World Journal of Fish and Marine Sciences 5 (3): 315-321, 2013
ISSN 2078-4589

DOI: 10.5829/idosi.wjfms.2013.05.03.72123

Detection and Identification of Different Streptococcosis Strains in Farmed Rainbow Trout in Boyerahmad and Dena Regions (North South of Iran)

Edris Rahimi Kia and Yadollah Mehrabi

Department of Agriculture, Islamic Azad University, Yasouj Branch, Yasouj, Iran

Abstract: A total of 280 moribund rainbow trout with clinical signs of a hyperacute hemorrhagic septicemia were collected from rainbow trout farms in Kohgiluyeh-Boyer Ahmad province in the southwest of Iran during spring to summer 2011 for detecting 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 42 fish. 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. 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

Streptococcus is a genus of bacteria containing some species that cause serious diseases in a number of different hosts. This disease causes significant economic losses in the aquaculture industry in the United States of America, Japan, Israel, South Africa, Iran, Australia, the Philippines, Taiwan, Bahrain and other countries.

A major identifying feature of Streptococcus is that they are Gram-positive. By contrast, most of the common disease-causing bacteria of fish are Gram-negative. Streptococcal (Strep) diseases of fish are not common; however, when they do occur, significant losses can result. Streptococcosis of fish, from a clinical point of view, is a generic term used to designate similar, but different, diseases in which any one of at least six different species of gram-positive cocci, including *streptococci*, *lactococci* and *vagococci*, are involved [1, 2]. The main pathogenic species responsible for these streptococcal infections are *Streptococcus parauberis*, *Streptococcus iniae*, *Streptococcus difficilis*, *Lactococcus garvieae*, *Lactococcus piscium*, *Vagococcus salmoninarum* and *Carnobacterium piscicola* [1, 3, 4].

Streptococcal disease in fish was first reported in 1957, affecting cultured rainbow trout in Japan [5]. Since then, numerous other species of fish have been found susceptible to infection, including salmon, rainbow trout [6, 7]; mullet, golden shiner, pinfish, eel, sea trout, tilapia [6, 8-10]; sturgeon, red drum (*Sciaenops ocellatus*) [4]; yellowtail (*Seriola quinquerodiata*) [11]; rabbit fish (*Siganus canaliculatus*) [12]; sea bass (*Dicentrarchus labrax*) [13]; Japanese flounder (*Paralichthys olivaceus*) [14]; ayu (*Plecoglossus altivelis*) [6]; barramundi (*Lateus calcifer*) [15] and striped bass [10, 16]. Strep has also been isolated from a variety of ornamental fish, including rainbow sharks, red-tailed black sharks, rosy barbs, danios, some cichlids and several species of tetras.

Iran is now one of the leading countries in trout production in freshwater with a total production of about 91519 tons in 2011 [17]. Despite significant losses due to this zoonotic bacterial disease in trout aquaculture in Iran, little information is available particularly on the epizootiology and the causative agents involved. In the present study, the disease epidemiology has been assessed in Kohgiluyeh and Buyer Ahmad Province one of the major trout-producing provinces (with a total production of about 8832 tones 1n 2010). Conventional bacteriology and polymerase chain reaction (PCR) were

Corresponding Author: Edris Rahimi Kia, Department of Agriculture, Islamic Azad University, Yasouj Branch, Yasouj, Iran.

used to compare the accuracy of disease detection. Isolated bacteria also were phylogenetically characterized and compared with available data.

MATERIALS AND METHODS

Fish: During spring and summer 2011, sampling from 14 rainbow trout (*O. mykiss*) farms in Kohgiluyeh and Buyer Ahmad Province (located in North West of Iran) was done. 280 rainbow trout (20 fish from each farm, 10-200 g each) suspected of having streptococcosis/lactococcosis from these farms with a water temperature of 15-19°C were examined for the presence of the etiological agent of the disease. Moribund fish with external and internal body hemorrhages, abdominal distension and exophthalmia were euthanized with a blow to the head and used immediately for bacteriological examination.

