

Detection of antibiotic resistance genes in some *Lactococcus garvieae* strains isolated from infected rainbow trout

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Abstract

The present study was done to evaluate the presence of antibiotic resistance genes in *Lactococcus garvieae* isolated from cultured rainbow trout, West Iran. The isolates were examined for antimicrobial resistance using disc diffusion method. Of the 24 strains tested, 21 were resistant to ampicillin (87.5%), 9 to erythromycin (37.5%) and 19 to tetracycline (79.1%). Fourteen strains were resistant to four antibiotics, 8 resistant to five antibiotics and 2 to six antibiotics. The strains were also characterized for their genotypic resistance profiles. The results revealed that all 24 isolates contained one to three of the antibiotic resistance genes. *StrA*, *tetS* and *ermB* genes coding for streptomycin, tetracycline and erythromycin resistance were found in 7, 10 and 9 isolates, respectively and sulfamethoxazole resistance gene, *sul2*, was not detected in the examined isolates. High levels of antibiotic resistance and detection of resistance genes in *L. garvieae* strains should be considered as a potential danger for trout culture as well as for public health.

Keywords: *Lactococcus garvieae*, Antimicrobial resistance genes, Rainbow trout.

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Introduction

Lactococcus garvieae is an emerging zoonotic pathogen which has been isolated from cattle (Collins *et al.*, 1989), from various species of fish (Elliott *et al.*, 1991; Carson *et al.*, 1993; Eldar *et al.*, 1996) and from human (Wang *et al.*, 2007). Lactococcosis has been defined as acute septicemia, causing economic losses in farmed fish. The mortality rate depends on physiological conditions of the host, environmental factors, water quality and temperature (Raissy and Ansari, 2011).

The clinical signs are similar to streptococcus which was described for the first time at the end of the 50s in Japan, where the first cases were diagnosed in rainbow trout (Elliott *et al.*, 1991). The disease has been reported in rainbow trout in several countries such as Australia, South Africa, Japan, Taiwan, England, Turkey, countries of the Mediterranean area and Iran (Ghittino and Prearo, 1992; Palacios *et al.*, 1993; Chen *et al.*, 2001; Chang *et al.*, 2002; Chen *et al.*, 2002; Diller *et al.*, 2002; Soltani *et al.*, 2008).

Antimicrobial resistance is an important public health problem that highly affects disease management and control (Raissy *et al.*, 2012). Resistance to common antibiotics such as tetracycline, doxycycline, erythromycin and streptomycin has been reported in *L. garvieae* in previous studies (Charpentier *et al.*, 1993; Raissy and Ansari, 2011). Soltani *et al.* (2008) reported the sensitivity of *L. garvieae* to

ampicillin and enrofloxacin. Sharifyazdi *et al.* (2010) found that the isolates were sensitive to erythromycin, enrofloxacin, chloramphenicol, clarithromycin and sulfadiazine. However, Raissy and Ansari (2011) reported the resistance of *L. garvieae* strains to erythromycin.

In this study, antibiotic resistance genes of *L. garvieae* isolated from cultured rainbow trout in Chaharmahalva Bakhtiari Province, Iran, are studied.

Materials and methods

Bacterial isolates

A total of 24 *L. garvieae* isolates were randomly taken from a collection of over 60 strains recovered from different outbreaks in cultured trout between October 2008 and November 2012. The strains were previously identified by standard morphological, physiological and biochemical tests (Chang *et al.*, 2002; Austin and Austin, 2007) and PCR as described by Zlotkin *et al.* (1998).

Antibiotic susceptibility test

Antibiotic susceptibility of the *L. garvieae* isolates was studied using the disc diffusion method on Mueller-Hinton agar (Oxoid) according to the instructions of Clinical Laboratory Standards Institute (CLSI). Discs (Oxoid) containing the following antibiotics were used: tetracycline (30µg), streptomycin (30µg), erythromycin (15µg), sulfamethoxazole (25µg), ampicillin (10µg), azitromycin (15µg), nalidixic acid (30µg), amikacin

(30 μ g), florfenicol (30 μ g), ciprofloxacin (5 μ g), enrofloxacin (5 μ g) clindamycin (15 μ g) and norfloxacin (10 μ g). The results were recorded as resistant or susceptible by measurement of the inhibition zone diameter according to the instruction of CLSI (2007).

