International Journal of Biological, Life and Agricultural Sciences
ISSN: 2415-6612
Vol:9, No:2, 2015

Detection of Tetracycline Resistance Genes in *Lactococcus garvieae* Strains Isolated from Rainbow Trout

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***Abstract*—**The present study was done to evaluate the presence of tetracycline 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 49 strains tested, 19 were resistant to tetracycline (38.7%), 32 to enrofloxacin (65.3%), 21 to erythromycin (42.8%), 20 to chloramphenicol and trimetoprim-sulfamethoxazole (40.8%). The strains were then characterized for their genotypic resistance profiles. The results revealed that all 49 isolates contained at least one of the tetracycline resistance genes. *Tet* (A) was found in 89.4% of tetracycline resistant isolates and the frequency of other gene were as follows: *tet* (E) 42.1%, *tet* (B) 47.3%, *tet* (D) 15.7%, *tet* (L) 26.3%, *tet* (K) 52.6%, *tet* (G) 36.8%, *tet* (34) 21%, *tet* (S) 63.1%, *tet* (C) 57.8%, *tet* (M) 73.6%, *tet* (O) 42.1%. The results revealed high levels of antibiotic resistance in *L. garvieae* strains which is a potential danger for trout culture as well as for public health.

Keywords*—Lactococcus garvieae,* rainbow trout, tetracycline resistance genes.

I. Introduction

LACTOCOCCOSIS caused by *Lactococcus garvieae* 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 [1]. *L. garvieae* is an emerging zoonotic pathogen which has been reported from cattle [2], from various species of fish [3]-[5] and from human [6].

The clinical signs are similar to streptococcosis which was described for the first time at the end of the 50 s in Japan, where the first cases were diagnosed in rainbow trout [5]. 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 [7]-[12]. Lactococosis leads to severe economic losses because of high mortality rates and reducing growing rate. In addition, infected fish will be unmarketable because of their appearance. The mortality rate depends on environmental conditions, water quality and fish density [7]. This diseases is endemic in rainbow trout fish farms in Iran and has been frequently reported from different areas of Iran.

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Antimicrobial resistance is an important public health problem that highly affects disease management and control [1]. Resistance to common antibiotics such as tetracycline, doxycycline, erythromycin and streptomycin has been reported in *L. garvieae* in previous studies [1], [13] although the respective genes are now studied. Soltani et al. [12] reported the sensitivity of *L. garvieae* to ampicillin and enrofloxacin. Sharifyazdi et al. [14] found that the isolates were sensitive to erythromycin, enrofloxacin, chloramphenicol, clarithromycin and sulfadiazine. However, Raissy and Ansari [1] reported the resistance of *L. garvieae* strains to erythromycin.

In this study, antibiotic resistance and tetracycline resistance genes of *L. garvieae* isolated from cultured rainbow trout in Chaharmahal va Bakhtiari Province, Iran is studied.

II. Materials and Method

A. Bacterial Isolates

A total of 300 fish were obtained from 30 rainbow trout farms between Sep 2012 and Feb 2014. The samples of the liver, kidneys and heart were placed on a 5% sheep blood agar (Himedia, India) with 1% yeast extract agar (Merck, Germany) plates and then incubated at 24°C and 37°C for 2-3 days under aerobic conditions. Standard physiological and biochemical tests recommended by [15] and [7] performed at 25°C. Identification of the *L. garvieae* isolates was confirmed by PCR assay as described by [16] with the primer sequence mentioned in Table I.

B. Antibiotic Susceptibility Test

Antibiotic susceptibility of the isolates was studied using the disc diffusion method on Mueller-Hinton agar (Oxoid) according to the instruction of Clinical Laboratory Standards Institute [10]. Discs (Oxoid) containing the following antibiotics were used: tetracycline (30 µg), streptomycin (30 µg), kanamycin (30 µg), erythromycin (15 µg), gentamicin (10 µg), penicillin (10 µg), trimethoprim-sulfamethoxazole (1.25, 23.75 µg), ampicillin (10 µg), amoxicillin (25 µg), enrofloxacin (5 µg), florfenicol (30 µg) and chloramphenicol (30 µg). The results were recorded as resistant or susceptible by measurement of the inhibition zone diameter according to the instruction of CLSI [17].

