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Characterization of atypical pathogenic *Aeromonas salmonicida* isolated from a diseased Siberian sturgeon (*Acipenser baerii*)

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ABSTRACT

Acipenser baerii (Siberian sturgeon) is native to Kazakhstan and is currently endangered and is listed within the first class of protected animals. Sturgeon aquaculture is becoming an important tool for the recovery of this endangered species. Nonetheless, diseases involving typical symptoms of skin ulceration and systemic bacterial hemorrhagic septicemia have occurred in cultured *A. baerii* on a fish farm located in Western Kazakhstan. In this study, an infectious strain of bacteria isolated from an ulcer of diseased *A. baerii* was identified as *Aeromonas salmonicida* (strain AB001). This identification involved analyses of 16S rRNA, *gyr*B, *rpo*D, and *fla*A genes' sequences. Even though strain AB001 belongs to *A. salmonicida*, it exhibited noticeable mobility and growth at temperatures of $\geq$37 °C. Profiling of virulence genes uncovered the presence of seven such genes related to pathogenicity. Antibiotic sensitivity testing showed that the strain is sensitive to aminoglycosides, amphenicols, nitrofurans, quinolones, and tetracyclines. Half-lethal doses (LD_{50}) of strain AB001 for *Oreochromis mossambicus* and *A. baerii* were determined: respectively 1.7×10^8 and 7.2×10^7 colony-forming units per mL. The experimentally induced infection revealed that strain AB001 causes considerable histological lesions in *O. mossambicus*, including tissue degeneration, necrosis, and hemorrhages of varied severity.

1. Introduction

Aeromonas salmonicida, known as the only nonmotile species of the genus *Aeromonas*, has been considered one of the major bacterial pathogens causing furunculosis in wild and farmed salmonids [1]. This bacterium used to be regarded as a salmon-specific pathogen, but considerable morbidity and mortality associated with *A. salmonicida* in numerous nonsalmonid species have also been reported [2–7]. It should be stressed that fish infected by *A. salmonicida* do not always show typical signs of furunculosis [8]. In most outbreaks among wild or farmed nonsalmon fish, this infection appears as skin ulcers and skin lesions as well as bacterial

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septicemia [9]. Although the lack of motility has been assumed to be one of reliable distinctive features of *A. salmonicida*, a motile form of this bacterium has been identified too [10–12].

So far, there are five officially acknowledged *A. salmonicida* subspecies: *achromogenes*, *salmonicida*, *masoucida*, *smithia*, and *pectinolytica* [13]. Strains of subspecies *salmonicida*, *smithia*, *achromogenes*, and *masoucida* have a psychrophilic lifestyle and therefore are unable to grow at temperature higher than ~25 °C and can infect many taxa of fishes [10]. Only the subspecies *pectinolytica* isolated from polluted rivers is considered mesophilic, and there are no reports of its pathogenicity [14]. Nonetheless, recent studies describe some *A. salmonicida* mesophilic strains capable of infecting fish, birds, humans, and other mammals [15–17]. Furthermore, it has been shown that mesophilic strains of *A. salmonicida* not only have a wide range of hosts but also are more genetically diverse than psychrophilic strains [18].

The 16S ribosomal RNA (rRNA) gene is widely accepted as a gene suitable for bacterial species identification [19]. On the other hand, the 16S rRNA gene is strongly conserved in the *Aeromonas* genus, and some of these taxa differ by only a few nucleotides [20]. Research on two or more housekeeping genes (*gyr*B and *rpo*D) could help to increase reliability of phylogenies of *Aeromonas* species [21]. Recently, in addition of housekeeping genes, several virulence-associated genes such as bacterial flagellum (*fla*A), virulence protein array (*vap*A), and aerolysin (*aer*A) sequences were used to define subspecies of *A. salmonicida* [22–24].

It is known that the key virulence factors in pathogenic *Aeromonas* strains are aerolysin, proteases, enterotoxins, hemolysin, and acetylcholinesterase [25]. The presence of multiple virulence factor–encoding genes acting either alone or in a synergistic manner determines disease severity in the host [26]. Recently, Chen et al. [27] demonstrated that the mesophilic *A. salmonicida* SRW-OG1 strain cultivated at 28 and 37 °C has higher hemolytic activity and virulence than that cultured at 18 °C. Further investigation showed that the observed increase in virulence is associated with significant upregulation of *hly*A and *aer*A (hemolytic genes encoding hemolysin and aerolysin) at 28 and 37 °C [27]. Investigation into the presence of virulence-associated factors in *A. salmonicida* isolates is important to clarify the epidemiology and pathogenesis of such infections [28].

The Siberian sturgeon (*Acipenser baerii)*, a sturgeon species from the family *Acipenseridae*, is an economically important fish prized as a source of valuable animal protein, including caviar and meat, and has high commercial value [29]. Even though the Siberian sturgeon is native to Kazakhstan [30], it is currently an animal that is endangered and is listed within the first class of protected animals of Kazakhstan. Moreover, it was characterized as a critically endangered fish species within the IUCN (International Union for Conservation of Nature and Natural Resources) Red List in 2019. Therefore, much effort has been made to support the recovery of this endangered species, including human-assisted reproduction and placement of the resultant fingerlings in the Ural River, which flows into the Caspian Sea [31]. In the last two decades, sturgeon aquaculture has greatly increased in Kazakhstan. Aquaculture needs successful preventive measures and effective treatments to reduce the prevalence of infectious diseases caused by various bacteria. This is especially true for sturgeon aquaculture because sturgeon farms are costly to maintain, and much time elapses before sturgeons spawn [32]. Indeed, pathogenic bacteria are the leading cause of mortality of these fish [33–35]; nonetheless, so far, information about these illnesses and fish health control has been quite limited. In the current article, data on isolation, identification, virulence genes, pathogenicity, and growth parameters of an *A. salmonicida* isolate are presented. The histopathological changes induced by this bacterium and antibiotic susceptibility were researched as well in order to manage this disease.

