Distribution and Molecular identification of some causative agents of streptococcosis isolated from farmed rainbow trout *(Oncorhynchus mykiss*, Walbaum) in Iran

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Abstract

Over the past few years, the syndrome of streptococcosis has been associated with outbreaks in rainbow trout (*Oncorhynchus mykiss*, Walbaum) and caused significant economic losses in the aquaculture industry in Iran. The main purpose of this work was molecular identification of some causative agents of streptococcosis in rainbow trout. A total of 520 samples were collected from the head kidney of diseased fish (weight, 50–200g) in 72 farms of 8 provinces in Iran, during 2008 to 2009. 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 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. for the rapid and specific detection and identification of this pathogen from different sources. Approximately 40% of specimens were infected to *Streptococcus spp*. Consequently, five pathogenic species have been identified, including *S. iniae* in Fars province, *S. faecium* in Mazandaran province, *S. agalactiae* in Gilan and Mazandaran provinces, *S. dysgalatiae* in Lorestan, Kohgiluyeh and Boyerahmad, Gilan and Kermanshah provinces and *S. uberis*, which was common in all provinces (except Mazandaran and Lorestan). The dominant species (based on important species index) were *S. uberis*, *S. dysgalactiae* and *S. agalactiae*, respectively.

Keywords: Iran, Streptococcosis, Rainbow trout, Syndrome, PCR, DNA

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Introduction

Nowadays, 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). The most prevalent disease-causing bacteria are Gram-negative; however, there are still several kinds of Gram-positive bacterial pathogens of fish, such as bacteria, which causes the syndrome of streptococcosis (Yang and Li, 2009).

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). In addition to bacteria in the genus *streptococcus*, there are several other closely related groups of bacteria that can cause similar diseases such as: *Lactococcus garvieae*, *L. piscium* and *Vagococcus salmoninarum* (Buller, 2004; Austin and Austin, 2007).

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 pathogens 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 Israel and 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.

Materials and methods

Sample collection and bacterial isolation

A total of 520 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 72 cold freshwater fish farms of 8 provinces (Chaharmahalbkhtiary, Mazandaran, Lorestan, Fars, Kohgiluyeh and Boyerahmad, Gilan, Kermanshah and Tehran in north, west and center of Iran, during 2008 to 2009 (Table1). Samples were streaked onto brain heart infusion agar (BHA; Merck, Germany) or triptycase soy agar (TSA; Merck, Germany) plates and incubated aerobically at 25°C± 3°C 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).

Table 1: Farmed rainbow trout production in different provinces of Iran (Iranian Fisheries Organization, Annual report, 2007)

No	Province	Production (tone)	No	Province	Production (tone)
1	Chaharmahal & bakhtiari*	10043	16	Eilam	960
2	Mazandaran*	8097	17	Ghazvin	933
3	Lorestan*	7994	18	Ardebil	745
4	Fars*	4600	19	Zanjan	659
5	Azarbaijan gharbi	4420	20	Khorasan shomali	431
6	Kohgiloyeh & boyerahmad**	3820	21	Kerman	382
7	Tehran**	2475	22	Ghom	332
8	Esfahan	1867	23	Yazd	265
9	Azarbaijan sharghi	1621	24	Golestan	263
10	Markazi	1601	25	Semnan	215
11	Hamedan	1576	26	Khorasan jonobi	156
12	Kermanshah**	1497	27	Sistan & Balochestan	71
13	Gilan**	1296	28	Hormozgan	12
14	Khorasan razavi	1240	29	Khozestan	5
15	Kordestan	1185	30	Booshehr	-
Total					58761

* These provinces were the main regions for sampling; because 80% of the total production of rainbow trout belongs to them.

** These provinces were also selected for sampling due to reports of disease outbreaks from these regions by farmers.

Table 2: Primers characteristics and sequences used in this study

Primer	Sequence (5`…..3`)	Gene	Annealing
STRP	GCCCAGACTCCTACGGGAGG CCGCCTGCGCTCGCTTTACG	16S RNA	69˚C
STRA	CTAACCCATTCGTTCTATGCC CATGACTGATGTTGTCAAAAC	Glucose kinas	64˚C
Bac RNA	CGAGCTGACGACAACCATGCACCACCTGTC GACCGAGCAACGCCGCGTGAGTGAAGAAG	16S RNA	66˚C
ENR	AGTCTGACCGAGCAACGCCG CAATTCCTTTGAGTTTCAACC	16S RNA	64˚C
STRP 1	CTCTGTCCCGAAGGAAAATC AACTAACCAGAAAGGGACGG	16S RNA	57˚C

Table 3: Sizes of DNA fragments in each species by different primers

Primer	Sizes of fragments (in base pairs)					
	S. iniae	*S. dysgalactiae*	*S. agalactiae*	*S. uberis*	*S. faecium*	*S. parauberis*
Bac RNA	-	675	-	675	675	675
ENR	-	-	-	-	540*	540*
STRA	-	-	430	-	-	-
STRP	-	260**	-	260**	-	-
STRP1	554	-	-	-	-	-

* The PCR product digests with restriction enzyme *XhoI*, if size of fragment is 163bp, the sample will be *S. parauberis* and if the enzyme doesn't have cut site, the sample will be *S. faecium*.

