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First isolation and identification of *Aeromonas veronii* and *Chryseobacterium joostei* from reared sturgeons in Fars province, Iran

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Abstract

The purpose of the present study was to isolate and identify the pathogenic agents in *Acipenser stellatus* (Pallas, 1771) and *Huso huso,* (Linnaeus, 1758) reared in the south of Fars province, Iran which have shown infectious disease signs. Samples from spleen and kidney of 32 fishes showing septicemia symptoms such as decreasing of appetite, unbalanced swimming, expanded wounds, and petechia on the body surfaces, pectoral fins rot, visceral hemorrhage, bleeding on the spleen, and heart ascites were collected. Then samples were cultured on brain heart infusion agar growth media, stain and biological and biochemical tests on purified bacteria were performed. On the other hand, 16S rDNA region of the isolated organism was amplified using PCR. The amplified gene fragment was sequenced and evolutionary history was inferred by phylogenetic tree construction using neighbor-joining method. Results indicated that two bacterial species including *Chryseobacterium joostei* which isolated from the kidney of stellate sturgeon (43.00%), and *Aeromonas veronii* which isolated from the spleen of both sturgeon species (75.00% and 31.00% from beluga and stellate sturgeon, respectively), were recognized. Phylogenetic tree analysis showed that Fars isolated organisms including *A. veronii* and *C. joostei* had highest similarity with *A. veronii bv veronii* and *C. joostei* isolated from France, respectively.

نخستین جداسازی و شناسایی باکتری‌های *آئروموناس ورونی* و *کرایزوباکتریوم جوستئی* از ماهیان خاویاری پرورشی در استان فارس، ایران

چکیده

پژوهش پیش رو با هدف جداسازی و شناسایی عامل بیماری عفونی در ماهیان اوزون برون و فیل ماهی پرورشی در جنوب استان فارس انجام گردید. بدین منظور، نمونه‌برداری از طحال و کلیه ۳۲ قطعه ماهی دارای علائم سپتی سمی نظیر کاهش اشتها، شنای نامتعادل، زخم‌های گسترده و خونریزی نقطه‌ای در سطح بدن، خوردگی باله‌های سینه‌ای، هموراژی احشایی، خونریزی در طحال و آب آوردگی قلب صورت گرفت. پس از کشت میکروبی روی محیط کشت عصاره قلب و مغز، مراحل رنگ‌آمیزی و تست‌های بیوشیمیایی از باکتری‌های خالص سازی شده انجام شد. جهت تائید ملکولی، ناحیه 16S rDNA باکتری جدا شده توسط PCR تکثیر شده و مورد خوانش سکانس قرار گرفت. همچنین تاریخچه تکاملی باکتری توسط ترسیم درخت فیلوژنی با استفاده از تکنیک اتصال-همسایگی مشخص شد. یافته‌ها حاکی از این بود که دو گونه باکتری شامل *کرایزوباکتریوم جوستئی* که از کلیه ماهی اوزون برون جدا شد (با ۴۳/۰۰ درصد جداسازی) و *آئروموناس ورونی* که از طحال هر دو گونه ماهی جدا شد (با ۷۵/۰۰ و ۳۱/۰۰ درصد جداسازی به ترتیب در فیل ماهی و اوزون برون) تشخیص داده شدند. ترسیم درخت فیلوژنی نیز جدایه فارس *آئروموناس ورونی* و *کرایزوباکتریوم جوستئی* را با بیشترین همسانی به ترتیب با *آئروموناس ورونی زیست‌جوره ورونی* و *کرایزوباکتریوم* جوستئی جدا شده در فرانسه نشان داد.

واژه های کلیدی: *آئروموناس ورونی*، استان فارس، اوزون برون، فیل ماهی، *کرایزوباکتریوم جوستئی*

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Introduction

Beluga, *Huso huso,* (Linnaeus, 1758) and stellate sturgeon, *Acipenser stellatus,* (Pallas, 1771) are belong to the family Acipenseridae. They have important roles in the Caspian Sea in related to biodiversity, ecosystem, commercial harvest and caviar production.[1,2] As the endangered species which have been introduced by international union for conservation of nature (IUCN), they were supervised from April 1998 by the convention on international trade in endangered species (CITES) of wild fauna and flora.[2,3] In fact, the main factor of stock reduction of these species is overfishing under illegal fisheries.[3] Hence, breeding and rearing of sturgeons have been expanded in Caspian littoral countries such as Iran and Russia, since the second half of the twentieth century. It's obvious that rearing condition, especially in high density, leads to an outbreak of infectious diseases that haven't already occurred.[4]

