The first outbreak of citrobacteriosis caused by *Citrobacter gillenii* in reared Russian sturgeon (*Acipenser gueldenstaedtii*) in Turkiye

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Article Info	Abstract
	Russian sturgeon (*Acipenser gueldenstaedtii*) is an endangered fish species and also an important resource for the sturgeon aquaculture industry in Turkiye. Recently, a fatal and persistent bacterial disease occurred in the reared sturgeon kept in a trout farm in Turkiye. The disease outbreak has been with notable external signs including petechial hemorrhages and systemic anemia. This outbreak lasted for six weeks, and cumulative mortality reached around 35.00 - 40.00%. In this study, no parasitic and viral agents were observed in the sturgeons. *Citrobacter gillenii* was isolated from the diseased fish and identified by biochemical and molecular methods including API 20E and 20NE and 16S rRNA gene region sequencing, respectively. As a result, *C. gillenii* was identified for the first time in Russian sturgeon in Turkiye. The sequence was also deposited under the Genbank with MW057770 accession number. According to the result of disc diffusion method, bacteria were sensitive to enrofloxacin, streptomycin, amoxicillin and oxytetracycline and resistant to penicillin, trimethoprim/sulfamethoxazole, florfenicol and erythromycin. Also, *ampC, sul1* and *floR* resistance genes were detected in the isolated bacteria. The results of this study provide important information for the diagnosis and treatment of this newly emerged disease of Russian sturgeon.

Introduction

Approximately half a century ago, aquaculture has started with rainbow trout (*Oncorhynchus mykiss*) farming in Turkiye. Especially, the carnivore species including trout, sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) became the most extensively cultured fish species.[1] On the other hand, sturgeon rearing has become an important and quickly expanding sector of aquaculture worldwide. Sturgeon farming has become increasingly important worldwide for its meat and production of caviar.[2] Sturgeons (Acipenseridae) are primarily distributed in the northern hemisphere. The number of wild sturgeon, however, has decreased dramatically in recent years, particularly in Eurasia. Since the late 1960s, there has been growing interest in sturgeon aquaculture for meat production.[3]

Several species are reared, among which the most attractive are Russian (*Acipenser gueldenstaedtii*) and Siberian (*Acipenser baerii*) sturgeons due to their short reproductive cycles and desirable products.[4] Sturgeons, like several fish species, are on the list of species to be protected, referred to the "Red List" of the International Union for Conservation of Nature.[5]

There is limited knowledge about diseases and patho-gens affecting sturgeon since the cultural practices of this species have not been at the desired level in Turkiye yet.[6] In recent years, sturgeon larvae (*A. gueldenstaedtii* and *A. baerii*) production was achieved successfully in Turkiye. Six sturgeon species also occur naturally along with the cost of the Black Sea in Turkiye entering the rivers for spawning.[5] Thus, it is possible to carry out more researches on these species. It is thought that sturgeons are relatively more resistant to fish diseases than other fish species including trout. However, several bacterial disease pathogens including the genus *Aeromonas, Pseudomonas* and *Acinetobacter* were often isolated from cultured sturgeons.[7,8] Also, many viral and parasitic disease agents have been isolated from sturgeons.[4]

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Citrobacter species are Gram-negative and motile bacilli known as opportunistic pathogens. Hence, some unfavorable situations including stress, over-density and environmental pollution have a key role in the occurrence of infection.[9] *Citrobacter* sp. was first reported as an emerging bacterial fish pathogen from aquarium sunfish (*Mola mola*) in Japan and has subsequently been found in most areas where fish species are cultured.[10] In the last decade, there has been an important awareness of the *Citrobacter* species in Turkiye. Fish diseases caused by this bacterium are frequently encountered in cultured fish.[11] *Citrobacter* sp., *C. freundii, C. braakii,* and *C. gillenii* were also isolated in rainbow trout (*O. mykiss*) in Turkiye.[12,13]

As for other *Citrobacter* species, reports of anti-microbial-resistant *Citrobacter* species have increased in recent years. Particularly, most of the *C. freundii* isolates were reported as significantly resistant to all beta-lactam antibiotics as well as aminoglycoside, fluoroquinolone, tetracycline, sulfonamide and nitrofurantoin.[13] Although many studies have been conducted on the phenotypic anti-microbial resistance of *Citrobacter* species, there is limited knowledge about their genetic resistance.

