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Isolation and Identification of *Streptococcus parauberis* From Freshwater Fish in Turkey

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ABSTRACT

Streptococcus parauberis is an alpha-hemolytic gram positive coccoid bacterium belonging to the *Streptococcaceae* family. This bacterium cause streptococcosis is a major disease in cultured fish due to the intensification of aquaculture and causes significant economic losses in fish farm industry. In our study, we isolated a total of 37 lactic acid bacteria from wild fish (*Sander lucioperca*, *Carassius gibelio*, *Cyprinus carpio*) of Lake Eğirdir and cultured fish (*Oncorhynchus mykiss*, Walbaum) of farm in Turkey. For the isolation of bacteria phenotypic and biochemical characteristics of the 37 isolates obtained from the colonies grown on MRS, TSA and M17 agar characterized by determining colony morphology, cell morphology, motility, gram staining and the production of cytochrome oxidase and catalase. Further biochemical characteristics were determined using conventional tests according to Bergey's Manual of Systematic Bacteriology. From the isolated lactic acid bacteria 25 of were identified as *S. parauberis*, 2 of as *Vagococcus* sp., 2 of as *Lactococcus garvieae* and 5 of as *Lactococcus lactis* by culture-based, biochemical test and 16Sr RNA gene sequencing techniques but 3 of them couldn't (F65, F49 and F50) identified. In this study, the main objective of us is to identify *S. parauberis* with conventional and 16Sr RNA gene sequencing techniques from wild fish in Lake Eğirdir and in cultured fish in fish farm of Egirdir-Turkey.

Keywords: 16Sr RNA, freshwater, molecular identification, fish disease, aquaculture.

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Streptococcus parauberis'in Türkiye'deki Tatlısu Balıklarından İzolasyon ve İdentifikasyonu

Öz: *Streptococcus parauberis* alfa hemolitik, gram pozitif kokoid bir bakteri olup *Streptococcaceae* familyasına aittir. Bu bakteri kültür balıklarında yoğun üretimden kaynaklı çok önemli bir hastalık olan streptokokozise neden olmaktadır ve balık çiftliği endüstrisinde önemli bir ekonomik kayba neden olmaktadır. Çalışmamızda Eğirdir gölü balıklarından (*Sander lucioperca*, *Carassius gibelio*, *Cyprinus carpio*) ve kültür balığı türü olan (*Oncorhynchus mykiss*, Walbaum)'dan 37 tane laktik asit bakterisi izole edilmiştir. Laktik asit bakterilerinin tanımlanması için MRS, TSA ve M17 agardan izole edilen 37 izolatın fenotipik ve morfolojik karakterleri koloni morfolojisi, hücre morfolojisi, hareketlilik, gram boyama, katalaz ve oksidaz üretimiyle yapılmıştır. Diğer biyokimyasal özellikler de Bergey's Manual of Systematic Bacteriology'e göre yapılmıştır. İzole edilen bakterilerinden 25 tanesi *S. parauberis*, 2 tanesi *Vagococcus* sp., 2 tanesi *Lactococcus garvieae* ve 5 tanesi de *Lactococcus lactis* olarak biyokimyasal yöntemler ve 16Sr RNA gen sekansıyla tanımlanmıştır fakat 3 tanesi (F65, F49 ve F50) tanımlanamamıştır. Çalışmamızda *S. parauberis*'in izolasyon ve identifikasyonu ilk olarak Türkiye'deki Eğirdir gölü balıklarından ve gökkuşağı alabalığından gerçekleştirildi.

Anahtar kelimeler: 16Sr RNA, Tatlısu, moleküler tanımlama, balık hastalığı, yetiştiricilik.

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Introduction

Aquaculture has become an economically important industry in the world which requires continued research with scientific and technical developments and innovation. So the purpose of global aquaculture is to maximize the efficiency of

production. The world aquaculture production in 2014 was approximately of 73.8 million tons, which represents around 41% of that obtained from extensive captures for human consumption. To meet the increased need of food in the World, there is an intensive production in fisheries and this makes inevitable the occurence of diseases (FAO 2016; Bondad-Reantaso et al. 2005; Kesarcodi-Watson et al. 2008; Subasinghe et al. 2009).

