Isolation and characterization of *Lactococcus garvieae* from diseased rainbow trout (*Oncorhynchus mykiss*, Walbaum) cultured in Iran

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Summary

A total of 200 moribund rainbow trout with clinical signs of a hyperacute haemorrhagic septicemia were collected from rainbow trout farms in Fars, Kohkiloyeh-Boyer Ahmad and Charmohal-Bakhtiari provinces in the south and southwest of Iran during summer 2002 to winter 2008 for detection of *Lactococcus garvieae*, the causative agent of lactococcosis. Fish kidney samples were cultured aseptically onto brain heart infusion agar plates and incubated at 25°C for 48 h. Using conventional biochemical tests, *L. garvieae* was detected from 32 fish (16% of total fish samples). Additionally, isolates were confirmed as *L. garvieae* using a specific PCR assay based on 16S rDNA gene by producing a single band of 1107 bp. Partial analysis of 16S rDNA revealed 100% sequence similarity for all Iranian isolates and there was a close genetic relationship among these isolates and those previously reported from mullet in Taiwan (AF352166) and yellowtail in Japan (AB267897) based on GenBank data. Results of antibiogram tests on *L. garvieae* isolates showed a high susceptibility to erythromycin, enrofloxacin, chloramphenicol and clarithromycin. In pathogenicity tests, immersion of fish in a bacterial suspension of 6×10^5 colony forming unit/ml of *L. garvieae* in challenge experiments showed 60% mortality during 14 days post-infection. Experimentally, infected fish showed typical blackening of skin haemorrhages, exophthalmia and wide haemorrhages on viscera. The present study provides useful molecular and biochemical information for *L. garvieae* isolates in Iran compared with those from different hosts and geographic locations.

Key words: *Lactococcus garvieae*, Rainbow trout, PCR, 16S rDNA, Iran

Introduction

Lactococcosis is an emerging disease which has been particularly devastating in the freshwater culture of salmonid fish and marine culture species and causes important economic losses both in marine and freshwater aquaculture all over the world, especially when water temperature increases over 15°C (Austin and Austin, 2007). Lactococcosis, in fact, is a kind of streptococcosis caused by *Lactococcus garvieae*. The bacterium previously described as *Streptococcus garvieae* was proposed as a new species, *Enterococcus seriolicida*, in order to bring together 12 isolates recovered from eels and streptococcosis outbreaks in Japanese yellowtail (*Seriola quinqueradiata*) in Japan (Kusuda *et al*., 1991). Lactococcosis has spread to many countries and aquatic organisms causing significant economic losses to the rainbow trout industry such as Italy (Ghittino and Prearo, 1992), Australia and South Africa (Carson *et al*., 1993), Turkey (Diler *et al*., 2002), England (Bark and McGregor, 2001), Portugal (Pereira *et al*., 2004), France and the Balkans (Eyngor *et al*., 2004).

Farming rainbow trout fish has been developed during the last decades in different suitable areas with numerous

springs that have allowed the aquaculture industry to expand quickly in Iran. Various infectious diseases have emerged due to imposed stress factors during fish production. The occurrence of streptococcosis in the cultured rainbow trout of Fars province, Iran was reported by Akhlaghi and Keshavarzi (2002). Also, the histopathology of the disease in some fish organs was conducted by Akhlaghi and Mahjor (2004). Recently the epizootic outbreak of lactococcosis caused by *L. garvieae* in farmed rainbow trout in Iran has been reported by Soltani *et al*. (2008), however a sequence comparison between isolates has not been undertaken.

This study describes the phenotypic and genetic characteristics of *L. garvieae* isolates from cultured rainbow trout with lactococcosis in the south and southwest of Iran and their comparison to the strains of *L. garvieae* isolated in some other countries.

Materials and Methods

Fish

Two hundred rainbow trout (22-240 g weight range) suspected of having streptococcosis/lactococcosis from rainbow trout farms cultured in Fars, Kohkiloyeh-Boyer Ahmad and Charmohal-Bakhtiari provinces in the south and southwest of Iran with a water temperature of 15-19°C were examined for the presence of the aetiological agent of the disease during summer 2002 to winter 2008. Moribund fish with external and internal body haemorrhages, abdominal distension and exophthalmia were euthanized with a blow to the head and used immediately for bacteriological examination.

