow trout	Gumushane, Turkey	2009
Kalis	Rainbow trout	Gumushane, Turkey	2014
235-16	Rainbow trout	Izmir, Turkey	2009
M1	Rainbow trout	Mugla, Turkey	2002
M2	Rainbow trout	Mugla, Turkey	2002
M3	Rainbow trout	Mugla, Turkey	2003
Lgper	Rainbow trout	Ordu, Turkey	2011
A30	Rainbow trout	Rize, Turkey	2007
Sider17	Rainbow trout	Rize, Turkey	2008
OM	Rainbow trout	Rize, Turkey	2008
Iysaf	Rainbow trout	Rize, Turkey	2009
Ser114	Rainbow trout	Trabzon, Turkey	2009
Trb	Rainbow trout	Trabzon, Turkey	2011
Akoluk	Rainbow trout	Trabzon, Turkey	2012
Trb-2	Rainbow trout	Trabzon, Turkey	2012
2398	Rainbow trout	France	1998
I2015	Rainbow trout	Iran	2015
M300	Goat cheese	Italy	2001
A-58	Rainbow trout	Italy	2002
G-27	Cow milk	Italy	2003
PP6O	Rainbow trout	Italy	2004
ATCC49156	Yellowtail	Japan	1974
1684	Rainbow trout	Spain	1997
164 A/03	Rainbow trout	Spain	2000
8053	Rainbow trout	Spain	2000
532	Rainbow trout	Spain	2001
FTPI	Rainbow trout	Spain	2001
498	Rainbow trout	Spain	2001
ATCC43921	Cattle	UK	1984
L. lactis	Cow milk	Isparta, Turkey	2005

yield, lysozyme treatment was extended to 1 h. The RNA/DNA calculator (spectrophotometer, Bio-Rad) was employed to measure optical density at 260 and 280 nm. Before checking for putative virulence genes and the CGC, the species identification of all isolates was confirmed by PCR as described by Altinok (2011).

Primers

Potential virulence genes of *L. garvieae* were listed by Miyauchi et al. (2012). The following putative virulence genes were studied: the CGC, hemolysins 1, 2, and 3 (*hly*1, -2, -3), NADH oxidase, superoxide dismutase (*sod*), phosphoglucomutase (*pgm*), adhesin Pav (*adh*Pav), adhesin PsaA (*adh*PsaA), enolase (*eno*), LPxTG-containing surface proteins 1, 2, 3, and 4 (LPxTG-1, LPxTG-2, LPxTG-3, LPxTG-4), adhesin clusters 1 and 2 (*adh*CI, *adh*CII) and adhesin (*adh*). Primers were designed (Tables 2 & 3) from these putative virulence genes by using the Primer-BLAST program available at NCBI. Nucleotide sequences of the *L. garvieae* virulence genes and the CGC were obtained from the *L. garvieae* Lg2 (NCBI RefSeq: NC_017490) and ATCC 49156 (NCBI RefSeq: NC_015930) genomes.

PCR assays for detection of putative virulence genes

Each PCR reaction mix (25 µl total) included 200 ng of DNA sample, 100 pmol of each primer, 12.5 µl of 2× Master Mix PCR mixture (Qiagen, Master PCR kit), and 9.5 µl of distilled water. DNA amplification was performed in a thermocycler (SensoQuest) under the following conditions: a 3 min initial denaturation at 94°C; followed by 35 cycles of denaturation at 94°C

Table 2. Putative virulence genes: primer sequences, target genes, locus tag, amplified product size, and annealing temperature. Nucleotide sequences of the *Lactococcus garvieae* putative virulence genes and capsule gene cluster were obtained from the *L. garvieae* Lg2 strain (NCBI RefSeq: NC_017490) genome

