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# Identification of some streptococcus species isolated from rainbow trout (Oncorhynchus msykiss) in Iran by using molecular method

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**ABSTRACT:** The syndrome of streptococcosis has been associated with outbreaks in rainbow trout (Oncorhynchus mykiss) and caused significant economic losses in the aquaculture industry in Iran in recent years. The main purpose of this work was molecular identification of some streptococcus species in rainbow trout. A total of 485 samples were collected from the head kidney of diseased fish (weight, 50–200g) in four provinces of Iran, during 2011 to 2012. DNA extraction was carried out from a single colony by using the extraction promega kit following the conditions described by the supplier. The PCR assay was developed based on the 16S rRNA and glucose kinase genes of Streptococcus spp. Consequently, four streptococcus species have been identified, including S. iniae in Fars province, S. agalactiae in Gilan province, S. dysgalatiae in Kohgiluyeh and Gilan provinces and S. uberis which was common in all these provinces. The dominant species (based on important species index) were S. uberis, S. dysgalactiae and S. agalactiae, respectively.

***Keywords*:** Iran, Streptococcus, Rainbow trout, PCR, DNA

## INTRODUCTION

Todays, with an increase in water pollution and intensive aquaculture expansion, there are increasingly numerous of fish diseases that appear in freshwater fishes and cause severe economic losses every year from countries around the world. Among fish diseases, bacteria are the most important causative agents of losses in fish farming industry (Yang and Li, 2009). Based on several reports, fish stretptococcosis is currently considered as one of the main limiting factors in the aquaculture industry, due to the significant economic losses (annually more than $150 million) that these infections cause in different cultured fresh and seawater fish species worldwide (Shoemaker et al., 2006; Garcia et al., 2008; Romalde et al., 2008).

To date, examples of Streptococcus species that have been associated with disease in fish include: S. iniae, S. agalactiae, S. parauberis, S. dysgalactiae S. faecium, S. milleri, S. uberis, S. ictaluri, S. phocae and S. faecalis, (Shewmaker et al., 2007; Romalde, et al., 2008; Yang and Li 2009). Streptococcal disease in fish was first reported in 1957, affecting cultured rainbow trout in Japan (Hoshina et al., 1958). Since then, numerous other species of fish have been found susceptible to this infection (Buller, 2004; Klesius et al., 2006; Vendrell et al., 2006; Austin and Austin, 2007). Streptococcosis in fish can cause high mortality rates (more than 50%) over a period of 3 to 7 days. (Yanong and Francis-Floyd ,2002) mentioned that some outbreaks are more chronic in nature and mortalities may extend over a period of several weeks, with only a few fish dying each day. Bunch and Bejerano (1997) suggested that Streptococcus spp. is an opportunistic pathogen because it is wide spread in the aquaculture environment and because of its dependence on stress to assert pathogenicity.

Molecular diagnostic techniques, such as PCR assays, are increasingly used to detect and identify many different bacterial species including the most significant fish pathogens such as Streptococcus species. Many of the PCR assays use the 16S rRNA gene as target molecule (Blanco et al., 2002; Mata et al., 2004). Molecular identification methods are a powerful alternative to the conventional differentiation of bacteria by plating especially when closely related species are analyzed. Detecting and identifying various species with rapid methods is also important for in vivo monitoring. At species level there are several reports on specific identification systems, mainly

based on 16S ribosomal RNA gene (rRNA) (Blaiotta et al., 2002). For instance, (Edler ,1997) reported that *S. iniae* is a well-known pathogen of both fish and humans that is difficult to identify by conventional biochemical tests. He also mentioned that the PCR was also effective in detecting the bacterium from inoculated tissue homogenates, suggesting its potential use for a rapid and accurate diagnosis of S. iniae infections. S. iniae isolated from tilapia and trout in the United States were subtyped by restriction length polymorphism (RFLP) based on PCR amplified 16S rDNA and by ribotyping. 16S rDNA RFLP discriminated between S. iniae and other fish pathogens but not between S. iniae strains (Mata et al., 2004). (Mian and co-worker ,2009) analyzed aspects of the epidemiology, transmission and virulence of S. agalactiae infections, nine outbreaks of meningoencephalitis and septicemia in Nile tilapia farms in Brazil. They isolated S. agalactiae from diseased fish from all farms, and 29 strains were identified by phenotypic tests and 16S rRNA gene sequencing.