Bacteriological Examination: Infected or suspected fishes were randomly sampled from selected farms and then organs such as brain, kidney, spleen and liver sterile swabs were streaked on brain heart infusion agar plate (BHI) supplemented with 1.5% NaCl. Plates, blood agar and MacConkey agar plates were transferred to the laboratory, where they were kept on ice and incubated at 25°C for 48 h. The bacteria were harvested by centrifugation at 3,500 *g*, 4°C for 10 min. Suspected bacterial colonies were subcultured onto BHI, blood agar (5% sheep blood) and identified using the conventional biochemical system [18]. Antibiogram tests using the disk diffusion method [19] were performed to determine the antibiotic susceptibility of isolates. Pure cultures were then sampled and subjected to morphologic and cellular fatty acid analysis.

Biochemical Characterization: After pure colonies of bacteria are obtained and identified as Gram-positive cocci, biochemical tests are performed. Table 1 presents a comparison of biochemical tests used in diagnosis of the pathogens responsible for streptococcal disease, in cultured fish. Biochemical characterization was performed with minor modifications according to Buller [20]. Specifically, hemolytic experiments were conducted at 25°C and 37°C on plates of sheep blood agar (SBA). In addition the following tests were also carried out: growth on MacConkey media, growth in 6.5% NaCl with triptycase soy broth (TSB), growth at a wide range of temperature (10°C, 25°C, 37°C, 45°C, 50°C) nitrate reduction, simon citrate utilization, urease production, voges proskauer reaction, catalase production, arginine dihydrolase (ADH), oxidation and fermentation of glucose (OF), production of ß-galactosidase, indole and H_2S, observation of motility on SIM (SH_2, Indole, Motility) media, degradation of gelatin, hippurate sodium and aesculin hydrolysis, acid production from carbohydrates namely: glucose, sorbitol, arabinose, trehalose, manose, xylose, salicin, inositol, maltose and manitole. All these examinations were read after incubation at 25°C for 24h.

Isolation of Bacterial DNAs: Bacterial chromosomal DNA used in PCR assay was extracted by the phenol-chloroform method described previously [21]. Purified DNA was dissolved in 100 µl of distilled water and then stored at -20°C until use.

DNA from the isolates was extracted using Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instructions.

PCR Assay: Genomic DNAs of the isolated bacteria were extracted using boiling method [22]. 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*. [23]. Primer sequences were pLG-1 (5'-CATAACAATGAGAATCGC-3') and pLG-2 (5'-GCACCCTCGCGGGTTG-3') to identify *L. garvieae*, also for *Streptococcus iniae* F((5'-CTAGAGTACACATGTACTAAG-3') and R((5'-GGATTTCCACTCCCATTAC-3'). 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 Swas tested at least in duplicate and sterile water used as the negative control.

Gel Electrophoresis: A gel consisted of 1% agarose in 1× TBE running buffer was prepared. Following that, the PCR product solution was mixed with 3 µl of 6× loading dye solution (Fermentas, Lithuania) and electrophoresed at 100 V for 45 min in a MT-108 Wide Mini Horizontal Gel Electrophoresis System. The gels were stained with 1 µg/ml ethidium bromide (Sigma, USA) for 30 min and visualized with an ultraviolet transilluminator.

Data Analysis: Target DNA amplicon were scored (1 for band visible, 0 for no band visible) for each suitable marker (bp). Faint bands were considered. Results were then entered in NTedit 1.2a software according to the markers selected. Construction of a phylogenetic tree was done using NTSYSpc 2.10j (Numerical Taxonomy and Multivariate Analysis System version 2.1j) software.

RESULTS

Disease outbreaks were observed in fish of 10 to 200 g in farms in North West of Iran (Kohgiluyeh and Buyer Ahmad Province). The disease occurred during spring and summer when water temperature increased above 14°C. Sluggish movement, darkening of body, bilateral exophthalmia sometimes together with cataract and hemorrhage, abdominal distention and prolaps of anal area with hyperemia/hemorrhage were observable in most affected fish. Also, accumulations of bloody fluids in abdominal cavity, hemorrhage in intestinal lumen were seen in dissection examination.

In most cases, the affected fish farms were using rivers as the main source of their water with water temperature in the range of 14.3-22.7°C, dissolved oxygen of 6.68-8.92 mg/l, pH of 7.85-8.16.

After bacterial isolation and primary testing by Gram stain, %3 KOH and catalase tests on all samples (280 samples), 258 positive specimens were obtained. Following biochemical characterization of pure or dominant colonies, *L. garvieae* were determined. Biochemical 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 1).

