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Isolation and Characterization of *Lactococcus garvieae* from Diseased Rainbow Trout (*Oncorhynchus mykiss*, Walbaum) Cultured in Northern Iran Based on the Nucleotide Sequences of the 16s rRNA Gene

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Abstract

This study was done to determine the molecular and biochemical identification of some causative agents of lactococcosis in farmed rainbow trout in Mazandaran provenience (northern Iran). A total of 200 moribund rainbow trout, suspected of lactococcosis from 10 rainbow trout farms in Mazandaran province, were collected during spring 2012 to winter 2012. Sampling was done from the kidney, spleen, liver and brain and cultured aseptically onto brain heart infusion (BHI) agar plates and incubated at 25 °C for 24 - 48 h. Results of bacteriological cultures of these organs showed 19 % *Lactococcus garvieae* (38 fish), 9 % *Streptococcus* spp., (18 fish), 17 % *Yersinia* spp. (36 fish), and 55 % of fish were culture negative. The PCR assay was developed based on the 16s rRNA gene of *L. garvieae* for the rapid and specific detection and identification of this pathogen from different sources. Two pairs of primers were designed based on the nucleotide sequences of the 16s rRNA gene of *L. garvieae*. After PCR assay on isolated bacterial colonies, DNAs extracted from 38 *L. garvieae* gave the expected 1107 bp PCR fragment of 16S rDNA sequences, which is specific for *L. garvieae.* The results of this study suggest the use of molecular methods along with current biochemical methods are effective diagnostic tools in the identification of *L. garvieae*. The combination of these methods for diagnosis of other bacterial disease is recommended.

Keywords: *Lactococcus garvieae*, *Oncorhynchus mykiss*, 16s rRNA, PCR, Iran

Introduction

Streptococcosis/lactococcosis is one of the most important bacterial fish pathogen with symptoms such as anorexia, uni or bilateral exophthalmia, blackening of the skin, abdominal distension and haemorrhages in the internal and external organs. The main pathogenic species that have been associated with disease include: *Streptococcus iniae*, *S. agalactiae*, *S. parauberis*, *S. dysgalactiae S. faecium*, *S. milleri*, *S. uberis*, *S. ictaluri*, *S. phocae*, *S. faecalis*, *L. garvieae*, *L. piscium*, *Carnobacterium piscicola and Vagococcus salmoninarum.* The total annual loss due to this disease in trout farming has been

estimated to be about 150 million USD [1-3]. Different results showed that *S. iniae* and *L. garvieae* are the major pathogens of streptococcosis and lactococcosis in the cultured rainbow trout in Iran [4,5].

L. garvieae (*Enterococcus serilicida*) has been isolated from rainbow trout farms in different parts of Iran, especially Mazandaran province, in northern Iran. Recently the epizootic outbreak of lactococcosis caused by *L. garvieae* in farmed rainbow trout in Iran has been reported by Fadaeifard *et al.* [6], Rahimi-Kia and Y Mehrabi [7], Sharifiyazdi *et al.* [8] and Soltani *et al.* [9].

Among the fish species, salmonids and especially rainbow trout (*Oncorhynchus mykiss*) are most susceptible to lactococcosis. The host range of this bacterium is not only limited to fish but also bacteria has been isolated from cattle, buffalo [10], dogs and cats and also raw animal products, including milk, beef and poultry meat [6,11,12]. Also, this bacterium was described as an important zoonotic bacterial disease that causes cellulitis and endocarditis in humans [13].

Losses due to lactococcal septicemias in cultured fish, the expense involved in using antimicrobial compounds and the reported increasing drug resistance of the causative Gram-positive cocci pointed out the need for complete study about this pathogens.

Despite significant losses due to this zoonotic bacterial disease in trout aquaculture in Iran, little information is available particularly from northern Iran. Therefore, this study aimed at identifying *L. garvieae* in rainbow trout from northern Iran by using conventional biochemical analyses and molecular methods (PCR).

