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RESEARCH PAPER

Disease of Russian sturgeon (*Acipenser gueldenstaedtii*) caused by *Aeromonas* sp.

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Abstract

In this study, the bacteriological examination was performed to investigate the cause of juvenile sturgeon (*Acipenser gueldenstaedtii*) mortality, kept in a fish farm, in Turkey. During the disease outbreak, 15% of the sturgeon (2.7-4.2 g) died. The most important clinical signs were swimming upside-down in the water and no feed intake. The fish have exhibited an enlarged abdomen due to the swim-bladder gas problem. Aseptic liver and head-kidney of affected fish were streaked on Tryptic Soy Agar. The pure colonies were characterized by several biochemical tests and rapid commercial test kit (API 20NE). Genetic identification was performed by 16S rRNA gene sequencing. The antibacterial susceptibility of bacteria against six different antibiotics was also evaluated by disk diffusion method. Based on biochemical characteristics, the causative bacteria were identified as *Aeromonas hydrophila* (API 20NE, profile: 5477754). Bacteria were identified as *Aeromonas* sp. by molecular analysis. The bacteria were sensitive to enrofloxacin, florfenicol, oxytetracycline, sulfamethoxazole+trimethoprim, gentamycin and resistant to erythromycin. The most effective antibiotics were enrofloxacin and florfenicol. In this study, an infection caused by *Aeromonas* sp. was reported in Russian sturgeon. Overall this study revealed that *Aeromonas* sp. may be considered as a causative agent of inflation of the swim bladder in Russian sturgeon.

Keywords: Fish, sturgeon, swim bladder, *Aeromonas*, DNA sequencing.

Introduction

Aquaculture is a quite new industry for Turkey, which started with rainbow trout (*Oncorhynchus mykiss*) culture. Aquaculture has grown notably over the past 30-40 years. The carnivore species including sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) became the most extensively cultured fish species (TUIK, 2017).

The sturgeon aquaculture is underdeveloped in Turkey. Therefore, there is limited information on their diseases and pathogen profiles. In recent years, sturgeon larvae production was achieved successfully in our country. Therefore, more scientific studies on this species have been carried out. Sturgeon (Acipenseridae) are species of fish which are endangered all over the world. Many species are listed on the list of species to be protected, referred to as the "Red List" of the International Union for Conservation of Nature (IUCN). It is known that six sturgeon species are naturally found on the Turkish Black Sea coastal waters and entering the rivers including Kızılırmak, Yeşilırmak, Sakarya, and Çoruh for spawning. The sturgeon species have also been strictly protected throughout Turkey since 1997 (Ustaoglu & Okumus, 2004; Akbulut *et al*., 2011). Reproduction studies of three species, Russian sturgeon (*Acipenser gueldenstaedtii*), Beluga (*Huso huso*), and Stellate (*Acipenser stellatus*) have mainly focused on building up broodstocks, sperm collection, cryopreservation and larval rearing (Akbulut *et al*., 2011).

In sturgeons, generally, researchers focused on bacterial and parasitic diseases in Turkey. In a recent study, fish pathogenic bacteria including *Acinetobacter radioresistens*, *Bacillus mycoides*, *Aeromonas* and *Pseudomonas* species were isolated from Russian and Siberian sturgeon (*Acipenser baerii*) in Turkey (Kayis, Er, Kangel, & Kurtoglu, 2017). In another study, bacterial hemorrhagic septicemia outbreaks caused by *A. hydrophila* were reported with low mortality in Russian sturgeon (Timur, Akaylı, Korun, & Yardımcı, 2010).

Aeromonads are species of bacteria which are Gram-negative, short straight rod, facultatively anaerobic, and usually motile by a flagellum, occur in fresh and brackish water and they have a broad host

range. They are considered as opportunistic pathogens. Some species are pathogenic for both fish and humans (Holt, Krieg, Sneath, Staley, & Williams, 1994). Species of motile *Aeromonas* exhibits a significant risk for aquaculture facilities in Turkey. *A. hydrophila*, *A. sobria*, *A. veronii*, *A. caviae*, and *A. schubertii* were reported as causative agents of various diseases in economically important fish species including rainbow trout, sea bass, sea bream and sturgeon in Turkey (Ozturk & Altinok, 2014). However, the inflation of swim bladder associated with *Aeromonas* has not been reported in sturgeon species. In this study, *Aeromonas* sp. infection was reported in Russian sturgeon with low mortality. The purpose of this study is to understand the cause and find a solution for inflation of swim bladder in sturgeon fry.