** 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*.

Biochemical characterization

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

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 from genomic DNA of 172 wild streptococcal gram-positive catalase-negative strains isolated from 8 provinces (Table 2).

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 3).

Results

Bacterial isolation and biochemical characterization

After bacterial isolation and primary testing by Gram stain, %3 KOH and catalase tests on all samples (520 samples), 206 positive specimens (various species of *streptococcus*) were obtained. Following biochemical characterization of pure or dominant colonies, five species were determined including *S. faecium S. uberis,* S. *agalactiae, S. dysgalactiae* and *S. iniae* (Table 4).

Molecular identification

Following PCR assay 5 pathogenic species have been identified and confirmed, including *S. iniae* species in Fars province, *S. faecium* species in Mazandaran province, *S. agalactiae* in Gilan and Mazandaran provinces, *S. dysgalatiae* in Lorestan, Kohgiluyeh, Gilan and Kermanshah provinces and *S. uberis* species which was common in all provinces (except Mazandaran and Lorestan) (Fig. 1 and Table 5). Based on important species index (ISI) (Rushforth and Brock, 1991), the dominant species were *S. uberis*, *S. dysgalactiae* and *S. agalactiae*, respectively.

Table 4: Biochemical characterizations of observed *Streptococcus* species

Parameters	*S. faecium*	*S. uberis*	*S. agalactiae*	*S. dysgalactiae*	*S. iniae*
Gram- staining	+	+	+	+	+
Catalase	-	-	-	-	-
haemolysis	α	-	-	+	+
arginine dihydrolase	+	+	-	-	-
Motility test	-	-	-	-	-
Nitrate reduction	-	-	-	-	-
Indole production	-	-	-	-	-
Citrate utilization	-	-	-	-	-
Urease production	-	-	-	-	-
Voges proskauer reaction	+	+	+	-	-
Aesculin hydrolases	+	+	-	-	+
Degradation of gelatin	-	-	-	-	-
ß-galactosidase production	+	-	-	-	-
Oxidative/ fermentative test	+	+	+	+	+
Acid production from: arabinose	+	-	-	-	-
" glucose	+	+	+	+	+
" inositol	-	-	-		-
" maltose	+	+	+	+	+
" manitol	+	+	-	-	+
" mannose	+	+	+	+	+
" salicin	+	+	-	-	+
" sorbitol	-	+	-	-	-
" trehalose	+	+	+	+	+
" xylose	-	-	-	-	-
H_2s	-	-	-	-	-
Growth at macconkey	+	-	-	-	-
Temperature	10 -50^{oC}	10 -37 ^{oC}	25 -37 ^{oC}	25 -37 ^{oC}	25 -37 ^{oC}
Growth on NaCl	0-%6.5	0-%6.5	0-%3	0-%4	2-%4
Hipporate sudium	+	+	+	-	-

Table 5: 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*
Mazandaran	1	-	-	430	-	-	-	*S. agalactiae*
Mazandaran	4	675	540	-	-	-	*XhoI*: no anded	*S. faecium*
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*
Kermanshah	3	675	-	-	260	-	*DraIII*: no anded	*S .uberis*
Kermanshah	7	675	-	-	260	-	*DraIII: 110 and 150 bp*	*S. dysgalactiae*
Lorestan	20	675	-	-	260	-	*DraIII: 110 and 150 bp*	*S. dysgalactiae*
Fars	16	675	-	-	260	-	*DraIII*: no anded	*S .uberis*
Fars	11	-	-	-	-	554	-	*S. iniae*

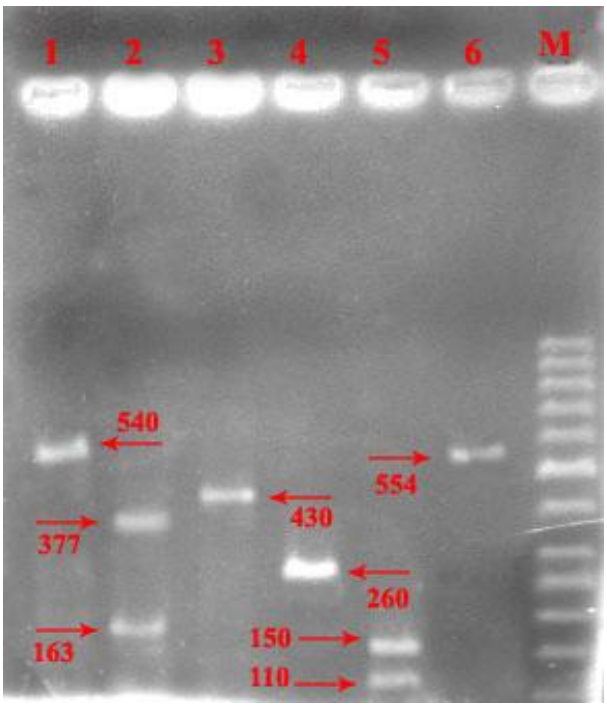

Fig. 1: Representative PCR products from template DNA of the streptococcos colonies following the bacterial isolation from diseased rainbow trout. Lane 1: *S. Faecium*, Lane 2: *S. parauberis*, Lane 3: *S. agalactiae*, Lane 4: *S. uberis*, Lane 5: *S. dysgalactiae*, Lane 6: *S. iniae*, Lane M: Marker