To our knowledge, *C. gillenii* was not reported in cultured sturgeon in Turkiye. The study reported herein investigated the etiology of this fatal and chronic disease. Russian sturgeon (*A. gueldenstaedtii*) is an endangered fish species, and also an important resource for the sturgeon aquaculture industry in Türkiye. Recently, a fatal and persistent bacterial disease occurred in the cultured sturgeon being kept in freshwater fish farms in Turkiye. The disease outbreak lasted for six weeks with notable external signs. The results of this study could provide significant information for the diagnosis and treatment of this recently emerged disease of Russian sturgeon.

Materials and Methods

All examinations on animals were performed following National and Institutional Guidelines for the protection of animal welfare.[14] This study was also conducted with the permission of the Local Ethical Committee of the Trabzon Central Fisheries Research Institute (CFRI), Trabzon, Turkiye, with the protocol number of 42208298-040-04-02. Sturgeon (*A. gueldenstaedtii* and *A. baerii*) production is performed in a private trout farm in Rize province, Turkiye, under CFRI supervision. About 10,000 juveniles are produced annually in a flow-freshwater-based system. In the late summer of 2020, the disease outbreak occurred with notable external signs including petechial hemorrhages, lethargy and systemic anemia in Russian sturgeon. The etiological agent of this fatal disease was investigated in this study. A total of 30 moribund Russian sturgeons (weighing 5.00 - 15.00 g) showing the clinical signs of disease were sampled.

Fish samples were aseptically collected and transported to the fish diseases laboratory for viral, bacterial, and parasitic examinations. The physical and chemical parameters of the water during the outbreak event are presented in Table 1.

Table 1. Physical and chemical charactersitics of the farm water during the investigation.

Parameters	Inlet water	Rearing water
Dissolved oxygen (mg L^{-1})	9.44	8.62
pH	7.47	7.81
Temperature (℃)	16.90	16.80
Ammonia (mg L^{-1})	< 0.02	0.06
Nitrite (mg L^{-1})	0.01	0.01
Nitrate (mg L^{-1})	5.28	4.40
Electrical conductivity (s m^{-1})	70.90	75.10

Viral and parasitic examinations. Viral examination of fish was performed as described by Altuntaş and Öğut, previously.[15] Briefly, after the diluted samples (kidney, spleen and liver) were centrifuged, the supernatant was inoculated in triplicate into a monolayer culture of Bluegill Fry-2 cell lines. The cultures were incubated at 15.00 ℃ for 14 days and checked every day. At the end of the incubation period, the samples showing no cytopathic effect were assumed as a virus negative. For specificity controls, infectious pancreatic necrosis virus (IPNV) provided by our laboratory was used as positive controls.

For parasitic examination, fish samples were prepared through skin and gills scraping. Samples were examined microscopically at a magnification of 20× and 40×.[8]

Bacteriological examinations. For this purpose, 30 moribund fish (Russian sturgeon) were collected randomly from fish tanks. The liver and kidney of fish were inoculated in Tryptic Soy Agar (Merck, Darmstadt, Germany) and incubated at 28.00 ℃ for three days. After incubation, typical bacterial colonies were selected from the plate and inoculated on the same medium to ensure the purity of the bacteria. Thereafter, biochemical properties of pure colonies were determined by following tests: Gram stain, catalase, cytochrome oxidase, hemolytic activity and motility. Analytical profile indices (API 20E and 20NE) were also performed to identify and characterize the bacteria.[16]

Anti-microbial susceptibility determination. Antibiotic susceptibility of the isolated bacterium was tested by the disk diffusion method on Mueller Hinton Agar (Merck). The test was performed and evaluated according to the Clinical and Laboratory Standards Institute Guidelines.[17] The following anti-microbial discs (Oxoid, Basingstoke, UK) were used: penicillin G (10.00 units), erythro-mycin (15.00 µg), enrofloxacin (5.00 µg), florfenicol (30.00 µg), amoxicillin/clavulanic acid (30.00 µg), sulphametho-xazole/ trimethoprim (25.00 µg), oxytetracycline (30.00 µg) and streptomycin (10.00 µg).

