**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 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.

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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. 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. In fact, the main factor of stock reduction of these species is overfishing under illegal fisheries. 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.

Background information on micro-organisms related to sturgeons are mostly about the microbial flora of sturgeon's digestive tract and other organs. The information about sturgeon's dominant diseases and pathogenic agents and their prevalence, distribution, originality and ecotypes are limited. However, some studies could identify some bacterial pathogens that cause infectious diseases in these animal species; for instance, *Aeromonas hydrophila*, *A. veronii*, *A. veronii bv. sobria*, *Flavobacterium johnsoniae*, *F. columnare*, *Flavobacterium aquaticum*, *Vibrio vulnificus*, *V. alginolyticus* and *Pasteurella sp.*, have been reported. Also, *Yersinia ruckeri*, *F. columnare*, *F. psychrophilum* and *Renibacterium salmoninarum*, have been isolated from sturgeons reared in recirculating aquaculture system. 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. 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. 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, Lj 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; 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.

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: 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, H₂S, 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, xylene, melicitose, 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. 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. The primer sequences which described previously were FUP, 5’-AGA GTTTGATCCTGGCTCAG and RUP 5’-ACGGCTACCTTGTT ACGACTT-3’. 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.
multiple-aligned with a number of bacterial sequences retrieved from the GenBank using Clustal W, (version 2.0.12; UCD Conway Institute, Dublin, Ireland).

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.).

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).

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                   | +                | +         |
| H₂S                           | +                | +         |
| Lysine decarboxylase          | -                | -         |
| Ornithine decarboxylase       | -                | -         |
| Methyl red test               | -                | -         |
| Voges-Proskauder test         | -                | +         |
| Nitrate reduction             | -                | -         |
| Utilization of sodium citrate | -                | -         |
| Degradation:                  |                  |           |
| 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).

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. 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. 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. Likewise, *A. hydrophila* as a
pathogenic agent has been isolated from Amur sturgeon (*Acipenser schrenckii*) in China. In addition to this kind of fishes, *A. veronii* bv. sobria was a causative agent of mass mortality in cultured Nile tilapia in Egypt. *A. veronii* bv. sobria was detected in both diseased common carp and grass carp but not silver carp. 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.

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

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. Some species of this genus such as *C. indologenes* could cause disease in newborns and adults with the deficient immune system. 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. Some novel *Chryseobacterium* sp. were recovered from different fishes which could be pathogenic, such as *C. aquaticum*, *C. daecheongense*, *C. piscicola*, *C. indologenes*, *C. Chaponense*, and *C. viscerum*.

According to the growing number of infection outbreaks 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.

