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Isolation, identification, and characterization of pathogenic *Aeromonas hydrophila* from critically endangered *Acipenser baerii*

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ABSTRACT

The Siberian sturgeon (*Acipenser baerii*) is a long-lived and late-maturing fish; its natural populations are considered endangered and listed in the first class of protected animals in Kazakhstan. To date, attempts have been made to increase the number of sturgeons through artificial reproduction in aquaculture and a release of the resultant juveniles into the Ural River, which flows into the Caspian Sea. This species faces an increased risk of *Aeromonas* infections. *Aeromonas hydrophila* is the most important sturgeon pathogen in Kazakhstan, but studies on *A. hydrophila* infection in Kazakhstan are still inconclusive. In the present work, our purpose was to isolate and characterize dominant bacteria in diseased *A. baerii*. This isolate, tentatively named AB005, was identified as *A. hydrophila* in an analysis of its morphological, physiological, and biochemical features and 16S ribosomal-RNA and *gyrB* gene sequences. A pathogenicity test was carried out for the isolate on healthy *Oreochromis niloticus* and *Acipenser ruthenus* via intraperitoneal injection along the caudal peduncle of the fish. Half-lethal doses (LD_{50}) of isolate AB005 for *O. niloticus* and *A. ruthenus* were determined: 8.37×10^5 and 2.89×10^6 colony-forming units per milliliter, respectively. Virulence gene profiling revealed the presence of seven virulence genes related to pathogenicity (acyltransferase, phospholipase A, serine protease, heat-stable cytotonic enterotoxin, nuclease, and aerolysins A and B) in this *A. hydrophila* isolate. Drug sensitivity testing showed that the isolate is sensitive to quinolones, aminoglycosides, nitrofurans, amphenicols, and tetracyclines. The present findings will lay the foundation for future research on this pathogen in Siberian-sturgeon aquaculture.

1. Introduction

The sturgeon is the common name for 27 species of fish belonging to the genus *Acipenser*. They are regarded as the most ancient fish species (Birstein et al., 1997). The sturgeon has substantial economic value as an animal protein source, including caviar and meat. Ninety percent of the world production of sturgeon caviar had come from countries that surround the Caspian Sea, including Kazakhstan, until the year 2000 (Catarci, 2004). The dissolution of the Soviet Union in the early 1990s led to the collapse of existing management and control systems. Subsequently, due to large amounts of illegal catches, water pollution, and destruction of the habitat, the sturgeon population has declined significantly (Orlov et al., 2021; van Uhm and Siegel, 2016). The *Acipenser baerii* geographic range has also decreased at an alarming rate leading to the categorization of *A. baerii* as endangered by the IUCN Red List. This fish is considered endangered in Kazakhstan, Russia, China, and Mongolia (Lagutov, 2008). Consequently, the Kazakhstan government, just as the leadership of the other Caspian countries, has attempted to increase the stocks of sturgeons via artificial reproduction and a release of the resulting fingerlings into the Ural River flowing into the Caspian Sea (Bartley and Rana, 1998). Sturgeon aquaculture may be a solution to the decline of the sturgeon population and may restore the almost interrupted life cycle of the species. In addition, aquacultural production of sturgeon meat and caviar satisfies the needs of the market,

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and as a result, the fishing pressure on natural stocks of sturgeon fishes can be reduced. On the other hand, rapid development of aquaculture has been accompanied by outbreaks of diseases caused by bacterial infections (primary due to unhygienic and often stressful conditions of cultivation) that lead to high mortality and catastrophic economic losses in sturgeon aquaculture.

Aeromonas hydrophila is the major pathogen of cultured sturgeon species, and in some cases, the mortality even reaches 100% (Jiang et al., 2016). *A. hydrophila* is a facultative anaerobic, gram-negative, rod-shaped bacterium occurring in all bodies of water worldwide (Cabral, 2010). Outbreaks of *A. hydrophila* infectious diseases have been identified in various countries and previously well described as the pathology causing septicemia with an open dermal ulcer, gastroenteritic hemorrhage, ascites, and cloacal hemorrhaging (El-Son et al., 2019; Elsheshtawy et al., 2019; Pridgeon and Klesius, 2011; Zhang et al., 2013). However, the symptoms and pathological lesions in infected fish differ depending upon the particular bacterial isolates or strains. The ability of this bacterium to cause disease, depends largely on presence of various virulence factors, which include aerolysin (*aer*), serine protease (*ser*), elastase (*ahyB*), cholesterol acyltransferase (*gcaT*), type III secretion system (*ascV*), DNases (*exu*), polar flagella (*fla*), cytotonic enterotoxins (*act*, *alt*, *ast*), lipase (*lip*) (Allan and Stevenson, 1981; Pattanayak et al., 2020; Rasmussen-Ivey et al., 2016). It was reported that aerolysin, cytotoxic enterotoxin and extracellular serine protease to be the most critical genes for identifying potentially pathogenic *A. hydrophila* strains (Li et al., 2011; Sha et al., 2002). However, the data available in this respect indicate, that the pathogenicity of *A. hydrophila* is probably multifactorial, and does not arise from a single gene, but it is likely the result of synergistic effects of several virulence genes (Beaz-Hidalgo and Figueras, 2013; Hu et al., 2012; Sha et al., 2009; Zhao et al., 2020).

Most of sturgeon species in Kazakhstan are cultured, and bacterial pathogens are the main cause of mortality in these fish (Ginayatov et al., 2016; Nurzhanova et al., 2021); however, to date, information about diseases and fish health control is rather limited. To our knowledge, there is no record of *A. hydrophila* infection among cultured sturgeons in Kazakhstan.

The largest amount of sturgeon products in Kazakhstan is produced in the Uralsk region on a full-system sturgeon farm called the LLP Educational and Scientific Complex of Pilot Industrial Production of Aquaculture (Uralsk, Kazakhstan). This enterprise maintains the largest sturgeon brood stock in Kazakhstan. The breeding of marketable products and the maintenance of brood stock is carried out by means of recirculating aquaculture systems. By comparing microbial communities in a recirculating aquaculture system, in a recent study, we demonstrated that the abundance of *Aeromonas* species dramatically increases at the optimal rearing temperature (18 °C) of *A. baerii* (Sergaliev et al., 2021). Lately, diseases with characteristic symptoms of generalized bacterial hemorrhagic septicemia and skin ulceration have been occurring among cultured *A. baerii* fishes on this farm. After isolation of the prevalent bacteria from diseased *A. baerii*, a typical bacterial isolate has been identified as *A. hydrophila*. In the present article, the results on isolation, identification, pathogenicity, virulence genes, and growth characteristics of this *A. hydrophila* isolate are described. The histopathological alterations produced by these bacteria and antibiotic sensitivity were additionally investigated to manage the disease.

2. Materials and methods

2.1. Fish sampling

Diseased *A. baerii* individuals were sampled in a sturgeon fish farm located in Western Kazakhstan (Uralsk), which employed recirculating aquaculture systems technology (RAS). The fish hatchery employed water recirculation at a renewal rate of 5–10% of total system volume per day. Briefly, fresh water was pumped from a well and was strongly aerated and sand-filtered before entering each RAS, where mechanical filtration is also carried out. After mechanical filtration, water flows to a biofilter tank for biological filtration and is subsequently pumped back to a protein skimmer where it reenters the system. In fish farm oxygen levels in the fish tanks were kept to an average of 7.3 mg/L, the water temperature at 22 °C and the pH at 7.0. Diseased fish exhibited anorexia, lethargic swimming mostly at the water surface, hemorrhagic spots, and anus inflammation. Anemia was evident from the pale color of the gills, while most affected fish characterized by the presence of multiple ulcers of various surface areas and penetrating deep into muscle.

A total of 7 diseased *A. baerii* were collected and were transferred to the Laboratory of Fisheries Research (Ichthyology and Aquaculture) in Zhangir Khan West Kazakhstan Agrarian Technical University (Uralsk, Kazakhstan) in a special vessel supplied with oxygen for the clinical and bacteriological examinations. The average of diseased fish body weight was 1080 ± 150 g, and total length ranged from 56 to 68 cm.