The appearance and development of a fish disease is the result of the interaction among pathogen, host and environment. It is important to point out that diseases classically considered as typical of freshwater aquaculture, such as furunculosis (*Aeromonas salmonicida*), bacterial kidney disease (BKD) (*Renibacterium salmoninarum*) and some types of streptococcosis are today important problems. Streptococcal infection is a disease affecting most of fish including wild and cultured fish (Toranzo et al. 2005).

The important fish pathogens which have importance are *Lactococcus garvieae (*synonym *Enterococcus seriolicida), Lactococccus piscium, S. iniae (*syn. *S. shiloi), S. agalactiae* (syn. *S. difficile*), *Streptococcus parauberis* and *Vagococcus salmonina*rum. Therefore, streptococcosis of fish should be regarded as a complex of similar diseases caused by different genera and species capable of inducing a central nervous damage characterised by suppurative exophthalmia and meningoencephalitis. While warm water streptococcosis (causing mortalities at temperatures above 15˚C) typically involves *L. garvieae, S. iniae, S. agalactiae* and *S. parauberis*, cold water streptococcosis (occurring at temperatures below 15 ˚C) is caused by *L. piscium* and *V. salmoninarum*. It is important to report that the etiological agents of warm water streptococcosis are considered also as potential zoonotic agents capable to cause disease in humans (Domenech et al. 1996).

Streptococcus is a genus of bacteria containing some species that cause serious diseases in a number of different hosts. This disease causes significant economic losses in the aquaculture industry in the United States of America, Japan, Israel, South Africa, Iran, Australia, Philippines, Taiwan, Bahrain, Turkey and other countries. Streptococcal disease in fish was first reported in 1957 (Hoshina et al. 1958; Baeck et al. 2006; Rahimi and Yadollah 2013).

S. parauberis seems to be endemic of cultured turbot (Toranzo et al. 1994). *S. parauberis* was first identified as a fish pathogen after an outbreak in 1993 in cultured turbot (*Scophthalmus maximus*) in Spain (Domenech et al. 1996). It was also responsible for streptococcosis in olive flounder (*Paralichthys olivaceus*) from a fish farm on Jeju Island, Korea during 2005 (Baeck et al. 2006). Prior to these reports, *S. parauberis* was known primarily as an etiologic agent of mastitis in dairy cows (Williams and Collins 1990).

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* (Yang and Li 2009).

Unfortunately, conventional biochemical tests do not allow for the precise identification and classification of streptococcal isolates, because of differences in growth rates, inoculum levels, and incubation periods (Facklam and Elliott 1995). Consequently, the number and the nature of bacteria species associated with fish streptococcosis remains controversial (Romalde JL et al. 2008).

Molecular techniques to diagnose fish streptococcosis are powerful method have been applied. The techniques based on amplification of 16S rRNA (Zlotkin et al. 1998a,1998b; Nho et al. 2009; Lämmler 1998) seem to be of choice as a standard method for diagnosis of these gram positive cocci. In the case of *S. parauberis*, detection can be performed using the procedures that described for mammals by Lämmler et al. (1998) which combines PCR amplification and endonuclease restriction. Here we report *S. parauberis* from freshwater fish and Rainbow trout (*Oncorhychus mykiss*, Walbaum) which are important economically. Especially, rainbow trout faced with diseases due to the intensive production and culture condition. For hindered of these diseases the one of the natural solution is probiotic bacteria. During the bacterial detection for probiotic bacteria *S. parauberis* obtained in significant densities by biochemical methods. Molecular techniques to diagnose fish streptococcosis are powerful method have been applied. The techniques based on amplification of 16Sr RNA seem to be of choice as a standard method for diagnosis of these gram positive cocci. So we made the identification of these bacteria by 16Sr RNA gene sequence analysis. Because the fast and correct identification is very important in fish farm for hindered and transport of disease from one fish to another. In this study, the main objective of us is to identify *S. parauberis* with conventional and 16Sr RNA gene sequencing techniques in wild fish in Lake Eğirdir and in cultured fish in fish farm of Egirdir-Turkey.