Rainbow trout is a large economic fish in Iran and there are increasingly artificial breeding farms of this fish species. However, with rapid expanding production, problems of their diseases become more complex and serious. Over the past few years, Streptococcus spp. has been associated with outbreaks of disease in this species. Streptococcosis was first reported from cultured rainbow trout in Mazandaran province (north of Iran) by (Ghiasi et al.,2000). Since then, the disease has been reported from some other provinces (Akhlaghi and Keshavarzi, 2002; Soltani et al., 2005, 2008; Saeedi et al., 2009; Pourgholam et al., 2010). The aim of the present study was distribution and molecular identification of some causative agents of streptococcosis isolated from main rearing regions of farmed rainbow trout in Iran

The aim of present study was Identification of some streptococcus species isolated from farmed rainbow trout (Oncorhynchus msykiss, Walbaum) in Iran by using molecular method.

## MATERIALS AND METHODS

### *Sample collection and bacterial isolation*

A total of 485 samples were collected aseptically from the head kidney of diseased and moribund or freshly dead rainbow trout, Oncorhynchus mykiss, Walbaum (weight, 50–200 g) in cold freshwater fish farms of 4 provinces (Chaharmahal, Fars, Kohgiluyeh and Gilan) during 2011 to 2012 (Table1). Samples were streaked onto brain heart infusion agar (BHA; Merck, Germany) or soy agar (TSA; Merck, Germany) plates and incubated aerobically at 25ºC± 3ºC to determine the presence or absence of the bacterial isolates in the fish according to previously published procedures (Buller, 2004; Austin and Austin, 2007). Final results were read 72h after inoculation and compared with the biochemical profiles. All isolates, selected from pure or dominant colonies on TSA, were subjected to primary testing by Gram stain, %3 KOH and catalase tests. Pure culture of three isolates per plate was stored at −80˚C in %20 glycerol (final concentration) supplied with nutrient broth (NB).

### *Biochemical characterization*

Biochemical characterization was performed with minor modifications according to Buller (2004). Specifically, hemolytic experiments were conducted at 25ºC 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.

### *DNA isolation and PCR amplification*

Bacterial isolates representing morphology and biochemical profiles of streptococcus spp. were further confirmed by polymerase chain reaction (PCR). DNA extraction was carried out from a single colony by using extraction kit (Promega, USA) following the conditions described by the supplier. A PCR assay based on the 16S rRNA and glucose kinase genes of *Streptococcus spp*. was developed for the rapid and specific detection and identification of this pathogen from different sources. Five primers were designed to amplify the 16S rRNA and glucose kinase genes by Generuner software (Table 1).

Amplification of each DNA sample was performed in a 25 µl reaction mixture containing X PCR buffer (10 mM Tris- HCl pH 9.0, 50 mM KCl, 0.01% gelatin), 0.2 mM dNTPs, 2.0 mM $MgCl_2$, 5 pmol of each primer, 1.5 units of Taq DNA polymerase and 25–50 ng of DNA. The amplifications were carried out in a Quanta biotech thermal cycler set with the following parameters: 5 min of initial denaturation at 94˚C followed by 30 cycles of 30 s at 94˚C, 45s at the

annealing temperature and 1 min at 72˚C. A final elongation of 4 min at 72˚C was added. The amplified products were stored at 4˚C. Band patterns were photographed under UV light. The amplified products were resolved on 2% agarose gels using a TBE buffer system. The size of the restriction fragments was estimated by comparison to a 50-bp-size ladder (Table 2).