Genomic DNA preparation and polymerase chain reaction (PCR) assay. The genomic DNA of Gram-negative bacteria was isolated from pure culture through a boiling technique with minor modifications.[18] The quantity and quality of genomic DNA were checked by RNA/DNA calculator (QIAexpert, Hilden, Germany) and gel electrophoresis method. The average DNA concentration was adjusted to 100 ng µL^{-1} before genetic analysis. The genetic characterization of the isolate was performed by DNA sequencing of 16S rRNA gene with a forward primer (fDl) and reverse primer (rP2; Table 2). The PCR was performed with AmpliTag Gold 360 Master Mix (Thermo Fisher Scientific, Vilnius, Lithuania) in a thermocycler (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's recommendations. The PCR products were confirmed/analyzed by 1.20% agarose gel electrophoresis in 1× Tris-Borate-EDTA (AppliChem, Council Bluffs, USA) buffer at 100 V for 30 min of staining with ethidium bromide. The DNA fragment length was observed with the migration of a 100-bp DNA ladder (Bioneer, Oakland, Canada) and visualized by ultraviolet transillumination.

PCR assays for detection of antibacterial resistance genes (ARGs). The presence of ARGs, including sulfonamides (sul1 and sul2), tetracycline (tetA and tetB), trimethoprim (dhfrAI), erythromycin (ereA), florfenicol (floR) and b-lactam (blaTEM and ampC) was determined by simplex PCR. Each PCR mixture (25.00 µL) contained 100 ng (1.00 µL) of DNA sample, 10.00 pmol (1.00 µL) of each primer (Table 2), 12.50 mL of AmpliTag Gold Master Mix (Thermo-Fisher Scientific) and 10.50 mL of distilled water. As a negative control, PCR mixture without DNA sample was used; while, for the positive control, known bacterial DNA containing different resistance genes was used.[16] The DNA amplification was carried out in a thermocycler

(Applied Biosystems) following the instructions of the manufacturer's master mix. Each PCR product was subjected to electrophoresis and visualized as described below. The PCR products belonging to different ARGs were also confirmed by DNA sequence.

Sequencing of PCR product. The products belonging to positive ARGs and presumptive bacteria were also confirmed by DNA sequence. The sequencing reaction was done using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems), according to the manufacturer's instructions. The ABI PRISM 3500 Genetic Analyzer and POP-7 polymer were applied as the separation machine and matrices, respectively. The sequence data were further analyzed using the Bio Edit Sequence Alignment Editor Software (version 7.1.11; Ibis Therapeutics, Carlsbad, USA).[27] The sequence data were analyzed by ABI Prism DNA Sequencing Analysis Software v5.1. The 16S rRNA gene sequence was aligned using BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with previously published data in GenBank and percentage similarities of sequences were determined.

Molecular characterization. A phylogenetic tree was constructed by the sequence of the bacteria from this study for validation of the sequencing data. The sequence was aligned with several bacterial sequences retrieved from the GenBank using Clustal W Multiple Alignment tool (BioEdit Sequence Alignment Editor Software).[27,28] After the data were entered into Mega-X software, the best substitution model analysis was performed.[29] A neighbor-joining phylogenetic tree was constructed using the Kimura 2-parameter model, with a gamma-distributed parameter, the nearest-neighbor-interchange tree inference option.[30,31] The reliability of tree topology was assessed by bootstrap analysis with 1,000 replicates.[32]

Table 2. Primers used in this study.

| Primers	Sequence (5´- 3´)	Target gene	Fragment length (bp)	References	Annealing temperature (˚C)
TetA	F: GCTACATCCTGCTTGCCTTC R: CATAGATCGCCGTGAAGAGG	*tetA*	210	19	55
TetB	F: TTGGTTAGGGGCAAGTTTTG R: GTAATGGGCCAATAACACCG	*tetB*	659	19	54
ereA	F: AACACCCTGAACCCAAGGGACG R: CTTCACATCCGGATTCGCTCGA	*ereA*	420	20	58
Sul1	F: CGGCGTGGGCTACCTGAACG R: GCCGATCGCGTGAAGTTCCG	*sul1*	433	21	60
Sul2	F: GCGCTCAAGGCAGATGGCATT R: GCGTTTGATACCGGCACCCGT	*sul2*	293	21	60
Flor	F: TATCTCCCTGTCGTTCCAG R: AGAACTCGCCGATCAATG	*floR*	399	22	50
dhfr1	F: CTGATATTCCATGGAGTGCCA R: CGTTGCTGCCACTTGTTAACC	*dhfr1*	433	23	56
TEM	F: ATGAGTATTCAACATTTCCG R: CAATGCTTAATCAGTGAGG	*bla*TEM	859	24	45
AmpC	F: TTCTATCAAMACTGGCARCC R: CCYTTTTATGTACCCAYGA	*ampC*	550	25	49
D1 P2	F: AGAGTTTGATCCTGGCTCAG R: ACGGCTACCTTGTTACGACTT	*16S rRNA*	1,500	26	60