C. Detection of Resistance Genes

The methods used to extract DNA have been described by [4]. The bacteria were grown overnight at 30°C in Trypic 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 µl of Tris-EDTA buffer (Merck, Germany) (10 mMTris–HCl, 1 mM EDTA, pH 8.0), followed by addition of 30 µl of 10% (w/v) sodium dodecyl sulfate (Merck, Germany) and 3 µl of proteinase K (Cinnagen, Iran) (20 mg/ml) and incubation for 1 h at 37 °C. The samples were treated with 100 µl of 5 M NaCl and 80 µl 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 1 ml of 70% cold ethyl alcohol. The DNA pellet was dried at room temperature for 30 min and resuspended in TE (10 mMTris-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 260 and 280 nm wavelengths. The DNA concentration for PCR reaction was adjusted to 50 ng/µl.

Sequence of primers used for detection of resistance genes and cycling conditions are listed in Table II. The PCR operation was performed with PTC-100 Eppendorf thermal cycler in a 50 µl volume consisting of 2 µl of extracted genomic DNA (50 ng/µl), 5 µl of 10× PCR buffer (100 mMTris–HCl, pH 8.3, 500 mMKCl, 60 mM MgCl2, 0.1% gelatin and 1% Triton X-100), 1 µl of the primers (50 pmol/µl), 1 µl each of the 10 mMdNTPs, 0.2 µl units Taq DNA polymerase (5 units/µl) and 40 µl of sterile distilled water. 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.

III. Results

The antimicrobial resistance test showed multidrug resistance in the examined isolates as the resistance to tetracycline was found in 19 isolates (38.7%), enrofloxacin (32, 65.3%), erythromycin and streptomycin (21, 42.8%), chloramphenicol and trimetoprim-sulfamethoxazole (20, 40.8%), kanamycin (24, 48.9%), gentamicin (34, 69.3%), penicillin (26, 53.8%), ampicillin (10 µg), amoxicillin (25 µg), florfenicol (27, 55.1%).

The presence of antimicrobial resistance genes was also studied in 49 *L. garvieae* isolates collected from diseased fish. The obtained results showed that 19 isolates contained at least one of the 12 tetracycline resistance genes. Number of genes in the examined isolates varied between 3 and 9. According to the results, 89.4% of tetracycline resistant isolates contained *Tet* (A). Other gene were found in the isolates as follow: *tet* (E) 42.1%, *tet* (B) 47.3%, *tet* (D) 15.7%, *tet* (L) 26.3%, *tet* (K) 52.6%, *tet* (G) 36.8%, *tet* (34) 21%, *tet* (S) 63.1%, *tet* (C) 57.8%, *tet* (M) 73.6%, *tet* (O) 42.1%.

TABLE I
SEQUENCE OF PRIMERS USED FOR DETECTION OF *LACTOCOCCUS GARVIEAE*

Targeting gene	Primer sequence	PCR Amplicon	References
16S rRNA	CATAACAATGAGAATCGC GCACCCTCGCGGGTTG	1100 bp	[16]

TABLE II
SEQUENCE OF PRIMERS USED FOR DETECTION OF ANTIBIOTICS RESISTANCE GENES [21]