Primer	Primer sequence (5′–3′)	Target gene	Locus	Product size (bp)	Annealing temp. (°C)
H1-F	CCTCCTCCGACTAGGAACCA	Hemolysin 1	LCGL 0323	521	54
H1-R	GAAAAGCCAGCTTCTCGTGC				
H2-F	TCTCGTGCACACCGATGAAA	Hemolysin 2	LCGL 0374	492	53
H2-R	TGAACTTCGGCTTCTGCGAT				
H3-F	AACGCGAGAACAGGCAAAAC	Hemolysin 3	LCGL 0597	291	56
H3-R	CCCACGTCGAGAGCATAGAC				
NADHO-F	TGCGATGGGTTCAAGACCAA	NADH oxidase	LCGL 0664	331	53
NADHO-R	GCCTTTAAAAGCCTCGGCAG				
SOD-F	GCAGCGATTGAAAAACACCCA	Superoxide dismutase	LCGL 0664	80	54
SOD-R	TCTTCTGGCAAACGGTCCAA				
PG-F	AAGTTTACGGCGAAGACGGT	Phosphoglucomutase	LCGL 0285	997	53
PG-R	TTTTCTGGTGCATTGGCACG				
AP-F	CCTGTCGGGCGCTTTTATTG	Adhesin Pav	LCGL 1330	232	56
AP-R	TCCCGGAAGAAGAGTACGGT				
APSA-F	GTTGCAACAGCTGGACACAG	Adhesin PsaA	LCGL 1533	180	54
APSA-R	ATACGGTTGAGTTGGGCTGG				
E-F	CAAGAGCGATCATTGCACGG	Enolase	LCGL 1514	201	54
E-R	CATTCGGACGCGGTATGGTA				
LP1-F	GTGAACGTGGAGCTTCCAGA	LPxTG-1	LCGL 1005	878	54
LP1-R	CCACTCACATGGGGGAGTTC				
LP2-F	GCCAGTGAGAGAACCGTTGA	LPxTG-2	LCGL 1410	767	54
LP2-R	CAGGTTCAAGTGCAACTGCC				
LP3-F	TTAAGCACAACGGCAACAGC	LPxTG-3	LCGL 1585	231	54
LP3-R	CACGCGAAATGATGGTGCAT				
LP4-F	GGGAGCACCGGATTCACTTT	LPxTG-4	LCGL 1672	928	52
LP4-R	ACAAAGCCGCAGACCTTACA				
AC1-F	TTGGGCACATCAGACTGGAC	Adhesin cluster 1	LCGL 0842	264	54
AC1-R	AGCATCATCAGCTGCCAAGT				
AC2-F	CTGCGAGTGGCATCTCCATT	Adhesin cluster 2	LCGL 0843	160	52
AC2-R	TCAACACTGCGACCTTCTGT				
AF-F	CAGCCAGCACCAGGTTATGA	Adhesin	LCGL 0196	358	54
AF-R	CTCCTGCGTTGACATGGACT				

Table 3. *Lactococcus garvieae* capsule gene cluster primer sequences and amplified product size. Nucleotide sequence of the *L. garvieae* capsule gene cluster was obtained from the *L. garvieae* Lg2 strain (NCBI RefSeq: NC_017490) genome

Primer	Primer sequence (5′–3′)	Product size (bp)
1020-F	ACCTTCACTTGCATTCATAGGGT	304
1323-R	TTGTCCCAGAGGGTTCTCCT	
851-F	TAGGAGGTGTTCCTGGGAGG	549
1399-R	TGTCCCACTCCTACTGTCGT	
6329-F	AAAAACGGAGGGCAACAAGC	785
7175-R	CACTTGTACAGGCCACTGGT	
5358-F	TGGAGGGTATTGCCTACCGA	650
6007-R	CCACAGCAGCTTCTTCACCT	

for 30 s, annealing at 52–56°C (see Table 2) for 30 s, extension at 72°C for 60 s; and final extension at 72°C for 10 min. Controls consisted of the PCR mixture containing (1) no DNA template (reagent control) and (2) DNA from *L. garvieae* with *L. garvieae*-specific primer (positive control). After the PCR, 10 µl of PCR products were subjected to electrophoresis in 1.2% (w/v) agarose gel prepared with 1× Tris-borate-EDTA (TBE) buffer and run at 100 V for 45 min. Then, the DNA bands were stained with RedSafe (Intron Biotechnology) and viewed by UV transillumination. The sizes of the PCR products were determined by comparing them with the migration of a 100 bp DNA ladder (Bio Basic). Each of the 17 putative virulence gene PCR products was also confirmed by DNA sequencing.