2. Materials and methods

2.1. Bacterial isolation

Diseased *A. baerii* specimens were collected from a sturgeon fish farm in Uralsk, West-Kazakhstan region, Kazakhstan. The most consistent external signs seen in infected fish (87–91 cm in length and body weight 1555 ± 130 g [mean ± SD]) were skin ulcers apparently penetrating deep into muscle, hemorrhagic spots, anus inflammation, and pale gills. Swabs were taken from external lesions throughout their entire depth 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 7.0) and incubated at 30 °C for 24 h. Dominant colonies from the plates were restreaked on LB agar plates three times to obtain pure culture. The homogeneous dominant isolate was designated as AB001. The AB001 isolate culture was stored frozen in LB/20% glycerol at −80 °C.

2.2. Physiological and biochemical tests

A single colony of isolate AB001 was picked from the LB agar plate and inoculated into LB broth until optical density at 600 nm (OD_{600}) of the bacterial suspension reached 1.0. This suspension (0.5 mL) was then used to inoculate fresh LB broth for subsequent identification. The AB001 isolate was cultured at 150 rpm, and growth was monitored for 48 h at 600 nm every 1 h by calculation of the optical density. The effect of pH on the growth was investigated in fresh LB broth with initial pH levels adjusted to 3.0, 5.0, 7.0, and 9.0. For determining the effects of NaCl concentrations on this isolate's growth, the culture was inoculated into fresh LB broth supplemented with NaCl at various concentrations (1–5%). Tested temperatures were 13, 18, 27, 32, 37, and 42 °C. All assays were performed as three independent experiments with three biological replicates in each. Gram staining and biochemical characterization procedures were based on a manual for the identification of common bacterial systems [36]. These experiments were carried out three times independently.

2.3. Motility assessment

To test the motility of *A. salmonicida* isolate AB001, the bacterium was cultured in semisolid LB (0.25% agar) with NaCl added to a final concentration of 1%. Bacterial cells from an overnight culture were inoculated, using sterile toothpicks, into the substrate (see above). The plates were incubated at 15, 20, 30 or 37 °C, and motility was monitored for 24 h by measurement of the diameter of swimming zones. The motile bacterium *Escherichia coli* and nonmotile bacterium *Staphylococcus aureus* served as a positive and negative control, respectively. Moreover, swimming motility was assessed by light microscopy in liquid media.

2.4. Phylogenetic analyses of the 16S rRNA, gyrB, rpoD, and flaA genes

Genomic DNA of isolate AB001 was extracted with the Genomic DNA Extraction Kit (TransGen, China) and was used as a template for PCR amplification. PCRs were conducted using primers that target the *Aeromonas* 16*S* rRNA, *gyr*B, *rpo*D, and *fla*A genes (Table 1). 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). All the obtained sequences were submitted to NCBI, and GenBank accession numbers are given in phylogenetic trees (see figures below). 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. [37].

2.5. Experimental infections

Healthy *Oreochromis mossambicus* (Mozambique tilapia) individuals with the average length of 11 ± 0.9 cm were collected and transferred alive to al-Farabi Kazakh National University to be used for an experimental challenge. Siberian sturgeons (*A. baerii*) with average length 27.7 ± 3.1 cm were supplied by the LLP Educational and Scientific Complex of Pilot Industrial Production of Aquaculture (Uralsk, Kazakhstan). The *A. baerii* individuals were the offspring of artificially propagated sturgeons. All fish were reared in 100 L aquaria containing 50 L of static water with aeration at 20–22 °C (*A. baerii*) or 25–27 °C (*O. mossambicus*). They were allowed to acclimate to glass aquaria for 14 days. The fish were then randomly subdivided into six groups with 10 fish in each. Five groups were intraperitoneally injected with 0.5 mL of an isolate AB001 suspension of different concentrations (10^6, 10^7, 10^8, 10^9, or 10^{10} colony-forming units [CFU]/mL). The fish in the remaining group (control) were injected with 0.5 mL of phosphate-buffered saline. All fish were reared for 4 days at 20–27 °C for the purpose of observation and recording of the symptoms.

A negative control group was additionally maintained under similar conditions in which the fishes did not receive any injection. The survival rates were measured daily over a period of 4 days of the experimental challenge, and the relative percentage of survival

Table 1
Sequences of primers used.

Target gene	PCR primer sequence (5′ → 3′)	Product size (bp)	T_m, °C optimized	References
16S rRNA	AGAGTTTGATCCTGGCTCAG GGCTACCTTGTTACGACTT	1492	55	[38,39]
*gyr*B	TCCGGCGGTCTGCACGGCGT TTGTCCGGGTTGTACTCGTC	1127	55	[40]
*rpo*D	ACGACTGACCCGGTACGCATGTAYATGMGNGARATGGGNACNGT ATAGAAATAACCAGACGTAAGTTNGCYTCNACCATYTCYTTYTT	903	58–63	[41]
*fla*A	CACCCCNTTGTTCCATCT GCTTAGGAGAATGGTTATG	988	45–50	[22]
*vap*A	GGCTGATCTCTTCATCCTCACCC CAGAGTGAAATCTACCAGCGGTGC	421	57	[42]
hlyA	GGCCGGTGGCCCGAAGATACGGG GGCGGCGCCGGACGAGACGGG	595	55,3	[43]
aerB	CCGGAAGATGAACCAGAATAAGAG CTTGTCGCCACATACCTCCTGGCC	451	55,3	[44]
ast	TCTCCATGCTTCCCTTCCACT GTGTAGGGATTGAAGAAGCCG	331	55,3	[45]
pla	ATCTTCTCCGACTGGTTCGG CCGTGCCAGGACTGGGTCTT	382	52,5	[45]
ahpB	ACACGGTCAAGGAGATCAAC CGCTGGTGTTGGCCAGCAGG	513	45,6	[45]
alt	TGACCCAGTCCTGG GGTGATCGATCACC	442	50,9	[25]
ahe2	ACGGGGTGCGTTCTTCCTACTCCAG CCGTTCATCACGCCGTTATAGTCG	211	50,9	[46]
nucl	CAGGATCTGAACCGCCTCTATCAGG GTCCCAAGCTTCGAACAGTTTACGC	504	50,9	[46]
gcaT	CTCCTGGAATCCCAAGTATCAG GGCAGGTTGAACAGCAGTATCT	237	50,9	[47,48]
aerA	CAAGAACAAGTTCAAGTGGCCA ACGAAGGTGTGGTTCCAGT	309	55	[49]

(RPS) was calculated as follows: RPS = (1 − % mortality in test group/% mortality in control group) × 100 [50].