2.2. Bacterial strains and culturing conditions

The fish were dissected after their skin was cleaned with 75% ethyl alcohol. Three diseased fish with apparent clinical signs were used for isolation of bacterial strains. Bacteria were isolated with a sterile loop from different organs of each fish (liver, kidney, spleen, and external lesions) and were inoculated onto the Luria–Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 15 g/L agar; pH 7) and incubated at 30 °C for 24 h. Bacterial colonies were purified by streaking onto the LB plates (at least three times).

A single bacterial colony was selected and inoculated into LB broth at 37 °C or 30 °C for 18 h incubation and was then preserved at − 80 °C in the LB medium containing 20% (v/v) of sterile glycerol. The dominant isolate was designated as AB005. Of note, that no significant difference was observed in bacterial growth on LB agar plates and LB broth medium at 37 °C and 30 °C. Therefore, in subsequent experiments AB005 isolate were grown in LB or LB agar at 37 °C, unless specified otherwise.

2.3. Physiological and biochemical characterization

Pure cultures of AB005 were inoculated onto the LB medium for overnight incubation at 37 °C, and morphological characteristics such as shape, size, Gram staining, flagellation, and motility were examined. Simultaneously, physiological characteristics were studied by observing the growth of an isolated colony at various temperatures, NaCl concentrations, and pH levels. Freshly inoculated bacterial culture in LB broth was incubated at temperatures ranging from 13° to 42°C for 48 h with constant orbital rotation. To evaluate the influence of pH on growth characteristics, LB culture media with initial pH levels adjusted to 3.0, 5.0, 7.0, and 9.0 were prepared with 0.5 N HCl or NaOH. For evaluation of growth in response to salt concentration, LB broth was supplemented with NaCl at various concentrations (1–5%). After that, the inoculated media were incubated at 37 °C for 48 h with constant orbital rotation. In both culture manipulations, bacterial growth was determined by measuring optical density at 600 nm (OD_{600}) on a spectrophotometer. All assays were performed as three independent experiments with three biological replicates in each. Biochemical characterization procedures were based on a manual for the identification of common bacterial systems (Holt et al., 1994). These experiments were carried out in triplicate.

2.4. 16S ribosomal RNA (rRNA) and gyrB gene sequence analysis

Genomic DNA of isolate AB005 was extracted using a commercial DNeasy PowerLyzer Microbial kit (Qiagen, Germany). A pair of universal primers [27F: 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R: 5′-GGCTACCTTGTTACGACTT-3′ (Lane, 1991)] was employed for the amplification of the 16S rRNA gene. Two pairs of primers [gyrB-F: 5′-GAAGTCATCATGACCGTTCTGCA(TC)GC(TCAG)GG(T CAG)GG

(TCAG)AA(AG)TT(TC)GA-3′ and gyrB-R: 5′-AGCAGGGTACGGATGTG CGAGCC (AG)TC(TCAG)AC(AG)TC(TCAG)GC(AG)TC(TCAG)GTCAT-3′ (

Yamamoto and Harayama, 1995); *gyr*B3F: 5′-TCCGGCGGTCTGCA CGGCGT-3′ and *gyr*B14R: 5′-TTGTCCGGGTTGTACTCGTC-3′ (Yáñez et al., 2003)] was used for the amplification of the *gyrB* gene. PCR was carried out in a 30 μL reaction mixture on a Mastercycler EP Gradient S thermocycler (Eppendorf, Germany). The amplicons were examined and then sequenced at Biofidal (Vaulx-en-Velin, France; http://www.biofidal-lab.com). A BLAST search for sequences was performed via the NCBI website. Phylogenetic trees were constructed by the neighbor-joining method in the MEGA XI software according to Han et al. (2017).

2.5. Antibiotic-sensitivity tests

The antibiotic-sensitivity pattern of AB005 was determined by the disc diffusion method on Mueller Hinton Agar (Condalab, Spain; Hudzicki, 2009) according to the Clinical and Laboratory Standards Institute (CLSI) guideline for the following antibiotics: oxacillin, penicillin G, ampicillin, amoxicillin, enrofloxacin, norfloxacin, cefazolin, gentamicin, streptomycin, nitrofurantoin, tetracycline, oxytetracycline, erythromycin, lincomycin, rifampicin, novobiocin, chloramphenicol, florfenicol, and trimethoprim+sulfamethoxazole.

2.6. Detection of virulence genes

The presence of 10 virulence genes [aerolysin (*aerA*), heat-stable cytotonic enterotoxin (*ast*), phospholipase A (*pla*), serine protease (*ahe2*), extracellular nuclease (*nucl*), phospholipid-cholesterol acyltransferase (*gcaT*), aerolysin (*aerB*), hemolysin (*hlyA*), elastase (*ahpB*), and cytotoxic enterotoxin (*alt*)] was analyzed by PCR. The primers utilized for the amplification of these genes are presented in Table 1. The volume of the PCR reaction mixture was 20 μL, including: 10 μL of the 2x PCR Master Mix (Thermo Scientific), 2 μL of each primer (10 pmol/μL), 2 μL of template DNA (250–500 ng) and 6 μL of nuclease-free water (Thermo Scientific). Each PCR reaction started with denaturation at 94 °C for 10 min, followed by 30 cycles of amplification, and extension at 72 °C for 5 min was implemented in the last cycle. Each cycle was as follows: 94 °C denaturation for 1 min, annealing of the primers at 45.6–55.3 °C for 1 min, and 72 °C extension for 1 min. PCR was carried out using the Mastercycler EP Gradient S thermocycler (Eppendorf, Germany). The PCR products were run on 1% agarose gel stained with ethidium bromide (10 μg/mL) in Tris-acetate-EDTA buffer (TAE) using a Power Pac universal Electrophoresis unit (Bio-Rad) and visualized with a UV Transilluminator with the help of Molecular Imager Gel DOC™ with the Image Infinity software (Vilber, France).

Table 1
PCR primers, targets, and amplicon sizes.

Target gene	PCR primers sequence (5′→3′)	Product size (bp)	References
aerA	F: CAAGAACAAGTTCAAGTGGCCA R: ACGAAGGTGTGGTTCCAGT	309	Wang et al. (2003)
hlyA	F: GGCCGGTGGCCCGAAGATACGGG R: GGCGGCGCCGGACGAGACGGG	595	Zhu et al. (2007)
aerB	F: CCGGAAGATGAACCAGAATAAGAG R: CTTGTCGCCACATACCTCCTGGCC	451	Falcón et al. (2006)
ast	F: TCTCCATGCTTCCCTTCCACT R: GTGTAGGGATTGAAGAAGCCG	331	Sen and Rodgers (2004)
pla	F: ATCTTCTCCGACTGGTTCGG R: CCGTGCCAGGACTGGGTCTT	382	Sen and Rodgers (2004)
ahpB	F: ACACGGTCAAGGAGATCAAC R: CGCTGGTGTTGGCCAGCAGG	513	Sen and Rodgers (2004)
alt	F: TGACCCAGTCCTGG R: GGTGATCGATCACC	442	Li et al. (2011)
ahe2	F: ACGGGGTGCGTTCTTCCTACTCCAG R: CCGTTCATCACGCCGTTATAGTCG	211	Nam and Joh (2007)
nucl	F: CAGGATCTGAACCGCCTCTATCAGG R: GTCCCAAGCTTCGAACAGTTTACGC	504	Nam and Joh (2007)
gcaT	F: CTCCTGGAATCCCAAGTATCAG R: GGCAGGTTGAACAGCAGTATCT	237	Thornton et al. (1988)

2.7. Experimental infections

Nile tilapias (*Oreochromis niloticus*) were obtained from a commercial fish farm in Almaty, Kazakhstan. Sterlet sturgeons (*Acipenser ruthenus*) were supplied by the LLP Educational and Scientific Complex of Pilot Industrial Production of Aquaculture (Uralsk, Kazakhstan), where various species of sturgeon are grown. The *A. ruthenus* individuals were the offspring of artificially propagated sturgeons. Sixty *O. niloticus* individuals (with average length 12.6 ± 0.7 cm) were subdivided into six groups (of which one served as the control group), each containing 10 fish. The *A. ruthenus* individuals (with average length 27.4 ± 1.4 cm) were also randomly distributed into six groups with each containing ten fish. All fish were reared in 100 L aquaria with 50 L of static water with aeration at 20–22 °C (*A. ruthenus*) or 25–27 °C (*O. niloticus*).