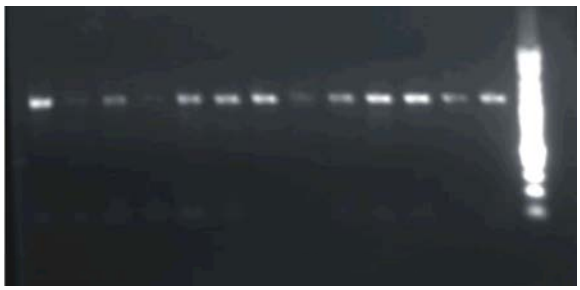

Fig. 1: Electerophoretic analysis (1.2% agarose gel) of DNA amplified fragments from 13 isolates in this experiment. Lane 1-13, the isolated bacteria from 13 rainbow trout farms (1107 bp). Lane 14: standard sample

1 ttgatgatcc cgcgttgtat tagctagttg gtagtgtaaa ggactaccaa ggcgatgata
61 catagccgac ctgagagggt gatcggccac actgggactg agacacggcc cagactccta
121 cgggaggcag cagtagggaa tcttcggcaa tgggggcaac cctgaccgag caacgccgcg
181 tgagtgaaga aggttttcgg atcgtaaaac tctgttgtta gagaagaacg ttaagtagag
241 tggaaaatta cttaagtgac ggtatctaac cagaaaggga cggctaacta cgtgccagca
301 gccgcggtaa tacgtaggtc ccaagcgttg tccggattta ttgggcgtaa agcgagcgca
361 ggtggtttct taagtctgat gtaaaaggca gtggctcaac cattgtgtgc attggaaact
421 gggagacttg agtgcaggag aggagagtgg aattccatgt gtagcggtga aatgcgtaga
481 tatatggagg aacaccggag gcgaaagcgg ctctctggcc tgtaactgac actgaggctc
41 gaaagcgtgg ggagcaaaca ggattagata ccctggtagt ccacgccgta aacgatgagt
601 gctagctgta gggagctata agttctctgt agcgcagcta acgcattaag cactccgcct
661 ggggagtacg accgcaaggt tgaaactcaa aggaattgac gggggcccgc acaagcggtg
721 gagcatgtgg tttaattcga agcaacgcga agaaccttac caggtcttga catactcgtg
781 ctatccttag agataaggag ttccttcggg acacgggata caggtggtgc atggttgtcg
841 tcagctcgtg tcgtgagatg ttgggttaag tcccgcaacg agcgcaaccc ttattactag
901 ttgccatcat taagttgggc actctagtga gactgccggt gataaaccgg aggaaggtgg
961 ggatgacgtc aaatcatcat gccccttatg acctgggcta cacacgt

Fig. 2: Multiple alignments of the L. garvieae 16S rRNA gene sequences

In the specific PCR assay, DNAs extracted from all 28 *L. garvieae* gave the expected 1107-bp PCR fragment of 16S rDNA sequences, which is specific for *L. garvieae* [23] (Fig. 1). The 1107 bp band was not observed with distilled water and DNA obtained from non- *L. garvieae* bacteria.

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 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 Gen Bank 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).

All results are based on the pairwise analysis of 6 sequences (Fig. 2). Analyses were conducted using the Maximum Composite Likelihood method in MEGA4 [26].

DISCUSSION

The considerable diversity of streptococcus bacteria associated with fish may explain the difficulties encountered when identification procedures are based only on phenotypic characteristics. The identification schemes for the causative agents, based on biochemical and antigenic features can barely differentiate these bacterial pathogens from other low virulent Gram-positive cocci such as *L. lactis* [18, 27]. Studies involving the phenotypic characterization of the virulent species of these *Strepotococcus*/*Lactococcus*, collected from different fish species and countries, have been conducted using conventional methods and miniaturized systems and have given variable results [18, 28, 29]. Thus, final identification of the bacteria requires the support of genetic data [4].

Clinical signs and gross findings in *L. garvieae* infection in rainbow trout described here are similar to those reported in rainbow trout and other fish [30, 31]. But, these lesions are similar to some fish diseases caused by different agents in rainbow trout 24. Therefore, a presumptive diagnosis of the *lactococcosis* in rainbow trout cannot be made on the basis of clinical signs and gross findings.