Deceased fish specimens were subjected to standard microbiological and pathological examinations, and a half-lethal dose (LD_{50}) was calculated by Probit analysis in Microsoft Excel 2019 [51]. All fish-handling procedures in this 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).

2.6. Antibiotic sensitivity tests

Antibiotic sensitivity was tested by the disk diffusion method on Mueller Hinton Agar (Condalab, Spain) [52] using oxacillin, penicillin G, ampicillin, amoxicillin, enrofloxacin, norfloxacin, cefazolin, gentamicin, streptomycin, nitrofurantoin, tetracycline, oxytetracycline, erythromycin, lincomycin, rifampicin, novobiocin, chloramphenicol, florfenicol, and trimethoprim + sulfamethoxazole as described by Bakiyev et al. [33].

2.7. Detection of virulence Factor–Encoding genes by PCR

Isolate AB001 was tested for the presence of 10 genes encoding 10 virulence factors by PCR with gene-specific primers (Table 1): SSU enterotoxin (*ast*), phospholipase A1 (*pla*), serine protease Ahe2 (*ahe2*), extracellular nuclease (*nucl*), phospholipid-cholesterol acyltransferase (*gca*T), aerolysins (*aer*A and *aer*B), hemolysin (*hly*A), elastase (*ahp*B), and cytotonic enterotoxin (*alt*). PCR amplification was performed in a 20 μL reaction mixture consisting of 10 μL of the 2 × PCR Master Mix (Thermo Scientific), 1 μL of each primer (10 pmol/μL), 2 μL of template DNA (250–500 ng), and 6 μL of nuclease-free water (Thermo Scientific). PCRs were carried out on a thermal cycler (Mastercycler EP Gradient S thermocycler, Eppendorf, Germany) under the following cycling conditions: initial denaturation at 94 °C for 10 min; 30 cycles of denaturation at 94 °C for 1 min, annealing at optimized temperature of 45.6–55.3 °C appropriate for each primer pair for 1 min, and extension at 72 °C for 1 min; followed by final extension at 72 °C for 5 min. The amplicons were separated electrophoretically in a 1% agarose gel stained with ethidium bromide (10 μg/mL). The electrophoresis was performed in a unit containing Tris-acetate-EDTA buffer (TAE) for 30 min at 100 V, and bands were viewed by means of a UV-transilluminator (Vilber, France).

2.8. Histopathological analysis

A gill, kidney, the intestine, liver, and spleen were excised from fish facing imminent death and from 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

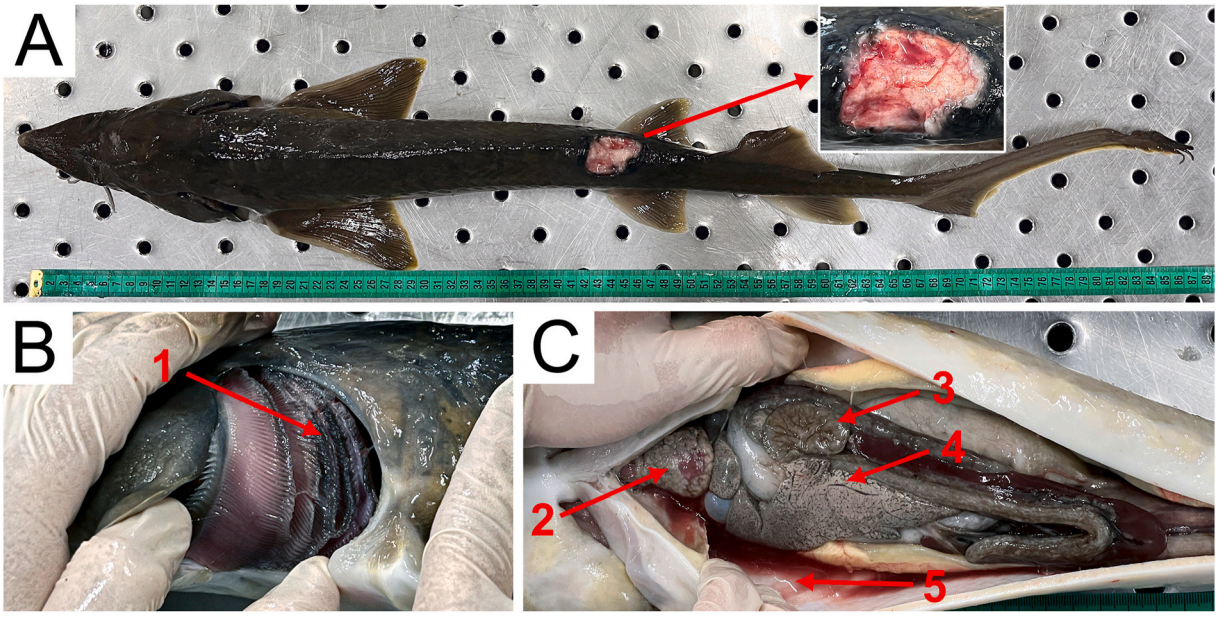

Fig. 1. Clinical examination and autopsy of diseased specimens of *A. baerii*. (A) Muscle necrosis (deeply penetrating) in the dorsal region; (B) pale color of gills; peripheral parts of gill filaments are necrotic (1). (C) Clinical symptoms of internal organs during postmortem examination. Pericarditis with petechial hemorrhage (2). The spleen (3) and liver are grayish-white in color (4). The build-up of bloody exudates within the abdominal cavity (5). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

dehydration (50–100% ethanol series), 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 symptoms

It was found that the diseased specimens of *A. baerii* are characterized by sluggish movement and loss of appetite and remain at the bottom of the pool. Clinical signs in the affected individuals were the presence of deep penetrating ulcers (Fig. 1). Marked necrosis of gill filaments was also observed (Fig. 1A and B). After dissection, hemorrhages of internal organs, pale gills, the liver grayish-white in color, and the presence of a bloody liquid within the abdominal cavity were documented (Fig. 1C).

