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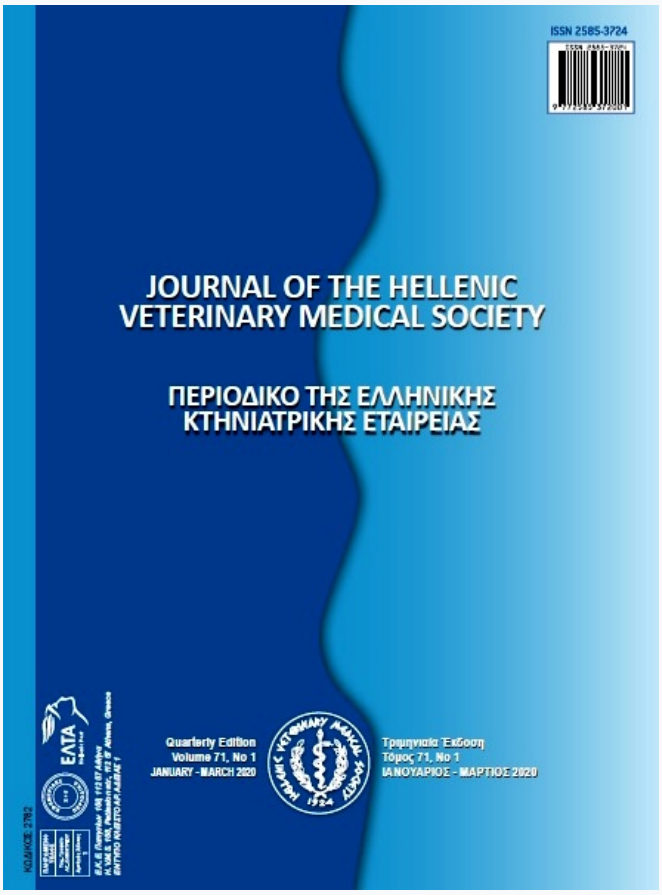


# Genetic characterization of Lactococcus garvieae isolated from farmed rainbow trout by random amplified polymorphic DNA-PCR in Iran

*F. FADAEIFARD, M. RABIEI, M. F. SHARIFPOUR*

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# Genetic characterization of *Lactococcus garvieae* isolated from farmed rainbow trout by random amplified polymorphic DNA-PCR in Iran

**F. Fadaeifard[1], M. Rabiei[2], M.F. Sharifpour[3]**

[1]*Faculty of Veterinary Medicine, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran*

[2]*Department of pathobiology, infectious disease and public health, School of Animal and Veterinary Science, The University of Adelaide, Adelaide, Australia*

[3]*School of Animal and Veterinary Science, The University of Adelaide, Adelaide, Australia*

**ABSTRACT.** Lactococcosis is one of the main bacterial infections of fish around the world. *Lactococcus garvieae* has been a major cause of rainbow trout losses in freshwater farming. This study aimed to genotype and determine the variability of *L. garvieae* isolated from infected farmed rainbow trout in Iran by the RAPD-PCR method. Bacterial samples were collected from 12 farms located in the western part of Iran and suspected to carry Lactococcus infection. Two hundred bacterial cultures containing cocci shaped bacteria were cultured in Trypticase soy agar (TSA) and blood agar mediums. All bacterial cultures were tested by conventional microbiological and biochemical tests, and PCR assay to identify *L. garvieae* by 16S rDNA genes. The RAPD-PCR method was used to determine the genetic pattern of all isolates. The sample strain pattern of the isolates was analyzed in the NTSYS program. According to a similarity coefficient index of 70%, all *L. garvieae* isolates were separated into two groups with four RAPD profile types. The highest and the lowest genetic pairwise similarity among the isolates were 98% and 54%, respectively. The results of the present study revealed that RAPD-PCR is an applicable method to describe the genetic diversity of different strains of *L .garvieae* among farmed fish.