Results

In the late summer of 2020, the disease outbreak was recorded in Russian sturgeon with notable external signs including petechial hemorrhages and systemic anemia. The gills and skin of the affected fish were very pale. There were petechial hemorrhages at the base of the anal and pectoral fins. The abdominal area was transparent and an amount of yellowish fluid was seen in the body cavity. Internally, hemorrhages were also present in the liver and kidney (Fig. 1). The disease was persistent and caused high mortality (approximately 35.00 - 40.00%). During the study period, no parasitic and viral agents were found in the sturgeons. However, bacteria were isolated from 21 of the 30 examined fish (70.00%). Isolated bacteria were short and rod-shaped, Gram-negative, motile, non-oxidative and catalase-positive. One out of the 21 bacteria exhibiting common properties was selected for further studies such as biochemical and molecular characterizations. Bacteria were characterized as *Citrobacter freundii* and *Pseudomonas luteola* by API 20 E test (Profil: 1604573, % ID: 99.80) and API 20 NE test (Profil: 5467743, % ID: 98.20), respectively. However, bacteria were identified as *Citrobacter gillenii* using the molecular test. A comparison of some biochemical characteristics of bacteria isolated from Russian sturgeon in this study with the description by Duman *et al.*,[13] is presented in Table 3.The PCR product size of 16S rRNA for the isolate was about 1,500 bp (Fig. 2). A comparison of the 16S rRNA sequence of the isolate with 16S rRNA sequences in Genbank database showed that the isolate shares 99.00% sequence similarity with the 16S rRNA gene sequence of *C. gillenii*. The generated 16S rRNA gene sequence was deposited in Genbank under the MW057770 accession number. Moreover, the phylogenetic tree based on the 16S rRNA sequence of various strains and Gram-negative bacteria (*Aeromonas hydrophila* and *Escherichia coli*) indicated that our isolate clustered with the *C.gillenii* strains (Fig. 3). Therefore, our isolate belongs to *C. gillenii* species.

Table 3. Comparison of some phenotypic features of *Citrobacter gillenii* isolated from *Acipenser gueldenstaedtii* in this study with the previously published report.

Biochemical features	This study	Duman *et al.*[13]
Gram stain	-	-
Cytochrome oxidase	-	+
Catalase	+	+
Motility	+	+
Pigmentation	-	-
Hemolytic activity	Gamma	Not performed
B-galactosidase	+	+
Arginine dihydrolase	-	+
Lisin decarboxylase	Not clear	+
Ornithine decarboxylase	Not clear	+
Reduction of nitrates to nitrites	+	+
Indole production	-	+
Fermentation of glucose	+	+
Urease	-	-
Esculin	+	+
Gelatin	-	-
H$_2$S production	+	+
Tryptophan deaminase	-	-
Voges-Proskauer	-	-
Assimilation of		
Glucose	+	+
Arabinose	+	+
Mannose	+	+
Mannitol	+	+
Inositol	-	-
Sorbitol	+	-
Rhamnose	+	-
Saccharose	+	+
Melibiose	+	Not performed
Amygdalin	+	+
N-acetyl-glucosamine	+	+
Maltose	+	+
Gluconate	+	+
Capric acid	-	-
Adipic acid	-	-
Malate	+	+
Trisodium citrate	+	+
Phenylacetic acid	+	+
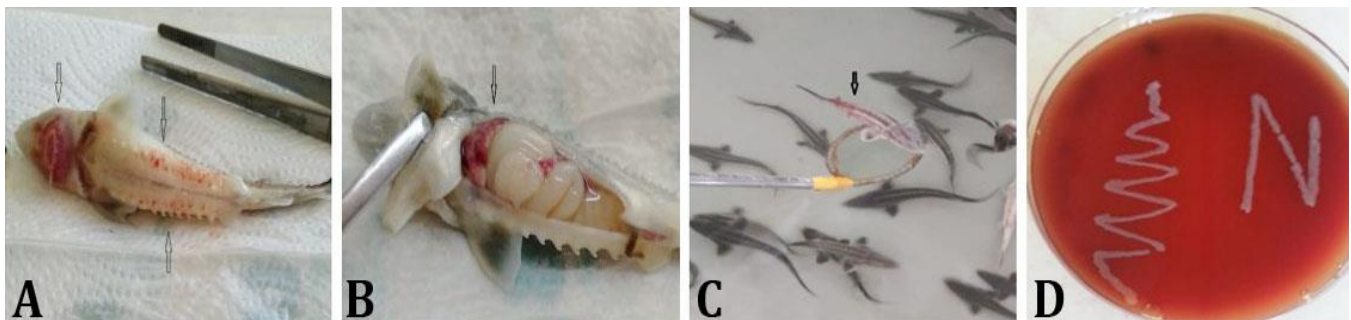
Fig. 1. External and internal disorders in the sturgeon caused by bacteria. **A)** Hemorrhages around the mouth and abdominal area (arrows); **B)** Hemorrhages in the liver (arrow); **C)** Lethargy, swimming disorder and very pale appearance (arrow); **D)** *Citrobacter gillenii* growth on the blood agar.
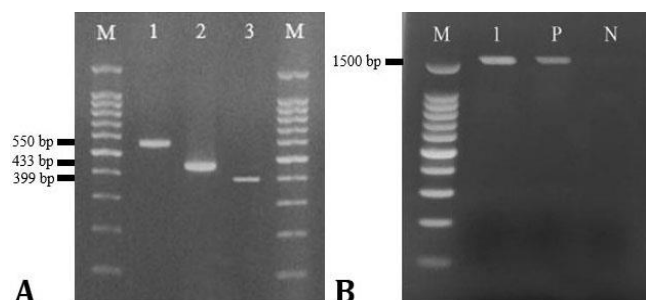
Fig. 2. A) Gel electrophoresis image of different resistance genes. M: 100 bp DNA marker; lane 1: *ampC* (550 bp); lane 2: *sulI* (433 bp); lane 3: *floR* (399). **B)** The image of polymerase chain reaction product belonging to *Citrobacter gillenii* strains. lane 1: Bacteria; P: Positive control; N: Negative control.