Background information on micro-organisms related to sturgeons are mostly about the microbial flora of sturgeon's digestive tract and other organs.[5] The information about sturgeon's dominant diseases and pathogenic agents and their prevalence, distribution, originality and ecotypes are limited.[6] However, some studies could identify some bacterial pathogens that cause infectious diseases in these animal spieces; for instance, *Aeromonas hydrophila*,[7-9] *A. veronii*,[10] *A. veronii bv. sobria*,[11] *Flavobacterium johnsoniae*,[12] *F. hydatis*,[9] *Chryseobacterium aquaticum*,[13] *Vibrio vulnificus*,[14] *V. alginolyticus* and *Pasteurella* sp.,[15] have been reported. Also, *Yersinia ruckeri, F. columnare*, *F. psychorphilum* and *Renibacterium salmoninarum*, have been isolated from sturgeons reared in recirculating aquaculture system.[16] Moreover, some experimental acipenserids are vulnerable to bacterial hemorrhagic septicemia (caused by *Aeromonas* and *pseudomonas* genera) and vibriosis (caused by *V. anguillarum*) if they had stocked in fresh and salt water, respectively. *Cytophaga* infection was expected more at lower stocking intensity.[7] Despite a number of researches which have been undertaken on isolation and identification of bacterial pathogens in these commercial and valuable fishes, however, further information still needed.[4] Therefore, the main purpose of the present study was to isolate and identify the pathogenic agents in sturgeons (*Acipenser stellatus and Huso huso*) reared in Fars province, Iran, which have shown infectious disease signs.

Materials and Methods

In a sturgeon farming site located in the south of Fars province, Estahban county, Ij area (29° 02' N, 54° 24' E) an infectious disease with a severe mortality rate was reported to the department of clinical sciences, School of Veterinary Medicine, Shiraz University) in May 2016. The farm was visited and a number of moribund stellate sturgeons (n = 16) and belugas (n = 16) showing infection symptoms, randomly have been sampled and weighed. Clinical examination and autopsy were adopted based on Noga's Instruction;[17] likewise, some parameters of water such as temperature, dissolved oxygen (DO), pH, ammonia, and nitrite were measured according to the methods proposed by American public health association.[18]

Microbial sampling and cultivating. Samples from spleen and kidney of fishes were streaked aseptically over brain heart infusion agar (BHIA) plates and were incubated at 25 ˚C for 48 hr. Suspected bacterial colonies were identified and purified, and the following biological and biochemical tests were carried out:[19] 1) Gram stain, shape, motility (24 ˚C), and colony pigmentation; 2) Growth in 0, 0-3 and 6.50% of NaCl, and at 4, 12, 24 and 37˚C, and under aerobic condition; 3) Biochemical tests including Catalase, oxidase, indole, phosphatase, H_2S, lysine decarboxylase, ornithine decarboxylase, methyl red, Voges-Proskauer, nitrate, utilization of sodium citrate; 4) Degradation of aesculin, blood (hemolysis), gelatin, tween 80 and 5) Acid production from some sugars such as cellobiose, fructose, xylose, melecitose, lactose, arabinose, sucrose, galactose, raffinose, glucose, mannitol, and sorbitol.

Polymerase chain reaction (PCR), sequencing and phylogenetic analysis. Bacterial colonies identified as genera *Chryseobacterium* and *Aeromonas* using biochemical tests were subjected for DNA extraction. DNA was extracted based on Holmes and Quiqley with some modifications.[20] The PCR was conducted using Accupower PreMix PCR kit (BioNeer, South Korea). An amount of extracted DNA (~40 ng) and 20 picomoles of each 16S rDNA primers were used in each PCR reaction according to PCR kit's manufacturer instructions.[21] The primer sequences which described previously were FUP, 5´-AGA GTTTGATCCTGGCTCAG-3´ and RUP 5´-ACGGCTACCTTGTT ACGACTT-3´.[22] Amplification reaction was carried out in a gradient thermocycler (MG 5331; Eppendorf, Hamburg, Germany). Thermal condition included an initial denaturation cycle at 94 ˚C for 5 min, and 35 cycles of denaturation at 94 ˚C for 1 min, annealing at 58 ˚C for 1 min, extension at 72 ˚C for 1 min and a final extension at 72 ˚C for 7 min. The resulting PCR products were electrophoresed on 1% agarose gel and visualized using red safe (Intron Biotechnology, Seoul, South Korea). In each PCR reaction both positive and negative controls were included.