Multiplex PCR for the CGC

A number of primer pairs were designed from the capsule gene of *L. garvieae* using NCBI Primer-BLAST. Primer pairs were tested (data not shown) against different Gram-positive and Gram-negative bacterial species.

Different annealing temperatures and $MgCl_2$ concentrations were tested to obtain the optimal specificity and sensitivity of the multiplex (mPCR) assay. Intensity of the amplicons for each target DNA, as well as the absence of nonspecific bands, was considered in selecting the optimal mPCR conditions. Each 25 µl mPCR reaction mixture (prepared on ice) contained 100 ng of the sample DNA, 12.5 µl of 2× Multiplex PCR mixture containing 3 mM $MgCl_2$ (QIAGEN Multiplex PCR kit, Qiagen Molecular Biochemicals), 2.5 µl of 5× Q solution (QIAGEN Multiplex PCR kit), and 100 pmol of each primer. Thermal cycling was performed with a SensoQuest PCR machine under the following conditions: an initial denaturation cycle at 95°C for 5 min; followed by 30 cycles of amplification (denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 60 s); and a final 10 min extension period at 72°C. Controls consisted of the PCR mixture containing (1) no DNA template (reagent control), (2) DNA from *L. lactis* (negative control), or (3) DNA from *L. garvieae* with specific *L. garvieae* primer (positive control). After the PCR, products were transferred to a 2.0% agarose gel, electrophoresed, and DNA was visualized by RedSafe staining. PCR products were confirmed by DNA sequencing.

DNA sequencing

To confirm that the correct products were amplified, PCR product from each gene was directly sequenced by Macrogen Inc. The derived nucleotide sequences were analyzed and aligned with Lasergene MegAlign (DNAStar). The results of the sequencing were used for homology searches.

RESULTS

A total of 34 strains of *Lactococcus garvieae* isolated from different sources and locations were tested for the presence or absence of putative virulence genes. All strains of *L. garvieae* had the *hly*1, -2, -3, NADH oxidase, *pgm*, *adh*Pav, LPxTG-2, LPxTG-3, *sod*, *eno*, *adh*PsaA, *adh*CI, and *adh*CII genes, while only the Lg2 strain contained the CGC (Table 4). The presence of *adh*, LPxTG-1, and LPxTG-4 genes varied between isolates. All isolates contained *adh* except for isolates from Izmir (235-16), Trabzon (Akoluk, Trb-2), and Gumushane (671-14, Kalis). The most virulent (i.e. containing the greatest number of putative virulence genes) strain (Lg2) contained all 17 virulence genes including *adh*, LPxTG-1, and LPxTG-4. Two Turkish strains (2/19), 6 Spanish strains (6/6), 1 French strain (1/1), and 2 Italian strains (2/4) had the LPxTG-1 gene, while 6 Turkish strains and 5 Spanish strains, but none of the Italian, French, and Iranian strains contained LPxTG-4 (Table 4).