Detection of resistance genes

The methods used to extract DNA have been described by Eldar *et al.* (1996). The bacteria were grown overnight at 30°C in Tryptic Soy Broth containing 1% sodium chloride. The bacteria (1.5 mL) was centrifuged for 10 min at 12000g, and the cell pellets were resuspended in 567 μ L of Tris-EDTA buffer (Merck, Germany) (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), followed by the addition of 30 μ L of 10% (w/v) sodium dodecyl sulfate (Merck, Germany) and 3 μ L of proteinase K (Cinnagen, Iran) (20 mg/mL) and incubation for 1h at 37°C. The samples were treated with 100 μ L of 5 M NaCl and 80 μ L of hexadecyltrimethyl ammonium bromide (CTAB)/NaCl (Sigma, Germany), and incubated at 65°C for 10 min. The obtained mixture was extracted with an equal volume of phenol-chloroform- isoamyl alcohol (25:24:1, v/v) and DNA was precipitated with 0.6 volume of cold isopropanol (Sigma, Germany) and washed with 1mL of 70% cold ethyl alcohol. The DNA pellet was dried at room temperature for 30min

and resuspended in TE (10 mM Tris-HCl, 100 mM EDTA, pH 7.8) buffer and stored at -20°C. The quantity of the extracted DNA was evaluated by measuring optical densities at wavelengths of 260 and 280 nm. The DNA concentration for PCR reaction was adjusted to 50 ng/ μ L.

The sequence of primers used for detection of resistance genes including *ermB*, *tetS*, *strA* and *sul2* are listed in Table 1. The PCR operation was performed with PTC-100 Eppendorf thermal cycler in a 50 μ L volume consisting of 2 μ L of extracted genomic DNA (50 ng/ μ L), 5 μ L of 10 \times PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 60 mM MgCl₂, 0.1% gelatin and 1% Triton X-100), 1 μ L of the primers (50 pmol/ μ L), 1 μ L each of the 10 mM dNTPs, 0.2 μ L units Taq DNA polymerase (5 units/ μ L) and 40 μ L of sterile distilled water. Cycling conditions were as follows; initial denaturation at 95°C for 5 min was followed by 30 cycles of 94°C for 1 min, 60°C for 40 seconds and 72°C for 40 seconds with a final extension at 72°C for 7 min and cooling to 4°C. Amplified PCR products were separated by electrophoresis in 1.5% agarose gels at 90 V for 50 min after staining with ethidium bromide. The product bands on gels were visualized and photographed with a UV transilluminator.

Table 1: Sequence of primers used for detection of antibiotics resistance genes.

Primer	Sequence (5'-3')	Target gene	Amplicon size	Reference
<i>ermB</i> -F	AGACACCTCGTCTAACCTTCGCTC	<i>ermB</i>	640	(Raissy <i>et al.</i> , 2012)
<i>ermB</i> -R	TCCATGTACTACCATGCCACAGG			
<i>tetS</i> -F	ATCAAGATATTAAGGAC	<i>tetS</i>	590	(Kim <i>et al.</i> , 2004)
<i>tetS</i> -R	TTCTCTATGTGGTAATC			
<i>Sul2</i> -F	AGGGGGCAGATGTGATCGAC	<i>Sul2</i>	271	(Raissy <i>et al.</i> , 2012)
<i>Sul2</i> -R	TGTGCGGATGAAGTCAGCTCC			
<i>strA</i> -F	TTGATGTGGTGTCCCGCAATGC	<i>strA</i>	267	(Raissy <i>et al.</i> , 2012)
<i>strA</i> -R	CCAATCGCAGATAGAAGGCAA			