They were allowed to acclimate to these experimental conditions for 10 days. Next, fish of five groups were intraperitoneally injected with five concentrations of bacterial cells (expressed in colony-forming units [CFU]/mL) at a dose of 500 μL/fish, respectively. The remaining control group received a 0.9% NaCl solution (saline with pH 8.5). All fish were reared for 4 days at 20–27 °C for the purpose of observing and recording the symptoms. The occurrence of disease symptoms and morbidity were recorded. Bacteria from the liver and spleen of the experimentally infected fish were reisolated. The half-lethal dose (LD_{50}) was determined by Probit analysis in Microsoft Excel 2019. All fish-handling procedures involved in the study complied with the principles for biomedical research involving animals. The experimental protocol was approved by the Kazakh National University Committee for the Ethical Care and Use of Animals in Experiments (authorization No. IL-51–8–2019).

2.8. Histopathological analysis

A gill, kidney, the intestine, and spleen were extracted from fish facing imminent death and control fish. The tissue specimens were fixed with formaldehyde for 24 h at room temperature. Then, the samples were dehydrated through a graded ethanol series (50–100%), cleared in xylene, and embedded in paraffin wax. For staining, the sectioned material was deparaffinized in xylene, rehydrated through a graded ethanol series, and stained with hematoxylin and eosin. Permanent histological slides were obtained by dehydration (50–100% ethanol), clearing in xylene, and sealing of the tissue sections in a neutral resin. The sections were examined under a MicroOptix MX30 microscope (West Medica, Austria).

3. Results

3.1. Clinical examination

The diseased fish from the sturgeon farms manifested lethargy, sluggishness, and swimming near the surface of the water. Clinical examination of the diseased sturgeons revealed multiple ulcers of various surface areas and penetrating deep into muscle (Fig. 1A), hemorrhages in the abdominal region and in the lateral bugs (Fig. 1B). An autopsy of the diseased fish revealed septicemia represented by pale gills and kidney congestion, hemorrhagic spots in the liver, and accumulation of bloody exudates in the abdominal cavity (Fig. 1C and D).

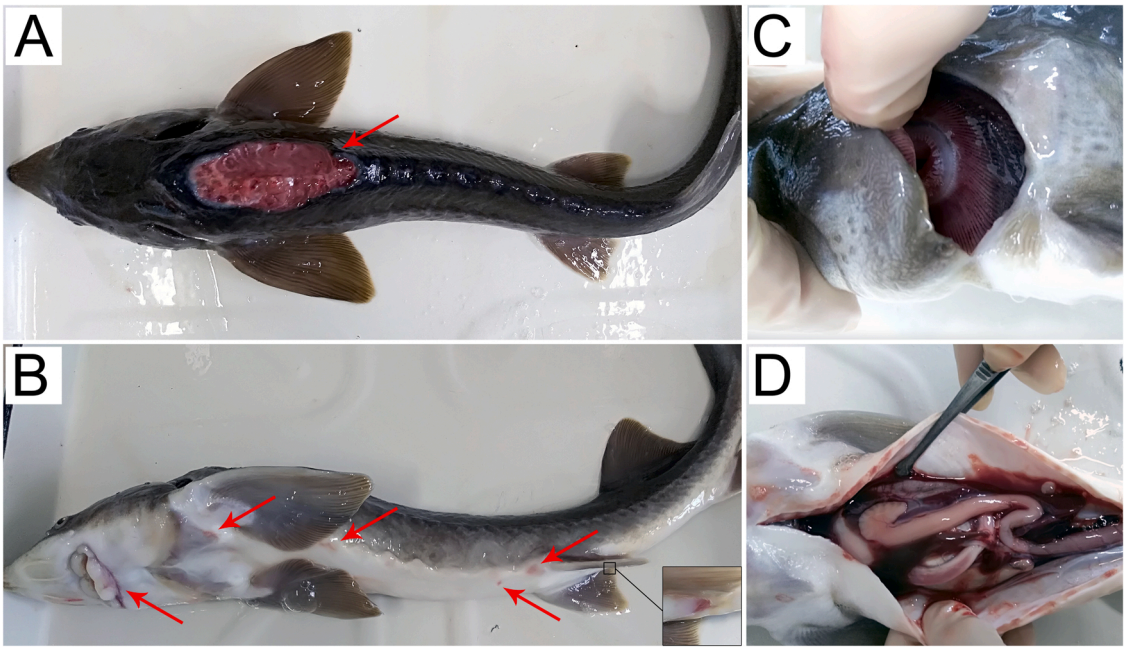

Fig. 1. Clinical symptoms in *A. baerii*. (A) Deeply penetrating muscle necrosis in the dorsal region. (B) Redness in the mouth, pectoral fins, and abdominal regions and anus inflammation. (C) Branchial ischemia. (D) The presence of a bloody fluid in the abdominal cavity.

3.2. Morphological, physiological, and biochemical characteristics

The AB005 strain was found to be a gram-negative motile bacterium capable of growing in an environment with NaCl concentration of **0–4%** and pH 5.0–9.0 at 13–42 °C (Table 2). The strain yielded positive results in the oxidase assay, methyl red assay, Voges-Proskauer test, arginine dihydrolase assay, and O-nitrophenyl-beta-D-galactopyranoside (ONPG) test and formed acid from sucrose and trehalose. A negative result was obtained in tests for ornithine decarboxylase and for D-xylose and indole production. AB005 proved to be capable of hydrolyzing gelatin and esculin and forming hydrogen sulfide. The strain yielded varied results in two tests: the lysine decarboxylase assay and acid formation from lactose. According to the results of the (Oxidative/fermentation) O/F test, the bacterium metabolizes carbohydrates oxidatively, by fermentation.

3.3. Sequence analysis of the 16S rRNA and gyrB genes

The amplification of the 16S rRNA gene from the genomic DNA of the AB005 strain as a template resulted in a DNA band of ~1.4 kbp (Fig. 2). The 16S rRNA sequence was deposited in the GenBank database and assigned accession No. OK634406. The *gyrB* gene sequence turned out to be 1.1 kbp in length (Fig. 2; GenBank accession No. ON124027). BLAST results showed that isolate AB005 shares 99% identity with other *A. hydrophila* strains. In the phylogenetic trees built on the 16S rRNA and *gyrB* gene sequences (Fig. 3A and B), isolate AB005 clearly grouped with a cluster of known strains of *A. hydrophila*. The BLAST and phylogenetic-analysis results confirmed that AB005 is *A. hydrophila*.

3.4. Virulence factors and antibiotic sensitivity

Isolate AB005 was tested for the presence of virulence genes (Fig. 4). The analysis showed that out of the 10 virulence genes under study, isolate AB005 contains seven [aerolysin (*aerA*, amplicon: 309 bp), acyltransferase (*gcaT*, 237 bp), aerolysin (*aerB*, 451 bp), phospholipase A (*pla*, 382 bp), serine protease (*ahe2*, 211 bp), heat-stable cytotonic enterotoxin (*ast*, 331 bp), and nuclease (*nucl*, 504 bp)]. The sensitivity of isolate AB005 to 19 antimicrobial agents was evaluated next (Table 3). AB005 was found to be resistant to oxacillin, penicillin G,

Table 2
Morphological, physiological, and biochemical characteristics of AB005.