Bacteriological examination

Fish kidney samples were cultured aseptically by streaking a loop onto brain heart infusion agar (BHI), blood agar and MacConkey agar plates and incubated at 25°C for 48 h. Suspected bacterial colonies were subcultured onto BHI, blood agar (5% sheep blood) and identified using the conventional biochemical system (Austin and Austin, 2007). Antibiogram tests using the disk diffusion method (Bauer *et al*., 1966) were performed to determine the antibiotic susceptibility of *L. garvieae* isolates.

Pathogenicity test

In this study a challenge protocol was designed to evaluate the pathogenicity of *L. garvieae* isolates using the challenge experiment procedure described by Akhlaghi *et al*. (1996) with some modification. Briefly, 5 groups of rainbow trout (10 fish in each group, mean weight of 56 g) including a control group were placed into six isolated 300 L fiberglass tanks to acclimate for a week before the challenge procedure. For the challenge, the fish were immersed in a bacterial suspension of 6×10^5 colony forming unit/ml of the *L. garvieae* for 30 min with appropriate aeration. Samples were taken from each dilution of the bacterial suspension used in the challenge and processed for plate count. Fish were kept in aerated tanks with an average water temperature of 19°C and watched for two weeks. Dead fish due to lactococcosis were examined and kidney samples were cultured on BHI agar.

PCR assay

Genomic DNAs *Lactococcus garvieae* were extracted using boiling method (Holmes and Quiqley, 1981). The DNA extracted by this method was visualized by gel electrophoresis on a 0.9% agarose gel before being stored at -20°C.

The PCR assay used was previously developed for definitive identification of *L. garvieae* based on the 16S rDNA sequence of *L. garvieae* by Mata *et al*. (2004). Primer sequences were pLG-1 (5'-CATAACAAT GAGAATCGC-3') and pLG-2 (5'-GCACCCTCGCGGGTTG-3'), to identify *L. garvieae*. The specificity of these primers was checked on all sequences available from the GenBank database using the BLAST program. The primers were commercially synthesized by the Cinnagen Company (Iran).

The following PCR conditions were applied to each assay; 50 mM KCL, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 200 µM dNTPs, 20 pmol of each primer, and 2 U *Taq* DNA polymerase (Fermentas) per 50 µl

reaction using 4 µl of DNA extracted as the template. A gradient thermocycler (Bio-Rad Laboratories Inc., Hercules, CA, USA), was used to determine an optimal annealing temperature for the specific binding of the primer set to the template DNA. The temperature was adjusted from 36 to 62°C and later from 56 to 62°C. It was determined that 55°C was optimal for species-specific PCR. The optimal thermal parameters were as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 1 min and extension at 72°C for 1 min. A final extension at 72°C for 5 min at the end of the amplification cycles was included. Each sample was tested at least in duplicate and sterile water was used as the negative control. In addition, DNAs from other bacterial fish pathogens including *Vibrio anguillarum*, *Flavobacterium psychrophilum*, *Streptococcus iniae*, *Streptococcus agalactia* and *Yesinia ruckeri* were used to ensure any cross reactivity. The amplification of 16S rDNA was confirmed by running the amplification product in 1.2% agarose gel electrophoresis.

16S rDNA sequence analysis

The PCR products of 16S rDNA of the 6 isolates (2 samples from each region) were sequenced. Sequencing of each PCR product was performed using a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Forward and reverse nucleic acid sequence data were used to construct a continuous sequence of inserted DNA. Further comparison of the continuous sequences was made with previously available sequences in NCBI (National Center for Biotechnology Information) using BLAST (Basic Local Alignment Search Tool) (Table 1). Multiple-sequence alignment analysis and construction of a phylogenetic tree were performed using the MEGA 4 program via FASTA algorithms, Phylogenetic trees were constructed on the basis of the UPGMA method (Sneath and Sokal, 1973) and the evolutionary distances were estimated by using MEGA version 4 (Tamura *et al*., 2007).