Materials & Methods

Microbiological Examinations

The study was approved by the Local Ethical Committee of the Central Fisheries Research Institute (CFRI, Protocol No: 42208298-040-04-02). Ten russian sturgeon were sampled for bacterial examination in a fish farm located in northeastern part of Turkey. Infected fish were four-months-old and size of the fish was range between 2.7 and 4.2 g. All fish were examined both externally and internally. The bacterial examination was performed in the Fish Diseases Laboratory. Head-kidney and liver were aseptically streaked onto Tryptic Soy Agar (TSA, Merck, Germany) and incubated at room temperature for 48 hours. After incubation, typical colonies were selected and subcultured into the same media to check the purity of bacteria. The pure cultured colonies were biochemically characterized by rapid test kit (API 20NE, bioMerieux, France) and several biochemical tests including Gram staining, motility, cytochrome oxidase and catalase (Altinok, Balta, Capkin, & Kayis, 2007).

Extraction and Sequencing of DNA from Cultured Bacteria

The bacteria was further confirmed by partial 16S ribosomal RNA gene (16S rRNA) sequencing. For this purpose, extraction of genomic DNA from bacteria was performed for the PCR assay using a boiling technique as previously described (Ture, Misir, Altuntas, & Kutlu, 2018). The concentrations of DNA were measured by RNA/DNA calculator (Thermo Fisher Scientific, USA). Average, DNA concentrations were adjusted to 100 ng/µl.

The universal primers specific for 16S rRNA gene of a wide range of bacteria (fD1-AGAGTTTGATCCTGGCTCAG and rP2-ACGGCTACCTTGTTACGACTT) were used for PCR amplification (Weisburg, Barns, Pelletier, & Lane, 1991). These primers were used to yield approximately 1500 bp amplification product by PCR. PCR was conducted in a thermocycler (Applied Biosystems, USA). For amplification, AmpliTaq Gold 360 Master Mix (Thermo Fisher Scientific) was used according to the manufacturer's recommendations. Analysis of PCR product was performed using electrophoresis in 1.5% agarose gel with 1×TBE (Tris-Borate-EDTA) buffer containing SYBR Green alongside a 100-bp DNA ladder (Bio Basic, Canada).

The PCR product was purified, and the sequencing reaction was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA), according to the manufacturer's instructions. ABI PRISM 3500 Genetic analyzer and POP-7 polymer were used as the separation machine and matrices. The derived nucleotide sequences were described and aligned by NCBI (www.ncbi.nlm.nih.gov/genome/microbes). The obtained sequences were compared with previously published data in GenBank. The relationships among isolates were estimated using the neighbor-joining (NJ) method in Mega 5.0. The phylogenetic tree was created by using the CLUSTALW program (Tamura *et al.*, 2011).

Antibiotic Susceptibility

The antibacterial susceptibility of bacteria against six different antibiotics was determined with a disc diffusion method using commercial discs (Oxoid, Germany) on Mueller Hinton Agar (MHA, Merck) plates. The test was evaluated according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2014). The antibiotic discs used in this study are trimethoprim-sulfamethoxazole (SXT; 25 µg), gentamycin (CN; 10 µg), erythromycin (E; 15 µg), florfenicol (FFC, 30 µg), oxytetracycline (OT; 30 µg) and enrofloxacin (ENR, 5 µg). The plates were incubated at 30°C for 22h. The isolate was characterized as susceptible or resistant to the antibiotics.

Results

The cumulative mortality was approximately reached 15% between May and August 2018 in juvenile sturgeon (2.7-4.2 g). Externally, diseased fish had anorexia, lethargy, enlarged abdomen, dark pigmentation and hemorrhage at the base of the anal fins. One of the most important clinical sign was the floating upside-down at the water surface (Figure 1). Internally, clinical signs included empty stomach and inflation of the swim bladder. Due to the swim bladder problem, feed in take decreased dramatically.

The presence of *Aeromonas* sp. was detected in internal organs (liver and kidney) of affected sturgeons. Bacteria were isolated from all fish samples. Biochemically, the isolate was Gram-negative, oxidase