Materials and methods

Sampling

Two hundred moribund juvenile rainbow trout suspected to lactococcosis were gathered. These samples were obtained from 10 rainbow trout farms in Mazandaran province, northern Iran, during spring 2012 to winter 2012. After sampling, infected or suspected fish were transported to the central laboratory Veterinary Organization of Sari for bacteriological examination.

Isolation of bacterium and bacteriological examination

Sampling of the kidney, spleen, brain and liver were done in aseptic conditions, and then were directly streaked by sterile swabs on brain heart infusion (BHI, at pH 5 - 9.5) agar. Plates were incubated at 25 °C for 24 - 48 h. After macroscopic and microscopic observation of the colonies, single colonies with pure culture growth were subcultured onto BHI and identified by using the conventional biochemical tests (**Table 1**). Finally, isolated bacterium was tested for determination of the sensitivity of *L. garvieae* isolates by using antibiogram tests.

DNA extraction

DNA was extracted using a DNA isolation kit (MBST, Iran) according to the manufacturer's instructions. First, the samples (bacterial colonies isolated from the kidneys of fish) were lysed in 180 µL lysis buffer, and then the proteins were degraded with 20 µl proteinase K for 10 min at 55 °C. After addition of 270 µl bindings buffer and incubation for 10 min at 70 °C, 320 µL ethanol (100 %) was added to the solution and after vortexing, the complete volume was transferred to the MBST-column. The MBST column was first centrifuged and then washed twice with 500 µl washing buffer. Finally, DNA was eluted from the carrier with an elution buffer.

Primers

Two pairs of primers were designed based on the nucleotide sequences of the 16s rRNA gene of *L. garvieae* including: F: (5′- CAT AAC AAT GAG AAT CGC–3′) and R: (5′- GCA CCC TCG CGG GTT G–3) in order to identify the *L. garvieae*. Primers were synthesized by the Cinna Gen company (Tehran, Iran).

PCR amplification

The PCR was performed in a total reaction volume of 50 µL containing: 50 mM KCl, 10mM Tris-HCl (pH 9.0), 1.5 mM, $MgCl_2$, 200 µM dNTPs, 20 pmol of each primer and 2 U *Taq* DNA polymerase per 50 µl reacti and 4µl of template DNA. The reaction was repeated for 37 cycles under the following conditions: 4 min at 94 °C (1 Hz), 1 min at 94 °C, 1 min at 58 °C, 1.5 min at 72 °C (35 Hz) and finally, PCR was completed with the final extension step at 72°C for 10 min. Distilled water was used as a negative control in each PCR reaction. Each sample was tested in duplicate. In order to decrease the errors *S. iniae* was used as the negative control. Also, DNAs from other *Streptococcus* species and *Yersinia* spp were used to ensure any cross reactivity.

Gel Electrophoresis

PCR products were separated on 1.5 % agarose gel in 0.5× Tris-borate-ethylene diamine tetraacetic acid (EDTA) buffer and visualized using ethidium bromide and a UV illuminator.

Results and discussion

The mean weight of the fish were 220±80 g. Water temperature during sampling was 12 - 28 °C, dissolved oxygen of 5.80 - 8.72 mg/l and a pH of 6.12 - 8.35. Results of bacteriological cultures of fish kidneys showed 19 % *L. garvieae* (38 fish), 9 % *Streptococcus* spp., (18 fish), 17 % *Yersinia* spp. (36 fish), and 55 % were culture negative. Most outbreaks were reported during the warm season, late spring till mid autumn, and the time when the water temperature of trout farming increases up to 15 °C particularly in those fish farms that use rivers as the source of water. In this study, the most and the least infections were observed in summer (with 68.2 %) and winter (with 44.2 %), respectively.