Results

Bacteriological cultures of the kidney samples of moribund fish showed 16% *L. garvieae* (32 fish), 2% *Streptococcus* spp. (4 fish), 12% *Aeromonas hydrophila* (24 fish) and 70% (140 fish) were culture negative. Results of biochemical tests on the *L. garvieae* isolated in this study were compared with the published results of biochemical tests of *L. garvieae* isolated by other researchers (Table 2).

Antibiogram tests showed that *L. garvieae* isolates were highly susceptible to erythromycin, enrofloxacin, chloramphenicol and clarithromycin (Table 3).

In the pathogenicity tests, the fish immersed in the bacterial suspension by challenge experiments showed 60 ± 4.3% (mean ± SE) mortality during 14 days post-infection. Experimentally infected fish showed erratic and circular swimming at the water surface, a reluctance to eat feed and revealed blackening of the skin, uni or bilateral exophthalmia. No mortality was observed in the control group. *L. garvieae* colonies were detected on BHI plates from all the dead fish kidneys.

In the specific PCR assay, DNAs extracted from all 32 *L. garvieae* gave the expected 1107-bp PCR fragment of 16S rDNA sequenses, which is specific for *L. garvieae* (Mata *et al*., 2004) (Fig. 1). The 1107 bp band was not observed with distilled water and DNA obtained from non-*L. garvieae* bacteria.

Table 1: Data on the *L. garvieae* strains analyzed in phylogenic analysis

Accession No.	Country	Strain	Host/Source	Year
EU727199	Iran	Fars 87	Rainbow trout (*Oncorhynchus mykiss*)	2008
AF352166	Taiwan	FLG	Mullet (*Mugil cephalus*)	2002
AB267897	Japan	Lg2	Yellowtail (*Seriola quinqueradiata*)	2006
AF283499	Taiwan	–	Giant freshwater prawn (*Macrobranchium rosenbergii*)	2001
AB018211	Japan	E1	Common carp (*Cyprinus carpio*)	2000
DQ010113	China	T030817-1	Flounder (*Paralichthys olivaceus*)	2005

Table 2: Biochemical characteristic of fish pathogenic *Lactococcus garvieae* and *L. garvieae* ATCC 43921

Character	Our study (EU 727199)	Austin and Austin (2007)	Soltani *et al*. (2008)	Eldar *et al*. (1999)	ATCC 43921*
Cell morphology	Ovoid cocci		Cocci		
Motility	-	-	-		-
TSI	A/A-				
O/F	F	F			
Production of:					
Catalase	-	-	-		
Oxidase	-	-	-		
H_2S	-	-	-		
Indole	-	-	-		
Lysine decarboxylase	-		-		
Ornithine decarboxylase	-		-		
Arginine hydrolase	+	+	+	+	+
Methyl red test	+	+	+		
Nitrate reduction	-	-	-		
Voges-Proskauer reaction	-	+	-	+	+
Growth on/at:					
0% sodium chloride	+	+			
5% sodium chloride		+			
6.5% sodium chloride	+	+			
pH (5-9.5)	+	+	+	+	
10°C	+	+		+	+
37°C	+	+		+	
45°C	+			+	+
Degradation of:					
Blood (haemolysis)	α	α	α	α	
Aesculin	+	+	+		+
Gelatin	-	-	+		
Starch	-	-	-		
Urea	-				
Utilization of sodium citrate	-		+		
Production of acid from:					
Cellobiose	+	+		+	
Glucose	+	+	+	+	
Glycerol	-	-			-
Inositol	-	-	-	-	
Lactose	+	-	+		+
Maltose	+	+	+	+	+
Galactose	+	+		+	
Mannitol	+	+	v	+	+
Raffinose	-	-		-	-
Rhamnose	-	-		-	
Salicin	+	+		+	
Sorbitol	+	+			-
Ribose	v			+	+
Sucrose	+	-	v		-
Trehalose	+	+		+	+
Xylose	-	-	-	-	

*(Teixeira *et al*., 1996). A/A- = acid/acid no gas, F = fermentation, and v = variable