Sequencing results from PCR products confirmed that the primers did amplify the correct products, with the amplified fragment matching exactly the database sequence of the targeted gene (Fig. 1). Each of the 4 pairs of oligonucleotide primers for the

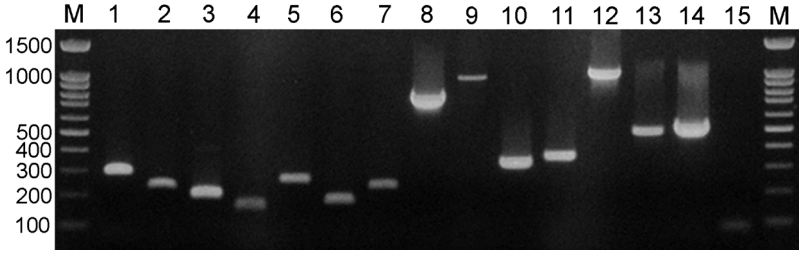

Fig. 1. Identification of different virulence genes in *Lactococcus garvieae* isolates. Lane M: 100 bp DNA ladder. Lanes 1–15: hemolysin 3, LPxTG-containing surface protein 3 (where LPxTG means Leu-Pro-any-Thr-Gly), enolase, adhesin cluster 2, adhesin cluster 1, adhesin PsaA, adhesin Pav, LPxTG-2, LPxTG-4, NADH oxidase, adhesin, phosphoglucomutase, hemolysin 2, hemolysin 1, and superoxide dismutase

CGC exclusively amplified the capsule gene cluster of *L. garvieae*. Amplification products, of the expected sizes, ranged between 304 and 785 bp (Fig. 2).

Table 4. Presence of virulence genes in *Lactococcus garvieae* isolates and the *L. lactis* isolate. Hemolysins 1, 2, and 3, NADH oxidase, phosphoglucomutase, adhesin Pav, LPxTG-containing surface proteins 2 and 3 (where LPxTG means Leu-Pro-any-Thr-Gly), superoxide dismutase, adhesin PsaA, enolase, and adhesin clusters 1 and 2 were found in all isolates. '+' indicates gene is present; '–' indicates gene is absent

Isolate	Gene(s)			
	Capsule gene cluster	Adhesin	LPxTG-1	LPxTG-4
Lg2	+	+	+	+
225-1	–	+	–	+
399-18	–	+	+	–
637-5	–	+	–	–
671-14	–	–	–	–
K9	–	+	–	–
Kalis	–	–	–	–
235-16	–	–	–	+
M1	–	+	–	+
M2	–	+	–	+
M3	–	+	–	–
Lgper	–	+	–	–
A30	–	+	–	–
Sider17	–	+	–	–
OM	–	+	–	–
Iysaf	–	+	–	+
Ser114	–	+	+	–
Trb	–	+	–	+
Akoluk	–	–	–	–
Trb-2	–	–	–	–
2398	–	+	+	–
I2015	–	–	–	–
M300	–	+	+	–
A-58	–	+	–	–
G-27	–	+	+	–
PP6O	–	+	–	–
ATCC49156	–	+	+	+
1684	–	+	+	+
164A/03	–	+	+	+
8053	–	+	+	–
532	–	+	+	+
FTPI	–	+	+	+
498	–	+	+	+
ATCC43921	–	–	–	–
L. lactis	–	–	–	+

All of the expected-sized fragments (Fig. 2) were amplified in both the simplex PCR and multiplex PCR.

Capsule staining, reflecting the mPCR results, showed that a capsule was only present in the Lg2 strain while the other strains did not have a capsule.

DISCUSSION

Lactococcus garvieae is one of the most important infectious fish pathogens, with significant economic losses for fish farms in many countries (Eyngor et al. 2004). *L. garvieae* has been considered to show increasing clinical significance in both veterinary and human medicine; however, the pathogenic mechanisms of this agent are poorly understood. It has previously been thought that virulence of *L. garvieae* relies on its ability to form a capsule (Ooyama et al. 2002). *L. garvieae* isolated from fish is serologically divided into 2 groups, encapsulated (KG^-) and noncapsulated (KG^+) strains (Kang et al.