The amplified PCR products were extracted from gel using QIAquick gel extraction kit (Qiagen, Hilden, Germany) as described by the kit manufacturer. The purified PCR products were subjected to sequencing (Macrogen, Seoul, South Korea). The resultant sequences were BLAST searched and compared with the similar sequences in GenBank at NCBI, to determine genus and species of infecting pathogens.[21] The sequences were

multiple-aligned with a number of bacterial sequences retrieved from the GenBank using Clustal W, (version 2.0.12; UCD Conway Institute, Dublin, Ireland).[23] The pairwise genetic distances for the examined isolates were estimated using MEGA, (version 4.0, MEGA Inc., Englewood, USA). The evolutionary history was inferred by phylogenetic tree construction using neighbor-joining method. The neighbor-joining tree was constructed using program MEGA (MEGA Inc.).[24] Reliability of the inferred trees was tested by 1000 bootstrap replications.

Results

Mortality rate and clinical examination. Mortality rate that is calculated based on the daily cumulative casualties counts, was estimated about 40% only within one week (4775 from 12000 stocked fishes) as a high moderately trend. Clinical examination and autopsy of stellate sturgeon (average weight = 9.70 ± 1.10 kg) and beluga (average weight = 20.70 ± 3.80 kg) indicated some symptoms include decreasing of appetite, unbalanced swimming, expanded wounds and petechia on body surfaces, pectoral fins rot, visceral hemorrhage, bleeding on the spleen, and heart ascites, without any difference between the fish species (Fig. 1).

Fig. 1. Clinical examination and autopsy findings of infected sturgeon (A) Expanded wounds on body surfaces, (B) Petechia on body surfaces, (C) Pectoral fins rot, (D) Visceral hemorrhage, (E) Bleeding in the spleen, (F) Heart ascites.

Water quality. Based on the results of water analysis including temperature (mean = 24 ˚C), dissolved oxygen (DO; 5.30 mg L^{-1}), pH (7.5), ammonia (0.45 mg L^{-1}), and nitrite (0.17 mg L^{-1}), it was revealed that the water parameters were in normal range.

Biological and biochemical tests. The results demonstrated that two bacterial genera including *Chryseobacterium* with 43.00% frequency was isolated from the kidney of stellate sturgeon, and *Aeromonas* with 75.00% and 31.00% frequency in beluga and stellate sturgeon, respectively was isolated from the spleen of both sturgeon species (Table 1).

Table 1. Biochemical characteristics of bacteria isolated from diseased sturgeons.

Tests	*Chryseobacterium*	*Aeromonas*
Gram stain	-	-
Shape	rod	rod
Pigmentation	yellow	Brown
Motility	+	+
Growth:		
in 0.00% NaCl	+	+
in 0.00-3.00% NaCl	+	+
in 6.50% NaCl	-	-
at 4˚C	+	+
at 12˚C	+	+
at 24˚C	+	+
at 37 ˚C	+	-
Aerobic growth	+	+
Production of:		
Catalase	+	+
Oxidase	+	+
Indole	-	+
Phosphatase	+	+
H_2S	+	+
Lysine decarboxylase	-	-
Ornithine decarboxylase	-	-
Methyl red test	-	-
Voges-Proskauer test	-	+
Nitrate reduction	-	-
Utilization of sodium citrate	-	-
Degradation of:		
Blood hemolysis	+	-
Aesculin	-	+
Gelatin	+	+
Tween 80	-	+
Production of acid from:		
Cellobiose	+	-
Fructose	+	+
Melecitose	-	-
Lactose	+	-
Arabinose	+	-
Raffinose	-	-
Xylose	+	-
Glucose	-	+
Galactose	+	+
Sucrose	+	+
Mannitol	+	+
Sorbitol	-	-
Salicin	-	+

Polymerase chain reaction and sequencing. The PCR product size of 16S rDNA for *Chryseobacterium* and *Aeromonas* were about 1480 and 1508 bp, respectively (Fig. 2). Alignment of nucleotide sequences of 16S rDNA from isolated bacteria with those available 16S rDNA sequences in Genbank showed that the sequences were belonged to two bacterial species including *Chryseobacterium joostei* (with 99.00% similarity) and *Aeromonas veronii* (with 97.00% similarity).