Primer	Nucleotide sequence (5′–3′)	Cycling conditions
*tet*A (F)	GTA ATT CTG AGC ACT GTC GC	95°C, 30 s, 62°C, 30 s, 72°C, 45 s (23 cycles)
*tet*A (R)	CTG CCT GGA CAA CAT TGC TT	
*tet*B (F)	CTC AGT ATT CCA AGC CTT TG	95°C, 30 s, 59°C, 30 s, 72°C, 20 s (25 cycles)
*tet*B (R)	CTA AGC ACT TGT CTC CTG TT	
*tet*C (F)	TCT AAC AAT GCG CTC ATC GT	95°C, 30 s, 62°C, 30 s, 72°C, 30 s (30 cycles);
*tet*C (R)	GGT TGA AGG CTC TCA AGG GC	
*tet*D (F)	ATT ACA CTG CTG GAC GCG AT	95°C, 30 s, 59°C, 30 s, 72°C, 20 s (25 cycles)
*tet*D (R)	CTG ATC AGC AGA CAG ATT GC	
*tet*E (F)	GTG ATG ATG GCA CTG GTC AT	95°C, 30 s, 62°C, 30 s, 72°C, 45 s (23 cycles)
*tet*E (R)	CTC TGC TGT ACA TCG CTC TT	
*tet*G (F)	TTC AAG CCG GCT TGG AGA G	95°C, 1 m, 56°C, 1 m, 72°C, 2 m (30 cycles)
*tet*G (R)	TTG TTT GAG AGC ATT GCC TGC	
*tet*K (F)	TTA GGT GAA GGG TTA GGT CC	95°C, 1 m, 55°C, 1 m, 72°C, 1·30 m (25 cycles)
*tet*K (R)	GCA AAC TCA TTC CAG AAG CA	
*tet*L (F)	CAT TTG GTC TTA TTG GAT CG	95°C, 1 m, 55°C, 1 m, 72°C, 1·30 m (25 cycles)
*tet*L (R)	ATT ACA CTT CCG ATT TCG G	
*tet*M (F)	GTT AAA TAG TGT TCT TGG AG	94°C, 30 s, 55°C, 30 s, 2°C, 1·30 m (30 cycles)
*tet*M (R)	CTA AGA TAT GGC TCT AAC AA	
*tet*O (F)	GAT GGC ATA CAG GCA CAG AC	95°C, 1 m, 60°C, 1 m, 72°C, 1·30 m (25 cycles)
*tet*O (R)	CAA TAT CAC CAG AGC AGG CT	
*tet*S (F)	TGG AAC GCC AGA GAG GTA TT	95°C, 1 m, 60°C, 1 m, 72°C, 1·30 m (25 cycles)
*tet*S (R)	ACA TAG ACA AGC CGT TGA CC	
*tet*34 (F)	ATG AAA ACG AAC GCT AAT TAA CCA	95°C, 1 m, 56°C, 1 m, 72°C, 2 m (30 cycles)
*tet*34(R)	ACA TAG AGA TCG ATG CTA GTA CTA	

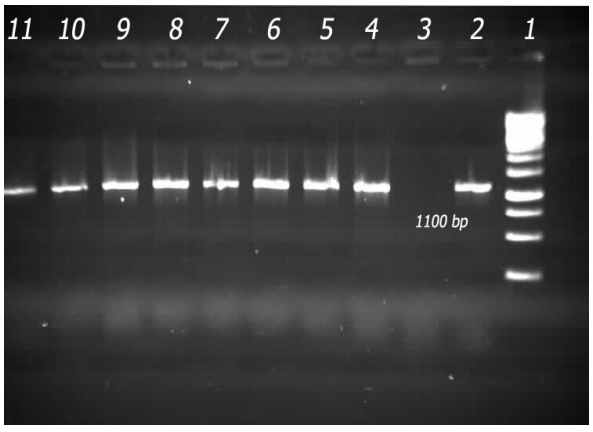

Fig. 1 Electrophoresis of *L. garvieae*. 1: ladder. 2: positive control. 3: negative control. 4-11: positive samples

IV. Discussion

Many fish species except common carp are susceptible to *L. garvieae*. Rainbow trout is the most sensitive species compared to other fish species [1]. *L. garvieae* have also been isolated from human in several cases, suggesting that it could be cataloged as a potential zoonotic agent [3].

In recent years, trout culture industry has been widely developed in Iran especially in Chaharmahal va Bakhtiari Province as the production rate reached to 16200 and 18000 tons/yr in 2012 and 2013, respectively. Along with development of rainbow trout culture in recent years, epizootic outbreak of some infectious diseases such as streptococcosis and lactococcosis resulted in many economic losses in aquaculture trade in this area.

Spreading streptococcosis and lactococcosis between fish farms resulted in uncontrolled using of antibiotics by fish farmers. Overusing antibiotics in recent years has increased antimicrobial resistance to common antibiotics [1]. Resistance of *L. garvieae* to common antibiotics including tetracycline, erythromycin, enrofloxacin, amoxicillin and florfenicol has been reported in many countries [1], [12], [18].

