# Molecular identification of some causative agents of warm-water streptococcosis by M-PCR in cultured rainbow trout, Chaharmahal - Bakhtiari Province, Iran

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**Abstract**

Streptococcosis has been defined as a hemorrhagic septicemia in many fish species especially rainbow trout. This disease causes serious economic losses due to high mortality in fish and the bacteria responsible is also considered as a zoonotic pathogen. The aim of this study was identification of different causative agents of warm water streptococcosis and evaluation of the capsule gene in *Lactococcus garvieae* isolates collected from rainbow trout in Chaharmahal - Bakhtiari Province, Iran. A total of 430 clinically suspected fish were collected from 43 rainbow trout farms. The samples were analyzed using biochemical methods and multiplex-PCR. The results indicated that samples of 36 fish farms (83.7%) were infected with one of the studied bacteria. The infection by *L. garvieae* was found to be more than that by other species (30 of 36 farms, 83%). Infection by *S. iniae* and *S. uberis* was found in nine and one fish farm, respectively. Presence of virulence genes was studied in *L. garvieae* isolates by PCR assay. According to the results 34 of 50 isolates (68%) contained the capsule gene. The results revealed high prevalence of virulent *L. garvieae* in rainbow trout farms which should be considered in preventional policies such as vaccine production and also in public health.

**Keywords:** Streptococcosis, Virulence gene, Rainbow trout, PCR, Iran

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## Introduction

Streptococcosis in fish is a general term used to describe diseases caused by different species of Gram-positive cocci including *Streptococcus*, *Lactococcus*, and *Vagococcus* in both cold and warm water. The main pathogenic species responsible for warm water streptococcosis are *S. iniae*, *S. parauberis*, *S. uberis*, *S. difficilis* and *L. garvieae*. On the other hand, *V. salmoninarum* and *L. piscium* cause cold water streptococcosis (Klijn *et al*., 1995; Mata *et al*., 2004).

Streptococcosis was described for the first time at the end of the 50s in Japan, where the first cases were diagnosed in the intensive production of rainbow trout (Hoshina *et al*., 1958). Since then, 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 also Iran (Ghittino and Prearo, 1992; Palacios *et al*., 1993; Bark and Mc Gregor, 2001; Chang *et al*., 2002; Chen *et al*., 2002; Diler *et al*., 2002; Soltani and Tarahomi, 2008). *L. garvieae* and *S. iniae* are known as two important causative agents of warm water streptococcosis and the other species are less reported. In recent years, several cases of lactococcosis caused by *L. garvieae* have been reported in Iran (Akhlaghi and Keshavarzi, 2003; Soltani and Tarahomi, 2008; Raissy and Ansari, 2011; Soltani *et al*., 2011). This species is an emerging zoonotic pathogen which has been isolated from different species of fish including rainbow trout (Carson *et al*., 1993; Eldar *et al*., 1996; Elliot and Facklam, 1996; Soltani and Pirali, 2012). It has also been collected from cattle (Collins *et al*., 1983), poultry meat (Barakat *et al*., 2000), and from human (Wang *et al*., 2007).

Regardless the causative agent, warm water streptococcosis leads to severe economic losses because of high mortality rates and reducing the growth rates. In addition, infected fish will be unmarketable because of their appearance (Ghittino and Prearo, 1992). The mortality rate depends on environmental conditions, water quality and fish density (Prieta *et al*., 1993).

The aim of this study was to identify different causative agents of warm water streptococcosis and detect the virulence genes in the *L. garvieae* isolates collected from rainbow trout in Chaharmahal - Bakhtiari Province, Iran.

## Materials and method

### *Sampling*

A total of 430 moribund fish were collected from 43 rainbow trout farms during summer and autumn 2013. The samples were immediately transferred to the Fishery Research Center, IAU, Shahrekord in appropriate conditions.

### *Bacterial strains*

Samples of kidney, spleen and liver were aseptically cultured on brain heart infusion agar medium (BHIA) supplemented with 5% defibrinated sheep blood. Inoculated plates were incubated at 25°C for 24-72 h for microbiological studies.

Standard morphological and biochemical tests were performed at 25°C according to Austin and Austin (1999) (Table 1). The isolates were then confirmed using PCR technique as described by Zlotkin *et al*. (1998) (Table 2).