Results

The antimicrobial resistance test showed multidrug resistance in the examined isolates as the resistance to tetracycline was found in 19 isolates (79.1%), ampicillin (21, 87.5%), streptomycin (7, 29.1%), erythromycin (9, 37.5%), norfloxacin (15, 62.5%), nalidixic acid (9, 37.5%), azitromycin (8, 33.3%), enrofloxacin (8, 33.3%), ciprofloxacin (3, 12.5%), amikacin (2, 8.3%), clindamycin (4, 16.6%), sulfamethoxazole and florfenicol (0). According to the results, 14 strains were resistant to four antibiotics, 8 resistant to five antibiotics and 2 to six antibiotics.

The presence of antimicrobial resistance genes was also studied in 24 *L. garvieae* isolates collected from diseased fish. The obtained results

showed that 16 isolates contained at least one of the antibiotic resistance genes (Table 2). *StrA*, *tetS* and *ermB* genes coding for streptomycin, tetracycline and erythromycin resistance were found in 7, 10 and 9 isolates, respectively. Sulfamethoxazole resistance gene, *sul2*, was not detected in the examined isolates.

Discussion

Many fish species except common carp are susceptible to *L. garvieae*. Rainbow trout is the most sensitive species compared to other fish species (Vendrell *et al.*, 2006). *L. garvieae* have also been isolated from human in several cases, suggesting that it could be cataloged as a potential zoonotic agent (Carson *et al.*, 1993).

Table 2: Antibiotic resistance genes in *Lactococcus garvieae* isolates.

Isolates	Antibiotic resistance pattern	Antibiotic resistance genes detected
<i>L. garvieae</i> 1	2	<i>tetS</i>
<i>L. garvieae</i> 2	2	-
<i>L. garvieae</i> 3	6	<i>strA</i>
<i>L. garvieae</i> 4	2	-
<i>L. garvieae</i> 5	4	<i>ermB</i>
<i>L. garvieae</i> 6	2	-
<i>L. garvieae</i> 7	4	<i>tetS,ermB</i>
<i>L. garvieae</i> 8	2	-

Table 2 continued:

<i>L. garvieae</i> 9	6	<i>strA</i>
<i>L. garvieae</i> 10	2	<i>tetS</i>
<i>L. garvieae</i> 11	5	-
<i>L. garvieae</i> 12	5	-
<i>L. garvieae</i> 13	4	<i>ermB</i>
<i>L. garvieae</i> 14	3	<i>tetS,ermB,strA</i>
<i>L. garvieae</i> 15	4	<i>tetS, ermB</i>
<i>L. garvieae</i> 16	5	-
<i>L. garvieae</i> 17	4	<i>tetS,ermB</i>
<i>L. garvieae</i> 18	2	<i>tetS</i>
<i>L. garvieae</i> 19	1	<i>ermB,strA</i>
<i>L. garvieae</i> 20	7	<i>strA</i>
<i>L. garvieae</i> 21	2	<i>tetS</i>
<i>L. garvieae</i> 22	7	-
<i>L. garvieae</i> 23	1	<i>tetS,ermB,strA</i>
<i>L. garvieae</i> 24	4	<i>ermB</i>

Legend: 1- AMP, STR, TET, ERY, NOR.

2- AMP, TET, NOR, NAL.

3- NAL, STR, TET, ERY.

4- TET, ENRO, AZT, AMP, ERY.

5- NOR, AMP, AK, CIP.

6- STR, AZT, NOR, ENRO, AK, CLI.

7- AMP, TET, STR, CLI.

AMP, ampicillin; SUL, sulfamethoxazole; AZT, azitromycin; NAL, nalidixic acid; NOR, norfloxacin; STR, streptomycin; TET, tetracycline; ERY, erythromycin; CIP, ciprofloxacin; AK, amikacin; CLI, clindamycin; ENRO, enrofloxacin.