Alves D'azevedo et al. [20] unlike Diler et al. [10] found that *L. garvieae* was resistant to erythromycin, although Kav and Eganis [18] reported that all examined isolates were sensitive to erythromycin. *L. garvieae* isolates in the study of [14] were found to be sensitive to erythromycin, enrofloxacin, chloramphenicol, clarithromycin and sulfadiazine. However, [12] reported that their isolates were only sensitive to ampicillin and enrofloxacin. These differences in antibiotic resistance pattern of bacteria may be due to the differences of *L. garvieae* isolate and antibiotics usage in different areas.

The results of this study showed that 19 of 49 examined isolates had one or more tetracycline resistance genes. Resistance to tetracycline has been previously reported by [19]. 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. The isolates were screened for the presence of antibiotic resistance genes including tetracycline, erythromycin, streptomycin, clindamycin and nitrofurantoin. The results showed the presence of antibiotic resistance genes in *L. garvieae* which is 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 [21]. In another study 30 strains out of 35 tetracycline resistant isolates were found to be positive for *tet* (M) [22]. In the current study 19 of 49 examined isolates were found to be resistant to tetracycline (38.7%). This antibiotic is widely used against fish disease by fish farmers explaining the high prevalence of tetracycline resistant bacteria.

V. Conclusion

A relatively high prevalence of multi-drug resistant *L. garvieae* was found in this study 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 a better knowledge.

References

[1] M. Raissy, and M. Ansari, "Antibiotic susceptibility of *Lactococcus garvieae* isolated from rainbow trout (*Oncorhynchus mykiss*)". *Afr. J. Biotechnol*, vol. 8, 2011, pp. 1473-1476.

[2] M.D. Collins, C. Ash, J.A.E. Farrow, S. Wallbanks, and A.M. Williams,"16S ribosomal ribonucleic acid sequence analyses of lactococci and related taxa. Description of *Vagococcusfluvialis* gen. nov.sp. nov". *J. Appl. Bacteriol*, vol. 67, 1989, pp. 453-460J.

[3] N. Carson, B.Gudkovs, and B. Austin, "Characteristics of an *Enterococcus*-like bacterium from Australia and South Africa, pathogenic for rainbow trout (*Oncorhynchus mykiss* Walbaum)". *J. Fish. Dis*. vol. 6, 1993: 381-8.

[4] A. Eldar, C. Ghittino, L. Asanta, E. Bozzetta, M. Goria, M. Prearo, and H. Bercovier, "*Enterococcus seriolicida* is a junior synonym of *Lactococcus garvieae*, a causative agent of septicemia and meningoencephalitis in fish. *Current*". *Microbiol*, vol. 32, 1996, pp. 85-88.

[5] J.A. Elliott, M.D. Collins, N.E. Pigott, and R.R. Facklam, "Differentiation of *Lactococcus lactis* and *Lactococcus garvieae* from humans by comparison of whole-cell protein patterns". *J. Clin. Microbiol*, vol. 29, 1991, pp. 2731-2734.

[6] C.Y. Wang, H.S. Shie, S.C. Chen, J.P. Huang, I.C. Hsieh, M.S. Wen, F.C. Lin, D. Wu,"*Lactococcus garvieae* infections in humans: possible association with aquaculture outbreaks". *Int. J. Clin. Prac*, vol. 61, 2007, pp. 68-73.

[7] P.H. Chang, C.W. Lin, and Y.C. Lee, "*Lactococcus garvieae* infection of cultured rainbow trout, *Oncorhynchus mykiss*, in Taiwan and associated biophysical characteristics and histopathology". *Bull. Euro. Assoc. Fish Pathol*. Vol. 22, 2002, pp. 319-27.

[8] S.C. Chen, Y.D. Lin, L.L. Liaw, and P.C. Wang,"*Lactococcus garvieae* infection in the giant freshwater prawn *Macrobranchium rosenbergii* confirmed by polymerase chain reaction and 16S rDNA sequencing". *Dis. Aqua. Organs*, vol. 45, 2001, pp. 45-52.

