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Lactococcus garvieae Isolates from Rainbow Trout (*Oncorhynchus mykiss*, W.) Compared by PLG and SA1B10 PCR Primer Pairs

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Abstract

The aim of this study was to identify and compare Lactococcus garvieae strains using the PLG primer pair and SA1B10 primer pair. Also, the antibiotic resistance of the strains was investigated in the study. For this aim, commercial trout farms at Kemer, Korkuteli, and Manavgat, Antalya Province, Turkey were visited every month from June to September 2018. Thirty sick fish were sampled in the study. Lethargy, anorexia, darkening of skin color, unilateral or bilateral exophthalmos, opacification and hemorrhages in the eyes of the sick fish, and abdominal dropsy were observed. At necropsy, hemorrhages in the internal organs, splenomegaly, and darkening of the spleen and ascites were present. Seventy-five strains from sick fish samples verified on the basis of the biochemical characterization and PCR studies. The strains showed homogeneity in terms of phenotypic characteristics and all strains were identified as Lactococcus garvieae. The PCR technique was applied to 50 of 75 strains using PLG primer pair. While 47 of 50 strains gave positive results, amplification was not observed in 3 strains. When the PCR technique using SA1B10 primer pair was applied to a total of 25 strains, 3 of which were negative, 22 of which gave positive amplification, and 25 strains gave positive results. Resistance was determined by the disk diffusion method. All seventy-five strains were resistant to ampicillin and all strains were susceptible to bacitracin and tetracycline. It was found that the resistance and susceptibility of the strains showed variation to other antibiotics used in the study.

Keywords: Lactococcus garvieae; Rainbow Trout; PCR; Antibiotic

INTRODUCTION

Infectious diseases in culture conditions are an important obstacle for the development and continuity of aquaculture. Factors such as fish losses due to contagious diseases, treatment costs and decrease in product quantity and quality increase production costs (Idowu et al. 2017). Generally speaking, healthy fish are resistant to diseases; however, infections can develop in fish when the innate immune system of fish is weakened and water quality is poor for fish (Idowu et al. 2017).

Lactococcosis is a bacterial infection and it affects various fish species worldwide, resulting important economic losses by causing serious epidemics (Sharifi Yazdi et al. 2010; Aron 2017). Lactococcosis was first identified as septicemia in rainbow trout (*Oncorhynchus mykiss*) kept under intensive production conditions in the late 1950s in Japan (Ferrario et al. 2013). Then, the disease has been observed and informed in many countries including Portugal (Pereira et al. 2004), Greece (Savvidis et al. 2007) and Iran (Kia and Mehrabi, 2013). The first *L. garvieae* infection in Turkey was reported from a rainbow trout farm in the Aegean region of Turkey (Diler et al. 2002). Since 2002, the disease has become one of the most common diseases among trout farms located in different geographical regions, especially in summer months when the pond water temperature is 15 °C and above (Kav and Erganiş, 2008; Altun et al. 2010; Timur et al. 2011; Korun et al. 2018). The bacterium *Lactococcus garvieae* is the causative agent of the disease. Although *L.*

garvieae is the primary pathogen of rainbow trout, the bacteria have also been isolated from yellowtail (*Seriola quinqueradiata*), amberjack (*S. dumerili*), kingfish (*S. lalandi*), grey mullet (*Mugil cephalus*) and giant freshwater shrimp (*Macrobranchium rosenbergii*) (Tsai et al. 2013; Fukushima et al. 2017).Lactococcosis is characterized by *hemorrhagic septicemia*. Most cases in rainbow trout are hyper acute with signs of anorexia, lethargy, loss of balance, irregular swimming, melanosis, unilateral or bilateral exophthalmos, abdominal dropsy and rectal prolapse (Vendrell et al. 2006).

The Streptococcaceae family includes Streptococcus, Lactovum and Lactococcus genera. *L. garvieae, L. lactis subsp. lactis and L. lactis subsp*. cremonis are the most important species of Lactococcus genus (Vendrell et al. 2006; Abachi et al. 2016). L. garvieae was previously identified as *Streptococcus garvieae.* After isolation of the bacterium *L. garvieae* from yellow tail (*S. quenqueradiata*) and eel fish (*Anguilla anguilla*) in Japan, *L. garvieae* was proposed as *Enterococcus seriolicida* as a new species (Aguado-Urdo et al. 2010; Ferrario et al. 2013). Therefore, PCR-based techniques targeting the 16S rRNA gene of the bacterium for the molecular identification of *L. garvieae* (Kim et al. 2011). Today, primer pairs include PLG-1 and PLG-2 (Zlotkin et al. 1998) and SA1B10-1-F and SA1B10-1-R primer pair (Kim et al. 2011) are used.