Material and Methods

Isolation of bacteria

Gut and spleen samples obtained from healthy rainbow trout (*O. mykiss*) in fish farm during summer in Isparta province, and gut and spleen samples of carp (*Cyprinus carpio*), silver crucian carp

(*Carassius gibelio*) and sander (*Sander lucioperca*) living in Egirdir lake were diluted for the isolation of lactic acid bacteria. Isolation sources and incubation conditions of bacterial isolates were given in Table 1. The isolates were inoculated in the selective mediums. All the fish were treated in 1 liter of water containing 0.5 ml phenoxyethanol for 2 min and killed. After one gram of sample obtained from the fish gut was placed in 10 ml PBS (phosphate-buffered saline) and was diluted 10^{-7} times, 0.1 ml dilutions were seeded on TSA (Tryptic Soy Agar-Merck 1.05458), MRS (De Man, Ragosa and Sharpe Agar- Merck 1.10660) and M17 (Merck 1.15108) agar and incubated at 22 °C under aerobic and anaerobic conditions for 24-48 hours. Then those whose morphologies resembling lactic acid bacteria were selected from petri dishes and stocked in 15 % of TSA at – 80 °C (Perez-Sanchez et al. 2011).

Table 1. Isolation sources and incubation conditions of bacterial isolates

Strain No	Fish	Organ	Medium	Aerobic/Anaerobic	Temperature	Incubation time
51	*C. gibelio*	Gut	MRS	Aerobic/Anaerobic	22˚C	24-48h
34	*Trout*	Spleen	M17	Aerobic/Anaerobic	22˚C	24-48h
57	*C. gibelio*	Gut	M17	Aerobic/Anaerobic	22˚C	24-48h
35	*O. mykiss*	Spleen	M17	Aerobic/Anaerobic	22˚C	24-48h
61	*C. carpio*	Gut	M17	Aerobic/Anaerobic	22˚C	24-48h
56	*C. gibelio*	Gut	M17	Aerobic/Anaerobic	22˚C	24-48h
73	*C. gibelio*	Gut	MRS	Aerobic/Anaerobic	22˚C	24-48h
42	*C. gibelio*	Gut	TSA	Aerobic/Anaerobic	22˚C	24-48h
28	*C. carpio*	Gut	TSA	Aerobic/Anaerobic	22˚C	24-48h
23	*C. gibelio*	Gut	TSA	Aerobic/Anaerobic	22˚C	24-48h
48	*C. gibelio*	Gut	MRS	Aerobic/Anaerobic	22˚C	24-48h
52	*C. gibelio*	Gut	TSA	Aerobic/Anaerobic	22˚C	24-48h
41	*C. gibelio*	Gut	TSA	Aerobic/Anaerobic	22˚C	24-48h
46	*C. gibelio*	Gut	TSA	Aerobic/Anaerobic	22˚C	24-48h
43	*C. gibelio*	Gut	TSA	Aerobic/Anaerobic	22˚C	24-48h
64	*S. lucioperca*	Gut	M17	Aerobic/Anaerobic	22˚C	24-48h
26	*S. lucioperca*	Gut	M17	Aerobic/Anaerobic	22˚C	24-48h
22	*C. gibelio*	Gut	TSA	Aerobic/Anaerobic	22˚C	24-48h
58	*C. gibelio*	Gut	M17	Aerobic/Anaerobic	22˚C	24-48h
62	*C. carpio*	Gut	M17	Aerobic/Anaerobic	22˚C	24-48h
59	*C. gibelio*	Spleen	M17	Aerobic/Anaerobic	22˚C	24-48h
47	*C. gibelio*	Gut	M17	Aerobic/Anaerobic	22˚C	24-48h
44	*C. gibelio*	Gut	M17	Aerobic/Anaerobic	22˚C	24-48h
45	*C. gibelio*	Gut	M17	Aerobic/Anaerobic	22˚C	24-48h
63	*C. carpio*	Gut	M17	Aerobic/Anaerobic	22˚C	24-48h
19	*C. carpio*	Gut	M17	Aerobic/Anaerobic	22˚C	24-48h
18	*C. carpio*	Gut	M17	Aerobic/Anaerobic	22˚C	24-48h
25	*C. gibelio*	Gut	M17	Aerobic/Anaerobic	22˚C	24-48h
24	*C. gibelio*	Gut	M17	Aerobic/Anaerobic	22˚C	24-48h
60	*C. gibelio*	Gut	M17	Aerobic/Anaerobic	22˚C	24-48h
31	*C. gibelio*	Gut	M17	Aerobic/Anaerobic	22˚C	24-48h
55	*C. gibelio*	Gut	M17	Aerobic/Anaerobic	22˚C	24-48h
32	*C. gibelio*	Gut	M17	Aerobic/Anaerobic	22˚C	24-48h
29	*C. gibelio*	Gut	M17	Aerobic/Anaerobic	22˚C	24-48h

Biochemical and phenotypic characterization

Phenotypic and biochemical characteristics of the 37 isolates obtained from the colonies grown on MRS, TSA and M17 agar characterized by determining colony morphology, cell morphology, motility, gram staining (Figure 1) and the production of cytochrome oxidase and catalase. Further biochemical characteristics were determined using

conventional tests according to Bergey's Manual of Systematic Bacteriology (Schleifer 1986).