***Keywords:*** *Lactococcus garvieae*; Genetic characterization; RAPD-PCR; Rainbow trout

*Corresponding Author:*
F. Fadaeifard, Islamic Azad University, Shahrekord Branch, PO Box 166
E-mail address: fadaeifard@gmail.com

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## INTRODUCTION

*Lactococcus garvieae* is a causative agent of lactococcosis that affects different types of fish and crustaceans in freshwater and marine cultures around the world (Vendrell *et al*., 2006). This pathogen was first isolated from some saltwater fish species in Japan that was initially called *Enterococcus seriolicida* (Kusuda *et al*., 1991). Evidence shows that the outbreak of lactococcosis increases with rising water temperatures from about 18 °C and could be controlled when the temperature drops to 13°C (Gibello *et al.*, 2016). In addition to fish and other animals (Villani *et al*., 2001), some infections associated with *L. garvieae* have also been reported in humans (Elliot *et al*., 1991; Li *et al*., 2008; Chan *et al*., 2011). The handling and eating of raw fish are important routes leading to human infections and have resulted in extending the zoonotic status of the pathogen (Gibello *et al*., 2016). This bacterium is also considered an emerging pathogen in both veterinary and human medicine (Meyburgh, 2017). *L. garvieae* can be identified by several laboratory methods from conventional microbiological tests to different kinds of molecular tests such as PCR, pulsed-field gel electrophoresis, ribotyping, random amplified polymorphic DNA (RAPD) PCR, sau-PCR and amplified fragment length polymorphism methods (Altun *et al.*, 2013). The multilocus sequence typing test (MLST) has also been used to improve knowledge of the evolutionary history and the genomic complexity of *L. garvieae* (Ferrario *et al*., 2013). In epidemiological studies of lactococcosis outbreaks, the RAPD-PCR method can be carried out as a potential test for identifying genetic variation of the causative agent. It is made applicable by using a single optional primer in a PCR reaction, resulting in the amplification of many discrete DNA products (Altun *et al*., 2013; Ravelo *et al*., 2003). *L. garvieae* strains are divided into three epidemiological groups according to genotypic variations determined by RAPD-DNA technique (Ravelo *et al*., 2003).

Lactococcosis is known as a significant infectious disease of farmed fish all over the world. In recent years, it has been one of the main causes of economic losses among rainbow trout farms in Iran. The first outbreak of Streptococcosis was reported in 2005 (Soltani *et al*., 2005) and then other researchers studied different subjects relating to the isolation, identification, pathogenicity and molecular evaluation of some streptococcal agents (Sharifiyazdi *et al*., 2010; Soltani *et al*., 2008; Fadaeifard *et al.*, 2012). The aim of the present study was genotyping of forty-three *L. garvieae* isolates obtained from rainbow trout farms in Iran. In this study, the RAPD-PCR method was used to determine genetic variability and relationship among isolates from different geographic areas of this country.

## MATERIALS AND METHODS

### Sampling and bacterial isolation

Samples were obtained from 12 rainbow trout farms of Iran during 2016-2018. These farms are located in the western part of this country (Fig 1). They had been chosen based on primary evaluations of suspected fish to clinical signs of Streptococcosis/ Lactococcosis disease. Samples were obtained by the stratified cluster sampling method. The target farms were chosen from four high trout-producing regions in Iran. In each region, three farms and from each farm five specimens were randomly collected. The study was done according to the research project with the approval certificate number of EC/0181. Sterile swabs were obtained from the kidney, spleen, and liver of each fish inoculated aseptically on blood agar (with 5% sheep red blood cells) and Trypticase soy agar (TSA, Merck) media and subsequently incubated aerobically at 22°C for 24 to 48 hours. Gram stain and cellular morphologies were examined at 1000×. All gram-positive cocci shaped colonies were chosen for further tests.


**Figure 1.** Location and geographical distribution of rainbow trout farms sampled in Iran

### Phenotypic and biochemical characterization

An overnight culture of the pure colony was subjected to the morphological, physiological and biochemical tests recommended by Austin & Austin (2012) which are described in Table 1. The condition of bacterial growth was determined in the four different ranges of temperatures (15°C, 20°C, 37°C, and 40°C) and pH (5-9.5) by culturing in nutrient broth

(NB). The hemolytic reaction of the isolates was examined on nutrient agar containing 5% sheep red blood cells. Acid production was checked by the carbohydrate fermentation procedure using the inoculation of the isolates into nutrient broth containing the respective carbohydrates such as glucose, lactose, and sucrose.