Table 1. Primers characteristics and sequences used in this study

| Primers | Sequence (5`…..3`) | Gene | Annealing |
|---|---|---|---|
| STRP | GCCCAGACTCCTACGGGAGG<br>CCGCCTGCGCTCGCTTTACG | 16S RNA | 69˚C |
| STRA | CTAACCCATTCGTTCTATGCC<br>CATGACTGATGTTGTCAAAAC | Glucose kinas | 64˚C |
| Bac RNA | CGAGCTGACGACAACCATGCACCACCTGTC<br>GACCGAGCAACGCCGCGTGAGTGAAGAAG | 16S RNA | 66˚C |
| ENR | AGTCTGACCGAGCAACGCCG<br>CAATTCCTTTGAGTTTCAACC | 16S RNA | 64˚C |
| STRP1 | CTCTGTCCCGAAGGAAAATC<br>AACTAACCAGAAAGGGACGG | 16S RNA | 57˚C |

Table 2. Sizes of DNA fragments in each species by different primers (in base pairs)

| Primers | S. iniae | S. dysgalactiae | S. agalactiae | S. uberis | S. parauberis |
|---|---|---|---|---|---|
| Bac RNA | - | 675 | - | 675 | 675 |
| ENR | - | - | - | - | 540 |
| STRA | - | - | 430 | - | - |
| STRP | - | 260* | - | 260* | - |
| STRP1 | 554 | - | - | - | - |

* The PCR product digests with restriction enzyme DraIII, if size of fragment is 110bp and 150bp, the sample will be S. dysgalactiae and if the enzyme doesn't have cut site, the sample will be S. uberis

### *Results*

Bacterial isolation and biochemical characterization After bacterial isolation and primary testing by Gram stain, %3 KOH and catalase tests on all samples (485 samples), 206 positive specimens (various species of streptococcus) were obtained. Following biochemical characterization of pure or dominant colonies, four species were determined including S. uberis, S. agalactiae, S. dysgalactiae and S. iniae .

### *Molecular identification*

Following PCR assay 4 Streptococcus species have been identified and confirmed, including S. iniae species in Fars province, S. agalactiae in Gilan province, S. dysgalatiae in, Kohgiluyeh and Gilan provinces and S. uberis species which was common in all these provinces (Figure1 and Table 3). Based on important species index (ISI) (Rushforth and Brock ,1991), the dominant species were S. uberis, S. dysgalactiae and S. agalactiae, respectively.

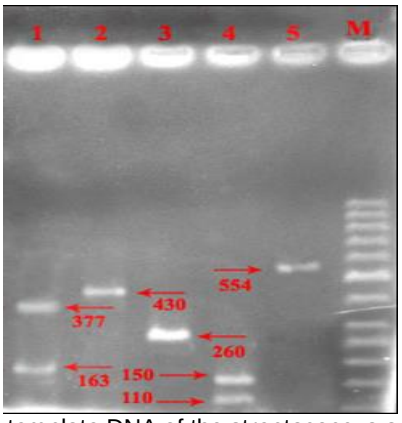


Fig. 1. Representative PCR products from template DNA of the streptococcus species following the bacterial isolation from rainbow trout. Lane 1: S. parauberis, Lane 2: S. agalactiae, Lane 3: S. uberis, Lane 4: S. dysgalactiae, Lane 5: S. iniae, Lane M: Marker