№	Characteristics	Reaction	
		AB005	*A. hydrophila*[a]
1	Gram stain	–	–
2	Morphology	rod	rod
3	Motility	+	+
4	Oxidase	+	+
5	Methyl red	+	+
6	Voges-Proskauer test	+	+
7	O/F test	F	F
8	Hydrolysis of gelatin	+	+
9	Hydrolysis of esculin	+	+
10	H_2S formation	+	+
11	Indole formation	–	+
12	Lysine decarboxylase	+/-	+
13	Ornithine decarboxylase	–	–
14	Arginine dihydrolase	+	+
15	ONPG	+	+
Acid formation from:			
16	Sucrose	+	+
17	Trehalose	+	+
18	D-xylose	–	–
19	Lactose	+/-	–
Growth under conditions:			
20	0% NaCl	+	+
21	1% NaCl	+	+
22	2% NaCl	+	–
23	3% NaCl	+	–
24	4% NaCl	+	–
25	5% NaCl	–	–
26	13 °C	+	–
27	27 °C	+	+
28	32 °C	+	+
29	37 °C	+	+
30	42 °C	+	+
31	pH 3.0	–	–
32	pH 5.0	+	–
33	pH 7.0	+	+
34	pH 9.0	+	+

+ , positive; -, negative; F, fermentative.
[a] Data are from the following references: Altwegg et al. (1990), Minana-Galbis et al. (2002), Nhinh et al. (2021), Penha et al. (2017), Saidi et al. (2011), Suneeta and Yogendra (2019), Yu et al. (2015).

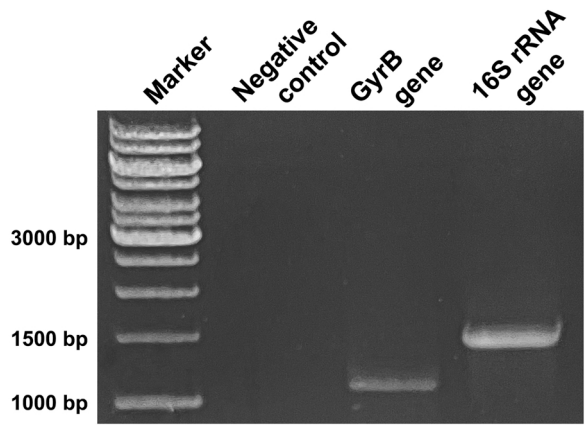

Fig. 2. Agarose gel electrophoresis of PCR products of the 16 S rRNA and *gyrB* genes of *A. hydrophila* AB005.

ampicillin, amoxicillin, cefazolin, erythromycin, lincomycin, rifampicin, and novobiocin. The following antibiotics proved to be toxic to AB005: enrofloxacin, norfloxacin, gentamicin, streptomycin, nitrofurantoin, tetracycline, chloramphenicol, florfenicol, and trimethoprim+sulfamethoxazole. Moderate (intermediate) sensitivity was registered for one antibiotic: oxytetracycline.

3.5. Growth characteristics

This isolate of *A. hydrophila* was able to grow in a temperature range from 13° to 42°C (Fig. 5A). Initial pH significantly affected growth latency of isolate AB005. Higher OD_{600} values were recorded in the medium with initial pH between 5 and 9 (Fig. 5B). By contrast, the latent phase of growth was markedly longer at pH 5. AB005 was unable to grow at pH 3. Among low concentrations of NaCl (1–2%), growth curves of isolate AB005 were similar: the beginning of the exponential phase at 2 h after inoculation and the stationary phase starting at 8 h (Fig. 5C). When the NaCl concentration was increased to 3%, the AB005 strain had a longer lag phase. NaCl at 4% negatively affected the growth of the isolate, and at 5%, the growth was completely inhibited.

3.6. Pathogenicity of A. hydrophila AB005 to O. niloticus

The *A. hydrophila* AB005 strain was found to be virulent because the challenged fish at 10^8 CFU/mL in the bacterial suspension experienced 100% mortality within 8 h of the challenge (Fig. 6). At 96 h post-injection (hpi), cumulative mortality was 80% for 10^7 CFU/mL, 60%

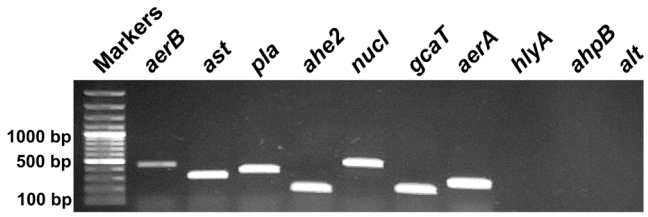

Fig. 4. Agarose gel electrophoresis of the amplicons of virulence genes. Seven virulence genes are present in isolate AB005.

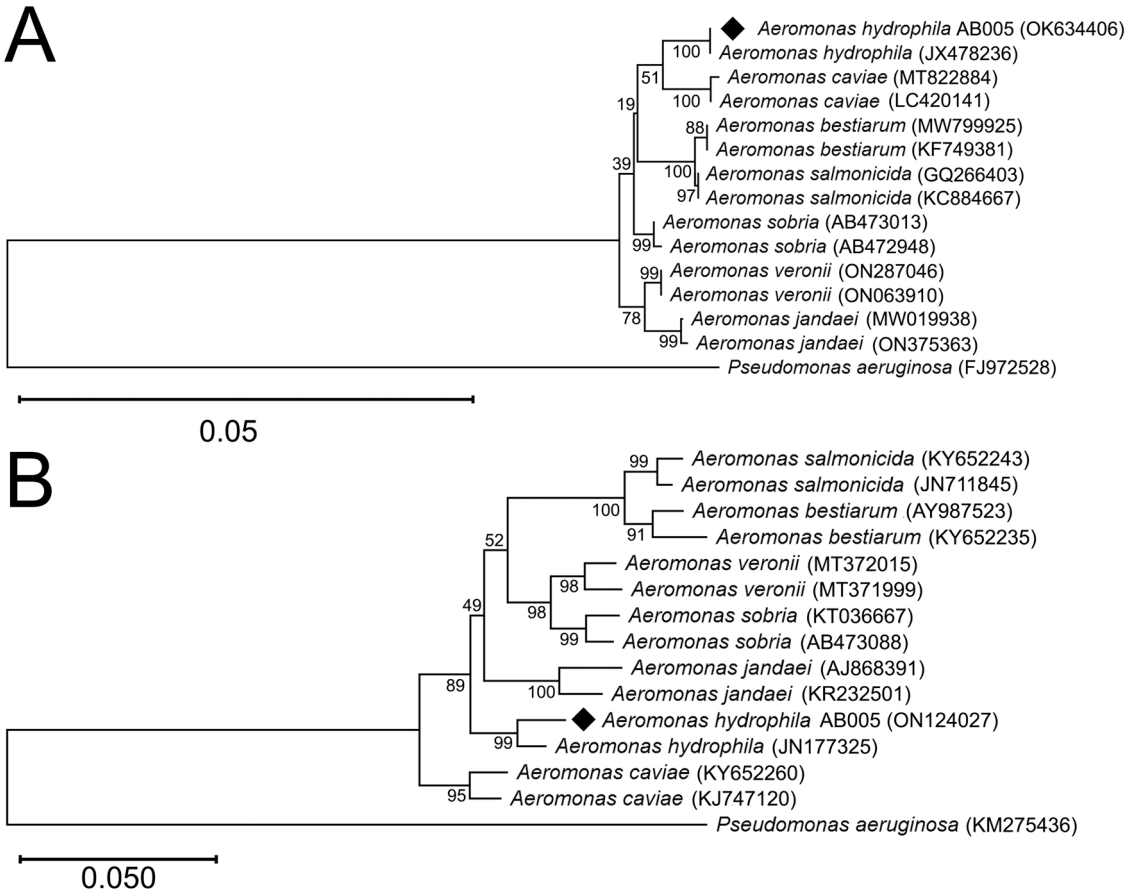

Fig. 3. Unrooted neighbor-joining phylogenetic trees based on 16S rRNA gene (A) and *gyrB* gene (B) sequences of *Aeromonas* strains. The phylogenetic trees showed clear-cut clustering of *A. hydrophila* isolate AB005 with some *A. hydrophila* strains whose sequences were retrieved from GenBank. Sequence accession numbers are given in parentheses.