**Molecular diagnosis**

The isolates were studied using the PCR method as a molecular diagnostic test. Genomic DNA was extracted from pure colonies in the TSB using a genomic DNA purification kit (Fermentas, Lithuania) according to the manufacturer's instructions. PCR reaction was performed with *L. garvieae* specific primer pairs (pLG-1:5′-CATAACAATGAGAATCGC-3′) and (pLG-2: 5′- GCACCCTCGCGGGTTG-3′) for genetic confirmation of isolates as described by Zlotkin *et al*. (1998). All isolates were identified with an amplification product of 1100 bp. The PCR assay was carried out in a total volume of 25 µl reaction mixture containing 2 µl of template DNA, with a 1 µM of each primer, 2 mM $MgCl_2$, 200 µM of each dNTP, 1 U of Taq DNA polymerase (Fermentas, Lithuania), and 2.5 µl of PCR buffer 10X. The amplification was performed in a thermal cycler (Flex Cycler, Germany) with an initial denaturation step at 94°C for 3 min, 35 cycles of a denaturation step at 94°C for 60s, primer annealing at 55°C for 60s and extension at 72°C for 90s. The final extension was performed in 10 min at 72°C. *L. garvieae* strain (CB10, Iranian strain) isolated from clinical samples of lactococcosis in Iran (Soltani *et al*., 2008) was chosen as a positive control and *Streptococcus iniae* (ATCC 29178) was used as the negative control. The PCR products were detected by running the amplification mixture in 2% agarose gel with 1X Tris-acetate-EDTA buffer and stained with ethidium bromide (0.5µg $ml^{-1}$) after the run.

**Genotyping by the RAPD method**

The RAPD-PCR test was used to identify genetic variation in 43 *L. garvieae* isolates which had already been confirmed by the PCR test. Amplification reactions were carried out in a 50 µl volume of 50mM KCl, 10 mM Tris- HCl (pH:8.3), 3 mM $MgCl_2$, 250 µM each dNTP, 1.5mM of primers, 4 units Taq polymerase enzyme and a 3 µl template DNA. In this reaction, M13 primer (5'-GAGGGTGGCGGTTCT-3') was used and the program was performed as previously described by Altun *et al.* (2013). The amplification program was run on initial denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 43°C for 40s and extension at 72°C for 2 min, followed by a final extension at 72°C for 7 min. The amplified product was run on a 1.5% agarose gel electrophoresis.

**Data analysis**

The RAPD test was performed three times on each isolate to ensure the accuracy of the results. After determining PCR products on the gel, they were sorted into: without any DNA (0) and with DNA (1). Thereafter they were analyzed with numerical taxonomy and a multivariate analysis system (NTSYS) software, version 2.2. Jacquard's similarity coefficient (JSC), Dice Similarity Coefficient (DSC) and a simple matching coefficient (SMC) were calculated to utilize the factors in defining a genetic similarity percentage among all isolates.

## RESULTS

**Physiological and Biochemical tests**

Obtained results were compared with the reference strain of *L. garvieae*. Results presented in Table.1 showed that many of the isolates were gram-positive cocci, catalase and oxidase negative, alfa and beta-hemolytic on blood agar (5% sheep's blood), non-motile, grow in a pH range of 5 to 9.5, and a temperature range of 15-40 °C, O/F positive, show no reaction in Indole, have no growth in urea and ornithine and have a fermentative reaction in glucose and lactose. The listed characteristics are highly similar to those found in comparable researches.

**Table 1.** Morphological and biochemical characteristic of isolated *Lactococcus garvieae* and comparison with published data

| | Present study | Austin and Austin, 2012 | Soltani *et al*, 2008 |
|---|---|---|---|
| Gram stain | Ovoid cocci | Ovoid cocci | Cocci |
| Morphology | + | + | + |
| Hemolysis | a/ß | a/ß | a/ß |
| Catalase | - | - | - |
| Oxidase | - | - | - |
| Motility | - | - | - |
| O/F | +/+ | +/+ | +/+ |
| Indole | - | ? | - |
| Glucose | + | + | + |
| Lactose | + | + | +/- |
| Sucrose | ? | ? | V |
| Ornithine | - | - | - |
| Urea | - | ? | - |
| Growth at: | | | |
| pH (5-9.5) | + | + | + |
| Temperature (15-40°C) | + | + | + |

V = variable results, ? = Not defined.

**PCR assay**

The PCR assay resulted in the amplification of the 1100 bp band (16S rDNA) for all cocci-shaped bacteria which had been previously confirmed by conventional laboratory tests. From 200 samples obtained from infected rainbow trout, 43 isolates were identified as *L. garvieae*. Amplified bands of *L. garvieae* isolates are shown in Fig 2.