Sequence analysis

The isolates were subjected to the 16S rDNA sequence analysis and identified all as *Lactococcus garvieae*. These strains shared 100% 16S rRNA gene sequence similarity. The sequencing result of the amplified

Table 3: Results of antibiogram test on *L. garvieae* isolate from rainbow trout farms, Iran (10 isolates were tested for each antibiotic)

	Antibiotic	Effective amount in disk (mcg)	Inhibition diameter (cm) (Mean±SE)
1	Erythromycin	15	2.7 ± 0.62
2	Enrofloxacin	5	2.4 ± 0.67
3	Chloramphenicol	30	2.2 ± 0.71
4	Clarithromycin	15	2.1 ± 0.65
5	Sulfadiazine	300	1.9 ± 0.47
6	Norfloxacin	10	1.7 ± 0.76
7	Vancomycin	30	1.6 ± 0.68
8	Streptomycin	10	1.5 ± 0.78
9	Ofloxacin	5	1.4 ± 0.57
10	Oxytetracycline	30	1.1 ± 0.64
11	Ampicilin	10	0.8 ± 0.64
12	Tetracycline	30	0.6 ± 0.74
13	Trimethoprim	5	0.4 ± 0.68

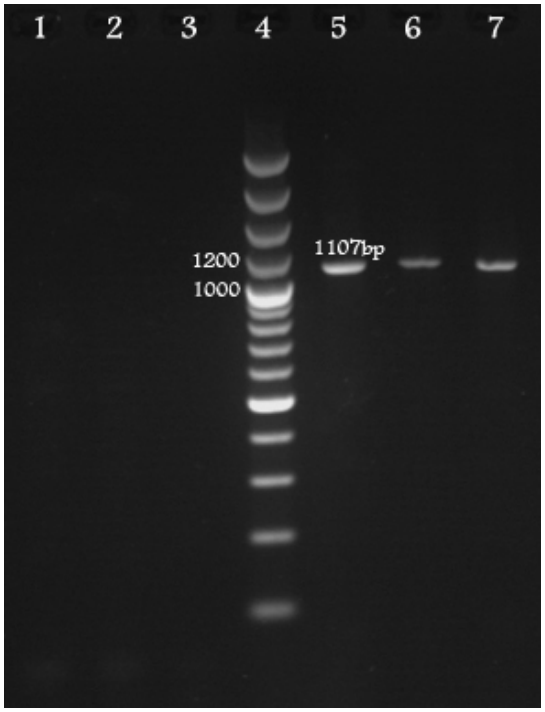

Fig. 1: Electerophoretic analysis (1.2% agarose gel) of DNA amplified fragments from 2 isolates in this study compared with standard strain. Lane 1: Negative control (distilled water), Lane 2: Negative control (DNA genomic of healthy fish), Lane 3: Negative control (*Streptococcus agalactiae*), Lane 4: Marker 100 base pair, Lane 5: Positive control, Lane 6 and 7: Positive samples

products obtained with pLG-1 and pLG-2 has been released as Iranian strain in GenBank under accession number EU727199. Moreover, these isolates showed 100 to 96% homology ranges with the other GenBank accession numbers of the *L. garvieae* in gene sequences analysis. Phylogenetic analysis, based on partial 16S rRNA gene sequencing, showed that this strain was similar (100% sequence similarity) to AF352166 from Taiwan and AB267897 from Japan. They also showed the lowest level of 16S rRNA gene sequence similarity (96%) to DQ010113 China isolate (Fig. 2).

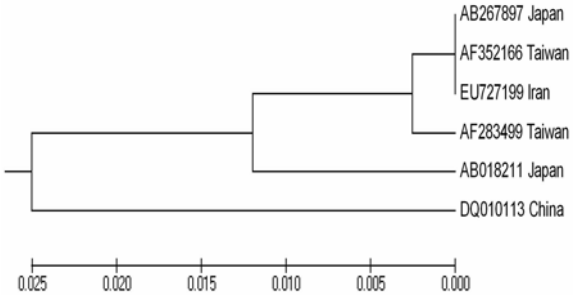

Fig. 2: Phylogenetic tree based on 16S rRNA gene sequences, constructed according to the UPGMA method, showing the position of Iran strain, other *Lactococcus garvieae* isolates. Bar, 1 substitution per 100 nucleotide positions

All results are based on the pairwise analysis of 6 sequences (Fig. 3). Analyses were conducted using the Maximum Composite Likelihood method in MEGA4 (Tamura *et al*., 2007).