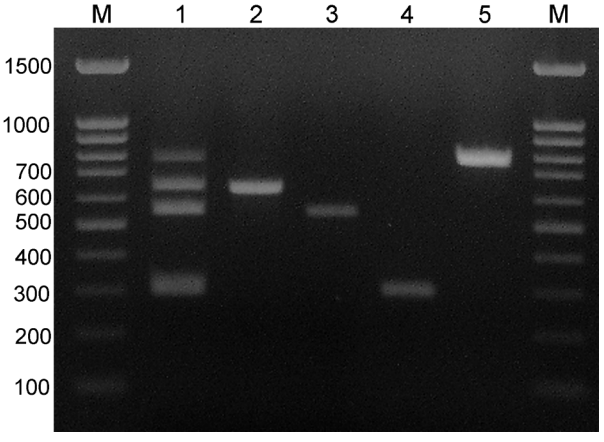

Fig. 2. Specificity of the multiplex PCR (mPCR) assay developed for detection of the capsule gene cluster of *Lactococcus garvieae*. Lane M: lambda DNA *Hin*dIII molecular size marker; Lane 1: mPCR with the 4-primer set; Lane 2: 5358-F and 6007-R primers (650 bp) alone; Lane 3: 851-F and 1399-R primers (549 bp) alone; Lane 4: 1020-F and 1323-R primers (304 bp) alone; Lane 5: 6329-F and 7175-R primers (785 bp) alone

2004). KG^- strains are more virulent than the noncapsulated KG^+ strains (Yoshida et al. 1997). Miyauchi et al. (2012) developed a PCR to detect the whole CGC that contains 17 genes (Morita et al. 2011). *L. garvieae* strain Lg2 had a 16.5 kb CGC that was absent in the ATCC 49156 strain, which had a 750 bp fragment. It is very difficult to amplify the whole 16.5 kb CGC in a reaction with a standard *Taq* polymerase enzyme. Therefore, we developed a multiplex PCR to detect the CGC of *L. garvieae* with 4 primer sets. The specificity of our assay was verified by performing the mPCR with DNA from the *L. garvieae* Lg2 strain and another 34 strains of *L. garvieae* and *L. lactis*. In all cases, no bands were visible on gel electrophoresis, and all yielded negative results, demonstrating that the mPCR assay did not cross-react with other *L. garvieae* strains which did not have CGC genes. Similar to the Morita et al. (2011) and Miyauchi et al. (2012) studies, the CGC was detected from the Lg2 strain but not from the other strains including ATCC 49156 in the present study. Miyauchi et al. (2012) reported that the CGC was found only in pathogenic *L. garvieae* strains as a genomic island. On the other hand, the noncapsulated *L. garvieae* ATCC 49156 strain and Lgper strain (the latter characterized during the current study) were virulent to rainbow trout with 98 and 89% mortality, respectively (Ture et al. 2014). Therefore, the CGC might not be responsible for the virulence of *L. garvieae* to rainbow trout, and other virulence genes may be responsible.

Miyauchi et al. (2012) proposed that *hly*1,-2,-3, NADH oxidase, *sod*, *pgm*, *adh*Pav, *adh*PsaA, *eno*, LPxTG-1, LPxTG-2, LPxTG-3, LPxTG-4, *adh*CI, *adh*CII, and *adh* are also responsible, in addition to the CGC, for the virulence of *L. garvieae*. However, possession of only a single or of a few virulence genes does not suffice to endow a strain with pathogenic status. A strain must have acquired the appropriate virulence gene combination in order to cause disease in a specific host species (Gilmore & Ferretti 2003). Adhesins are one of the most important types of virulence factors, and are bacterial cell surface attachments that simplify bacterial adhesion to other bacteria or to host cell surfaces. Adherence is a necessary step in bacterial pathogenesis, required for colonizing. In Gram-positive bacteria, a polysaccharide surface layer works as the specific adhesin (Coutte et al. 2003). Many bacterial pathogens are able to express different adhesins. During infection, expression of these adhesins plays an important role in adhesion-based virulence (Klemm & Schembri 2000). Miyauchi et al. (2012) reported that the human and fish isolates of *L. garvieae* contained specific adhesin genes. In the present study, all strains had the *adh*Pav, *adh*CI, *adh*CI, and *adh*PsaA genes, but 3 strains (235-16, Akoluk, 671-14) did not contain the *adh* gene.