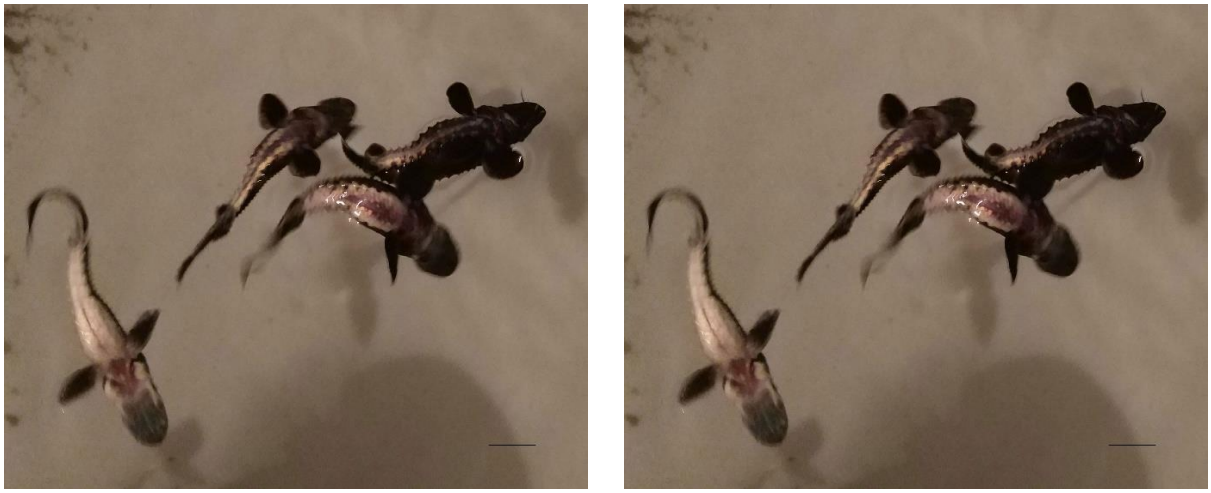

Figure 1. Enlarged abdomen and swim upside-down on the water surface in sturgeon (scale bar: 2 cm).

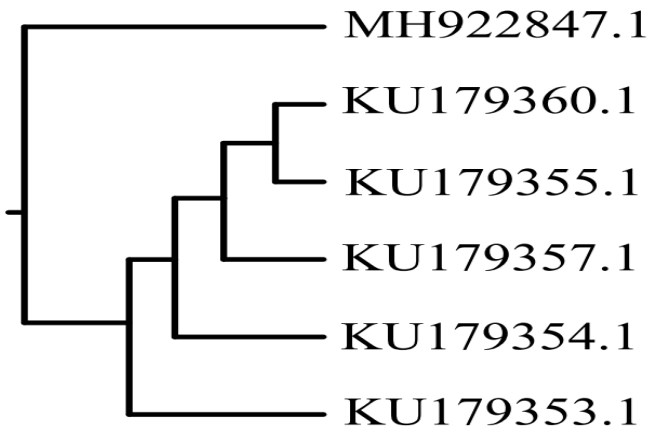

Figure 2. Phylogenetic tree based on 16S rRNA gene sequence comparison, obtained with the NJ method showing the *Aeromonas* strain with related taxa.

and motility positive, short rod-shaped and characterized as *A. hydrophila* by API 20NE test (Profil: 5477754, %ID: 99,9%). The other biochemical tests results were shown by comparison with the previously published study (Table 1).

An approximately 1500-bp DNA fragment was amplified from DNA extracted from a culture of pure bacteria. The sequencing results obtained from the 16S rRNA gene region were compared with other isolates that available in the Genbank database. The amplified nucleotide sequence expressed a≥98% homology with the 16 S rDNA sequence bacterium of *A. tecta*, *A. archeleia*, *A. hydrophila*, *A. veronii*, and *A. eucrenophilia*, GenBank accession number: KU179360.1, KU179355.1, KU179357.1, KU179354.1, and KU179353.1 respectively. Thus, bacteria isolated from sturgeon were evaluated as *Aeromonas* sp. 16S rRNA sequence of the bacteria have been deposited in the GenBank database (Accession Number: MH922847). The phylogenetic tree was shown in Figure 2.

Antimicrobial susceptibility test revealed that bacteria were sensitive to enrofloxacin, florfenicol, oxytetracycline, trimethoprim-sulfamethoxazole, and gentamycin and resistant to erythromycin. The most effective antibiotics were enrofloxacin and florfenicol respectively.

Discussion

In the present study, Russian sturgeon kept in a fish farm was investigated in term of bacterial pathogens in case of inflation of the swim bladder. *Aeromonas* sp. isolated from sturgeon with low mortality.

In recent years, aquaculture of sturgeons has increased, while decreases have been noticed of the natural populations in the World (Hoseinifar, Ringo, Masouleh, & Esteban, 2016). As world interest in the aquaculture of sturgeon stems from meat and caviar of these fish. In Turkey, the breeding of sturgeon fish is continued using local broodstocks and imported eggs.