In culture conditions, reducing stress factors such as poor water quality, high stocking density, overfeeding and insufficient nutrients for fish is often important for disease control; however, it would be difficult to reduce these stress conditions and the disease control requires an intense depended on antibiotics (Nakai and Park, 2002). Erythromycin, flumequin, oxytetracycline, enrofloxacin and amoxicillin are commonly used antibiotics for the treatment of lactococcosis (Savvidis et al. 2007; Fukushima et al. 2017). The aim of this study was to identify and compare *Lactococcus garvieae* strains using PLG primer pair and SA1B10 primer pair. Also, antibiotic resistance of *L. garvieae* strains were investigated in the study.

MATERIALS AND METHODS

Sampling

Thirty moribund fish, weighing from 180 g to 350 g, for *L. garvieae* isolation were sampled from rainbow trout fish farms located at Kemer, Korkuteli and Manavgat, Antalya Province, Turkey. The samples were collected every month from June to September 2018. Sick fish showing behavior and clinical findings including lethargy, anorexia, darkening of the skin, unilateral or bilateral exophthalmos, opacification and hemorrhage in the internal organs and ascites were used for isolation of the causative agent of lactococcosis. Pond water temperature (°C), dissolved oxygen (mg/l) and pH were measured and recorded using a portable measuring device (Hanna, Germany) (Table 1). The approval of Akdeniz University Animal Experiments Local Ethics Committee was obtained for the study (Protocol Number: 2018.01.030)

Table 1. Pond water parameters in sampling fish farms

Parameters	Unit	Kemer	Korkuteli	Manavgat
Temperature	°C	18	15	21
pH		7.5	7.5	7.5
Dissolved oxygen	mg/l	7.0	7.2	7.6

Phenotypic characterization of the isolates

Samples were aseptically taken from spleen, liver and head kidney of moribund fish and were streaked onto brain heart infusion agar (BHIA) (Merck, Germany) plates. The plates were incubated at 25 ± 2 °C for 72 h. After incubation period,

single presumptive colonies were selected from pure colonies and were done subcultures of them. Seventy five bacterial isolates which were non-motile, Gram-positive, cytochrome oxidase and catalase negative and fermentative cocci were characterized by using a set of biochemical tests including citrate utilization, methyl red (MR) test, Voges-Proskauer (VP) (acetoin production) test, production of indole, gelatin hydrolysis, starch hydrolysis, ortho-nitrophenyl-galactoside oxidase (ß-galactosidase production, onpg), production of hydrogen sulphide in triple sugar iron (TSI), growth in nutrient broth containing 0%, 2%, 4% and 6.5% NaCl and growth at 4 °C, 20 °C and 30 °C (Vendrell et al. 2006; Austin and Austin, 2012). All media which were used for determination of phenotypic characteristics of the isolates were incubated at 25 ± 2 °C for 24 to 72 h (except onpg, MR-VP, temperature and salinity tolerance tests). Biochemical test results of each strains were evaluated according to the phenotypic characteristics of which were assigned to *L. garvieae* described by Vendrell et al. (2006).

Molecular identification of L. garvieae isolates

The genomic DNA from fifty of 75 isolates was extracted using DNA isolation kit (Hibrigen, Turkey) according to the manufacturer's instructions. The primer pair, PLG-1 and PLG-2, amplifying the 1100 bp segment of 16S rRNA (Zlotkin et al. 1998) and SA1B10-1-F and SA1B10-1-R, amplifying the 709 bp segment in the central region of a dihydropteroate synthase gene of *L. garvieae* (Aoki et al. 2000) were synthesized by a commercial firm. The primers used for the PCR amplification of the genes were summarized in Table 2.

Table 2. Primer pairs used in the PCR assays

Target gene	Primers	DNA sequences (5´-3´)	Product size	Literature
PLG-1	F	CATAACAATGACAATCGC	1100	Zlotkin et al. 1998
PLG-2	R	GCACCCTCGCGGGTTG		
SA1B10	F	CATTTTACGATGGCGCAG	709	Aoki et al. 2000
	R	CGTCGTGTTGCTGCAACA		

F: Forward; R: Reverse

PCR was performed in a total volume of 50 µl of reaction mixture containing 10 µl of 5 x My Taq reaction buffer, 5 µl of template DNA, 2 µl of each primers, 1 µl of MyTaq Hs DNA polymerase and 32 µl of ddH2O. The cycling temperature conditions were the following: denaturation at 95 °C for 3 min, annealing at 44 °C for 15 sec, extension at 72 °C for 10 sec, final extension at 72 °C for 10 min. Thirty cycles were carried out. The PCR products were subjected to electrophoresis (60 min, 100 V) on 1% agarose gel prepared in 1% Tris-boric acid-EDTA buffer (pH 8.3) and the gels were stained with ethidium bromide (0.2 µl), visualized on a UV light, and photographed (Zlotkin et al. 1998; Aoki et al. 2000).