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 [18]. Production of acid from sucrose and mannitol by *L. garvieae* were variable in the results of Soltani *et al*. [24] 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 1). The biochemical properties of bacteria isolated from rainbow trout in this study are very similar to those described in other studies [4, 18, 30, 32].

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

Table 1: Biochemical characteristic of fish pathogenic Lactococcus garvieae and L. garvieae ATCC 43921

Character	This study	[18]	[24]	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)	*Á*	*Á*	*Á*	
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*. [25]. A/A- = acid/acid no gas, F = fermentation and v = variable

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 hemorrhagic 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 98-100%. 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 [33]. 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.

ACKNOWLEDGEMENTS

This work was financially supported by a grant from Research Council of Islamic Azad University of Yasouj Branch, Yasouj, Iran. The authors appreciate the Clinical Sciences Department of the Veterinary School and the Research Council of Shiraz University, Shiraz, Iran for supporting experimental procedure.

REFERENCES

1. Bercovier, H., C. Ghittino and A. Eldar, 1997. Immunization with bacterial antigens: infection with streptococci and related organisms. Dev. Biol. Stand., 90: 153-160.
2. Muzquiz, J.L., F.M. Royo, C. Ortega, I. De Blas, I. Ruiz and J.L. Alonso, 1999. Pathogenicity of streptococcosis in rainbow trout (Oncorhynchus mykiss): dependence on age of diseased fish. Bull. Eur. Assoc. Fish Pathol., 19: 114-119.
3. Eldar, A., C. Ghittino, L. Asanta, E. Bozzetta, M. Goria, M. Prearo and H. Bercovier, 1996. Enterococcus seriolicida is a junior synonym of Lactococcus garviae, a causative agent of septicemia and meningoencephalitis in fish. Curr. Microbiol., 32: 85-88
4. Eldar, A. and C. Ghittino, 1999. Lactococcus garvieae and Streptococcus iniae infections in rainbow trout, Oncorhynchus mykiss: similar but different diseases. Dis. Aquat. Org., 36: 227-231.
5. Hoshina, T., T. Sano and Y. Morimoto, 1958. A Streptococcus pathogenic to fish. J. of Tokyo Uni. of Fish., 44: 57-68.
6. Kitao, T., T. Aoki and R. Sakoh, 1981. Epizootic caused by betahaemolytic *Streptococcus* species in cultured freshwater fish. Fish Pathol., 15: 301-307
7. Eldar, A., Y. Bejerano, A. Livoff, A. Horovitcz and H. Bercovier, 1995. Experimental streptococcal meningo-encephalitis in cultured fish. Vet. Mic., 43: 33-40.