In some fish, clinical signs included bilateral exophthalmia, blackening of the skin, abdominal distension, hemorrhages in the eyes, skin, gills and internal organs. The results of biochemical tests are shown in **Table 1** and were compared to Austin and Austin [1] and Sharifiyazdi *et al*. [8]. Results of the antibiogrammes test confirmed the sensitivity of *L. garvieae* isolated to erythromycin, enrofloxacin, fleumequin but not to lincomycin and oxytetracycline. After PCR assay, DNAs extracted from 38 *L. garvieae* gave the expected 1107 bp PCR fragment of 16S rDNA sequences, which is specific for *L. garvieae* and different from other Lactococcus species. Distilled water and DNA obtained from non-*L. garvieae* bacteria did not show the 1107 bp band (**Figure 1**).

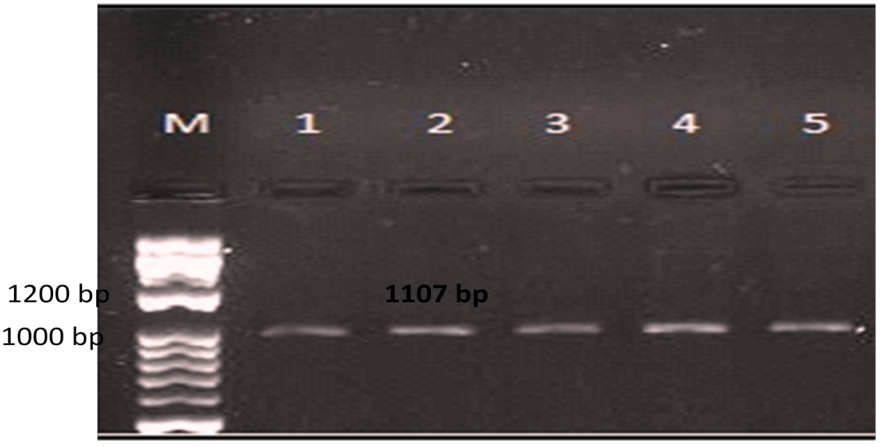

Figure 1 Electrophoretic analysis (1.5 % agarose gel) of DNA amplified fragments from 5 isolates in this experiment. Lane 1 - 4, the isolated bacteria (1107 bp). Lane 5: positive control.

Table 1 Biochemical characteristics of *Lactococcus garvieae*.

L. garvieae **Sharifiyazdi *et al.* [8]**	***L. garvieae*** **Austin and Austin [1]**	***L. garvieae*** **(our study)**	**Biochemical characteristics**
Ovoid cocci	-	Cocci	Cell morphology
+	+	+	Geram
-	-	-	Motility
-	*V	-	Production of Ornithine decarboxylase
-	-	-	Production of Indol
-	-	-	Production of H_2s
-	-	-	Production of Oxidase
-	-	-	Production of Catalase
+	+	+	Production of Arginine hydrolase
-	-	-	Nitrate reduction
+	+	+	Methyl red test
-	+	+	Voges-Proskauer reaction
F	F	F	O/F
-	-	-	Degradation of gelatin
-	-	-	Degradation of Starch
-	V	-	Degradation of Urea
+	+	+	Degradation of Aesculin
+	+	+	Production of acid from Maltose
+	-	-	Lactose production of acid from
+	-	+	Production of acid from Sucrose
-	-	-	Production of acid from Rhamnose
-	-	-	Production of acid from Inositol
+	+	+	Mannitol production of acid from
+	+	+	Production of acid from Glucose
+	+	+	Production of acid from Sorbitol
-	-	-	Xylose Production of acid from
+	+	+	Production of acid from Trehalose
-	-	V	Production of acid from Raffinose
-	-	-	Production of acid from Glycerol
α	A	A	Hemolysis (TSA with 5 % sheep erythrocytes)
+	+	+	Growth in: 10 °C
+	+	+	Growth in: 37 °C
+	+	+	Growth in: 45°C
+	+	+	Growth in 0 % NaCl
+	+	+	Growth in 2 - 5 % NaCl
+	+	+	Growth in 6.5 % NaCl
+	+	+	Growth at pH (5 - 9.5)

*(F: fermentation, V: variable, A: acid)

Table 2 Percent infection to *Lactococcus garvieae* in selective farms in Mazandaran province (n = 20).

