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Research Article

Identification of *Lactococcus garvieae* by PCR from Rainbow Trout and Investigation of Susceptibility to Antibiotics

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ABSTRACT

Lactococcus garvieae is the causative agent of Lactococcosis in rainbow trout and it is introduced as a significant strain among Gram positive cocci. The scope of this study is detection of lactococcosis in rainbow trout by genotypic method and determination antibiotic susceptibility of Lactococcus strains. In this research, 100 rainbow trout were collected randomly from fisheries of Aydın province and its surroundings. The specimens were brought to Adnan Menderes University Faculty of Veterinary Medicine Department of Microbiology in cold chain. Samples taken from liver, spleen, kidney and heart were inoculated to Tryptic Soy agar and *L. garvieae* strains were identified by genotypic methods. Antibiotic susceptibility of *L. garvieae* strains were determined by disk diffusion method after PCR application. The antibiotics used in this research were Penicillin G, Florfenicol, Amoxycillin-Clavulanic acid, Ampicillin, Cefoperazone, Erythromycin, Methicillin, Gentamicin, Oxacillin and Cloxacillin. *L. garvieae* strains were detected susceptible to Amoxycillin-Clavulanic acid (90%), intermediate susceptible to Florfenicol (65%) and resistant to other remaining antibiotics in this study.

Key words: *Lactococcus garvieae*, Rainbow trout, Identification, Antibiogram, PCR

INTRODUCTION

Trout farming in Turkey started in the 1970's with various cultural systems and has showed significant improvements since then. Today, there are many businesses operating in aquaculture after the use of cage systems in cultivation in marine and inland waters were legalized and state subsidies became available. Throughout years, technological advancements of the cage systems have contributed to the industry growth (Emre *et al*., 2008).

Lactococcus garvieae is a Gram-positive pathogen causing meningoencephalitis and septicaemia in sea and inland water's fish especially in summer with the increase in water temperature, and therefore serious economic losses (Barnes *et al*., 2002; Vendrell *et al*., 2006).

In 1974, *L. garvieae* was isolated from Yellowtail fish (*Seriola quinqueradiat*) in Japan (Ksuda *et al*., 1991). After the outbreak of summer 1991, it was reported to cause high mortality in rainbow trout in Italy and Spain (Barnes *et al*., 2002). Same agent has been reported to cause outbreaks in mullet fish and freshwater shrimp in Taiwan (Chen *et al*., 2001-2002; Chang, 2002). This disease has been reported to occur in a Rainbow trout's hatcheries in the Aegean Region in 2001 for the first time (Diler *et al*., 2002) and it has been observed in Rainbow trout farms in many regions since 2008.

Antibiotics have been used for infections in fish due to *Streptococcus* genus for years. However, erratic and unconscious use of antibiotics in fish has increased the resistance to antibiotics (Robinson and Meyer, 1996; Katao, 1982). Antibiotics are effective in vitro conditions against *L. garvieae*, however existence of resistant strains and anorexia that occurs in fish impact negatively on antibiotics so those are ineffective in vivo conditions (Bercovier *et al*., 1997). Erythromycin, Amoxycillin, Oxytetracycline and Doxycycline are usually used to control of *L. garviae* outbreaks in Rainbow trouts (Munday *et al*., 1994). In studies which were made with *L. garvieae* strains from different geographical origins, strains were found to be resistant to Oxolinic acid and Sulfamethoxazole-trimethoprim and sensitive to Enrofloxacin and Nitrofurantoin. The antimicrobial susceptibility against Chloramphenicol, Oxytetracycline, Erythromycin and Ampicillin shows difference according to origin of the strains (Ravelo *et al*., 2001).

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L. garvieae strains are detected as susceptible to Erythromycin, Ofloxacin, Ampicillin and Chloramphenicol but resistant to Penicillin and Clindamycin on the outbreak in Turkey (Diler *et al*., 2002). In recent years, the increase in the number of leukocytes has been detected due to use of probiotics with the increasing phagocytic activity, thus prevents infections in Lactococcosis (Brunt and Austin, 2005).