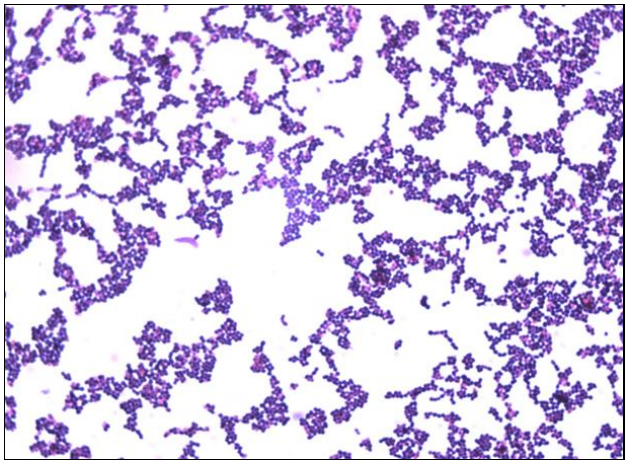

Figure 1. Gram staining of *S. parauberis* (F34) cultured on trypticase soy agar at 22˚C for 24h.

DNA isolation and molecular identification

DNA isolation was carried out with the rapid phylogenetic analysis (Liu 2000). For the molecular biological identification 16Sr RNA about 1.5 kb regions of the 37 isolates were amplified with 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) primers (216bp of 16S rRNA) using ARDRA PCR (Soto 2010). Representative amplification products of isolates were given in Figure 2.

Sequencing

The isolates were sequenced with the same primers used in PCR. The obtained sequence data were compared with the sequences in the GenBank database using the BLAST algorithm and then sent to NCBI (National Center for Biotechnology Information) to receive an access number.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of *S. parauberis* isolates was performed with the disc diffusion method in accordance with guidelines for the Clinical and Laboratory Standards Institute (CLSI), and the following antibiotics (Oxoid) were tested with the disk diffusion method: doxcycline (30 µg), enoxacine (10 µg), erythromycin (15 µg), florfenicol (30 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), enrofloxacin (5 µg), oxytetracycline (30 µg), chloramphenicol (30 µg), vancomycin (30 µg), penicillin (10 µg) . Antimicrobial susceptibility test of isolates were given in Table 2.

Experimental infection

For experimental infection the method by Haines et al. (2013) used after a minor modification. Pathogenicity test of isolates was conducted using healthy rainbow trout (9-10 g). To determine if *S. parauberis* (F34 and F35) isolates from fish would produce infection in rainbow trout, a group of 20 fish (n=10/tank) was inoculated intraperitoneally with 1 x 10^5 bacteria by optical density in a final volume of 10 ul sterile PBS for each isolate. Animals were held in 2 (PVC π 150 cm=880 L) maintained at 16 °C, supplied with freshwater and aeration and monitored daily for clinical signs of streptococcosis for two weeks, including evidence of external hemorrhage, exophthalmia, lethargy, and loss of appetite. Two weeks after inoculation all fish were euthanized and the spleens were aseptically removed (Figure 3) macerated and suspended in liquid media for re-isolation as described above. After experimental infection agent of disease (*S. parauberis*) was obtained from kidney of fish that have signs of streptococcosis and from the results of bacterial examination made with API 20 strep test *S.parauberis* reisolated from diseased fish.