8. Shoemaker, C.A. and P.H. Klesius, 1997. Protective immunity against enteric septicemia in channel catfish, Ictalurus punctatus (Rafinesque) following controlled exposure to Edwardsiella ictaluri. Journal of Fish Diseases, 20: 101-108.
9. Bowser, J., G.A. Wooster, R.G. Getchell and M.B. Timmons, 1998. Streptococcus iniae infection of tilapia (*Oreochromis niloticus*) in a recirculation production facility. J. World Aquac. Soc., 29: 335 339.
10. Shoemaker, C.A., P.H. Klesius and J.J. Evans, 2001. Prevalence of Streptococcus iniae in tilapia, hybrid striped bass and channel catfish on commercial fish farms in the United States. Am. J. Vet. Res. 62(2): 174-177.
11. Kaige, N., T. Miyazaki and S.S. Kubota, 1984. The pathogen and histopathology of vertebral deformity in cultured yellowtail. Fish Pathol., 19: 173-179.
12. Yuasa, K., N. Kitancharoen, Y. Kataoka and F.A. Al-Murbaty, 1999. Streptococcus iniae, the causative agent of mass mortality in rabbitfish Siganus canaliculatus in Bahrain. J. Aquat. Anim. Health, 11: 87-93.
13. Colorni, A., A. Diamant, A. Eldar, H. Kvitt and A. Zlotkin, 2002. Streptococcus iniae infections in Red Sea cage-cultured and wild fishes. Dis. Aquat. Org., 49: 165-170.
14. Nguyen, H.T., K. Kanai and K. Yoshikoshi, 2002. Ecological investigation of Streptococcus iniae in cultured Japanese flounder (*Paralichthys olivaceus*) using selective isolation procedures. Aquaculture, 205: 7-17.
15. Bromage, E.S., A. Thomas and L. Owens, 1999. Streptococcus iniae, a bacterial infection in barramundi Lates calcarifer. Dis. Aquat. Org., 36: 177-181.
16. Stoffregen, D.A., S.C. Backman, R.E. Perham, P.R. Bowser and J.G. Babish. 1996. Initial disease report of Streptococcus iniae infection in hybrid striped (sunshine) bass and successful therapeutic intervention with fluoroquinolone antibacterial enrofloxacin. Journal of the World Aquaculture Society, 27: 420-434.
17. Iranian Fisheries Organization, 2012. Fisheries statistic yearbook, Planning and development office, Fisheries public relation department, pp: 57.
18. Austin, B. and D.A. Austin, 2007. Bacterial fish pathogens, diseases of farmed and wild fish. 2nd Edn., Chichester, UK, Springer Praxis Publishing, pp: 552.
19. Bauer, A.W., W.M. Kirby, J.C. Sherris and M. Turck, 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol., 45: 493-496.
20. Buller, N.B., 2004. Bacteria from Fish and other Aquatic Animals: A Practical Identification Manual, CABI Publishing, Cambridge, pp: 37-162.
21. Blanco, M.M., A. Gibello, A.I. Vela, M.A. Moreno, L. Dom´ýnguez and J.F. Fernández-Garayzábal, 2002. Winter Disease outbreak in sea bream (*Sparus aurata*) associated with Pseudomonas anguilliseptica infection. Disease of Aquatic Organism, 50: 19-27.
22. Holmes, D.S. and M. Quiqley, 1981. A rapid boiling method for the preparation of bacterial plasmids. Ann. Rev. Biochem., 114: 193-197.
23. Mata, A.I., A. Gibello, A. Casamayor, M.M. Blanco, L. Domínguez and J.F. Fernández-Garayzábal, 2004. Multiplex PCR assay for detection of bacterial pathogens associated with warm-water streptococcosis in fish. Appl. Environ. Microbiol., 70: 3183-3187.
24. Soltani, M., G.H. Nikbakht, H.A. Ebrahimzadeh and N. Ahmadzadeh, 2008. Epizootic outbreaks of Lactococcus garvieae in farmed rainbow trout (*Oncorhynchus mykiss*) in Iran. Bull. Eur. Assoc. Fish Pathol., 28: 209-214.
25. Teixeira, L.M., V. Merquior, M. Viani, M. Carvalho, S. Fracalanzza, A.G. Steigerwalt, D.J. Brenner and R.R. Facklam, 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.
26. Tamura, K., J. Dudley, M. Nei and S. Kumar, 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol., 24: 1596-1599.
27. Agnew, W. and A.C. Barnes, 2007. Streptococcus iniae: an aquatic pathogen of global veterinary significance and a challenging candidate for reliable vaccination. J. Vet. Microbiol, 122: 1-15.

28. Zlotkin, A., H. Hershko and A. Eldar, 1998. Possible transmission of *Streptococcus iniae* from wild fish to cultured marine fish. Appl Environ. Microbiol., 64: 4065-4067.
29. Haghighi, S., M. Soltani, G. Nikbakhat-Brojeni, M. Ghasemi and H.F. Skall, 2010. Molecular epidemiology of zoonotic streptococcosis/ lactococcosis in rainbow trout (*Oncorhynchus mykiss*) aquaculture in Iran. Iranian J. Microbiol., 2: 198-209.
30. Chen, S.C., 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, 2002. Lactococcus garvieae, a cause of disease in grey mullet, Mugil cephalus L., in Taiwan. J. Fish Dis., 25: 727-732.
31. Eldar, A. and C. Ghittino, 1999. Lactococcus garvieae and Streptococcus iniae infections in rainbow trout, *Oncorhynchus mykiss*: similar but different diseases. Dis. Aquat. Org., 36: 227-231.
32. Diler, O., S. Altun, A.K. Adiloglu, A. Kubilay and B. Isikli, 2002. First occurrence of Streptococcosis affecting farmed rainbow trout (*Oncorhynchus mykiss*) in Turkey. Bull. Eur. Assoc. Fish Pathol., 22: 21-26.
33. Kawanishi, M., A. Kojima, K. Ishihara, H. Esaki, M. Kijima, T. Takahashi, S. Suzuki and Y. Tamura, 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.