Discussion

The considerable diversity of streptococcus bacteria associated with fish may explain the difficulties encountered

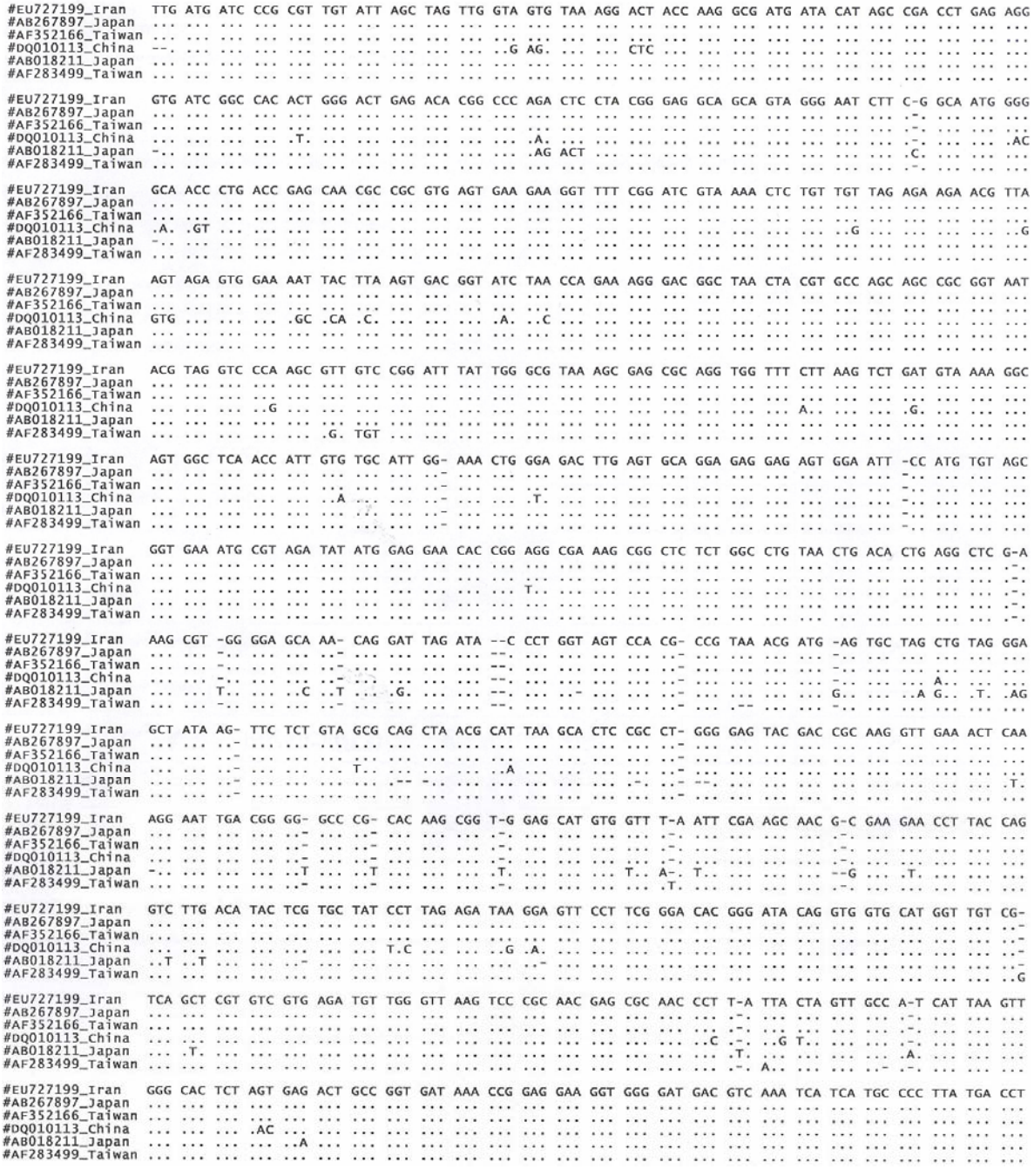

Fig. 3: Multiple alignments of the *L. garvieae* 16S rRNA gene sequences

when identification procedures are based only on phenotypic characteristics. Thus, final identification of the bacteria requires the support of genetic data (Eldar *et al*., 1999).