Antibiotic susceptibility test

The disc diffusion method was performed as described by Baker (1984) and EUCAST (2011). Inoculum was prepared in Mueller-Hinton Broth (MHB) (Merck, Germany) at a density adjusted to a McFarland No: 0.5 (1.5 ml x 108 CFU/ml) turbidity standard for the disc diffusion. The inoculum was streaked within 15 min of its preparation on Mueller-Hinton Agar (MHA) (Merck, Germany) plates using a sterile cotton swab. The plates were incubated at 28 ± 2 °C for 24-48 h. The diameters of the inhibition zones around the discs were measured, recorded and the average results were calculated. The isolates were evaluated as resistant, susceptible and intermediate resistant

according to the results of the disc diffusion test (Baker, 1984; EUCAST, 2011). Readymade commercial discs (Oxoid, UK) of ampicillin (10 µg), bacitracin (0.04 µg), erythromycin (15 µg), flumequin (30 µg), furazolidone (15 µg), kanamycin (30 µg), oxytetracycline (30 µg), streptomycin (10 µg) and trimethoprim (5 µg) were used for antibiotic susceptibility.

RESULTS AND DISCUSSION

Clinical findings

Lactococcosis outbreaks are related to high water temperatures (>21 °C) in rainbow trout farms. In Northern Portugal, outbreaks from trout farms in summer months of 2002 and 2003 were reported. Sick fish showed unilateral and bilateral exophthalmos and per ocular hemorrhage. In some cases, eye losses in moribund fish were observed. Internally, acidic fluid accumulation in the abdominal cavity and hemorrhage in the muscle of the affected fish were noted (Pereira et al. 2004). Savvidis et al. (2007) reported *L. garvieae* infections at high water temperatures between 2003 and 2006 in Greece. The authors informed exophthalmos and in some cases, losses of eyes or one eye in sick fish. At necropsy, hemorrhage in the abdominal cavity, pale liver and spleen were also observed in moribund fish (Savvidis et al. 2007). In the present study, behavioral findings such as lethargy, decreased feed intake, loss of appetite, swimming near the surface and mortality were observed in sick fish affected by lactococcosis in the summer months when the water temperature increased. There were darkening of the skin, especially on the head and dorsal parts of the body, including the fins (Figure 1), pallor of the gills, hemorrhage at the base of the pectoral, pelvic and anal fins, and lesions in the upper jaw. Unilateral or bilateral exophthalmos in the eyes of the affected fish (Figure 2), and unilateral exophthalmos, eye losses and hemorrhage in the orbit of one eye (Figure 3) in some fishes were observed. The liver was hemorrhagic, the spleen and kidney were dark, and the spleen was enlarged (splenomegaly) (Figure 4) in all fish affected by the disease. Also, acidic fluid accumulation in the abdominal cavity of the sampled fish, while the intestine was empty in terms of feed. The clinical findings observed in fish affected by lactococcosis in this study were similar to those reported by other investigators (Pereira et al. 2004; Kia and Mehrabi, 2013).

Fig. 1. Darkening of the skin including fins in fish affected by lactococcosis

Fig. 2. Bilateral exophthalmos in the eyes of the sick fish

Fig. 3. Exophthalmos in one eye, eye loss in the other eye with eye socket haemorrhagy

Fig. 4. Hemorrhagic liver, darkening of the spleen and kidney, enlargement of the spleen (splenomegaly) in the sick fish

Phenotypic characterization of the isolates

L. garvieae is a zoonotic fish pathogen. Various identification techniques such as phenotypic, molecular and proteomic techniques in vitro conditions are used to identify the bacterium *L. garvieae*. Although identification of fish isolates is not a problem, in some cases, the strains isolated from humans as *Enterococcus spp*. can be misidentified (Gibello et al. 2016).

In the present study, seventy five bacterial strains were isolated from moribund fish. The strains formed white and round colonies with a diameter of 0.5-1.0 mm at the end of 72 h of incubation at 25 ± 2 °C on BHIA (Figure 5). The all isolates were Gram-positive, non-motile, cytochrome oxidase and catalase negative and fermentative. Production of indole, VP and H2S production were negative in all strains. They were α (alpha) hemolysis on blood agar and showed amylase and gelatinase activities. The isolates were identified as *Lactococcus garvieae* by using the phenotypic characteristics which were listed in Table 3.