3.2. Pathogen isolation and characterization

Isolate AB001 extracted from the diseased *A. baerii* proved to be a rod-shaped and gram-negative bacterium (Fig. 2) and produced no pigment in the LB medium during 24 h cultivation. It tested positive for oxidase, catalase production, esculin hydrolysis, and gelatin but negative in terms of sucrose, Voges-Proskauer, and indole; detailed physiological and biochemical characteristics are summarized in Table 2. Moreover, isolate AB001 could grow at 13–42 °C and showed no growth at 4 °C as well as grew at 0–4% NaCl but not at 5% NaCl. It grew at pH 7.0–9.0 in a liquid medium. Optimal growth conditions of the AB001 isolate were found to be 37 °C, 1% NaCl, and pH 7.0 (Fig. 3).

Antibiotic sensitivity tests indicated that the AB001 isolate is highly susceptible to enrofloxacin (disk content: 5 μg), norfloxacin (10 μg), gentamicin (10 μg), streptomycin (10 μg), nitrofurantoin (300 μg), tetracycline (30 μg), chloramphenicol (10 μg), florfenicol (30 μg), and trimethoprim + sulfamethoxazole (25 μg). AB001 turned out to be resistant toward oxacillin (1 μg), penicillin G (10 μg), ampicillin (10 μg), amoxicillin (10 μg), cefazolin (30 μg), lincomycin (10 μg), rifampicin (5 μg), and novobiocin (30 μg). Medium sensitivity was observed for two antibiotics: oxytetracycline (30 μg) and erythromycin (15 μg) (Table 3).

In this study, we demonstrated that the motility of *A. salmonicida* isolate AB001 is tightly regulated by temperature. The extension of motility zones was significantly larger at 30 °C than at 37 °C (Fig. 4). For example, after 24 h of incubation, swimming zones' diameter was approximately 58 mm at 30 °C and 27 mm at 37 °C. The AB001 isolate showed intermediate swimming at 20 °C (47 mm) but nearly ceased swimming at 15 °C (Fig. 4).

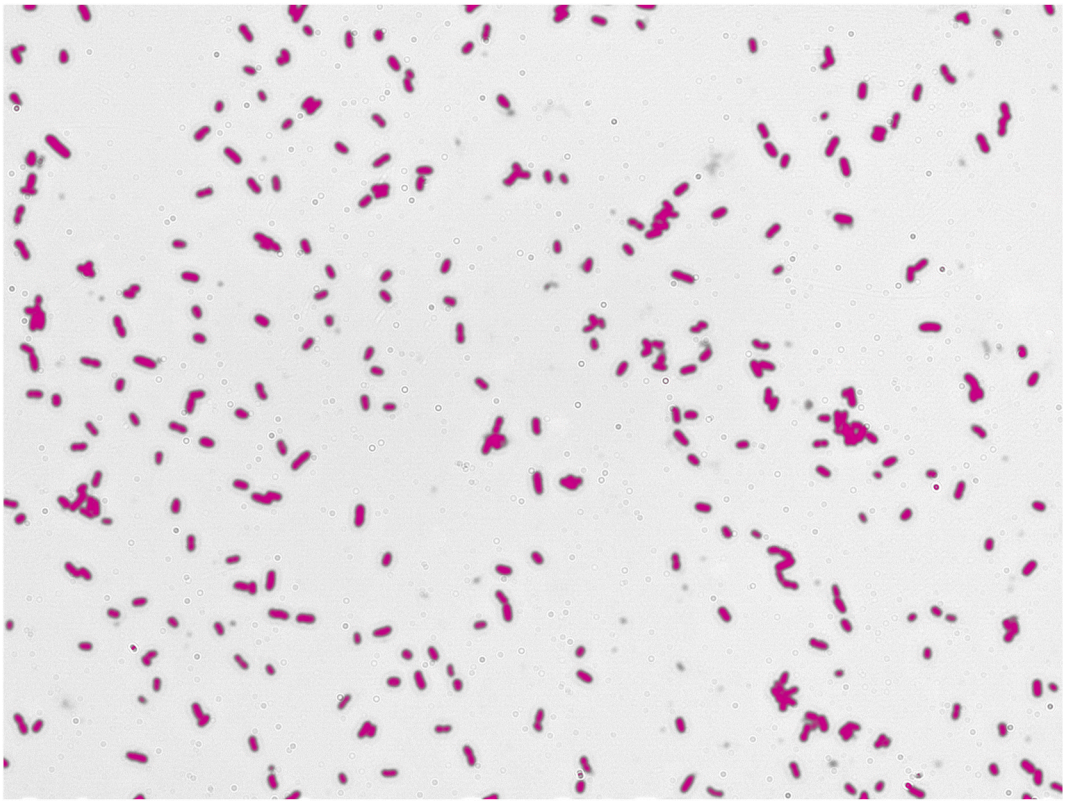

Fig. 2. Gram staining revealing gram-negative rods of the isolated *A. salmonicida* AB001.

Table 2
Biochemical characteristics of isolate AB001.

No.	Characteristics	Reaction	No.	Characteristics	Reaction
1	Gram staining	N	19	D-xylose	N
2	Morphology	rod	20	Lactose	N
3	Motility	P	Growth under conditions:		
4	Oxidase	P	21	0% NaCl	P
5	Methyl red	P	22	1% NaCl	P
6	Voges-Proskauer test	N	23	2% NaCl	P
7	O/F test	F	24	3% NaCl	P
8	Hydrolysis of gelatin	P	25	4% NaCl	P
9	Hydrolysis of esculin	P	26	5% NaCl	N
10	H2S formation	N	27	13 °C	P
11	Indole formation	N	28	18 °C	P
12	Lysine decarboxylase	N	29	27 °C	P
13	Ornithine decarboxylase	N	30	32 °C	P
14	Arginine dihydrolase	P	31	37 °C	P
15	ONPG	P	32	42 °C	P
16	Catalase	P	33	pH 3.0	N
Acid formation from:			34	pH 5.0	N
17	Sucrose	N	35	pH 7.0	P
18	Trehalose	P	36	pH 9.0	P

P, positive; N, negative; F, fermentative.