Phylogenetic analysis. According to the phylogenetic tree (Fig. 3), Fars Isolate has the most similarity with *C. joostei* isolated from France, and the least similarity was with Indian isolated. On the other hand, Fars isolate of *A. veronii* has maximum resemblance with *A. veronii bv. veronii* (Fig. 4).

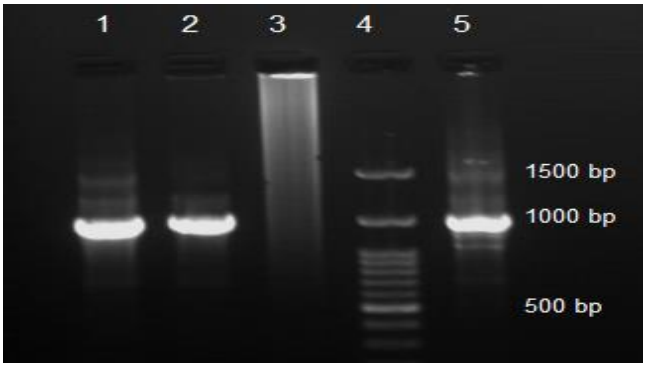

Fig. 2. PCR products electrophoresed on 1% agarose gel. Lane 1: *Chryseobacterium*, Lane 2: *Aeromonas*, Lane 3: Negative control, lane 4: Molecular marker (DNA ladder 100 bp +, SinaClon, Tehran, Iran), and lane 5: Positive control.

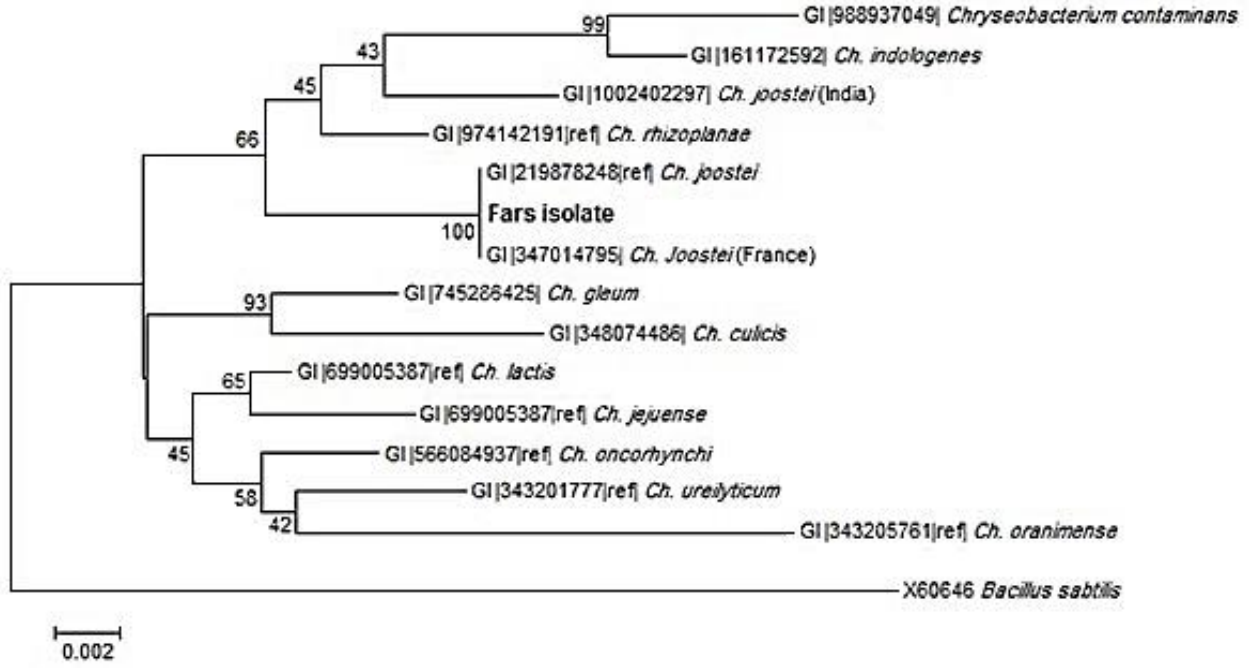

Fig. 3. Phylogenetic tree of *Chryseobacterium joostei*, Fars isolate from the present study and other *Chryseobacterium spp.* based on the 16S rDNA gene sequences. *Bacillus subtilis* was used as an out-group.