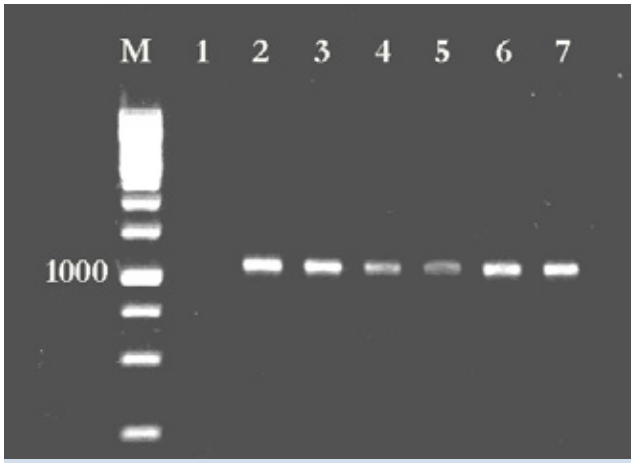


**Figure 2.** Amplification products from infected rainbow trout using PCR assay for the detection of *L. garvieae* (1,100 bp). Lane M, 1kb DNA ladder; lane 1, negative control; lane 2, positive control; lanes 3-7; test samples.

**RAPD- PCR test**

RAPD- PCR results showed the patterns obtained in different bands ranging from 220 to 2500 bp in size (Fig 3). Firstly, they were scored as 0 (with a band) and 1 (without a band) and then all obtained data were analyzed by NTSYS software. According to three different coefficients of data (SMC= 0.777, DSC= 0.688, and JSC=0.771), a Simple Match Coefficient at the value of 77% for the highest rate was used for calculating a genetic similarity percentage and for drawing the dendrogram. There was a 100% similarity only between isolates 35 and 39. The next highest genetic similarity was shown between isolates 41 and 43 (98 %) and the lowest was shown between isolates 1 and 22 (54%). In the dendrogram that was drawn for 43 isolates (Fig 4) with a 71% similarity coefficient, they were clustered into two groups A and B. Group A also divided into two subgroups A1 (isolate 1) and A2 (isolate 2). Furthermore, group B was divided into two subgroups, B1 (including 40 isolates) and B2 (isolate 9). It was found that using the 85% similarity coefficient, the number of subgroups rose, reaching 20 subgroups. Also, using the 93% similarity coefficient isolates clustered to 31 subgroups.

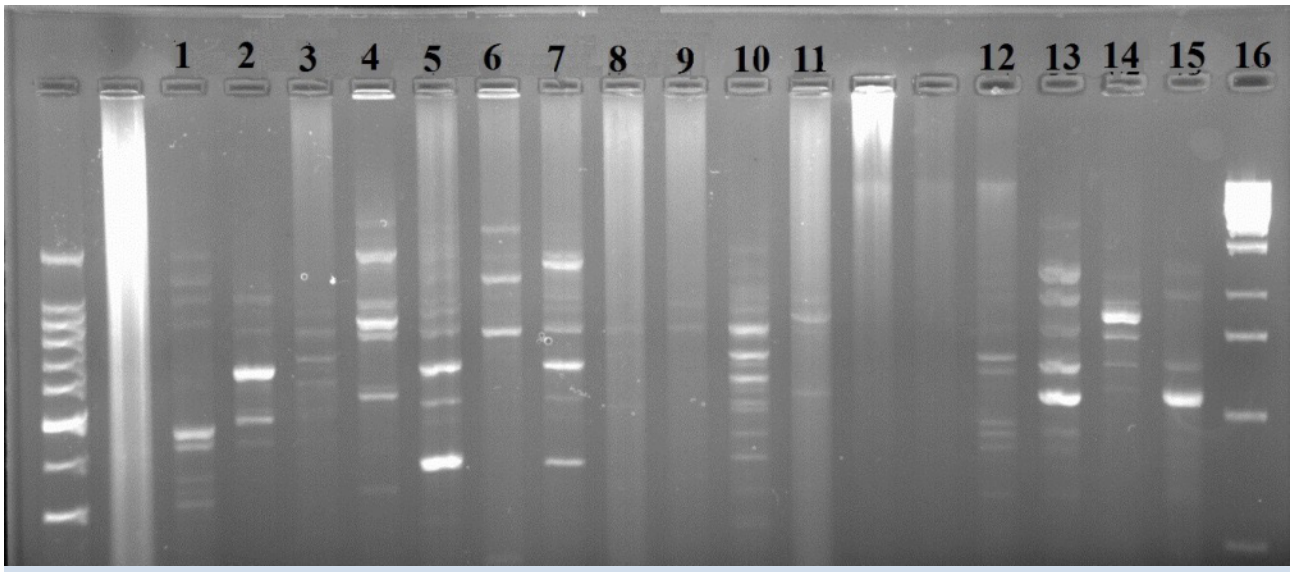


**Figure 3.** Agarose gel electrophoresis of RAPD-PCR products of *Lactococcus garvieae* isolates. A (lanes 1-16) that show different patterns of bands ranging from 220 to 2,500 bp in size, First lane on the left: 100bp DNA ladder and last lane on the right: 1kb DNA Ladder.