Table 3
Sensitivity of *A. hydrophila* AB005 to different antibiotics.

Group	Antibiotic	Disk content (μg)	AB005	
			Sensitivity	Zone diameter (mm)
Penicillins	Oxacillin	1	R	0
	Penicillin G	10	R	0
	Ampicillin	10	R	0
	Amoxicillin	10	R	0
Quinolones	Enrofloxacin	5	S	27 ± 1.32
	Norfloxacin	10	S	25 ± 0.87
Cephalosporins	Cefazolin	30	R	0
Aminoglycosides	Gentamicin	10	S	17.8 ± 0.29
	Streptomycin	10	S	17.3 ± 1.25
Nitrofurans	Nitrofurantoin	300	S	17.7 ± 0.29
Tetracyclines	Tetracycline	30	S	23.5 ± 0.5
	Oxytetracycline	30	I	22.5 ± 0.87
Macrolides	Erythromycin	15	R	12.2 ± 0.76
Lincomycins	Lincomycin	10	R	0
Rifamycins	Rifampicin	5	R	11.7 ± 0.58
Coumarins	Novobiocin	30	R	0
Amphenicols	Chloramphenicol	10	S	22.7 ± 0.76
	Florfenicol	30	S	28.3 ± 0.28
Folic acid synthesis inhibitors	Trimethoprim + sulfamethoxazole	25	S	21.8 ± 0.76

R: resistant, **I: intermediate**, S: sensitive.

for 10^6 CFU/mL, and 20% for 10^5 CFU/mL. There was no mortality at 10^4 CFU/mL up to 96 hpi. The calculated LD_{50} of *A. hydrophila* AB005 was 8.37×10^5 CFU/mL. The bacterium was reisolated from the infected fish, and the isolates had the same morphological and biochemical features as did AB005 (data not shown). The infected fish demonstrated erratic swimming behavior, stopped eating, and remained at the bottom of the aquarium. Moreover, the injected fish developed extensive hemorrhage in different body parts: in the eyes, at the base of opercula, and on the anal fin. The injected fishes' gills acquired pale colors because of necrosis. The autopsy of the diseased fish revealed disturbances of blood circulation and eroded internal organs (Figs. 7 and 8).

3.7. Histopathological observations

The liver, kidneys, and spleen of *O. niloticus* were found to be affected by the *A. hydrophila* infection (Fig. 9). Stasis of mononuclear leukocytes in dilated sinusoids and focal hepatocyte necrosis were registered in the liver. In addition, the necrosis of the pancreatic glandular epithelium, hepatocyte necrosis, foci of inflammatory infiltration with the presence of eosinophils were also noted in the liver (Fig. 9A and B). In the kidneys, edema and inflammatory-cell infiltration of the parenchyma as well as necrosis of renal glomerular and tubular epithelia were evident (Fig. 9C and D). In the spleen, severe necrosis of the splenic surface, edema of the spleen parenchyma, hemolysis of erythrocytes, and lymphocyte destruction was documented. There were also noticeable foci of hemosiderin deposition (Fig. 9E and F).

3.8. Pathogenicity of A. hydrophila AB005 to A. ruthenus

In the pathogenicity experiment, the earliest mortality was observed at 12 hpi in the groups infected with 10^8 CFU/mL *A. hydrophila* AB005 (Fig. 6). The highest cumulative mortality (100%) was seen in the group

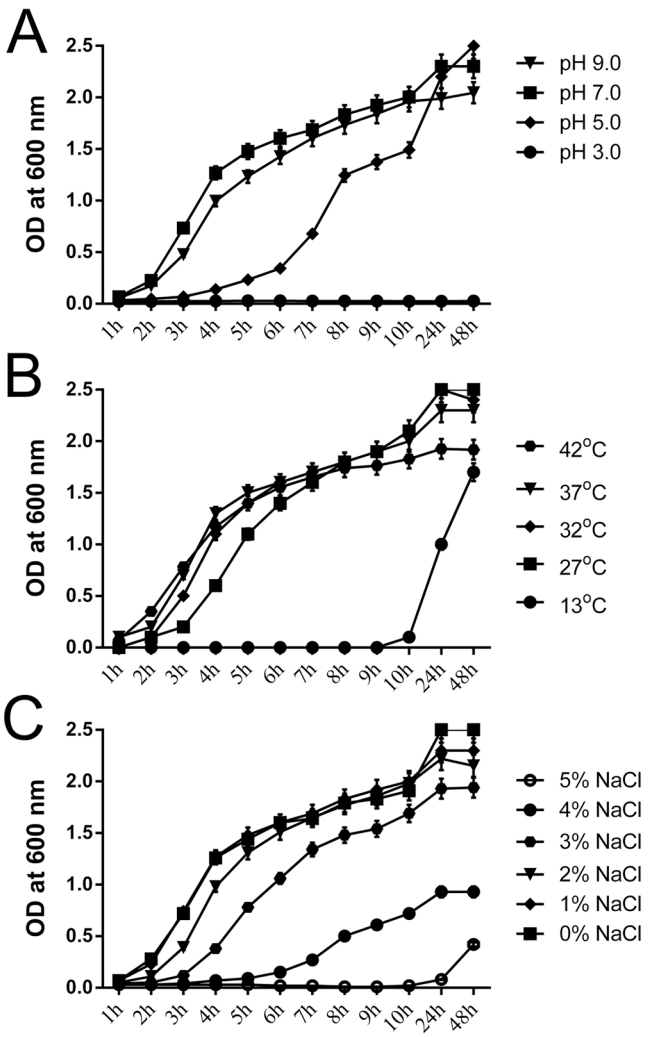

Fig. 5. Growth characteristics of AB005. (A) Growth at pH 3–9. (B) Growth at temperatures 13–42 °C. (C) NaCl concentrations 0–5%.

infected with 10^8 CFU/mL, followed by the groups infected with 10^7 CFU/mL (60%) and 10^6 CFU/mL (30%). There was no mortality at 10^5 and 10^4 CFU/mL up to 96 h postchallenge. In this work, the calculated LD_{50} of *A. hydrophila* was 2.89×10^6 CFU/mL. The bacterium was reisolated from the infected fish, and the new isolates manifested the same morphological and biochemical features as AB005 did (data not shown). Artificially infected *A. ruthenus* exhibited the following key clinical signs: lethargy, icteric appearance, petechial hemorrhages on the ventral body part, swelling and hyperemia of spleen, and intestinal tract (Fig. 10).