Table 3. Differentiation of various *Streptococcus* species according to PCR reaction

| Origin | No of samples | Primers | | | | | Digestion patterns | Final specification |
|---|---|---|---|---|---|---|---|---|
| | | Bac RNA | ENR | STRA | STRP | STRP1 | | |
| Chaharmahal | 30 | 675 | - | - | 260 | - | DraIII: no anded | S. uberis |
| Gilan | 3 | 675 | - | - | 260 | - | DraIII: no anded | S .uberis |
| Gilan | 19 | - | - | 430 | - | - | - | S .agalactiae |
| Gilan | 8 | 675 | - | - | 260 | - | DraIII: 110 and 150 bp | S. dysgalactiae |
| Kohgiluyeh | 15 | 675 | - | - | 260 | - | DraIII: no anded | S. uberis |
| Kohgiluyeh | 35 | 675 | - | - | 260 | - | DraIII: 110 and 150 bp | S. dysgalactiae |
| Fars | 16 | 675 | - | - | 260 | - | DraIII: no anded | S .uberis |
| Fars | 11 | - | - | - | - | 554 | - | S. iniae |

***Discusion***

Over the past few years, streptococcosis has been the most prevalent infectious bacterial disease in cold freshwater fish (rainbow trout) farms in Iran. This is a serious problem which causes economic losses every year in aquaculture industry, as it was reported by (Akhlaghi and Keshavarzi, 2002; Soltani et al., 2005, 2008; Saeedi et al., 2009; Pourgholam et al., 2010, in press) in some provinces of Iran.

Streptococcosis in rainbow trout is caused by many Streptococcus species such as S. iniae, and S. agalactiae and several other closely related groups of bacteria including L. garvieae, L. piscium; and Vagococcus salmoninarum (Buller, 2004). However, in the present study, approximately 40% of specimens were infected to Streptococcus species. Consequently, 4 streptococcus species have been identified, including S. iniae, S. agalactiae, S. dysgalatiae and S. uberis.

According to reports of other researchers, S. iniae is the main causative agent of streptococcosis in wild and farmed fish worldwide. It has been associated with disease outbreaks in aquaculture farms of different fresh and seawater commercial fish species (Shoemaker et al., 2006; Russo et al., 2006; Klesius et al., 2006; Pasnik et al., 2006; Evans et al., 2006; Klesius et al., 2007; Garcia et al., 2008; Suanyuk et al., 2008). Despite of reports with high frequency regarding S. iniae from other countries (Yang and Li, 2009) in the present study, it was isolated with low frequency compared to other observed species.

S. agalactiae infections have been reported in many fish species which are responsible for severe economic losses in wild and cultured fish worldwide.

S. dysgalactiae was isolated from moribund Amur sturgeon, Acipenser schrenckii, farmed with high density in central China by Yang and Li, 2009. This species was also isolated from cultured fish in Japan (Nomoto et al., 2004, 2006), despite the concerning reports of S. dysgalactiae with low frequency among Streptococcus species as fish pathogen (Yang and Li 2009). In the present research, S. uberis and S. dysgalatiae were isolated with high frequency compared to other observed species.

As much as we know, there are no reports regarding disease outbreaks of S. uberis in fish from other countries. In this work, it was isolated from diseased fish by significant clinical signs and sometimes with high mortality in most provinces. Therefore, this is probably the first report of disease outbreaks of S. uberis in this region.

Some isolates might be unidentified or misidentified by traditional identification system and biochemical characterization, thus we need to use more accurate procedures such as molecular identification. PCR assay and 16S rRNA gene sequencing could be a useful tool to identify and confirm the genus and species of Streptococcus. It is often very important for identifying pathogenic agents concerning disease diagnosis (Weinstein et al., 1997; Hassan et al., 2001; Blaiotta et al., 2002). In this method, we are able to detect the disease agents even before clinical signs would appear. To our knowledge, this is the first report of identification of the different Streptococcus species which was performed by designing special primers and the PCR method in Iran. The results of this study indicated that the PCR assay is a reliable, specific and sensitive method for accurate identification of this microorganism isolated from different sources

In conclusion, four streptococcus species have been identified, including S. iniae in Fars province, S. agalactiae in Gilan province, S. dysgalatiae in Kohgiluyeh and Gilan provinces and S. uberis common in all these provinces. In addition, the dominant species were S. uberis, S. dysgalactiae and S. agalactiae, respectively.

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