Discussion

The most prevalent bacterial and viral diseases of sturgeons around the world are columnaris diseases, cytophaga-like infections, motile *Aeromonas* septicemia, yersiniosis, vibriosis, white sturgeon adenovirus disease, white sturgeon herpesvirus disease, white sturgeon iridovirus disease, white sturgeon papova-like virus disease and epitheliocystis.[4,15,25] Results of the present study showed that *Aeromonas veronii* and *Chryseobacterium joostei* were isolated from sturgeons. These agents have been recognized based on biochemical tests followed by PCR detection of 16S rDNA gene and nucleotide sequencing for their specie confirmation.

Measured water parameters (temperature, DO, pH, ammonia and nitrite) were in normal range, so high mortality rate in the examined fish was probably due to the infectious agents. Observed clinical signs including hemorrhage and petechia in the head, around the mouth, operculum, anal and fins base, along with opened and severe wounds on the abdomen and dorsal areas of the body were expected to be seen in the infections caused by bacteria especially motile aeromonads.[17] Based on our biochemical and molecular tests, the isolated bacteria from both studied sturgeons was *Aeromonas veronii*. *A. veronii* (strain X106909) was identified as a pathogen and cause of Siberian sturgeon's (*Acipenser baerii*) mortality for the first time in China.[10] Likewise, *A. hydrophila* as a