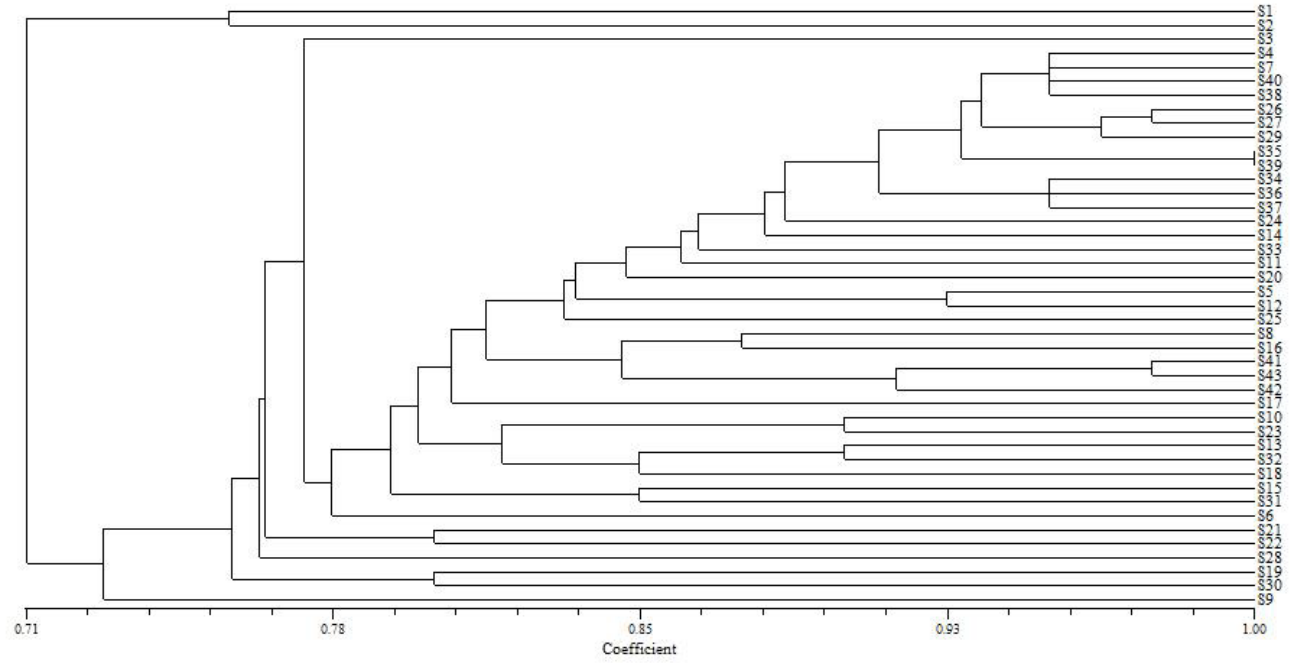


**Figure 4.** A dendrogram is drawn by using a Simple Match Algorithm with a Cofting coefficient of 0.777 for forty- three *L. garvieae* isolates.

## DISCUSSION

*Lactococcus garvieae* has been isolated from a wide range of fish, dairy animals and human samples (Vendrell *et al*., 2006; Fortina *et al*., 2007; Li *et al*., 2008). The pathogen has been recently associated with an increasing number of Lactococcosis infections in various kinds of fish particularly in farmed salmonid (Ravelo *et al*., 2003; Brunt and Austin, 2005). In recent years, it has also been the most significant cause of trout losses in Iran. There have been phenotypic typing and serotyping methods to identify and characterize the streptococcal isolates, but molecular assays such as classical ribotyping, PFGE, RAPD, and DNA sequencing techniques have been chosen as easy and fast techniques to detect and genotype *L. garvieae* strains (Magarinos *et al*., 2000; Altun et al., 2004; Ravelo et al., 2003). Among different mentioned methods, RAPD has been introduced as a reproducible test with a high differentiating rate, which has proved to be appropriate for the epidemiological analysis of a variety of bacteria, especially fish pathogens (Welsh *et al*., 1990; Ravelo *et al*., 2003; Foschino *et al*., 2008).