In recent years, trout culture industry has been widely developed in Iran especially in Chaharmahalva Bakhtiari Province as the production rate reached 16200 and 15000 tons in 2011 and 2012, respectively. Along with the development of rainbow trout culture in recent years, epizootic outbreak of some infectious diseases such as streptococcosis and lactococcosis have resulted in economic losses in aquaculture in this area.

The spread of streptococcosis and lactococcosis between fish farms resulted in the uncontrolled use of antibiotics by fish farmers. The overuse of antibiotics in recent years has increased antimicrobial resistance to

common antibiotics (Raissy and Ansari, 2011).

The results of this study showed that all examined isolates had one or more resistance genes. *StrA*, *tetS* and *ermB* genes were found in 7, 10 and 9 isolates, respectively coding for streptomycin, tetracycline and erythromycin resistance.

Resistance to tetracycline has been previously reported by Walther *et al.* (2008). They reported tetracycline-resistant *L. garvieae* which harbored *tetM* and *tetS*. A total of 31 *L. garvieae* isolated from bovine milk were tested in their study for susceptibility to 17 antibiotics and screened for the presence of antibiotic resistance genes

using a microarray. Resistance to tetracycline, erythromycin, streptomycin, clindamycin and nitrofurantoin were found. The results showed the presence of antibiotic resistance genes in *L. garvieae* which are in good agreement with the results of the current study.

Tetracycline resistance gene, *tetS*, was also detected in *L. garvieae* from cultured yellowtail in Japan and in *Vibrio* sp. from seawater in Korea (Kim *et al.*, 2004).

In this study 18 of 24 examined isolates were resistant to tetracycline (75%). This antibiotic was widely used against fish disease by fish farmers in recent years. This may explain the higher prevalence of *tetS* gene in the isolates.

According to the results, some of the studied strains did not contain *tetS* gene, although they were resistant to tetracycline which may be due to the presence of other genes encoding resistance to tetracycline such as *tetA*, *tetB*, *tetM* and *tetK*. This finding is similar to the results of Dang *et al.* (2006).

The results of this study also revealed that 10 of 24 examined isolates were resistant to erythromycin (41.6%) which is a recommended antibiotic against streptococcosis and lactococcosis in fish. The erythromycin resistance gene (*ermB*) has already been reported in lactococci from poultry, pork meat and bovine milk (Dang *et al.*, 2006; Vendrell *et al.*, 2006). It has also been reported from different *Vibrio* species (Raissy *et al.*, 2012).

Raissy and Ansari (2011) reported that more than 46% of the 52 *L. garvieae* strains were resistant to erythromycin. Resistance to this antibiotic should be seriously considered as this is the drug of choice for the bacteria. Alves D'azevedo *et al.* (2000) unlike Diler *et al.* (2002) found that *L. garvieae* was resistant to erythromycin, although Kav and Eganis (2008) reported that all examined isolates were sensitive to erythromycin. *L. garvieae* isolates in the study of Sharifyazdi *et al.* (2010) were found to be sensitive to erythromycin, enrofloxacin, chloramphenicol, clarithromycin and sulfadiazine. However, Soltani *et al.* (2008) reported that their isolates were only sensitive to ampicillin and enrofloxacin.

These different results may be due to the differences of *L. garvieae* isolates and antibiotics usage in different areas. Although many isolates were resistant to erythromycin, they remained susceptible to florfenicol which is now the most significant choice for antimicrobial treatment of lactococcosis. This drug is now one of the rarest useful antibiotics against lactococcosis and streptococcosis.

In this study, a relatively high prevalence of multi-drug resistant *L. garvieae* was found which may be due to excessive use of antibiotics by fish farmers. It may be suggested from the results that the application of antibiotics should be strictly controlled to prevent the dissemination of resistant bacteria which may transfer antibiotic resistance to other bacterial species. To our

knowledge, this is the first report available on the chromosomal antibacterial resistance in *L. garvieae* from Iran. Considering the possibility of transmission of the resistance genes to other bacteria, frequent assessment of antibacterial resistance profile either chromosomal or plasmid mediated will lead to better knowledge.

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