Figure 2. Rainbow trout (*O. mykiss*) showing clinical signs of streptococcosis a) Exopthalmus and haemorrhagic eyes, b) ascites, c) dark skin

Results

Upon the completion of the phenotypic tests, it was found that 25 of the 37 isolates belonged to the genus *Streptococcus*, 5 of them belonged to the genus *Lactococcus* and 2 of them belonged to the genus *Vagococcus*. After being amplified with universal primers definitive identification was made by sequence analysis. However, after the sequence analysis, while 34 of these 37 strains were identified, 3 were not (F65, F49 and F50). The identified 34 isolates with accession number obtained from genebank like as follows: F51 (Accession number KP137338), F34 (KP137328), F57 (KP137342), F35 (KP137329), F61 (KP137346), F56 (KP137341), F73 (KP137350), F42 (KP137331), F28 (KP137324), F23 (KP137320), F48 (KP137337), F52 (KP137339), F41 (KP137330), F46 (KP137335), F43 (KP137332), F64 (KP137349), F26 (KP137323), F22 (KP137319), F58 (KP137343), F62 (KP137347), F59 (KP137344), F47 (KP137336), F44 (KP137333), F45 (KP137334), F63 (KP137348) were identified as *S. parauberis*, F18 (KP137317), F19 (KP13731) as *L.*

garvieae, F24 (KP137321), F25 (KP137322) as *Vagococcus* sp. F60 (KP137345), F55 (KP137340), F29 (KP137325),F31 (KP137326),F32 (KP137327) as *L. lactis* subsp *lactis*.

Isolation sources and incubation conditions of bacterial isolates were given in Table 1. Bacteria isolated from fish were gram positive ovoid cells forming chains or single cells (Figure1) growth occured from 4˚C (except F34, F35 and F73) to 45˚C (except F23 and F48) with 0 to 6.5 % (except F24, F23, F48, F47, F22) NaCl. Antibiotic susceptibility test results are given in Table 2. Of the samples studied, except F18, F19, F26, F22 and F29 all isolates were sensitive to all antibiotics tested. Three isolates were multiple resistant against to 3 antibiotics which are F18 (en, o, c), F19 (en, o, c), F26 (do, sxt, c) respectively. The highest antibiotic resistance was to enoxacin, oxytetracycline and chloramphenicol. All the strains were sensitive to vancomycin and penicillin (except F29). In pathogenecity test, after inoculation with *S. parauberis* the 90-100 % of the fish species were died in two weeks.

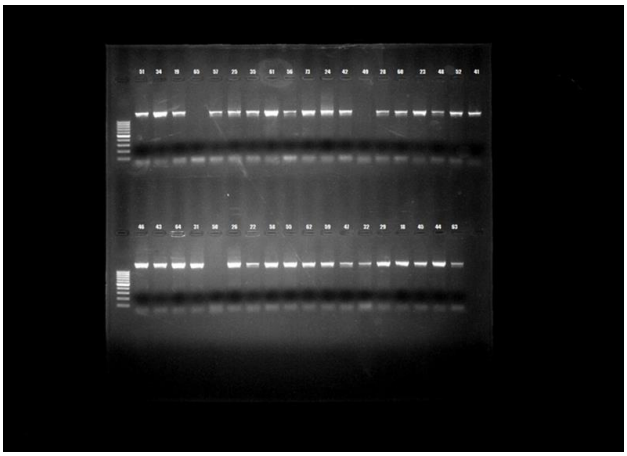

Figure 3. Representative amplification products of isolates (F51, F34, F57, F35, F61, F56, F73, F42, F28, F23, F48, F52, F41, F46, F43, F64, F26, F22, F58, F62, F59, F47, F44, F45, F63 are *S. parauberis*; F19 and F18 *L. garvieae*; F25 and F24 *Vagococcus* sp.; F60, F31, F55, F32, F29 are *L. lactis* subsp.*lactis*)

Table 2. Antimicrobial susceptibility test of isolates

Bacteria	do	en	e	flr	sxt	o	enr	c	v	p
F18	S	R	S	S	S	R	S	R	S	S
F19	S	R	S	S	S	R	S	R	S	S
F24	S	S	R	S	S	S	S	S	S	S
F25	S	S	S	S	S	S	S	S	S	S
F22	S	S	S	S	R	S	S	S	S	S
F26	R	S	S	S	R	I	I	R	S	S
F34	S	S	S	S	S	S	S	S	S	S
F42	S	S	S	S	S	S	S	S	S	S
F43	S	S	S	S	S	S	S	S	S	S
F47	S	S	S	S	S	S	S	S	S	S
F48	S	S	S	S	S	S	S	S	S	S
F51	S	S	S	S	S	S	S	S	S	S
F52	S	S	S	S	S	S	S	S	S	S
F57	S	S	S	S	S	S	S	S	S	S
F58	S	S	S	S	S	S	S	S	S	S
F59	S	S	S	S	S	S	S	S	S	S
F60	S	S	S	S	S	S	S	S	S	S
F29	S	S	S	S	S	S	S	S	S	R
F31	S	S	S	S	S	S	S	S	S	S
F32	S	S	S	S	S	S	S	S	S	S
F55	S	S	S	S	S	S	S	S	S	S

(*do:* doxycycline, *en:* enoxacin, *e:* erithromycin, *f:* florfenicol, *sxt:* trimethoprim *o*: oxytetracycline, *enr*: enrofloxacin, *c*: chloramphenicol (*ı*:intermediate sensistive, *s*:sensitive, *r*: resistance)).