The scope of this research is to determine *L. garvieae* infection in the culture rainbow trout by phenotypic and genotypic methods and determine antibiotic susceptibility of virulent *L. garvieae* strains.

MATERIALS AND METHODS

Sample collection

Of the 100 samples of Rainbow trouts (*Oncorhynchus mykiss* Walbaum, 1792) were collected from hatcheries in and around Aydin province managing culture fishing and brought to Adnan Menderes University Veterinary Faculty Department of Microbiology Routine Diagnosis Laboratory through cold chain. The experimental protocol was approved by the Animal Ethics Committee of Adnan Menderes University (2014/093).

Isolation of *Lactococcus sp.*

Fish samples were brought to the laboratory and opened technically. Bacterial culture was made by inoculating to Tryptic Soy agar (TSA) with 5% defibrinated sheep blood from liver, kidney, heart and spleen on aseptic conditions. Agar plates were incubated under aerobic conditions at 25°C for 72 hours (Mata *et al*., 2004). The colonies that made typically α-hemolysis were selected at the end of time and Gram staining was performed.

Oxidase and catalase tests were applied to colonies seen as Gram-positive cocci with Gram straining identification. Strains which were negative for oxidase and catalase reactions with *Lactococcus* sp. were considered suspicious and then passed to storage medium for identification and stored at -20°C for PCR tests.

Isolation of DNA

Bacterial colonies were collected from culture of samples obtained from viscera of 100 rainbow trout and passed to DNA extraction process for strains which are identified as to be *L. garvieae* (Mata *et al*., 2004). DNA isolations of strains have been done with genomic DNA extraction units (Fermentas®) suitable to procedure. Isolated DNAs has been protected with in cryo tubes in deep freeze at -20°C set.

Primers

Primers 16SrRNA used for fixing of *L. garvieae* by PCR method were designed in shape pLG-1 5'CATAACAATGAGAATCGC3' and pLG-2 5'GCACCCTCGCGGGTTG3 (Mata *et al*., 2004).

Positive control

Positive control DNAs, purified from standard strains of *L. garvieae* and used for polymerase chain reaction studies, was supplied from department stocks.

PCR

PCR method was performed in 50 µl reaction mixture containing DNA template (10 µl of DNA extracted from *L. garvieae* strains), 2mM $MgCl_2$, a1 µM concentration of pLG-1 and pLG-2 primers, a 0.25 Mm concentration of each deoxynucleoside trphosphate (Fermentas ®) and 1.5 U Taq DNA Polymerase (Fermentas®) along with its amplification buffer. The amplification was carried out in BIORAD thermal cycler with the following parameters: an initial denaturation step of 94°C 5 min; 30 cycles of a denaturation step of 92°C 1 min, annealing at 57°C 1 min and extension at 72°C 90 s; and final extension step of 72°C 7 min (Mata *et al*., 2004). A positive *L. garvieae* control and negative control (no template DNA) were included in each batch of PCRs. Then, PCR-generated products were detected by electrophoresis of 10 µl of each amplification mixture in 2% agarose gel including ethidium bromide (0.5 µg ml^{-1}).

Detection of the Amplification Product

The 10 µl amplified products were detected by staining with 0.5 µg/ml ethidium bromide after electrophoresis at 80 V for 40 min in 2% agarose gel. PCR products of 1150 base pairs were considered indicative for identification as *L. garvieae*.

Antimicrobial Susceptibility of *L. garvieae* strains

The antibiotic susceptibility tests for *L. garvieae* strains isolated from rainbow trout samples were carried out by disc diffusion as described by Kirby-Bauer Disc Diffusion method (CLSI, 2002-2003), using multidiscs (Oxoid) of Penicilin G (P-5U), Amoxycillin + Clavulanic acid (AMC-20 µg + 10 µg), Ampicillin (AMP-10 µg), Cefoperazone (CFP-30 µg), Erythromycine (E-15 µg), Gentamicin (CN-10 µg), Oxacillin (OX-5 µg) and Cloxacillin (OB-5 µg), Florfenicol (Mast Diagnostics) (FFC-30 µg). Each isolate 10^5 CFU/ml in 0.1 mL as determined by Kirby-Bauer Disc Diffusion method (CLSI, 2002-2003) was first poured on Mueller Hinton Agar. Then disks were placed on the Mueller-Hinton agar plates and then the plates were incubated at 37ºC for 24 h.