References

1. Norouzi M, Pourkazemi M, Fatemi M. Application of microsatellite markers to study the genetic structure of stellate sturgeon populations (Acipenser stellatus, Pallas, 1771) in the south Caspian Sea. Iran J Fish Sci 2009; 8(1):73-84.
2. IUCN red list of threatened species website. "Huso huso". International union for conservation of nature. Available at: http://www.iucnredlist.org/details/102690. Accessed 30 March 2014.
3. Pourkazemi M. Caspian Sea sturgeon conservation and fisheries: past present and future. J Appl Ichthyol. 2006; 22(Suppl. 1):12-16.
4. Bauer O, Pugachev O, Voronin V. Study of parasites and diseases of sturgeons in Russia: A review. J Appl Ichthyol 2002; 18(4-6):420-429.
5. Ghorbani-Choboghol H, Khosravi A, Sharifzadeh A, et al. Gastrointestinal microflora of captured stellate sturgeon (Acipenser stellatus, Pallas, 1771) from southeast Caspian Sea, Iran. Iran J Fish Sci 2014; 13(2):319-329.
6. LaPatra S, Subasinghe R, Schlotfeldt H. Present and future outlook on the ecology of wild sturgeon and fish health challenges associated with the management of these species. Bull Eur Assoc Fish Pathol 1999; 19(6): 289.
7. Zaharia T, Dumitrescu E. Disease detected at sturgeon reared in fresh and salt water. Indian J Geomarine Sci (National Institute for Marine Research and Development). 2011; 82(2): 671-685.
8. Meng Y, Xiao HB, Zeng LB. Isolation and identification of the hemorrhagic septicemia pathogen from Amur sturgeon, Acipenser schrenckii. J Appl Ichthyol 2011; 27(2):799-803.
9. Timur G, Akayli T, Korun J, et al. Study on bacterial haemorrhagic septicemia in farmed young Russian sturgeon in Turkey (Acipenser gueldenstaedtii). Turk J Fish Aquat Sci 2010; 25(1): 19-27.
10. Ma Z, Yang H, Li T, et al. Isolation and identification of pathogenic Aeromonas veroni isolated from infected Siberian sturgeon (Acipenser baerii). Acta Microbiol. Sin (Wei sheng wu xue bao) 2009; 49(10): 1289-1294.
11. Eissa IAM, El-lamel M, Sherif M, et al. Aeromonas veroni biovar sobria a causative agent of mass mortalities in cultured Nile Tilapia in El-Sharkia governorate. Egypt J Fish Sci J 2015; 12 (5): 90-97.
12. Karatas S, Ercan MD, Steinum TM, et al. First isolation of a Flavobacterium johnsoniae like bacteria from cultured Russian sturgeon in Turkey. Adv Anim Vet Sci 2010; 9(14): 1943-1946.
13. Bernardet JF, Vancanneyt M, Matte-Tailliez O, et al. Polyphasic study of Chryseobacterium strains isolated from diseased aquatic animals. Syst Appl Microbiol 2005; 28(7): 640-660.
14. Safari R, Adel, M, Ghiasi, M, et al. First isolation and identification of Vibrio vulnificus (biotype 2) from cultured beluga, Huso huso in Iran. Casp J Environ Sci 2014; 13(3): 275-281.
15. Costinar L, Hermani V, Pascu C, et al. Isolation and characterization of Vibrio alginolyticus and pasteurella spp. from Siberian sturgeon (Acipenser baerii). Lucr Stif Med Vet 2010; 1: 125-127.
16. Pelkola K, Vennerström P, Viljamaa-Dirks S, et al. Bacterial infections of farmed sturgeon in Finland. Fin Food Saf Auth Evira 2012; 12: 122-127.
17. Noga EJ. Fish disease diagnosis and treatment. 2nd ed. Ames, USA: Blackwell 2010; 5-78.
18. Eaton AD, Clesceri LS, Rice EW. Standard methods for examination of water and wastewater. 21st ed. Washington DC, USA: American Public Health Association 2005; 433-440.
19. MacFaddin JF. Biochemical tests for identification of medical bacteria. Philadelphia, USA: Lippincott Williams Wilkins 2000; 123-147.
20. Holmes DS, Quiqley M. A rapid boiling method for the preparation of bacterial plasmids. Ann Rev Biochem 1981; 114: 193-197.
21. Woo PC, Lau SK, Teng JL, et al. Then and now: Use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. Clin Microbiol Infect 2008; 14(10):908-934.
22. Kim KK, Lee KC, Oh HM, et al. Chryseobacterium aquaticum sp. nov., isolated from a water reservoir. Int J Syst Evol Microbiol 2008; 58(3): 533-537.
23. Larkin MA, Blackshields G, Brown NP, et al. Clustal W and Clustal X version 2.0. Bioinform 2007; 23(21): 2947-2948.
24. Kumar S, Nei M, Dudley J, et al. MEGA: A biologist-
centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform 2008; 9(4): 299-306.
25. Adkinson MA, Cambre A, Hedrick M. Identification of an iridovirus in Russian sturgeon (A. guldentstaedtii) from northern Europe. Bull Eur Assoc Fish Pathol 1998; 18: 29-32.
26. Modarres Mousavi Behbahani SM, Akhlaghi M, Sharifi-Yazdi H. Phenotypic and genetic diversity of motile aeromonads isolated from diseased fish and fish farms. Iran J Vet Res 2014; 15(3): 238-243.
27. Rahman M, Colque-Navarro P, Kihm I, et al. Identification and characterization of pathogenic Aeromonas veronii biovar sobria associated with epizootic ulcerative syndrome in fish in Bangladesh. App Env Microbiol 2002; 68(2): 650-655.
28. Monfort P, Baleux B. Dynamics of Aeromonas hydrophila, Aeromonas sobria, and Aeromonas caviae in a sewage treatment pond. App Env Microbiol 1990; 56(7): 1999-2006.
29. Janda JM, Abbott SL. Evolving concepts regarding the genus Aeromonas: An expanding panorama of species, disease presentations, and unanswered questions. Clin Infect Dis. 1998; 27(2): 332-344.
30. Loch TP. Identification of novel Flavobacteria from Michigan and assessment of their impacts on fish health. PhD Thesis. Michigan State University. Michigan, USA: 2012.
31. Lin JT, Wang WS, Yen CC, et al. Chryseobacterium indologenes bacteremia in a bone marrow transplant recipient with chronic graft-versus-host disease. Scand J Infect Dis 2003; 35(11-12): 882-883.
32. Kim KK, Bae HS, Schumann P, et al. Chryseobacterium daecheongense sp. nov., isolated from freshwater lake sediment. Int J Syst Evol Microbiol 2005; 55: 133-138.
33. Ilardi P, Fernández J, Avendaño-Herrera R. Chryseobacterium piscicola sp. nov., isolated from diseased salmonid fish. Int J Syst Evol Microbiol 2009; 59(12): 3001-3005.
34. Ilardi P, Abad J, Rintamäki P, et al. Phenotypic, serological and molecular evidence of Chryseobacterium piscicola in farmed Atlantic salmon, Salmo salar L., in Finland. J Fish Dis 2010; 33: 179-181.
35. Bernardet J-F, Segers P, Vancanneyt M, et al. Cutting a Gordian knot: Emended classification and description of the genus Flavobacterium, emended description of the family Flavobacteriaceae, and proposal of Flavobacterium hydatis nom. nov. (basonym, Cytophaga aquatilis Strohl and Tait 1978). Int J Syst Bacteriol 2010; 46(1): 128-148.
36. Lin YT, Jeng YY, Lin ML, et al. Clinical and microbiological characteristics of Chryseobacterium indologenes bacteremia. J Microbiol Immunol Infect 2010; 43: 498-505.
37. Kämpfer P, Fallschissel K. and Avendaño-Herrera R. Chryseobacterium chaponense sp. nov., isolated from farmed Atlantic salmon (Salmo salar). Int J Syst Evol Microbiol 2011; 61: 497-501.
38. Zamora L, Vela AI, Palacios MA, et al. Chryseobacterium viscerum sp. nov., isolated from diseased fish. Int J Syst Evol Microbiol 2012; 35(1): 24-29.