Discussion

Initially, main objective of us was detect and isolate lactic acid bacteria with potential probiotic properties. So we isolated bacteria from healthy fish. But from the molecular results also we isolated *S. parauberis* that is a pathogenic bacteria for fish especially in rainbow trout. Streptococcosis is associated with acute and chronic mortality in many aquaculture species (Nho et al. 2009). The considerable diversity of *streptococcus* bacteria associated with fish may explain the difficulties encountered when identification procedures are based only on phenotypic characteristics. The identification schemes for the causative agents, based on biochemical and antigenic features can barely differentiate these bacterial pathogens from other low virulent gram-positive cocci such as *L. lactis*. The study has been conducted using conventional

methods and miniaturized systems and have given variable results. Thus, final identification of the bacteria requires the support of genetic data.

The PCR method can be employed as a supplementary and complementary test for definitive identification of the bacteria cultured from suspected samples. Isolates identified through phenotypic methods did not support the sequence results obtained through 16Sr RNA sequencing. This result indicated that conventional method are not enough or useful in the identification of *S. parauberis* or the other lactic acid bacteria isolates (Domenech et al. 1996; Haines et al. 2013; Park et al. 2013). In some studies, 16Sr RNA sequencing technique was used for identification of *S. parauberis* and lactic acid bacteria (Haines et al. 2013; Pourgholam et al. 2013; Didinen et al. 2014). So we can say that 16Sr RNA technique is very powerful for discriminating of lactic acid bacteria and also *S. parauberis*. Also in our study we identified *Vagococcus* sp isolated by Didinen et al. (2011) from Turkey and *L. garvieae* isolated by Diler et al. (2002) and *L. lactis* subsp. *lactis*.

Antibiotic resistance and sensitivity in lactic acid bacteria vary depending on the strains and the source of isolation (Salminen 1998). Antibiotic susceptibility test results are given in Table 2. Of the samples studied, except F18, F19, F26, F22 and F29 all isolates were sensitive to all antibiotics tested. All *S. parauberis* isolates were sensitive to vancomycin and erythromycin. This result was similar with (Haines et al. 2013; Pitkälä et al. 2008). The highest antibiotic resistance was to enoxacin, oxytetracycline and chloramphenicol.

S. parauberis isolates were obtained from apparently healthy rainbow trout and experimentally injected trout managed to demonstrate symptoms of streptococcosis such as exophtalmus, hemorrhage, erratic swimming, dark skin. Haines et al (2013) report that they isolated *S. parauberis* from healthy stripped bass as we did, but when they infected fish with *S.parauberis* they failed to demonstrate the signs of streptococcosis. This could be explained as; the fish species we obtained the bacteria may not be susceptible to disease. Also we can think that, in the facility where we obtained pathogen bacteria from healthy fish species may have been an infection caused by *S. parauberis* previously and became a porter.

According to our knowledge this is the first report for isolation and molecular identification of *S.parauberis* from wild fish and fish farm in Turkey. To date, *S. parauberis* have been isolated from salmon, rainbow trout (Kitao et al. 1981; Eldar et al.1995) mullet, golden shiner, pinfish, eel, sea trout, tilapia sturgeon, red drum (*Sciaenops ocellatus*), yellowtail (*Seriola quinquerodiata*) (Kusuda et al. 1991), rabbit fish (*Siganus canaliculatus*) sea bass (*Dicentrarchus labrax*); Japanese flounder (*Paralichthys olivaceus*)], ayu (*Plecoglossus altivelis*), barramundi (*Lateus calcifer*) and striped bass (Rahimi and Yadollah 2013). Due to the *S. parauperis* cause major disease in fish species we must alarm the relevant bodies against any disesae outbreaks and we need to take precautions in fish farming Turkey. So this study can be helpful in the prevent of disease outbreaks and may help the researchers in further scientific works.

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