In this study, the phenotypic characteristics supported the conclusion that this strain is *L. garvieae*. This strain has different biochemical characterizations in producing acid from sucrose and Voges-Proskauer reaction when compared with those reported by Austin and Austin (2007). Production of acid from sucrose and mannitol by *L. garvieae* were variable in the results of Soltani *et al*. (2008), and gelatin and sodium citrate were also utilized by their isolate(s). Phenotypic heterogeneity of this strain with other strains is apparent, particularly when the Voges-Proskauer reaction and the production of acid from sucrose are considered (Table 2).

Lactococcus garvieae isolates in this research were sensitive to erythromycin, enrofloxacin, chloramphenicol, clarithro-

mycin and sulfadiazine. This is consistent with the study of Diler *et al*. (2002) on Turkish isolates and Ravelo *et al*. (2001) when *L. garvieae* from different geographic origins were subjected to antibiotic susceptibility. Soltani *et al*. (2008) only reported the sensitivity of their isolated *L. garvieae* to ampicillin and enrofloxacin. The affected trout farms currently use oxytetracycline, erythromycin and enrofloxacin. Due to the misuse of antibiotics such as oxytetracycline, this antibiotic is almost ineffective nowadays, presumably because of the development of resistant genes. The *tet(M)* gene was detected in 34 Japanese and Korean isolates out of 151 tetracycline-resistant bacterial isolates from fish and seawater, which included *Vibrio* sp., *L. garvieae*, *Photobacterium damsela* subsp. *piscicida*, and unidentified Gram-positive bacteria. The *tet(S)* gene was detected in *L. garvieae* from yellowtail in Japan and in *Vibrio* sp. from seawater in Korea (Kim *et al*., 2004).

In pathogenicity tests, our *L. garvieae* strain with 6×10^5 colony forming unit/ml showed 60 ± 4.3% mortality in challenge experiments during 14 days post-infection. It could be concluded that the isolate is quite virulent for rainbow trout. This is why Soltani *et al*. (2008) have not reported a mortality rate after experimental challenge with their isolate. *Lactococcus garvieae* (FLG strain from Taiwan, Table 1) caused 100% mortality with 1×10^7-10^8 and 50% mortality with 1.2×10^6 in mullet (Chen *et al*., 2002). The mortality rate with $<10^6$ *L. garvieae* (Lg2 strain from Japan) has been 100% in yellowtail (Kawanishi *et al*., 2007). Lactococcosis has been defined as a hyperacute and haemorrhagic septicemia (Bercovier *et al*., 1997). The typical clinical signs of the disease observed in rainbow trout are quite similar to those described for lactococcosis in other fish species like grey mullet (Chen *et al*., 2002).

Phenotypically diagnosed isolates in this study gave the expected 1107 bp PCR fragment of 16S rDNA sequences, confirming a definitive diagnosis of *L. garvieae*. Classical diagnosis in microbiology involved culture isolation and phenotypic identification, which takes several days to complete. In addition, *L. garvieae* identification based on biochemical profiles must be interpreted with caution. The PCR-based detection method tends to be more sensitive, but quicker than the traditional microbiological approach, since it can be performed in 8 h. Results of this study showed that this PCR procedure has high potential as a rapid screening test for the definitive detection of *L. garvieae* strains in Iran and differentiated it from other bacteria isolated in culture media, e.g. *Streptococcus* spp. and *Aeromonas hydrophila*, causing haemorrhagic septicemia in rainbow trout.

The PCR method can be employed as a supplementary and complementary test for definitive identification of the bacteria cultured from clinically suspected samples. In future studies, this PCR method can be used as a direct test for the detection of *L. garvieae* in tissues of infected trout.