Number farm	**Region/location**	**Number infection fish**	**Percent infection**
1	Lower part	7	40
2	Higher part	-	-
3	Lower part	4	20
4	Higher part	-	-
5	Higher part	2	10
6	Lower part	9	45
7	Higher part	2	5
8	Lower part	6	30
9	Lower part	3	15
10	Higher part	5	25

Mazandaran province, with 16,000 tons production of rainbow trout per year, is placed second in production of rainbow trout in Iran [14]. Economic losses and sanitary problems in trout farms of Iran during summers caused by Lactococcosis are significant. These facts confirm the results of Soltani *et al.* [16] in the aquaculture industry of Iran, especially in the northern parts.

L. garvieae along with some bacteria in the streptococcus genera, such as *S. inaei* are classified in streptococea family [1]. These bacteria could cause high mortality in rainbow trout farms. The first definite diagnosis of Lactococcosis in Iran, was reported in the cultured rainbow trout of Chahar Mahal-e Bakhtiari and Fars province [5]. Thereafter, in 2008 and 2009, epidemiology of this disease was studied in Lorestan, Mazandaran, Fars and Chahar Mahal-e Bakhtiari provinces [9,15].

Diagnosis of the causative agent of disease is important to specify a preventive strategy. In the current study, 38 *L. garvieae* isolates were diagnosed from 200 moribund fish for Lactococcosis. Biochemical and molecular results showed that 19 % of rainbow trout's of Mazandaran province were infected by *L. garvieae*. The results of Soltani *et al.* [16] showed that 4.6 % of rainbow trout were infected by *L. garvieae* in Mazandaran province. Sharifiyazdi *et al.* [8] by evaluation of 200 samples from fishes suspected of having Lactococcosis disease using bacteriological and biochemical tests, identified the *L. garvieae* in the Fars province. In addition, Fadaeifard *et al.* [6] have emphasized the importance of *L. garvieae* as a serious pathogen in Chaharmahal-va-Bakkhtiary province and its impact on the production rate.

In this study, the isolated bacteria was identified as *L. garvieae* using conventional biochemical tests. The biochemical properties of the isolated bacteria from rainbow trout were very similar to those described in other studies [1,8].

The results of the present study indicated that the rainbow trout in lower farms faced with higher bacterial infection in comparison with others in the higher ones (**Table 2**). This fact may be related to water quality parameters and also probable transmission of bacteria from higher farms to the lowers ones.

The effect of temperature and water quality are known as important agents in the incidence of disease [9]. As the water temperature increases from 15 °C, bacterial growth will increase. Pathogenicity of the disease will increase in low hygienic conditions. Results of the current study revealed that the incidence of disease will rise by increasing the temperature. The increase in water temperature together with the impact of polluted water sources will cause a significant decline in water quality parameters resulting in outbreaks by infectious diseases including lactococcosis.

To assure the accuracy of disease detection, conventional bacteriology and polymerase chain reaction (PCR) were applied. The results of this study suggest that the use of molecular methods along with current biochemical methods are effective diagnostic tools in the identification of *L. garvieae*. The combination of these methods in order to diagnose other bacterial disease is recommended.

Conclusions

The results of this study and previous studies showed that the mortality of rainbow trout farms of Iran caused by *L. garvieae* is increasing. This could be attributed to the low hygienic conditions in farms, therefore application of good manufacturing practice in farms is essential. Also, considering the contaminant sources of waters in farms, reduced stress conditions by improving environmental conditions improvement and public hygiene, density and good nutrition, use common disinfectants and strengthening the non-specific defense of fish by compounds such as vaccines, immunostimulants, probiotics, prebiotics and also, control of eggs, fish and breeder transportation, vaccination against this disease and a comprehensive monitoring of rainbow trout farms can be useful to control or reduce economic losses caused by *L. garvieae*.

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