**Table 1: Biochemical characteristics of *L. garvieae*, *S. iniae* and *S. uberis* isolates. V= variable results, += positive, -= negative.**

| Test | *L. garvieae* | *S. iniae* | *S. uberis* |
|---|---|---|---|
| **Gram staining** | + | + | + |
| **Morphology** | Cocci | Cocci | Cocci |
| **Hemolysis** | α | β | A |
| **Catalase** | - | - | - |
| **Oxidase** | - | - | - |
| **VP** | - | - | + |
| **Motility** | - | - | - |
| **Lactose** | V | - | + |
| **Glucose** | + | + | + |
| **Mannitol** | + | + | + |
| **Maltose** | + | V | + |
| **Arabinose** | - | - | - |
| **Sucrose** | + | + | + |
| **Arginine** | + | + | + |
| **Esculine** | + | + | + |
| **Urea** | - | - | V |
| **Sorbitol** | + | - | + |

**Table 2: Primer sequences, targeting gene and amplicon size of primers.**

| Bacterial species | Targeting Gene | Primer sequence | PCR Amplicon | References |
|---|---|---|---|---|
| *Streptococcus iniae* | *lctO* | F (5'- AAGGGGAAATCGCAAGTGCC -3')<br>R (5'- ATATCTGATTGGGCCGTCTAA -3') | 870 bp | Mata *et al.*, 2004 |
| *S. parauberis* | *23S rRNA* | F (5'-TTTCGTCTGAGGCAATGTTG-3')<br>R (5'-GCTTCATATATCGCTATACT-3') | 718 bp | Mata *et al.*, 2004 |
| *S. uberis* | *16S-23S RNA intergenic spacer* | F (5'- TAAGGAACACGTTGG TTAAG-3')<br>F (5'- TCCAGTCCTTAGACCTTCT-3') | 201 bp | Mata *et al.*, 2004 |
| *Lactococcus garvieae* | *16S rRNA* | F (5'-CATAACAATGAGAATCGC-3')<br>R (5'-GCACCCTCGCGGGTTG-3') | 1100 bp | Zlotkin *et al*., 1998 |
| *L. garvieae virulence gene* | *Capsule gene* | F (5'-GCTGTCATCATATTGTGTCCA-3')<br>F (5'-CTATGGCATTAGTCAGGAAG-3') | 747 bp | Miyauchi *et al*., 2012 |

*DNA Extraction and PCR assay*

The genomic DNA was extracted using phenol-chloroform method according to Ausubel *et al.* (1978). Briefly, the bacteria were grown in Trypic Soy Broth containing 1% sodium chloride at 30°C for one night. 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 mM Tris–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 hexadecyl trimethyl 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 mM Tris-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.

The PCR reaction was performed using PTC-100 thermo cycler (Eppendorf, Germany) in a 50 µL reaction system consisting of 2 µL of genomic DNA (50 ng/µL), 5 µL of 10× PCR buffer (100 mM Tris–HCl, pH 8.3, 500 mM KCl, 60 mM $MgCl_2$, 0.1% gelatin and 1% Triton X-100), 1 µL of each primer (50 pmol/µL), 1 µL each of the 10 mM dNTPs, 0.2 µL units Taq DNA polymerase (5 units/µL) and 40 µL of sterile distilled water. Multiplex-PCR operation for identification of the isolates was performed according to the following temperature-cycling parameters: an initial denaturation step at 94°C for 2 min; 25 serial cycles of a denaturation step at 92°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 90 s; and a final extension step at 72°C for 5 min. Amplified products were separated by electrophoresis in ethidium bromide stained with 1.5% agarose gels at 90 V for 50 min. The gels were visualized and photographed with a UV transilluminator. The sequences were then blasted against available sequences from GenBank database for exact identification.

*Detection of virulence genes*

Capsule gene which is important virulence factor was identified in the *L. garvieae* isolates by PCR. The PCR cycling conditions were as follows: 95˚C for 3min, followed by 30 cycles of 95˚C for 30 sec, 62˚C for 30 sec, 68˚C for 5min, and 72˚C for 10 min. The sequence of the specific primers and the amplicon size are mentioned in Table 2.

## Results

In this study, 430 fish from 43 rainbow trout farms (10 fish from each farm) were studied using biochemical tests and PCR. The majority of samples were positive with M-PCR in agreement with culture and biochemical analysis.