[9] S.C. Chen, L.L. Liaw, H.Y. Su, S.C. Ko, C.Y. Wu, H.C. Chaung, Y.H. Tsai, K.L. Yang, Y.C. Chen, T.H. Chen, G.R. Lin, S.Y. Cheng, Y.D. Lin, J.L. Lee, C.C. Lai, Y.J. Weng, and S.Y. Chu,"*Lactococcus garvieae*, a cause of disease in grey mullet, *Mugilcephalus* L. in Taiwan". *J. Fish Dis*, vol. 25, 2002, pp. 727-232.

[10] O. Diler, S. Altun, A.K. Adiloglu, A. Kubilay, and B. Isikli, "First occurrence of Streptococcosis affecting farmed rainbow trout in Turkey". *Bull. Europ. Assoc. Fish Pathol*, vol. 22, 2002, pp. 21-26.

[11] M.A. Palacios, M.J. Zamora, J. VaSquez Zamora, and A. Duran, "Streptococcosis in rainbow trout (*Oncorhynchus mykiss*) in Spain". *Boll. Soc. Ital. Patalog. Itt*, vol. 13, 1993, pp.11-6.

[12] M. Soltani, G.H. Nikbatht, H. Mousavi, and N. Ahmadzadeh, "Epizootic outbreak of *lactococcosis* caused by *Lactococcus garvieae* in farmed rainbow trout (*Oncorhynchus mykiss*) in Iran". *Bull. Europ. Assoc. Fish Pathol*, vol. 28, 2008, pp. 207-212.

[13] E. Charpentier, G. Gerbaud, and P. Courvalin, "Characterization of a new class of tetracycline-resistance gene *tet(S)*in *Listeria monocytogenes*BM4210". *Gene*, vol. 131, 1993, pp. 27-34.

[14] H. Sharifiyazdi, M. Akhlaghi, M. Tabatabaei, and S.M. MostafaviZadeh, "Isolation and characterization of *Lactococcus garvieae* from diseased rainbow trout (*Oncorhynchus mykiss*, Walbaum) cultured in Iran". *Iran. J. Vet. Res*, vol. 11, 2010, pp. 342-350.

[15] B. Austin, D.A. Austin, "Bacterial Fish Pathogens: Disease of Farmed and Wild Fish", 4th ed. Bristol: Springer, 2007.

[16] A. Zlotkin, A. Eldar, C. Ghittino, H. Bercovier, "Identification of *Lactococcus garvieae* by PCR". *J. Clin. Microbiol*, vol. 36, 1998, pp. 983-985.
[17] CLSI, Clinical and Laboratory Standards Institute. 2007: "Performance Standards for Antimicrobial Susceptibility Testing; Fifteenth Informational Supplement". CLSI document M100-S15. Clinical and Laboratory Standards Institute. Wayne, Pennsylvania.
[18] K. Kav, and O. Erganis. "Antibiotic Susceptibility of *Lactococcus garvieae* in rainbow trout (*Oncorhyncus mykiss*) Farms". *Bull. Vet. InsPul*, vol. 52, 2008, pp. 223-226.
[19] C. Walther, A. Rossano, A. Thomann, and V. Perreten, "Antibiotic resistance in *Lactococcus* species from bovine milk: Presence of a mutated multidrug transporter *mdt(A)* gene in susceptible *Lactococcus garvieae* strains". *Vet. Microbiol*, vol. 131, 2008, pp. 348-357.
[20] P. Alves D'azevedo, C. Perin, F.L. Becker, and G.Z Ramos, "Isolation and characterization of Lactococcus garvieae". Rev. Amr. Porto. Alegra. vol 44, 2000, pp. 81-84.
[21] S.R. Kim, L. Lisa, and S. Suzuki, "Occurrence of tetracycline resistance genes *tet(M)* and *tet(S)*in bacteria from marine aquaculture sites". *FEMS. Microbiol. Lett*, vol 237, 2004, pp. 147-156.
[22] O.G. Kim, O.H. Mitsuko, and S. Suzuk, "Occurrence of Tetracycline Resistant Bacteria and tet(M) Gene in Seawater from Korean Coast". *Environ. Poll. Ecotoxicol*, vol. 6, 2012, pp. 367-375.