The anti-microbial susceptibility test indicated that bacteria were sensitive to enrofloxacin, streptomycin, amoxicillin and oxytetracycline and resistant to penicillin, trimethoprim/sulfamethoxazole, florfenicol and erythro-mycin. The most effective antibiotic was enrofloxacin. A total of 3 different ARGs (multi-drug-resistant bacteria) were determined in a *C. gillenii* isolate. These genes were b-lactam, sulfonamides and florfenicol-related genes; ampC, sul1 and floR, respectively. The ARGs resulted in the amplification of DNA fragments with various band intensities, and their sizes were about 399, 433 and 550 bp, for floR, sul1 and ampC, respectively (Fig. 2). No other ARG was detected in bacteria. Sequencing of PCR products showed that sul1 and floR resistance genes of our bacteria isolate have 100% and 99.00% percentage identity with sul1 and floR resistance genes from *Citrobacter* and other enterobacteria species. These sequences were deposited in the GenBank database under accession numbers of MW123002 and MW123001, respectively. However, the sequencing of the *ampC* resistance gene of our isolate failed.

Discussion

The outbreak of the citrobacteriosis affecting Russian sturgeon occurred in a trout farm in Rize province, Turkiye. Despite the antibiotic treatment (20.00 mg kg^{-1} for three days enrofloxacin via intra-peritoneal route), this outbreak persevered for six weeks with high mortality (approximately 35.00 - 40.00%). In the present study, the following results were obtained: I) Isolated bacteria from the diseased Russian sturgeon were characterized and identified as *C. gillenii* by biochemical and molecular methods including API 20E and 20NE and 16S rRNA gene region sequencing, respectively. This study reports *C. gillenii* in Russian sturgeon in Turkiye, for the first time, and *C. gillenii* is assumed as a causative agent of the disease. II) The phenotypic and genotypic anti-microbial susceptibility of *C. gillenii* strain was determined.

Citrobacter sp. is known as an opportunistic pathogen. The outbreak with high mortality was related to a mixed infection with IPNV in a Spain trout farm.[9] *Citrobacter* sp. was also isolated from rainbow trout in Erzurum[33] and Trabzon.[12] In both cases, mortality rate was low. The *C. gillenii* was first reported from cultured rainbow trout in Turkiye; but, there is no information about mortality. However, in the reported study, a persistent disease with high mortality was observed. On other hand, the ammonia amount is within normal limits in the inlet water for sturgeon culture. However, the high ammonia amount of rearing water is due to the high density of fish. The physical and chemical parameters of the farm water should be considered among the causes of high fish mortality.