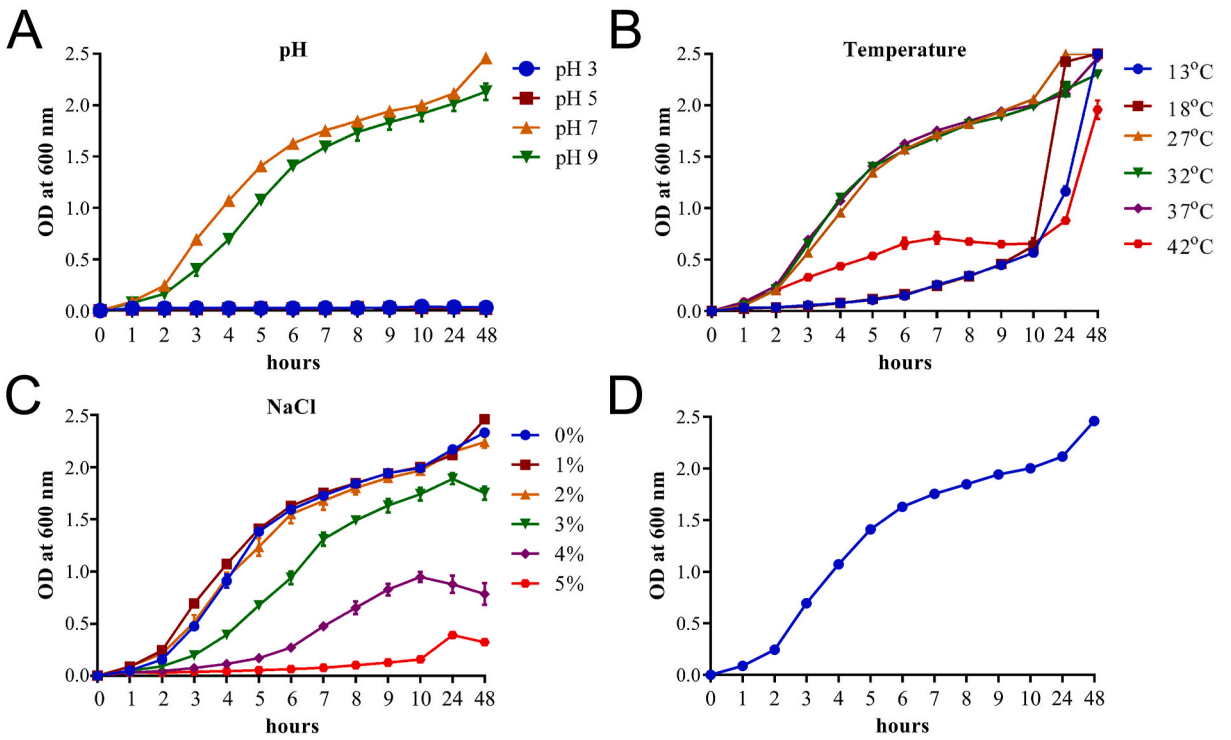

Fig. 3. Growth characteristics of AB001. (A) Growth at pH 3.0–9.0; (B) the growth at 13–42 °C; (C) the growth at 0%–5% NaCl; (D) a growth curve of *A. salmonicida* at pH 7.0, 37 °C, and 1% NaCl.

3.3. Molecular characterization via sequence analysis of the 16S rRNA, rpoD, gyrB, and flaA genes

Fragments of 16S rRNA, *gyr*B, *rpo*D, and *fla*A genes of isolate AB001 were PCR-amplified for sequencing. These fragments of genes 16S rRNA, *gyr*B, *rpo*D and *fla*A were 1492, 1127, 903, and 988 bp in length, respectively (Fig. 5; Please see more details in Supplementary file, Fig. S1). These sequences were deposited within database GenBank (accession numbers OK634025, ON124026, OQ144653, and OQ144652, respectively). BLAST alignments of the 16S rRNA fragment revealed that isolate AB001 shares the highest similarity (99.32%) with a type of an *A. salmonicida* strain, and genes *gyr*B and *rpo*D are also shared with this microbe at high identity (98.84% and 99.75%). Besides, analyses of phylogenetic trees of 16S rRNA, *gyr*B, and *rpo*D genes showed that isolate AB001 is closely

Table 3
Sensitivity of AB001 to different antibiotics.

Group	Antibiotic	Disk content (μg)	AB001	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	20.7 ± 1.2
	Norfloxacin	10	S	22.7 ± 1.5
Cephalosporins	Cefazolin	30	R	0
Aminoglycosides	Gentamicin	10	S	18 ± 1
	Streptomycin	10	S	16.7 ± 0.6
Nitrofurans	Nitrofurantoin	300	S	19 ± 1
Tetracyclines	Tetracycline	30	S	23.3 ± 0.6
	Oxytetracycline	30	I	20.7 ± 1.5
Macrolides	Erythromycin	15	I	14.3 ± 1.5
Lincomycins	Lincomycin	10	R	0
Rifamycins	Rifampicin	5	R	10 ± 1
Coumarins	Novobiocin	30	R	9.7 ± 1.2
Amphenicols	Chloramphenicol	10	S	24.3 ± 0.6
	Florfenicol	30	S	26.3 ± 0.6
Folic acid synthesis inhibitors	Trimethoprim + sulfamethoxazole	25	S	18 ± 1

I, intermediate; R, resistant; S, sensitive. Zone diameters are presented as mean ± SD.