The results revealed that samples of 36 fish farms (83.7%) were infected with one of the bacteria studied. The infection rate with *L. garvieae* was found to be more than other species (30 fish farms). *S. iniae* and *S. uberis* were isolated from fish in nine and one fish farm, respectively. *S. parauberis* was not isolated from fishes. Electrophoresis of the isolates is shown in Fig. 1.

Presence of the capsule gene which is a virulence factor was studied in *L. garvieae* isolates by PCR assay. The results indicated that 34 of the 50 isolates studied contained the capsule gene. The results are shown in Table 2. Results of electrophoresis of the virulence gene are indicated in Fig. 2.

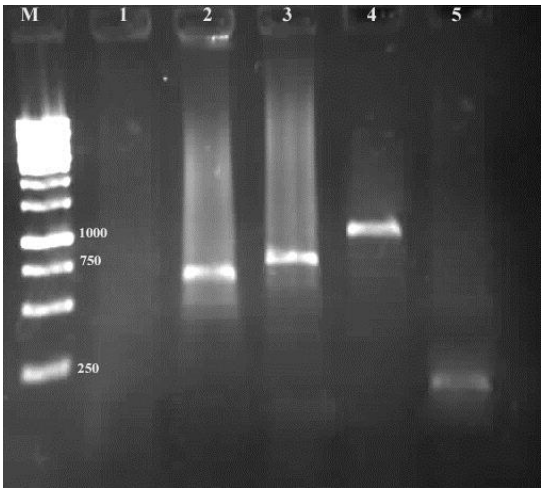


**Figure 1: Results of electrophoresis of the isolates. M: Marker (100bp), lane 1: negative control, lane 2: *S. Parauberis*, lane 3: *S. iniae*, lane 4: *L. garvieae*, lane 5: *S. uberis***

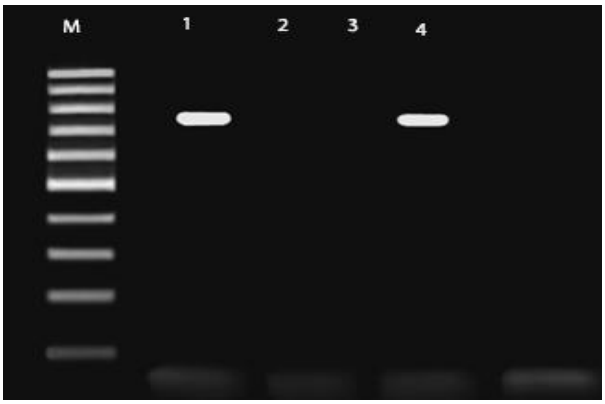


**Figure 2: Electrophoresis of the virulence genes of *L. garvieae* isolates. M: Marker (100bp), lane 1, 4: Positive samples, lane 2, 3: negative samples.**

**Discussion**

Warm water streptococcosis is an important bacterial disease associated with high mortality in fish and accordingly high economic losses. 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 (Ghittino and Prearo, 1992 Palacios *et al.*, 1993; Bark and Mc Gregor, 2001; Chang *et al*., 2002; Chen *et al*., 2002; Diler *et al*., 2002; Soltani and Tarahomi, 2008). In Iran, this species was firstly reported by Akhlaghi and Keshavarzi (Akhlaghi and Keshavarzi, 2003). In recent years, *L. garvieae* has been widely spread in rainbow trout fish farms mainly because of development of intensive culture so that it has been the most prevalent bacteria in rainbow trout fish farms in Iran (Raissy and Ansari, 2011).

Different species of *Streptococcus* including *S. iniae, S. uberis, S. parauberis, S. agalactiae, S. difficilis* and *L. garvieae* are known as the causative agents of streptococcosis in fish. From the above-mentioned species, *L. garvieae* and *S. iniae* have been reported more frequently (Soltani *et al.*, 2011). Lactococcosis and streptococcosis are characterized by similar clinical signs including darkening of skin, lethargy, exophthalmia, clouding of the cornea and hemorrhage of the internal organs. PCR is considered more accurate as it targets unique sequences of genome of the bacteria. PCR has been previously developed for the identification of *L. garvieae* using a pair of primers specific to target *16S rRNA* gene (Zlotkin *et al*., 1998). In recent years, M-PCR assay has been developed for simultaneous detection of *S. iniae*, *S. difficilis, S. parauberis*, and *L. garvieae* (Mata *et al*., 2004) and *S. iniae, S. parauberis* and *L. garvieae* (Baeck *et al*., 2006).