4. Discussion

The Siberian sturgeon (*A. baerii*) is a nonteleost ray-finned fish of the family *Acipenseridae*. Historically, *A. baerii* is native to China, Kazakhstan, Mongolia, and Russia (Ruban, 2018); however, due to its certain advantages, such as growth and maturation in a wide temperature range, omnivorous feeding, and hardiness, this fish has been used in aquaculture in different parts of the world. Among sturgeon species on fish farms, the Siberian sturgeon has been the most successfully reproduced species whose production in the world accounts for ~40% of the total sturgeon production (Orlov et al., 2021). This species is grown in more than 40 countries (Chebanov and Galich, 2018). Nevertheless, as in the cultivation of other fish species, there are bacterial infections of farmed sturgeons as a consequence of severe stress and high stock

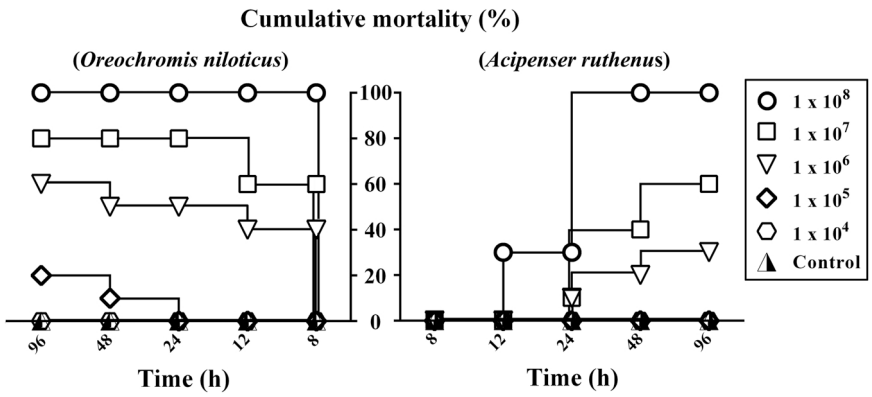

Fig. 6. Cumulative mortality of *O. niloticus* and *A. ruthenus* from infection at different doses of *A. hydrophila* AB005 in 96 h.

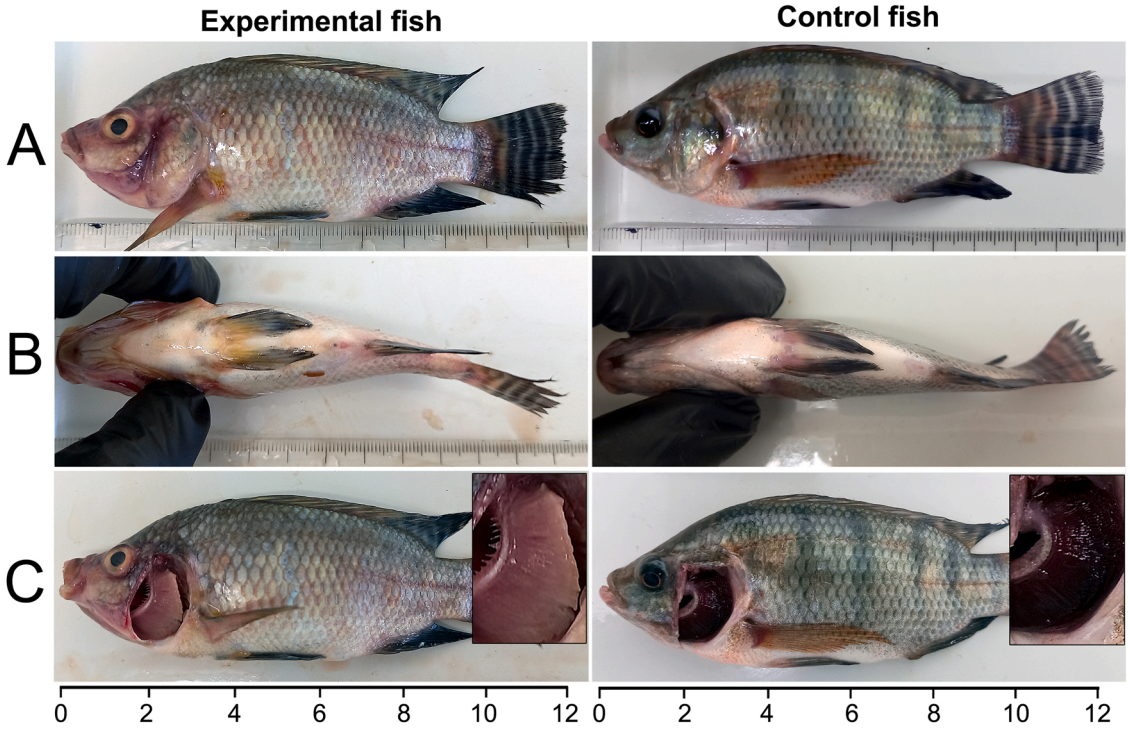

Fig. 7. Clinical features in tilapias infected with *A. hydrophila* AB005. (A) Side view. (B) View from the ventral side. (C) View of the gills. Experimental fish were injected with *A. hydrophila* isolate AB005; control fish were injected with saline. A ruler is presented below (cm).

densities (Ciulli et al., 2020).

It is well documented that *A. hydrophila* is a leading pathogen in aquatic habitats and causes serious infections in various freshwater fish species (Abd-El-Rhman, 2009; Jeney et al., 2009; Majumdar et al., 2007), including some sturgeon species, such as the Siberian sturgeon (Cao et al., 2010), the Persian sturgeon (*Acipenser persicus*) (Soltani and Kalbassi, 2001), the Russian sturgeon (*A. gueldenstaedtii*) (Timur et al., 2010), and the Amur sturgeon (*A. schrenckii*) (Zhou et al., 2018). To our knowledge, the current study is the first to report *A. hydrophila* infection among *A. baerii* fishes cultured in Kazakhstan. Clinical signs associated with the aeromonad infection observed in this work are single or multiple skin ulcers penetrating deep into muscle along the edges on the dorsal, abdominal, or tail parts of the fish and petechial hemorrhages on the ventral body part. Internally, the gills and kidneys were found to be congested. Hemorrhagic spots in the liver and accumulation of bloody exudates in the abdominal cavity were noted too (Fig. 1). Similar clinical signs, such as petechial hemorrhages and skin ulcers penetrating deep into muscle, have been observed in the common carp *Cyprinus carpio* L., rainbow trout *Oncorhynchus mykiss*, and largemouth bass *Micropterus salmoides* infected with *A. hydrophila*, especially when the fish are under stress (Huizinga et al., 1979; Kozińska and Pękala, 2012). Previously, we found that the quickest growth of *A. hydrophila* occurs at a continuous high-water temperature > 18 ℃ in industrial sturgeon aquaculture (Sergaliev et al., 2021). These results are consistent with the data from studies where in temperature climates, aeromonads were found in large numbers in late summer/early autumn when the temperatures were approximately 20–25 °C and were rarely detectable during the cold months (Gavriel et al., 1998; Kertsters et al., 1995). Our results indicate that this *A. hydrophila* isolate is well adapted to sturgeon habitats and can grow in a wide range of environmental temperatures 13–42 °C and a wide pH range, between 5 and 9 (Fig. 5). Growth rates of *A. hydrophila* isolate AB005 did not vary significantly at salt concentrations from 0% to 3%. By contrast, NaCl at 4% and higher concentration negatively affected the growth of the isolate, particularly at 5%, the growth was negligible. Our findings about its growth and proliferation under harsh conditions suggest that this isolate is well adapted to various

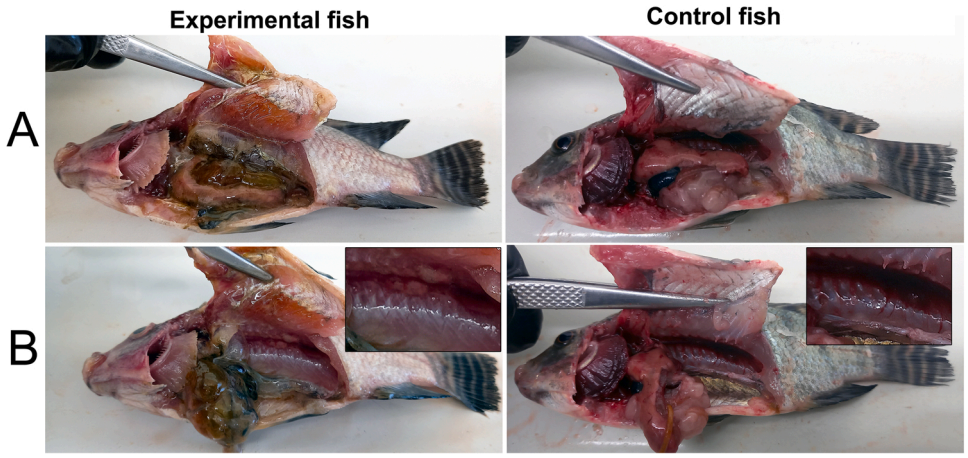

Fig. 8. Clinical features in tilapias infected with *A. hydrophila* AB005. (A) The condition of internal organs of the fish injected with *A. hydrophila* AB005 (experiment) and of fish injected with saline (control). (B) Disturbances of blood circulation and necrosis of internal organs in the fish injected with *A. hydrophila* AB005 (experiment), and the absence of circulatory disorders and necrosis of internal organs in the fish injected with saline (control).