Fig. 5. White, small, round and convex colonies on BHIA after 72 h incubation at 25 ± 2°C

Two hundred eighty rainbow trout affecting hyper acute septicemia from trout farms which were located in Kokilne-Bayer Ahmad Province, in the southwest of Iran were sampled. *L. garvieae* isolates from 42 of 280 fish by using conventional biochemical tests were identified. It was reported that the strains had ovoid-coccal shape, non-motile, fermentative, Gram-positive, cytochrome oxidase, catalase and indole production negative, H2S and VP negative and grow in 0% NaCl and 6.5% NaCl and at 37 °C. The strains were α (alpha) hemolytic in blood agar. Gelatinase and amylase productions of the strains were negative (Kia and Mehrabi, 2013). In our study, seventy five strains isolated from moribund rainbow trout showed similar phenotypic characteristics reported by Kia and Mehrabi (2013).

Molecular characterization of L. garvieae isolates

Lactococcosis is one of the most important and common diseases of the cultured rainbow trout in many countries. Therefore, rapid identification of the disease agent, *L. garvieae* is important for the control of the disease. Zlotkin et al. (1998) developed the specific primer pair (PLG-1 and PLG-2) based on the region carrying the 16S rRNA gene. The PLG-1 and PLG-2 primer pair targeting the chromosomal DNA of *L. garvieae* has been used by researchers (Aoki et al.

2000; Sharifi Yazdi et al. 2010; Fukushima et al. 2010; Korun et al. 2018). Bacterial DNA samples which were replicated by applying PCR to 50 of 75 strains were run in the gel electrophoresis. After the run, 47 out of 50 strains were gave positive results in the gel and 1100 bp amplification was detected (Figure 6). The amplifications in 3 out of 50 strains were not observed. According to the PCR study results, 47 strains were defined as *L. garvieae*. The results of the PCR performed by applying PLG-1 and PLG-2 primers for *L. garvieae* were similar to the findings of other researchers (Sharifi Yazdi et al. 2010; Fukushima et al. 2017).

Table 3. Phenotypic characteristics of 75 strains isolated from sick fish in the present study

Characteristics	Present study	Vendrell et al. 2006
Cell morphology	Ovoid-cocci	Ovoid-cocci
Gram-staining	+	+
Motility	-	-
Hemolysis	α	α
Citrate utilization	-	-
Nitrate reduction	-	-
Methyl red (MR)	+	.
Voges Proskauer (VP)	+	.
Indole production	-	-
H2S production	-	-
Growth on:		
4 °C	+	+
20 °C	+	+
30 °C	+	.
Growth in:		
0% NaCl	+	.
2% NaCl	+	.
4% NaCl	+	.
6.5% NaCl	+	.
Amylase production	-	.
Gelatinase production	-	.
ONPG	+	.

+: positive reaction; -: negative reaction, F: Fermentative, ONPG: o-nitrophenyl-ß-D, .: not stated.

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In recent years, the discovery of genome sequences in some of *L. garvieae* strains has provided specific information on genes related to virulence. Comparative genome analysis of *L. garvieae* strains showed that discovery of the capsule gene sequence was not a single factor related to the pathogenicity of the bacterium, and the adhesion surface proteins (fbp: fibronectin binding protein), hemolysis (hylIII) and resistance to antibiotics (pva: penicillin V; folP: dihydropteroate synthase) and these factors may be important in pathogenicity in both animals and humans (Eraclio et al. 2018). In order to verify the accuracy of the PLG-1 and PLG-2 primers for the identification of *L. garvieae* isolates, a total of 25, 3 of which were negative and 22 were positive, were used with the SA1B10-1-F and SA1B10-1-R primers. Three strains gave positive results in the PCR using SA1B10-1-F and SA1B10-1-R primer pair, while the strains were negative in the PCR study using PLG-1 and PLG-2 primer pair (Figure 7).