Table 1. Comparison of some phenotypic characteristics of *Aeromonas* sp. isolated from sturgeon in this study with the previously published study

Phenotypic features	*Aeromonas* sp. in this study	*Aeromonas* sp[a]
Gram stain	-	-
Reduction of nitrates to nitrites	+	+
Indole production	-	+
Fermentation (Glucose)	+	+
Arginine dihydrolase	-	+
Urease	-	-
Hydrolysis (Esculin)	+	+
Hydrolysis (Gelatin)	+	+
β-galactosidase (PNPG)	+	**?**
Assimilation of:		
Glucose	+	+
Arabinose	+	+
Mannose	+	+
Mannitol	+	+
N-Acetyl-Glucosamine	+	**?**
Maltose	+	+
Potassium gluconate	+	**?**
Capric acid	+	**?**
Adipic acid	-	**?**
Malate	+	**?**
Trisodium citrate	-	-
Phenylacetic acid	-	-
Cytochrome oxidase	+	+
1% NaCl, Growth	+	+
Catalase	+	+
Motility	+	+
Brown pigment	-	-

a: Phenotypic features of bacteria were obtained from Holt *et al*., (1994).
?: It was not done

In both cases, diseases cause 15-20% post-hatching mortality rates which significantly affect the production of these fish. In a previous study, Hyper-inflated swim bladder syndrome (HISB) was reported with a small percentage of mortality in Atlantic sturgeon (*Acipenser oxyrinchus*). According to that report, symptoms include loss of equilibrium and inability to maintain normal swimming, extended abdomen caused by inflation of swim bladder, and floating upside-down at the water surface. The causative agent of the syndrome has not been identified, however, an anaerobe bacterium which has been isolated from the intestine of sturgeon is suspect. It was thought that this bacterium produces hydrogen gas which may inflate the swim bladder. However, it expressed that reducing water temperatures down to 10° C and using injectable oxytetracycline will usually relieve symptoms in about 48 hours (Mohler, 2003). In our study, *Aeromonas* sp. isolated from the effected sturgeon. Enrofloxacin and florfenicol were determined as the most effective antibiotic. When symptoms persist, enrofloxacin was administrated (20 mg/kg/3 days active ingredient) intraperitoneally. However, affected fish did not recover.

In comparison, there is a little biochemical difference between the results of our study and Holt's manual (1994). In contrast to the results detailed in our study, indole production and arginine dihydrolase reaction tests were positive (Table 1). In this study, the bacterium was tried to identified by both API 20NE test and 16S rRNA gene sequencing methods. It was found that a weak relationship between the phenotypic and genotypic identification methods. According to the API 20NE result, bacteria were identified as *A. hydrophila*. However, the bacteria were identified to genus level through 16S rRNA gene sequencing methods. Since the 16S rRNA gene region has poor discriminatory power between closely related bacteria because of heterogeneity, identification of bacteria remained genus level. Therefore, bacteria were identified as *Aeromonas* sp.

Motil *Aeromonas* are Gram-negative and rod-shaped bacteria that can be divided into several distinct species, including *A. hydrophilia*, *A. putida*, *A. caviae*, *A eucrenophila*, *A. schubertii*, *A. sobria*, and *A. veronii*. Diseases caused by *Aeromonas* spp. occur in fish, human, amphibians, and reptiles and these animals may act as carriers of the bacteria. *Aeromonas hydrophila* is the most known species of bacterium present in freshwater environments. In fish, these bacteria cause diseases named motile Aeromonas septicemia, red pest, red fin disease, and infectious abdominal dropsy (Aberoum & Jooyandeh, 2010). Aeromonads are one of the most frequently reported bacteria species from sturgeons. In a previous study, *A. veronii* was isolated from beluga and stellate sturgeons in Iran (Gholamhosseini *et al*., 2018). The literature on bacterial diseases of sturgeons is limited in Turkey.

Bacterial pathogens including *A. hydrophila*, *Flavobacterium hydatis* (Timur *et al*., 2010), *Flavobacterium johnsoniae* (Karatas *et al*., 2010), *Acinetobacter radioresistens*, *Bacillus mycoides*, *Aeromonas* spp., and *Pseudomonas* spp., (Kayis *et al*., 2017) were reported in Russian sturgeon. In conclusion, cultured Russian sturgeon were investigated for bacterial fish pathogens in case of inflation of the swim bladder. *Aeromonas* sp. was isolated from sturgeon causing mortality in low rates. This study could further improve our understanding of the inflation of the swim bladder in sturgeon.

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