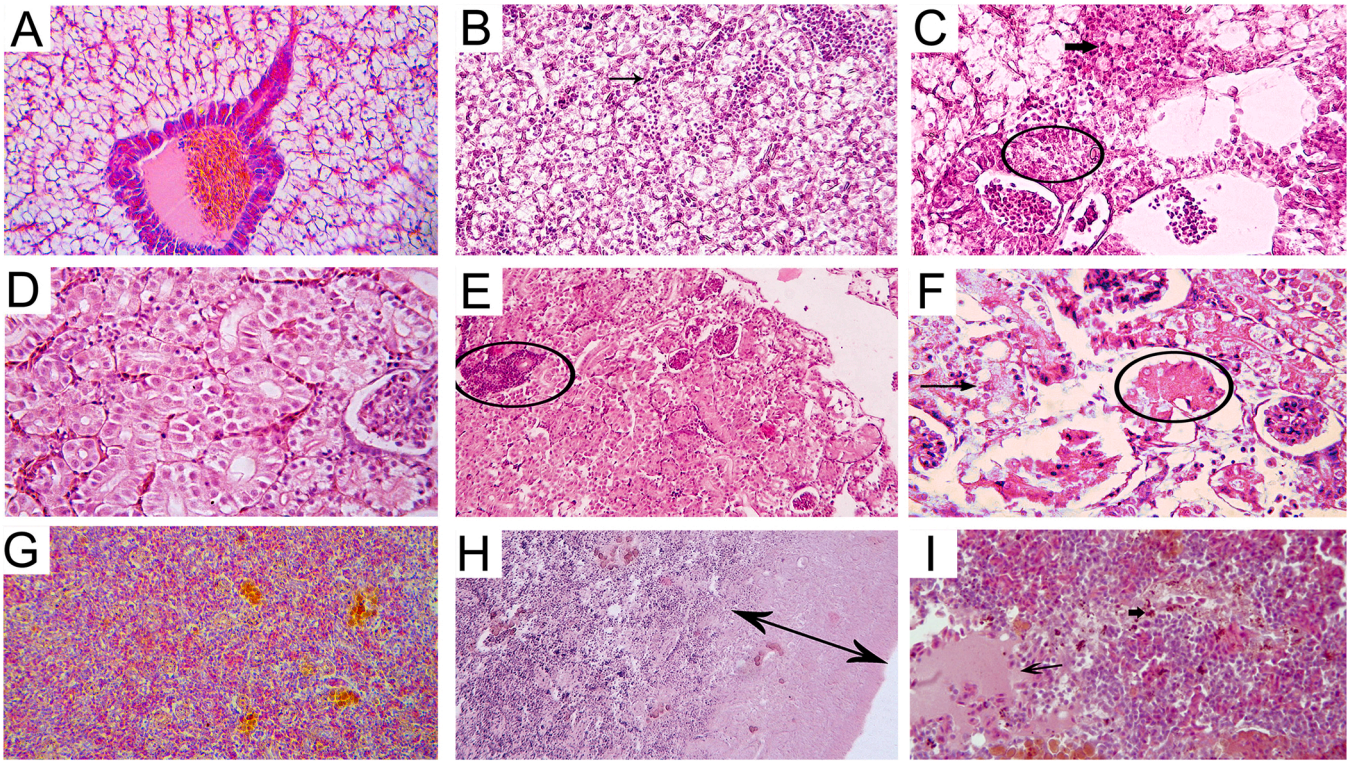

Fig. 9. Histopathological changes in *O. niloticus* subjected to artificial infection with *A. hydrophila*. (A) Liver of control fish. (B) Stasis of mononuclear leukocytes in dilated sinusoids (arrow) and focal hepatocyte necrosis. (C) Necrosis of the pancreatic glandular epithelium (circle), hepatocyte necrosis, and foci of inflammatory infiltration with the presence of eosinophils (arrow). (D) Kidney of control fish. (E) Inflammatory infiltration of the kidney parenchyma (circle) and necrosis of renal glomerular and tubular epithelia. (F) Edema of the kidney parenchyma, necrosis of renal glomerular (circle) and tubular (arrow) epithelia. (G) Spleen of control fish. (H) Severe necrosis of the splenic surface (double-headed arrow). (I) Edema of the spleen parenchyma (long arrow), hemolysis of erythrocytes, lymphocyte destruction, and foci of hemosiderin deposition (short arrow).

environments.

The biochemical characteristics of *A. hydrophila* observed in this study are consistent with those in other reports (Abbott et al., 2003), except for its inability to produce indole. Considering that indole production is usually present in *A. hydrophila* (Abbott et al., 2003), the indole test was repeated here to verify the results. It is worth mentioning that an *A. hydrophila* isolate negative for indole production has been noted previously (Sinha, 2004; Skwor et al., 2014). Despite the negative indole reaction, our phylogenetic analysis based on 16 S rRNA and *gyrB* gene sequences identified isolate AB005 as *A. hydrophila* (Fig. 3). Some investigators have shown that indole, a small regulatory molecule that is widely present throughout the bacterial kingdom, affects various processes in bacteria, including bacterial antibiotic tolerance (Melander et al., 2014). The AB005 strain assessed here turned out to be resistant to nine out of the nineteen antibiotics tested. AB005 is 100% resistant to β-lactam drugs but is still sensitive to aminoglycosides and amphenicols. The presence of β-lactamases' genes encoding penicillinase and cephalosporinase in *A. hydrophila* may be ascribed to the observed resistance

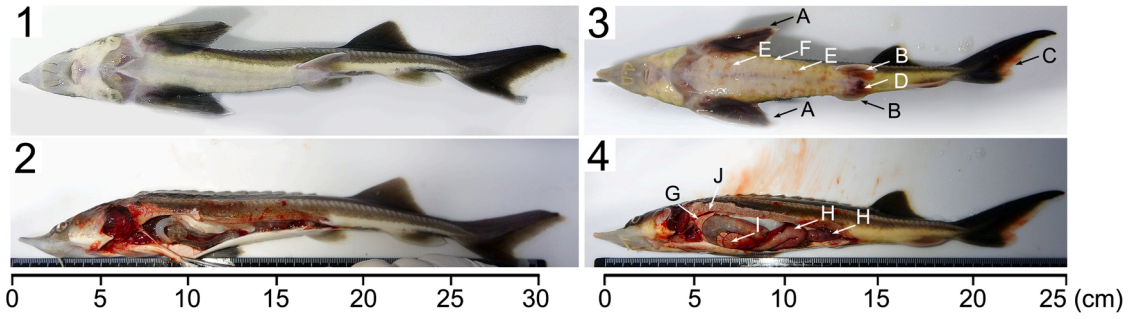

Fig. 10. Clinical symptoms of artificial infection of *A. hydrophila* AB005 in *A. ruthenus*. (1, 2) Healthy control fish showing no clinical signs and abnormalities. (3, 4) Clinical features in *A. ruthenus* infected with *A. hydrophila* AB005 showing (A, B and C) hemorrhage in the pectoral, pelvic and caudal fins. (D) septicemia and anus relapse. (E) pinpoint hemorrhages all over the skin. (F) icteric appearance of the ventral side. (G) liver pale in color, flabby consistency with signs of toxic dystrophy. (H and I) hemorrhagic inflammation of the intestine and enlargement and swelling of the spleen, flabby consistency. (J) muscles are saturated with edematous fluid.

to penicillin drugs and to cefazolin (Bertran et al., 2021). Its resistance to lincomycin, rifampicin, and novobiocin is in agreement with reports about the effects of these antibiotics on this pathogen (Kusdarwati et al., 2018; Revina et al., 2017). It is noteworthy that in the current study, the quinolone class of antibiotics strongly inhibited *A. hydrophila* growth; consequently, it can be utilized as a treatment on sturgeon farms. On the other hand, the use of antibiotics often reduces the immunity of fish to an infectious agent and may endanger human health because most of the antibiotics applied in aquaculture are used to treat human diseases (Arthur et al., 2000); hence, other treatments should be chosen to combat *A. hydrophila* infection.