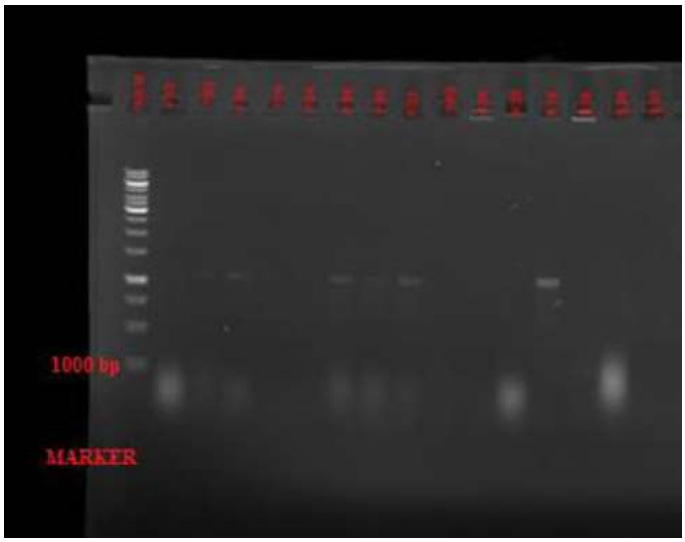

Fig. 6. PCR results of the bacterial strains isolated from moribund fish (Primers: PLG-1 and PLG-2)

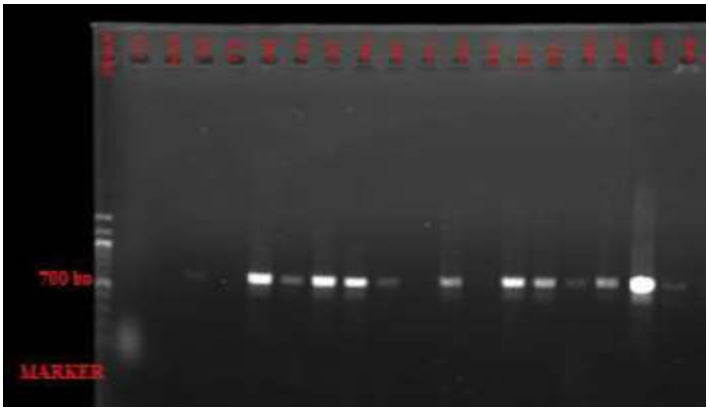

Fig. 7. PCR results of the bacterial strains isolated from moribund fish (Primers: SA1B10-1-F and SA1B10-1-R)

Antibiotic susceptibility test results of *L. garvieae* isolates

According to the antibiotic susceptibility test results, all 75 strains were found to be resistant to ampicillin and they were susceptible to bacitracin and tetracycline. Fifty five (73.33%) of the strains showed susceptibility to erythromycin. While 50 (66.66%) of the strains were susceptible to streptomycin, 25 (33.33%) of the strains were found to be resistant to kanamycin. Twenty strains (26.66%) were intermediate resistance to kanamycin. While 30 strains (40%) were resistant to flumequine and trimethoprim, 25 strains (33.33%) were intermediate resistance and 20 strains (26.66%) were susceptible to these antibiotics. Kav and Erganiş (2008) isolated and identified *L. garvieae* from sick fish in trout farms in Konya Province, Turkey. The authors reported that the strains were susceptible to ampicillin, chloramphenicol and erythromycin and they were resistant to bacitracin and sulphamethozaxole/trimethoprim. Sharifi Yazdi et al. (2010) informed that *L. garvieae* strains were susceptible to erythromycin, enrofloxacin and chloramphenicol according to the antibiotic test results. Kirkan et al. (2018) investigated lactococcosis in trout farms which were located in Aydın Province, Turkey and they informed that *L. garvieae* strains were resistant to ampicillin. In the present study, 75 *L. garvieae* strains were found to be resistant to ampicillin and susceptible to tetracycline and bacitracin according to the antibiotic susceptibility test results. Resistance and susceptibility of all strains to kanamycin, erythromycin, streptomycin, furazolidone, flumequine and trimethoprim varied depending on the strain.

CONCLUSION

For *L. garvieae* isolations, rainbow trout (O. mykiss) farms located in Kemer, Korkuteli and Manavgat, Antalya Province, Turkey were visited every month from June to September 2018. Because of lactococcosis is associated with high water temperatures, the disease was detected in moribund fish in farms where the water temperature was 15 °C and above. Seventy five strains isolated in the bacteriological studies showed homogeneity in terms of phenotypic characteristics. PCR was applied to 50 of 75 strains isolated from sick fish using PLG-1 and PLG-2 primer pair. While 47 of the strains gave positive results, amplification was not observed in 3 strains. When the PCR using the SA1B10-1-F and SA1B10-1-R primer pair was applied to a total of 25 strains, 3 of which were negative, 22 of which gave positive amplification, 25 strains gave positive results. When the antibiotic susceptibilities of the strains were evaluated, it was determined that the strains were resistant to ampicillin. They were susceptible to tetracycline and bacitracin and their resistance and susceptibility to other antibiotics varied depending on the strains.

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