Gram-positive bacteria have a thick cell wall including a multilayered peptidoglycan. These layers also consist of teichoic and lipoteichoic acids that are directly bound to the peptidoglycan. Also, the bacterial cell wall acts as a platform to support surface proteins, many of which play important physiological roles, e.g. in nutrient acquisition, sensing, and host interactions (Marraffini et al. 2006). LPxTG is a surface protein which covalently binds to the peptidoglycan isolated from many Gram-positive bacteria such as *Listeria monocytogenes*, *Staphylococcus aureus* (Mariscotti et al. 2012), *Streptococcus pneumoniae* (Perez-Dorado et al. 2012), and *Lactococcus garvieae* (Miyauchi et al. 2012); LPxTG proteins play essential roles in bacterial virulence. In this study, we investigated the presence of 4 types of LPxTG genes. LPxTG-1 and LPxTG-4 genes were detected in 37% of the isolates, while LPxTG-2 and LPxTG-3 were detected in all isolates.

Superoxide dismutases (SOD) are enzymes that catalyze the conversion of superoxide radicals into oxygen and hydrogen peroxide. They are important antioxidants and have powerful anti-inflammatory activity. During infection, some bacteria produce SOD to protect themselves from being killed. Some forms of SOD are present in humans, other mammals, and in bacteria, and are located in the cytoplasm, mitochondria, and extracellular area (Segui et al. 2004, Vanaporn et al. 2011). In this study, *sod* virulence genes were detected in all isolates.

Enolase (*eno*), phosphoglucomutase (PG), and NADH oxidase are other putative virulence genes, but their roles in virulence are not fully understood. Enolase and PG are metabolic enzymes, but they are also considered to be moonlighting proteins involved in the virulence of some bacteria (Amblee & Jeffery 2015). Surface-expressed enolase contributes to the pathogenesis of *Bacillus anthracis* (Agarwal et al. 2008), *Aeromonas hydrophila* (Sha et al. 2009), and *Streptococcus pneumoniae* (Kolberg et al. 2006). However, although *eno* and PG are immunogenic proteins in cell extracts of *L. garvieae* (Shin et al. 2009), the existence of these proteins on the cell wall of this microorganism has not been studied. In the present study, these 3 genes were present in all strains. Therefore, the virulence of bacteria that do not contain CGC may come from potential virulence

factors such as SOD, NADH oxidase, PG, and *eno* (Mitchell 2003, Buchanan et al. 2005, Morita et al. 2011).

Hemolysin is normally secreted by many bacteria and fungi. They are proteins and lipids that cause lysis of red and white blood cells by damaging their cell membrane during infection (Stipcevic et al. 2005). Bacteria are categorized according to visualization of hemolysis of red blood cells in agar as α, β, and γ hemolytic bacteria: *L. garvieae* is an α-hemolytic bacteria (Eldar et al. 1996). Hemolysin genes were also detected in *L. garvieae* isolates (Miyauchi et al. 2012). In the present study, the presence of 3 different hemolysin genes (*hly*1, -2, -3) was investigated, and all hemolysin genes were detected in all isolates.

The results of this study provide insights into understanding the virulence mechanisms of *L. garvieae* isolates. The frequency of the virulence genes *hly*, *adh*, *sod*, LPxTG, *eno*, NADH oxidase, and *pgm* remained high in *L. garvieae* isolates. Further studies could investigate other possible virulence genes. In conclusion, the CGC is not the only virulent factor in *L. garvieae*, because a strain that lacks the CGC is virulent to rainbow trout. Furthermore, it might be combinations of different genes rather than single genes that are responsible for the virulence of *L. garvieae*.

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