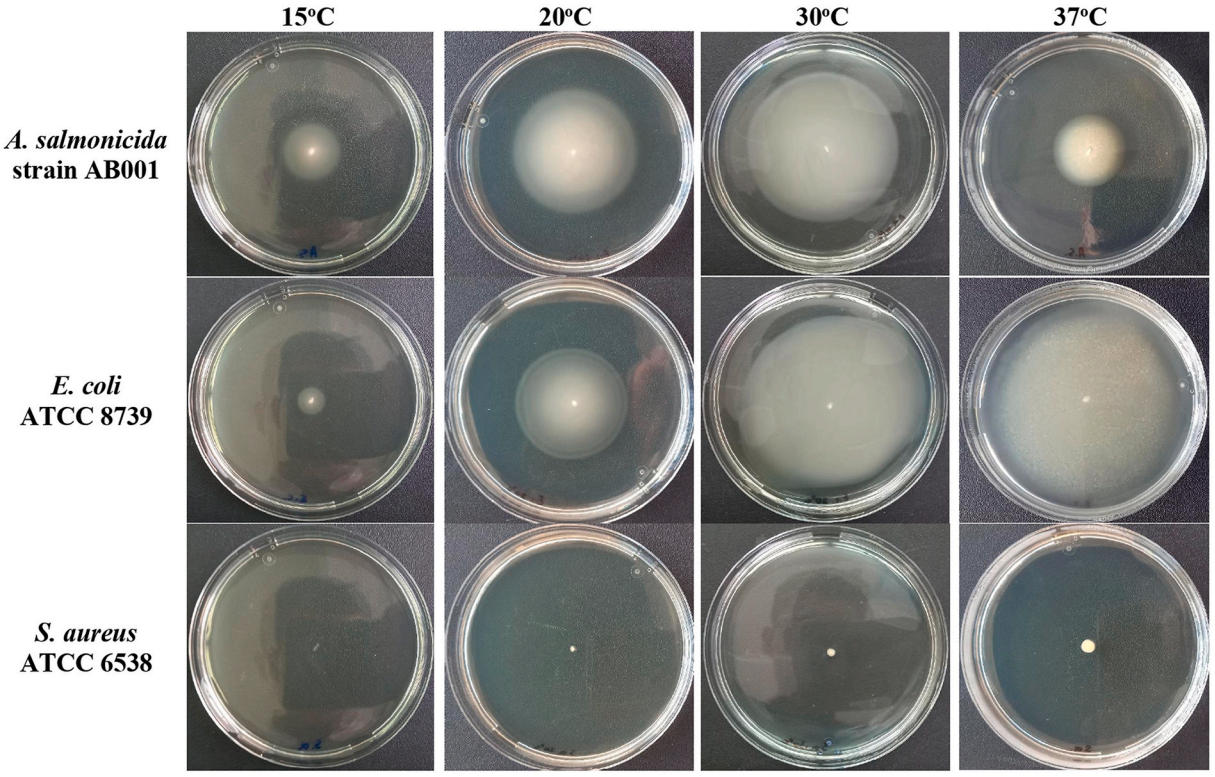

Fig. 4. Swimming motility of *A. salmonicida* isolate AB001 on semisolid agar. The motile bacterium *E. coli* and nonmotile bacterium *S. aureus* respectively served as a positive and negative control. The images of the motility assay were captured after 24 h incubation at 15, 20, 30, or 37 °C.

related to known strains of *A. salmonicida* (Figs. 6–8). In a recent study, researchers successfully identified subtypes of isolates of *A. salmonicida* via phylogenetic analyses of the *vap*A (virulence array protein) gene [23]. Our PCR tests were run with primers targeting the hypervariable *vap*A region (Table 1), but the amplification failed for AB001 (Fig. 5).

To obtain the full-length *fla*A gene, the PCR primers targeting its flanking regions (genes *yad*S and *fla*B) were utilized (Table 1) [22]. The primers amplified a single DNA fragment of ~912 bp from AB001 chromosomal DNA in the PCR. The amplicons were resolved by electrophoresis and were purified with a QIAquick® Gel Extraction Kit (Qiagen Corp.). The purified PCR products were sequenced. In the tree obtained by the neighbor-joining method, the AB001 strain is strongly associated with *A. salmonicida* subsp. *pectinolytica*

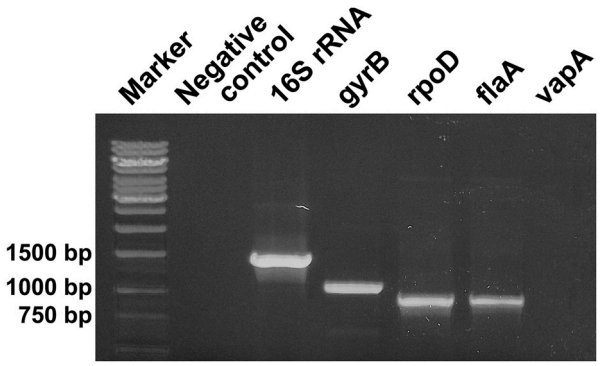

Fig. 5. Agarose gel electrophoresis analysis of PCR amplicons from 16S rRNA, *gyr*B, *rpo*D, *fla*A, and *vap*A genes of *A. salmonicida* strain AB001.

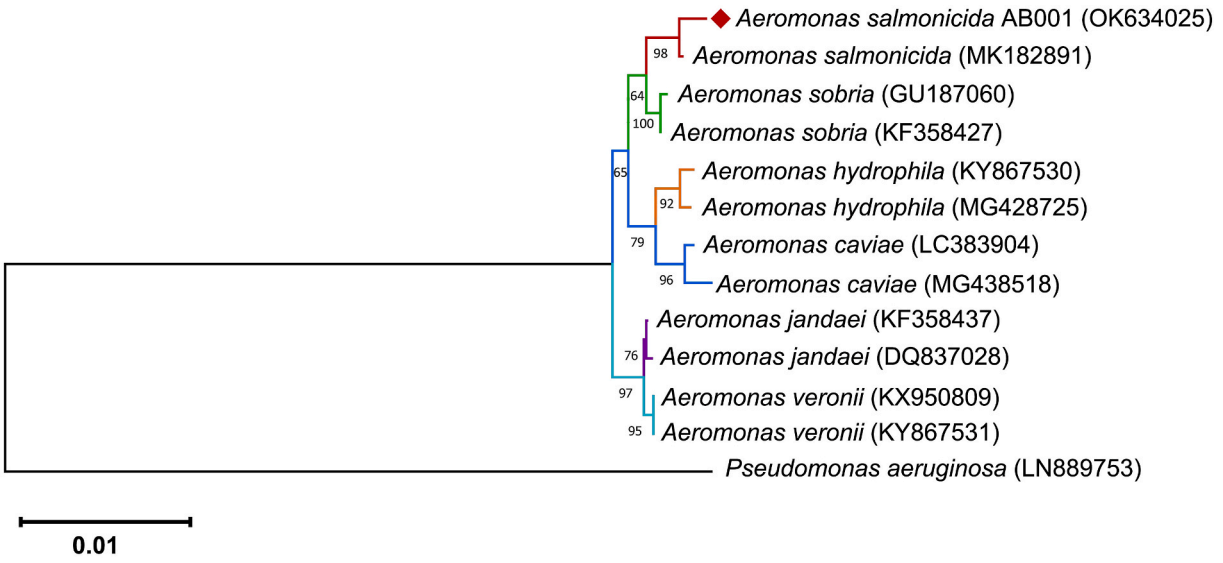

Fig. 6. Neighbor-joining phylogenetic trees (unrooted) constructed from the 16S rRNA gene sequence of *Aeromonas* strains. These phylogenetic trees revealed clear-cut clustering of *A. salmonicida* strain AB001 with some *A. salmonicida* strains whose sequences were downloaded by us from GenBank. *Pseudomonas aeruginosa* was used as an outgroup species. Sequence accession numbers are given in parentheses. The scale bar denotes 0.01 nt substitutions per site.