In the present study, specimens were collected from clinically infected rainbow trout in different geographical areas of Iran. Out of 200 isolates which were obtained from bacteriological tests, 43 isolates were identified as *L. garvieae* by PCR test and consequently characterized by RAPD-PCR assay with a universal M13 primer. Finally, the simple match coefficient was used to determine the genetic similarity of all isolates. Except for the 100% similarity observed between isolates 35 and 39, the lowest and highest percentages of similarity were found in the range of 54% and 98% among isolates. According to a dendrogram which has been drawn with a 71% similarity level, all isolates are placed into two groups A and B, whereas at an 85% similarity level, the number of branches has increased to 21 profiles (RAPD-types), which indicates a high genetic diversity among the studied isolates. Others have also studied the genetic characterization of this pathogen obtained from various sources of fish and animals with those different results previously being published. Our results displayed a close genetic relationship between isolates included in group B2 and obtained from just two sampling regions in Iran showing the highest genetic similarity between isolates in comparison with other regions. Pathogens can be distributed or transmitted by asymptomatic carriers or contaminated eggs into susceptible populations. Contaminated water and feed and also the presence of some leeches species in the aquaculture environments could have facilitated the *L. garvieae* dissemination (Woo and Bruno, 2011; Vendrell et al., 2006).

Foschino *et al.* (2008) used two universal M13 and P5 primers for genotyping of 81 *L. garvieae* strains from fish and dairy products using the RAPD-PCR method. M13 and P5 primers have the ability to differentiate 52 and 27 genotypes, respectively. All isolates were divided into 5 groups using the 52 RAPD types. They also showed that *L. garvieae* strains isolated from dairy samples were generally not related to those obtained from lactococcosis outbreaks in fish. The RAPD -PCR assay was used by Ravelo *et al*. (2003) to determine the genetic similarity of *L. garvieae* strains isolated from different geographic areas of the world. By applying two P5 and P6 primers, isolates were divided into three genogroups according to an analysis of 90%, 80% and75% similarity among different profiles. These isolates were isolated from rainbow trout and yellow-tail. Altun *et al.* (2013) evaluated RAPD PCR analysis for genotyping 12 *L. garvieae* isolates from Turkey, England and, Spain. After genotyping all isolates using ERIC2 primer, they were placed in three clusters according to a similarity coefficient index of 70%, and it was found that 66.6 % of isolates were related to the LG1 genotype.

Our results indicate that *L. garvieae* can originate from different sources with various genetic profiles. The M13 primer has been a highly productive potential primer to genotype *L. garvieae* isolates and the results agree with Foschino *et al*. (2008) and Altun et al. (2013). Bacterial samples were collected from different regions of Iran and this can increase the possibility of genetic variability of isolates derived from various sources of cultured freshwater and marine fish, cultured prawns, animals and food (Meyburgh *et al*., 2017; Wang *et al*., 2007; Tsai *et al*., 2012 ). On the other hand, trout farming is a fast-growing industry in Iran that it can increase distribution of the causative agent of lactococcosis in all aquaculture-related environment, as well as other places, have access to fish farms, and consequently raising the exposure of other animals to get the lactococcus infections and tend to enhances the pathogenesis and outbreak of the disease. According to Vela *et al*. (2000) phenotypic and genotypic characterization of *L. garvieae* isolates from some European countries originated from different sources of fish, animals, and human showed a low genetic similarity between them. That suggested

diverse infection sources for the different lactococcosis outbreaks. RAPD-PCR assay has been used as a highly robust test in genome scanning by genetic qualification and comparison among species and isolates (Belkum and Meis, 1994). This method enables the detection of genetic relationships amongst bacterial fish pathogens with a high differentiating rate (Magarinos *et al*., 2000; Foschino *et al*., 2008; Findik *et al*., 2011).

## CONCLUSION

High genetic diversity among all isolates indicates the necessity of studying cultured fish in different ways and according to their sources. This shows that the pathogen has an expanded genetic variation which plays a significant role in the prevalence of lactococcosis in the aquaculture industry. Knowledge of the geographical distribution of isolates and genetic diversity data of this pathogen will help us to design new products such as vaccines based on the DNA components of bacteria and continue to deliver practical ideas to control the disease. The finding of the present study suggests that RAPD-PCR can be a simple and efficient method to map the distribution of the pathogen.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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