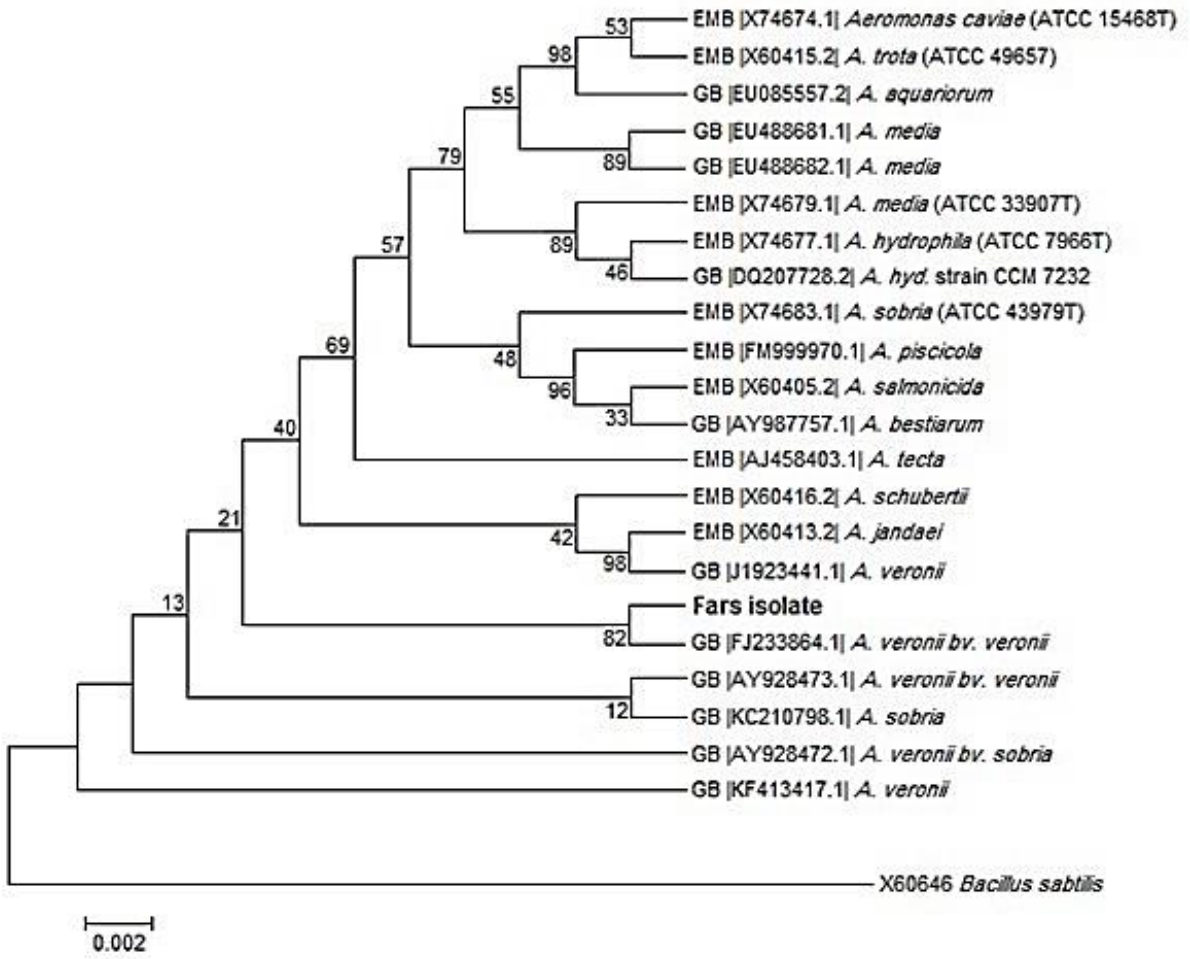

Fig. 4. Phylogenetic tree generated based on 16S rDNA gene sequences of *Aeromonas* isolates detected in the present study and the other *Aeromonas spp.* from Genbank. *Bacillus subtilis* was used as an out-group.

pathogenic agent has been isolated from Amur sturgeon (*Acipenser schrenckii*) in China.[8] In addition to this kind of fishes, *A. veronii bv. sobria* was a causative agent of mass mortality in cultured Nile tilapia in Egypt.[11] *A. veronii bv. sobria* was detected in both diseased common carp and grass carp but not silver carp.[26] Fars isolate of *A. veronii* (present study) had maximum similarity with *A. veronii bv. veronii*. *A. veronii* was also the causative agent of epizootic ulcerative syndrome (EUS) in fish in Bangladesh.[27]

The members of genus *Aeromonas* are usually circulating in aquatic environments,[28] and they have been reported as a significant pathogen in lower vertebrates such as fishes, amphibians, reptiles, and even human.[29] Some species of this genus has a wide host range and could cause hemorrhagic septicemia in fish.[26] *A. hydrophila* was identified via cultivating from epithelial wounds of sturgeons.[16] In Turkey, *A. hydrophila* was detected in Russian sturgeon (*Acipenser gueldenstaedtii*) as the cause of bacterial hemorrhagic septicemia and high rate of mortality.[9] In order to prevent the *Aeromonas* infection in sturgeon, tubifex (sewage worm) should be provided as natural and live food.[7] Amikacin, ciprofloxacin (Ciproxin), and gentamicin are the best antibiotics in eastern Asia are used against *Aeromonas* infections.[8]

Other results indicated that *Chryseobacterium joostei* isolated and identified from stellate sturgeons. The members of genus *Chryseobacterium* have been isolated from soil, water, and food sources.[30] Some species of this genus such as *C. indologenes* could cause disease in newborns and adults with the deficient immune system.[13,31] Most recently, some members of genus *Chryseobacterium* usually do not associate with infection in fish, although cases of disease related to these species are increasing. So that *C. balustinum*, *C. scophtalmum*, and *C. joostei* have been isolated until now.[13] Some novel *Chryseobacterium* sp. were recovered from different fishes which could be pathogenic, such as *C. aquaticum*,[22] *C. daecheongense*,[32] *C. piscicola*,[33-35] *C. indologenes*,[36] *C. Chaponense*,[37] and *C. viscerum*.[38]

According to the growing number of infection out-breaks in fishes, identification of sturgeon's pathogens, such as *A. veronii* and *C. joostei* in the present report, was

expected. The changes of Iranian sturgeons' habitat from Caspian Sea (natural habitat) to inland freshwater farms could relate to the increasing of their susceptibility to new diseases, especially under intensive condition. Therefore, for - the economic value of sturgeon, further researches on infectious pathogens are required.

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Conflicts of Interest

The authors of the paper do not declare a competitive financial interest.

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