(EU410321) (99% bootstrap value).

3.4. Virulence genes

The analysis of these genes indicated that out of the 10 tested virulence genes, strain AB001 has seven: phospholipase A1 *(pla)*, serine protease Ahe2 *(ahe2)*, phospholipid-cholesterol acyltransferase *(gca*T*)*, elastase *(ahp*B*)*, aerolysin *(aer*A*)*, hemolysin *(hly*A*)*, and cytotonic enterotoxin *(alt)* (Fig. 9).

3.5. Experimental infections and histopathological examination

O. mossambicus challenged with strain AB001 manifested fading of body pigmentation, a distended abdomen, and extensive skin hemorrhages including the base of opercula and fins. Moreover, the injected fish demonstrated unbalanced swimming and stayed near the bottom of the aquarium. Internally, the infected fish showed massive hemorrhaging of internal organs and branchial ischemia. The results indicated that the fish developed apparent clinical symptoms involving internal organs such as pale gills, necrosis of parenchymal organs, and ascites.

Artificially infected *A. baerii* exhibited such clinical signs as petechial hemorrhages on the ventral part of the body, an icteric appearance, and swelling and hyperemia of the spleen and of the intestinal tract (Fig. 10).

An infection dose of 10^{10} CFU/mL caused 100% mortality of *O. mossambicus* within 8 h postinfection after intraperitoneally injection (Fig. 11). The same dose of AB001 also resulted in 100% mortality of *A. baerii* within 24 h (Fig. 11). At 96 h postinjection, the

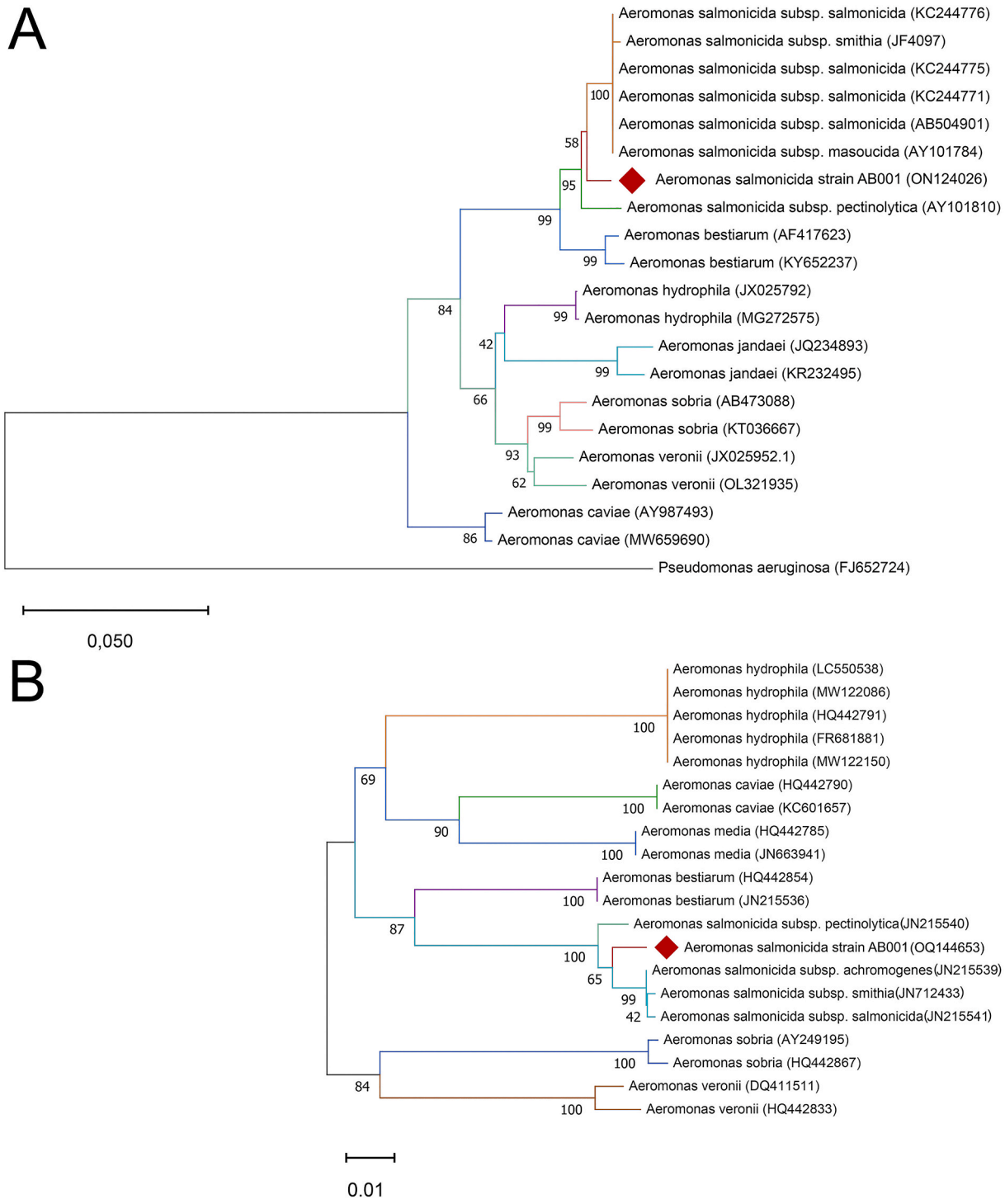

Fig. 7. Neighbor-joining phylogenetic trees (unrooted) that are based on *gyr*B gene (A) and *rpo*D gene (B) sequences from *Aeromonas* strains. The phylogenetic trees uncovered well-pronounced clustering of *A. salmonicida* strain AB001 with some *A. salmonicida* strains whose sequences were downloaded by us from GenBank. *P. aeruginosa* was used as an outgroup species. Sequence accession numbers are given in parentheses. The scale bar denotes 0.05 and 0.01 nt substitutions per site.