Discussion

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, 5 pathogenic species have been identified, including *S. iniae*, *S. faecium*, *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 (Evans et al., 2006; Klesius et al., 2006;

Pasnik et al., 2006; Russo et al., 2006; Shoemaker et al., 2006; Klesius et al., 2007; 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. In addition, Soltani et al. (2005) studied the biochemical and pathogenesis of *S. iniae* in farmed rainbow trout in Iran and indicated that, it was the causative agent of streptococcosis.

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.

To our knowledge, there are a few reports regarding disease outbreaks of *S. faecium* in fish from other countries. In this research, it was isolated from diseased fish by significant clinical signs and sometimes with high mortality, especially in Mazandaran province. Ghiasi et al. (2000) reported streptococcal disease in farmed rainbow trout from Mazandaran province. They suggested that *S. faecium* was the causative agent of disease outbreaks and the identification has been determined by morphological, physiological and biochemical assays. Austin and Austin (2007) noted that, the strain type of *S. faecium* has been determined to be pathogenic to salmonids in laboratory-based infectivity experiments. In addition, the pathogenicity of *S. faecium* was experimentally evaluated in rainbow trout by Pourgholam et al. (2010) which confirms the above discussion. Moreover, Pourgholam et al. (2010) also reported that, the histopathological and clinical findings were also similar to other *Streptococcus* 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.

Distribution of *Streptococcus* species was different in various ecological and geographical conditions. The difference was observed during our study in these provinces from northern, western and central parts of the country. Probably, this difference is somewhat related to various utilized water sources of cold water fish farms in these provinces. It means that some fish farms are supplied by river water, whereas some others use spring water. Distribution of various species in different climates and the relationship between species diversity and different ecological circumstances were obviously reported by Austin and Austin 2007; Suanyuk et al., 2008; Mian et al., 2009. It seems that, distribution of the

Streptococcus spp. is occurred more by transportation of eyed eggs, larva and fingerlings of trout in various geographical regions (different provinces), especially when a real quarantine facility is missing. Moreover, it can be spread to healthy fish by eating fish food made from streptococcal infected fish as reported by other researchers (McNultya et al., 2003; Russo et al., 2006).

These diseases maybe either chronic or acute and usually are associated with some type of stress. On the basis of culture conditions in the aforementioned provinces (water temperatures, water quality, stocking density and food quality) the mortality rate was between 20 to 50%, however, in acute cases it was sometimes increased to 70% as observed by Saeedi et al., 2009. Similar results have been reported by other researchers (Bunch and Bejerano, 1997; Shoemaker et al., 2000; Russo et al., 2006). Maximum outbreaks of disease and mortality were observed in warm seasons. Moreover, mortality rate during the summer was more in warm regions compared to cold regions (high lands and mountain areas). It was determined that there is a strong association between temperature and increased mortality. Similar results were reported by Agnew and Barnes 2007; Suanyuk et al., 2008; Bromage and Owens, 2009. The dominant symptoms were observed in all diseased fish in the above mentioned provinces, including darkening, hemorrhages in or around the eye, the gill plate, base of the fins or elsewhere on the body, exophthalmia (often in both eyes), corneal opacity (whitish eyes), loss of buoyancy control, lethargy, ascites and ulcerations.

Among these signs, hemorrhage, exophthalmia, darkening and rapidly progressing mortalities are the most frequent observations. Over 90% of these observations were similar to reports by other authors (Yanong and Francis-Floyd 2002; Russo et al., 2006; Suanyuk et al., 2008; Romalde et al., 2008). Moreover, in this study we observed less clinical signs because of meningoencephalitis such as erratic swimming in most farms. 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, five pathogenic species have been identified, including *S. iniae* in Fars province, *S. faecium* in Mazandaran province, *S. agalactiae* in Gilan and Mazandaran provinces, *S. dysgalatiae* in Lorestan, Kohgiluyeh,

Gilan and Kermanshah provinces and *S. uberis*, common in all provinces (except Mazandaran and Lorestan). In addition, the dominant species were *S. uberis*, *S. dysgalactiae* and *S. agalactiae*, respectively. However, the present research is not a comprehensive epidemiologic study and this objective remains incomplete. There is not enough information available to determine which species or strains of *Streptococcus* are more pathogenic to fish in Iran. Therefore further studies are necessary regarding the pathogenicity of various species of this bacterium in rainbow trout and other important farmed and wild fish.

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