On the basis of 16S rRNA gene sequence comparisons, our strain was found to be *L. garvieae*. The levels of similarity of the 16S rRNA gene sequence with other recognized *L. garvieae* were 96-100%. The analysis of genetic results indicated that outbreaks in Iran (EU 727199), Taiwan (AF352166) and Japan (AB267897) were produced by genetically related clones causing lactococcosis in rainbow trout (Iran, 2008), mullet (Taiwan, 2002) and yellowtail (Japan, 2006) respectively, suggesting the existence of the same sources of infection. Results of pulsed-field gel electrophoresis test to investigate the existing antimicrobial susceptibility and genetic characteristics of *L. garvieae* isolates from cultured *Seriola* in Japan showed that *L. garvieae* strains isolated in 2002 were closely related to ATTCC 49156 and ATCC 49157, which were isolated in 1974 in Japan (Kawanishi *et al*., 2005). These authors have suggested that isolates with the same origin have spread and caused lactococcosis in genus *Seriola* for 28 years in Japan. Iranian isolates displayed a great diversity with the strains isolated from flounder (China, 2005) and common carp (Japan, 2000).

The phenotypic and genetic results suggest the existence of diverse infection sources for lactococcosis affecting fish species worldwide. Thus, from the microbiological point of view and data

analysis in this study it could be concluded that a considerable diversity in phenotype and genetic characteristics among *L. garvieae* isolates is probably due to the host origin or geographical location of the organisms.

Based on the phenotypic and genetic characterizations, recent outbreaks of lactococcosis in Iran are suggested to be related to earlier outbreaks. Although a high genetic relationship was observed in the 16S rRNA sequence between recent and earlier isolates, other molecular typing methods (e.g. RAPD PCR and PFGE) with a higher discriminatory power are proposed for our further studies. Phenotypic and genetic characterizations of *L. garvieae* in this work may help researchers in further works such as epidemiological study and vaccine development.

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References

Akhlaghi, M and Keshavarzi, M (2002). The occurrence of streptococcosis in the cultured rainbow trout of Fars province. Iranian J. Vet. Res., 2: 183-189.

Akhlaghi, M and Mahjor, AA (2004). Some histopathological aspects of streptococcosis in cultured rainbow trout (*Oncorhynchus mykiss*). Bull. Eur. Assoc. Fish Pathol., 24: 132-136.

Akhlaghi, M; Munday, BL and Whittington, RJ (1996). Comparison of passive and active immunization of fish against streptococcosis (enterococcosis). J. Fish Dis., 19: 251-258.

Austin, B and Austin, DA (2007). *Bacterial fish pathogens, diseases of farmed and wild fish*. 2nd Edn., Chichester, UK, Springer Praxis Publishing. P: 552.

Bark, S and McGregor, D (2001). The first occurrence of lactococcosis in farmed trout in England. Trout News. 31: 9-11.

Bauer, AW; Kirby, WM; Sherris, JC and Turck, M (1966). Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol., 45: 493-496.

Bercovier, H; Ghittino, C and Eldar, A (1997). Immunization with bacterial antigens: infection with streptococci and related organisms. Dev. Biol. Stand., 90: 153-160.

Carson, J; Gudkovs, N and Austin, B (1993). Characteristics of an *Enterococcus*-like bacterium from Australia and South Africa, pathogenic for rainbow trout (*Oncorhynchus mykiss Walbaum*). J. Fish Dis., 6: 381-388.

Chen, SC; Liaw, LL; Su, HY; Ko, SC; Wu, CY; Chaung, HC; Tsai, YH; Yang, KL; Chen, YC; Chen, TH; Lin, GR; Cheng, SY; Lin, YD; Lee, JL; Lai, CC; Weng, YJ and Chu, SY (2002). *Lactococcus garvieae*, a cause of disease in grey mullet, *Mugil cephalus* L., in Taiwan. J. Fish Dis., 25: 727-732.

Diler, O; Altun, S; Adiloglu, AK; Kubilay, A and Isikli, B (2002). First occurrence of Streptococcosis affecting farmed rainbow trout (*Oncorhynchus mykiss*) in Turkey. Bull. Eur. Assoc. Fish Pathol., 22: 21-26.