RESULTS

Isolation results

Under laboratory conditions, 34 *Lactococcus* sp. were isolated from 100 rainbow trout (*Oncorhynchus mykiss Walbaum*, 1792) samples. The cultural, biochemical, and physiological characteristics tests were used in the identification of *L. garvieae* strains (Holt *et al*., 1994; Koneman *et al*., 1997).

PCR results

Molecular confirmation of all (n=34) *L. garvieae* strains were determined by using *L. garvieae* species-specific primer sets targeting the 16S rRNA genes. The amplification of PCR product of the expected size (1150 bp) confirmed the entity of the isolated bacteria as *L. garvieae*. All isolates were confirmed as *L. garvieae*. The PCR assay had a detection limit level of 2×10^3 cells/g for *L. garvieae*. The *L. garvieae* PCR results were shown at Figure 1.

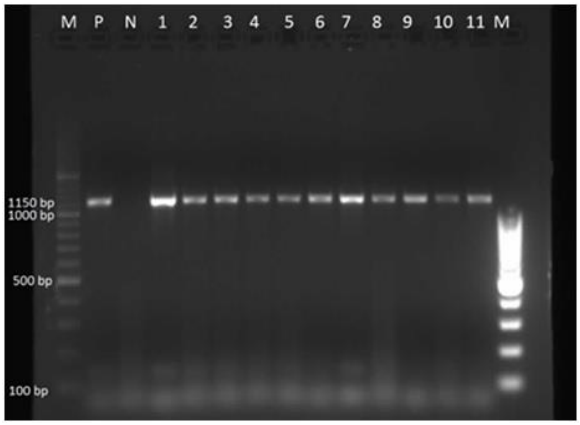

Fig. 1: *L. garvieae* PCR results, **M**:100 bp DNA ladder, **P**: *L. garvieae* positive control, **N**: Negative control, **1-11**: *L. garvieae* PCR positive samples

Table 1: Antimicrobial susceptibility results of *L. garvieae* strains (S: Susceptible, I: Intermediate susceptible, R: Resistant)

Antibiotics	Isolates of *L. garvieae* (n=34)		
	S	I	R
AMC	31	-	3
FFC	10	22	2
OX	-	2	32
CFP	4	9	21
AMP	8	-	26
P	4	-	30
CN	7	10	17
OB	7	12	15
E	-	7	27

Antimicrobial susceptibility results

L. garvieae strains were determined susceptible to Amoxicillin-Clavulanic acid in ratio of 90%, and Florfenicol in ratio of 65%. The strains were found resistant to Oxacillin in ratio of 94%, Cefoperazone in ratio of 62%, Ampicillin in ratio of 76%, Penicillin G in ratio of 88%, Gentamicin in ratio of 50%, Cloxacillin in ratio of 44%, Erythromycin in ratio of 80% and Methicillin in ratio of 88%. Antimicrobial susceptibility results of *L. garvieae* strains were given in Table 1.

DISCUSSION

Rainbow trout farm fishes, from 5g up to the 1 kg, were affected to the natural Lactococcosis infection (Diler *et al*., 2002; Pereira *et al*., 2004). A previous research performed in Italy reported that, *L. garvieae* were isolated in 7 samples of total 10 samples. In that research, phenotypical similarities were also determined between *L. lactis* and *L. garvieae* (Zlotkin *et al*., 1998). In another research conducted in Italy, 71 *L. garvieae* were isolated in epidemics more than 100 and occurred in different times (Eldar *et al*., 1999).

A total of 22 rainbow trout samples were examined with multiplex PCR previously at May 2005 and identified S. *iniae*, *S. parauberis*, *L. garvieae*. In this research, *L. garvieae* was identified 3 rainbow trout samples using multiplex PCR (Baeck *et al*., 2006).