The Nile tilapia, *O. niloticus* is a worldwide important species in aquaculture because of its fast growth, rapid reproduction rate, relatively undemanding to food diversity, and resilience in many environmental conditions (FAO, 2019). In the past several years, Nile tilapia aquaculture has been a fastest growing in Kazakhstan. Due to their tropical and subtropical origin, farmed tilapia production in Kazakhstan is increasingly performed using geothermal water with temperatures ranging from 26 °C to 30 °C (Mamilov et al., 2022; Moldagalieva et al., 2021; Syzdykov et al., 2020). To understand the pattern of pathogenicity of *A. hydrophila* strain AB005 isolated from *A. baerii*, initial experimental fish challenge studies were performed using *O. niloticus*.

The results of our experimental challenge test confirmed the virulence of our isolate toward healthy *O. niloticus*, as evidenced by 100% mortality at 8 hpi for 10^8 CFU/mL (Fig. 6). In this work, pale gills, extensive hemorrhage in different body parts, disturbances of blood circulation, and necrosis of internal organs were registered in *O. niloticus* infected with *A. hydrophila* isolate AB005. The histopathological changes in the internal organs of *O. niloticus* were also typical for *Aeromonas* spp. infections. In the liver, the changes manifested themselves as focal hepatocyte necrosis (Abdelhamed et al., 2017; Chen et al., 2018; Saharia et al., 2018). Obvious necrosis of the splenic surface was observed too, as reported by other authors (Abdelhamed et al., 2017; Chen et al., 2018). Necrosis of renal glomerular and tubular epithelia, inflammatory infiltration of the kidney parenchyma were noticed as well (Fig. 9).

A. baerii is an endangered species and listed among the first class of protected animals in Kazakhstan. In this regard, the pathogenicity of *A. hydrophila* AB005 was also researched here on a species closely related to *A. baerii* (sterlets, *A. ruthenus*). Sterlets are listed as vulnerable, although most of them reside in the Ural, Volga, and Danube rivers, where populations stabilized recently after a big decline before (Reinartz and Slavcheva, 2016).

The clinical signs observed in *A. ruthenus* infected with *A. hydrophila* AB005 are similar to those seen in infected *O. niloticus* but with lower severity. In our study, nearly 30% of *A. ruthenus* individuals were dead at 12 hpi, and the rate of mortality then sharply increased until mortality reached 100% at 24 hpi for the same bacterial-cell concentration. In this work, LD_{50} was found to be 8.37×10^5 CFU/mL for *O. niloticus* and 2.89×10^6 CFU/mL for *A. ruthenus*. The discrepant findings about the mortality rate suggest that although *A. hydrophila* is pathogenic for both fish species, *O. niloticus* is likely a more susceptible host. Previous experimental investigation into *A. hydrophila* infection in various hosts points to cumulative mortality ranging from 60% to 100%, depending on the challenge doses and route of infection (Hossain et al., 2013; Pauzi et al., 2020; Samayanpaulraj et al., 2019), indicating that *A. hydrophila* infectivity varies significantly among fish hosts.

It should be noted that the observed clinical signs of *A. ruthenus* infected with *A. hydrophila* AB005 differ from those of diseased fish from sturgeon farms (Fig. 10). Gross lesions such as a skin ulcer penetrating deep into muscle and accumulation of a bloody exudate in the abdominal cavity in artificially infected fish have not been registered. The pathological findings in the current study revealed that the lesions in naturally infected *A. baerii* are milder than those in experimentally infected *A. ruthenus*. Deaths at 12 h after the challenge imply that the infection caused by this pathogen has a short incubation period. Besides, other factors, such as the type and age of the host and the bacterial load on the host fish, can also influence the severity of the clinical symptoms (Hassan et al., 2017; Perretta et al., 2018; Zhang et al., 2016).

Some researchers demonstrated that hemolytic and cytotoxic activities and pathogenicity correlate with virulence genes in *Aeromonas* strains (Li et al., 2011). In our paper, AB005 was tested for 10 virulence genes (*hlyA*, *aerB*, *ast*, *alt*, *ahpB*, *gcaT*, *pla*, *nucl*, *ahe2*, and *aerA*) by the PCR assay. *A. hydrophila* isolate AB005 was found to possess genes of aerolysins, acyltransferase, phospholipase A, serine protease, heat-stable cytotonic enterotoxin, and nuclease. These virulence factors play major roles in infections (Sha et al., 2002; Soler, 2002). Variations in the distribution of potential virulence genes among *Aeromonas* isolates may contribute to their pathogenicity (Albert et al., 2000). Abrami et al. (1998) reported that the joint presence of the *ser* gene and *aer* enhances pathogenicity because aerolysin is activated by a serine protease. It has been reported that virulence factors GcaT, Lip, and Ser play a coherent integrated role in the overall pathogenicity of aeromonad infections (Lee and Ellis, 1990; Pemberton et al., 2006). Several virulence genes present in isolate AB005 imply that synergistic effects of combinations of these genes contribute to its pathogenicity.

In sum, an *A. hydrophila* isolate was extracted from diseased *A. baerii* and named AB005. Its characteristics mean that it has considerable virulence and results in acute infection in fish. A comparative infection trial revealed that the Nile tilapia is more susceptible to *A. hydrophila* AB005 infection than sturgeons are. Given that the infection with *A. hydrophila* causes a wide spectrum of symptoms among warm- and cold-blooded animal hosts, the virulence and physiological and biochemical characteristics of isolate AB005 underscore its pathogenicity and will provide the foundation for future research on this pathogen in Siberian-sturgeon aquaculture.

Ethics approval and consent to participate

The animal study protocol was reviewed and approved by the Local Ethics Committee of the Medical Faculty, Higher School of Public Health at al-Farabi Kazakh National University (Almaty, Kazakhstan).

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CRediT authorship contribution statement

Amangeldy Bissenbaev: Conceptualization, Methodology, Supervision, Writing – original draft. **Serik Bakiyev:** Methodology, Visualization, Investigation, Validation. **Izat Smekenov:** Software, Visualization, Investigation. **Irina Zharkova and Saidina Kobegenova:** Visualization, Investigation. **Nurlan Sergaliyev and Gaisa Absatirov:** Resources, Investigation. All authors contributed to critical review of the manuscript and approved the submitted version.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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The Effect of Non-Thermal Atmospheric Pressure Plasma Treatment of Wheat Seeds on Germination Parameters and α-Amylase Enzyme Activity

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Abstract:

This contribution presents the results of a study of the germination rate and growth parameters of wheat seeds after atmospheric pressure surface coplanar dielectric barrier discharge (DBD) plasma treatment. The germination rate and biometric parameters such as the root, shoot length, mass of the seedlings, and the α -amylase enzyme activity were studied at different plasma exposure time. The seed coat surface wettability and morphology were determined by apparent contact angle measurement and scanning electron microscope (SEM) analysis. Seed surface disinfection and the presence of filamentous fungi have also been investigated at different discharge parameters. It is shown that the optimal plasma treatment duration for increasing the growth parameters and enhancing the enzymatic activity is 5–15 s. It was found that the longer plasma exposure requires complete sterilization of the seed surface from pathogens, compared to the optimal treatment time for high germination, based on the obtained results,

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the possible mechanisms of the positive effect of plasma treatment on the enhanced germination of wheat seeds are discussed.

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