In the present study the M-PCR assay was used for detection of *S. iniae, S. uberis, S. parauberis* and *L. garvieae*, which cause great loss and are threats to fish farms.

The results revealed that fish from 30 farms were infected by *L. garvieae*. Soltani and Tarahomi (2008) reported that 20% of 600 Gram-positive cocci isolated from cultured rainbow trout were *L. garvieae* and the remaining were *Streptococcus* sp. In another study, 11 isolates of a total of 20 Gram-positive cocci isolated from rainbow trout in Chaharmahal - Bakhtiari Province was identified as *L. garvieae* which is in agreement with the results of this study (Mirzakhani, 2009).

In this study, the disease was mostly observed in fish farms located along the rivers at a close distance from each other. The majority of the fish farms along the river release their waste water without further treatment resulting in transferring the pathogens along the river to other fish farms at the lower parts of the river. Distribution of the isolates in the fish farms is mentioned in Table 3.

**Table 3: Distribution of the isolates in fish farms**

| ***Lactococcus garvieae* isolate** | **Fish farm** | **Capsule gene** |
|---|---|---|
| Lg1 & Lg2 & Lg3 | Fish farm 1 | + |
| Lg4 & Lg5 | Fish farm 2 | + |
| Lg6 & Lg7 | Fish farm 3 | + |
| Lg8 & Lg9 & Lg10 | Fish farm 4 | + |
| Lg11 & Lg12 & Lg13 & Lg14 | Fish farm 5 | - |
| Lg15 & Lg16 & Lg17 & Lg18 & Lg19 | Fish farm 6 | - |
| Lg20 & Lg21 & Lg22 | Fish farm 7 | - |
| Lg23 & Lg24 & Lg25 | Fish farm 8 | + |
| Lg26 | Fish farm 9 | + |
| Lg27 & Lg28 & Lg29 & Lg30 | Fish farm 10 | + |
| Lg31 & Lg32 & Lg33 | Fish farm 11 | + |
| Lg34 | Fish farm 12 | - |
| Lg35 &Lg36 | Fish farm 13 | + |
| Lg37 & Lg38 & Lg39 | Fish farm 14 | + |
| Lg40 | Fish farm 15 | + |
| Lg41 & Lg42 & Lg43 & Lg44 | Fish farm 16 | + |
| Lg45 & Lg46 & Lg47 | Fish farm 17 | + |
| Lg48 | Fish farm 18 | + |
| Lg49 & Lg50 | Fish farm 19 | - |

It is proved that the outbreak of streptococcosis in particular is mostly related to water quality and other environmental factors. In the present study, the obtained results strongly support the connection between water quality and outbreak of the disease. Because when the disease occurred, the mean water temperature was over 17°C (17.6°C) and the pools were also overcrowded (Bark and Mc Gregor, 2001).

The pathogenicity of *L. garvieae* is poorly understood. It has been proven that virulence of these bacteria is, in part, dependent on its ability to form a capsule (Villani *et al*., 2001). Barnes *et al*. (2002) reported capsule in some *L. garvieae* isolated and indicated virulence of the capsulated strains.

In this study 34 of 50 examined *L. garvieae* contained the capsule gene, however all the isolates were collected from the infected fish with clinical signs. The pathogenicity of non-capsulated strains is attributed to other virulence genes such as haemolysin, adhesion, phosphoglucomutase and superoxide dismutase (Miyauchi *et al*., 2012). Another study showed that no capsule was detected in *L. garvieae* isolated from human with endocarditis. The pathogenicity of non-capsulated strains in patients was attributed to adhesion genes producing adhesion proteins which may be involved in the host specificity differences of *L.*

*garvieae*. It is also possible that some strains lose the capsule gene cluster during subculturing in synthetic media (Kawanishi *et al*., 2007). These explain how non-capsulated strains may still be pathogenic for fish.

In this study, 3 species causing streptococcosis were identified by multiplex-PCR. The results revealed high prevalence of capsulated *L. garvieae* in rainbow trout farms which should be considered in preventional policies such as vaccine production.

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