L. garvieae were identified as a factor of Streptococcosis disease developing together pop eye syndrome; phenotypic characteristics of strains are homogenous; there are differences between phenotypical characteristics of field strains and reference strains around Konya province. *L. garvieae* was identified as to in ratios of 66.4% *L. lactis subsp. lactis*, 29.1% *E. feacalis*, 3.7% *E. fecium* and 0.9 % *E. durans* by API 20 strep. They isolated 32 strains of *L. garvieae* from 180 sick fish in a research conducted in and around Konya between 2002-2004. The researchers used to the *L. garvieae* species specific 16S rRNA pLG-1 and pLG-2 primers in the PCR (Kav and Erganis, 2007).

L. garvieae was identified from a trout farm in Marmara region using API 20 kit from 5 fishes weighted 180-200 gram taken (Timur *et al*., 2011).

It is known that *L. garvieae* isolated firstly in 2001 in Aegean region. Latest studies performed in Aegean region have shown that the incidence of *L. garvieae* increased to 52% (Gurpinar, 2013). But, in this research, isolation rate of *L. garvieae* was determined in the ratio of 34%. This situation may be a result of care and good maintenance conditions.

In northern Italy, a study of clinical isolates of *L. garvieae* strains reported that these strains were susceptible to Ampicillin, Amoxicillin, Oxytetracycline, Erythromycin and resistant to Oxolinic acid, Streptomycin, Trimethprim and Sulfadiazine (Mazzolini *et al*., 2003). In Turkey, *L. garvieae* strains were susceptible to Amoxicillin+Clavulanic acid, Enrofloxacin, Tetracycline, Doxycycline, Erythromycin and resistant to Kanamycin, Penicillin, Cefuroxime, Ciprofloxacine, Clindamycin and Trimethoprim+Sulfamethaxazole (Kubilay *et al*., 2005).

The strains were found susceptible to Enrofloxacin and Nitrofurantoins, resistant to Oxolinic acid and Trimethoprime-Sulfomethoxazole in previous studies performed with various geographical originated *L. garvieae* serotypes. However, the strains introduced variable susceptibly with regard to different originated serotypes (Ravelo *et al*., 2001).

The field strains of *L. garvieae* developed resistance against to Oxytetracycline, Erythromycin, Amoxicillin and Florfenicol (Altun *et al*., 2012). In another research, *L. garvieae* strains were resistant to Oxytetracycline but they are susceptible to Amoxicillin and they have intermediate susceptibility to Florfenicol and Erythromycin (Didinen *et al*., 2014).

L. garvieae is more susceptible to Ampicillin compared with Erytromycin, Oxytetracycline and Amoxicillin (Kum *et al*., 2004). The strains of *L. garvieae* were susceptible to Erytromycin, Chloramfenicol, Sulfadiazine, Clarithromycin and Enrofloxacin in Iran and some genes of isolated *L. garvieae* have developed resistance against to antibiotics like Oxytetracycline and this antibiotic is almost not effective because of the misuse. (Sharifiayazdi *et al*., 2010). Despite the high rate of susceptibility of Amoxicillin-Clavulanic acid, development of resistance against to other antibiotics was determined in this study.

Antibiotic susceptibility test were performed with 34 *L. garvieae* strains isolated in this research with Penicillin G, Florfenicol, Amoxycillin-Clavulanic acid, Ampicillin, Cefoperazone, Erythromycin, Methicillin, Gentamicin, Oxacillin and Cloxacillin. In conclusion, the strains were susceptible to Amoxicillin-Clavulanic acid in ratio of 90%, and Florfenicol in ratio of 65%. The strains were

resistant to Oxacillin in ratio of 94%, Cefoperazone in ratio of 62%, Ampicillin in ratio of 76%, Penicillin G in ratio of 88%, Gentamicin in ratio of 50%, Cloxacillin in ratio of 44%, Erythromycin in ratio of 80% and Methicillin in ratio of 88%.

Conclusions

L. garvieae, which has a significant role in lactococcosis disease seen in rainbow trout fisheries in our region, was detected by conventional and molecular methods in this study. Antibiotic susceptibilities were determined with various antibiotics that are commercially available, thus it was contributed to aquaculture and literature. Intensive aquaculture is increasing nowadays. Appropriate preventive medicine and therapy methods should be developed in this process. Determination of bacterial infection and the contribution to the right antibiotic is important.

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