Eldar, A; Goria, M; Ghittino, C; Zlotkin, A and Bercovier, H (1999). Bidiversity of *Lactococcus garvieae* strains isolated from fish in Europe, Asia and Australia. Appl. Environ. Microbiol., 65: 1005-1008.

Eyngor, M; Zlotkin, A; Ghittino, C; Prearo, M; Douet, DG; Chilmonczyk, S and Eldar, A (2004). Clonality and diversity of the fish pathogen *Lactococcus garvieae* in Mediterranean countries. Appl. Environ. Microbiol., 70: 5132-5137.

Ghittino, P and Prearo, M (1992). Report of Streptococcosis in rainbow trout (*Oncorhynchus mykiss*) in Italy: preliminary note. Boll. Soc. Ital. Pathol. Ittica. 8: 4-9.

Holmes, DS and Quiqley, M (1981). A rapid boiling method for the preparation of bacterial plasmids. Ann. Rev. Biochem., 114: 193-197.

Kawanishi, M; Kojima, A; Ishihara, K; Esaki, H; Kijima, M; Takahashi, T; Suzuki, S and Tamura, Y (2005). Drug resistance and pulsed-field gel electrophoresis patterns of *Lactococcus garvieae* isolates from cultured *Seriola* (yellowtail, amberjack and kingfish) in Japan. Lett. Appl. Microbiol., 40: 322-328.

Kawanishi, M; Yoshida T; Kijima, M; Yagyu, K; Nakai, T; Okada, S; Endo, A; Murakami, M; Suzuki, S and Morita, H (2007). Characterization of *Lactococcus garvieae* isolated from radish and broccoli sprouts that exhibited a KG^+ phenotype, lack of virulence and absence of a capsule. Lett. Appl. Microbiol., 44: 481-487.

Kim, SR; Lisa, L and Suzuki, S (2004). Occurrence of tetracycline resistance genes *tet(M)* and *tet(S)* in bacteria from marine aquaculture sites. FEMS Microbiol. Lett., 237: 147-156.

Kusuda, K; Kawai, K; Salati, F; Banner, CR and Fryer, JL (1991). *Enterococcus seriolicida* sp. nov., a fish pathogen. Int. J. Syst. Bacteriol., 41: 406-409.

Mata, AI; Gibello, A; Casamayor, A; Blanco, MM; Domínguez, L and Fernández-Garayzábal, JF (2004). Multiplex PCR assay for detection of bacterial pathogens associated with warm-water streptococcosis in fish. Appl. Environ. Microbiol., 70: 3183-3187.

Pereira, F; Ravelo, C; Toranzo, AE and Romalde, JL (2004). *Lactococcus garvieae*, an emerging pathogen for the Portuguese trout culture. Bull. Eur. Assoc. Fish Pathol., 24: 274-279.

Ravelo, C; Magarinos, B; Romalde, JL and Toranzo, AE (2001). Convencional versus miniaturizad systems for the phenotypic characterization of *Lactococcus garvieae* strains. Bull. Eur. Assoc. Fish Pathol., 21: 136-144.

Sneath, PHA and Sokal, RR (1973). *Numerical taxonomy*. 1st Edn., San Francisco, USA, Freeman. P: 573.

Soltani, M; Nikbakht, GH; Ebrahimzadeh, HA and Ahmadzadeh, N (2008). Epizootic outbreaks of *Lactococcus garvieae* in farmed rainbow trout (*Oncorhynchus mykiss*) in Iran. Bull. Eur. Assoc. Fish Pathol., 28: 209-214.

Tamura, K; Dudley, J; Nei, M and Kumar, S (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol., 24: 1596-1599.

Teixeira, LM; Merquior, V; Viani, M; Carvalho, M; Fracalanzza, S; Steigerwalt, AG; Brenner, DJ and Facklam, RR (1996). Phenotypic and genotypic characterization of atypical *Lactococcus garvieae* strains isolated from water buffalos with subclinical mastitis and confirmation of *L. garvieae* as a senior subjective synonym of *Enterococcus seriolicida*. Int. J. Syst. Bacteriol., 46: 664-668.