In the current study, the etiology of this outbreak with high mortality was investigated using biochemical and molecular tools. Bacteria were characterized as *C. freundii* and *P. luteola* by API 20 E and API 20 NE tests, respectively. However, bacteria were identified as *C. gillenii* by 16S
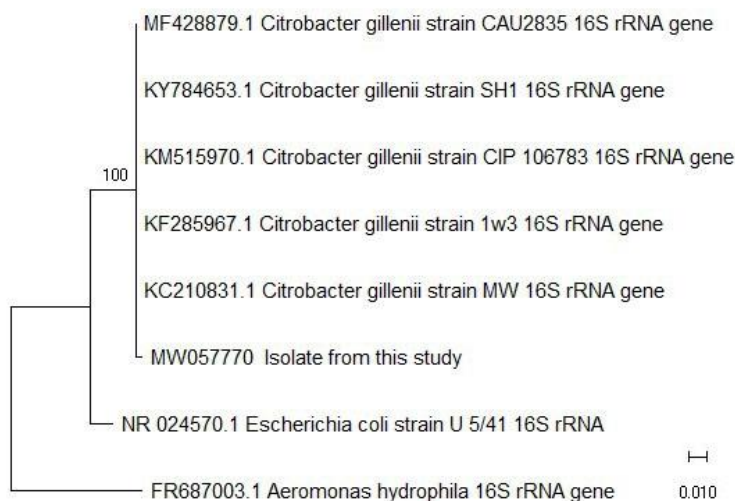
Fig. 3. The phylogenetic tree based on 16S rRNA gene sequence of our isolate and various related microorganisms generated by neighboor-joining method. The numbers on branches indicate the percentage of bootstrap values of the 1,000 bootstrap replicates.

rRNA gene region sequencing. All three bacteria belonging to Enterobacteriaceae. Previously, there were some confusions regarding the species of this genus; but, the reorganization of the genus including 12 *Citrobacter* species represented an important advancement in the identification of *Citrobacter* species. The complex of *C. freundii* was divided into eight different species, including *C. gillenii* sp. Probably because of limitations of biochemical analysis, the API 20 E test showed *C. freundii*. As a result, there is a poor relationship between phenotypic and genotypic identification methods. It is known that molecular methods including DNA fingerprinting are more discriminative than the other methods. Also, the phylogenetic analysis confirmed our isolate is a *C. gillenii* making a group of *C. gillenii* isolates from different countries. On other hand, in a previous study, typical and atypical *Citrobacter* sp. and *C. gillenii* species were defined by molecular methods in Turkiye. In contrast to the results detailed in our study, it was detected that some of the *Citrobacte*r species show oxidase-positive reactions.[13]

Antibiotics are usually used for treating bacterial diseases in humans and animals all over the world. In aquaculture, used antibiotics can accumulate into the aquatic environment and may trigger the development of anti-microbial resistance. Thus, after a bacterial disease outbreak, anti-bacterial therapy may fail.[34] In our study, according to the anti-microbial test results, bacteria were sensitive to enrofloxacin, streptomycin, amoxicillin and oxytetracycline. However, in another study, it was reported that *C. braakii* isolate, another *Citrobacter* species, was resistant to enrofloxacin, amoxicillin and oxytetracycline.[13] In this study, resistance genes (*ampC, sul1* and *floR*) were also determined in *C. gillenii* isolate. Whereas, in a previous study, *sulI, tetA, tetB* and *tetD* genes were identified in *C. gillenii* isolates.[13] In the last decade, florfenicol was one of the most commonly used antibiotics in the aquaculture sector of Turkiye. It was also one of the most preferred antibiotics for the treatment of bacterial fish diseases.[35] According to the anti-microbial susceptibility test, bacteria were resistant to florfenicol. Bacterial DNA also harbored the *floR* gene. Additionally, our isolate was resistant to trimethoprim/ sulfamethoxazole and carried the *sul1* resistance gene. These results could indicate that the common use of a combination of sulfamethoxazole and trimethoprim in aquaculture increases resistance against these antibiotics in Turkiye.

In conclusion, The *C. gillenii* was identified for the first time in Russian sturgeon by biochemical and molecular methods in Turkiye. The phenotypic and genotypic anti-microbial susceptibility of *C. gillenii* strain was also determined. The results of this study supply useful information for the diagnosis and treatment of this newly emerged disease of Russian sturgeon.

Conflict of interest

The authors declare there is no conflict of interest.

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