mortality rate was 70% and 90% for *O. mossambicus* and *A. baerii*, respectively, at 10^9 CFU/mL. When the pathogen concentration was 10^7 CFU/mL, the mortality rate of *O. mossambicus* and *A. baerii* was very low, no more than 10%. There was no mortality at 10^6 CFU/mL up to 96 h postinfection. Calculated LD_{50} of *A. salmonicida* AB001 was 1.7×10^8 CFU/mL for *O. mossambicus* and 7.2×10^7 CFU/mL

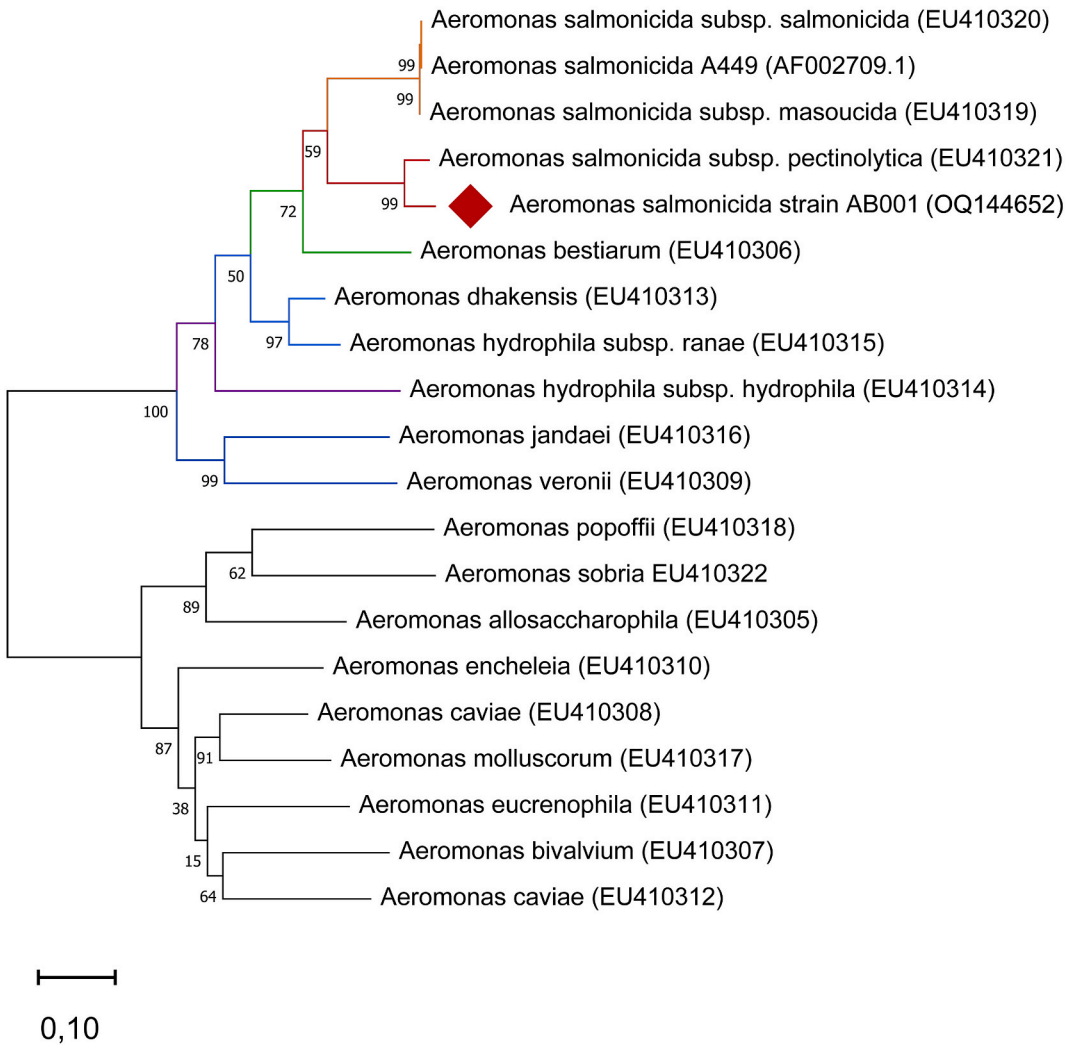

Fig. 8. Neighbor-joining phylogenetic tree generated based on *fla*A gene sequences (from the *A. salmonicida* strain detected in the present study and from other *Aeromonas* spp. from GenBank). Sequence accession numbers are given in parentheses. The scale bar represents 0.10 nucleotide substitutions per site.

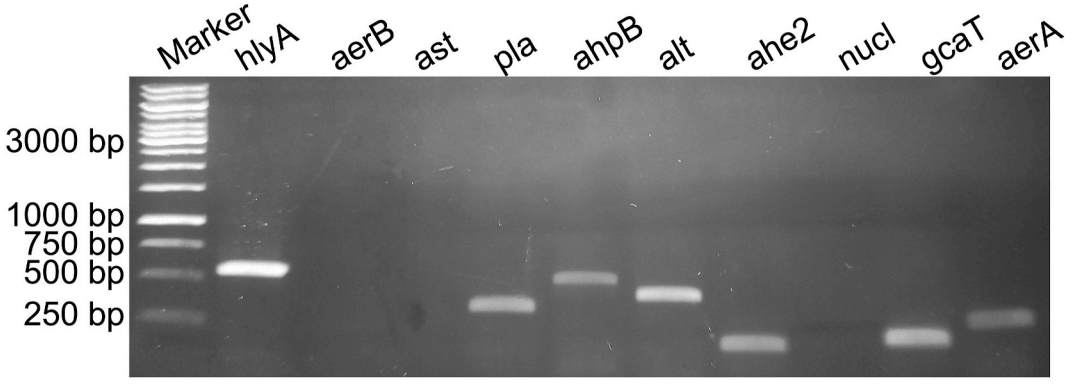

Fig. 9. Agarose gel electrophoresis of amplified fragments of virulence genes. M: markers, 100 bp DNA Ladder (Thermo Fisher Scientific).

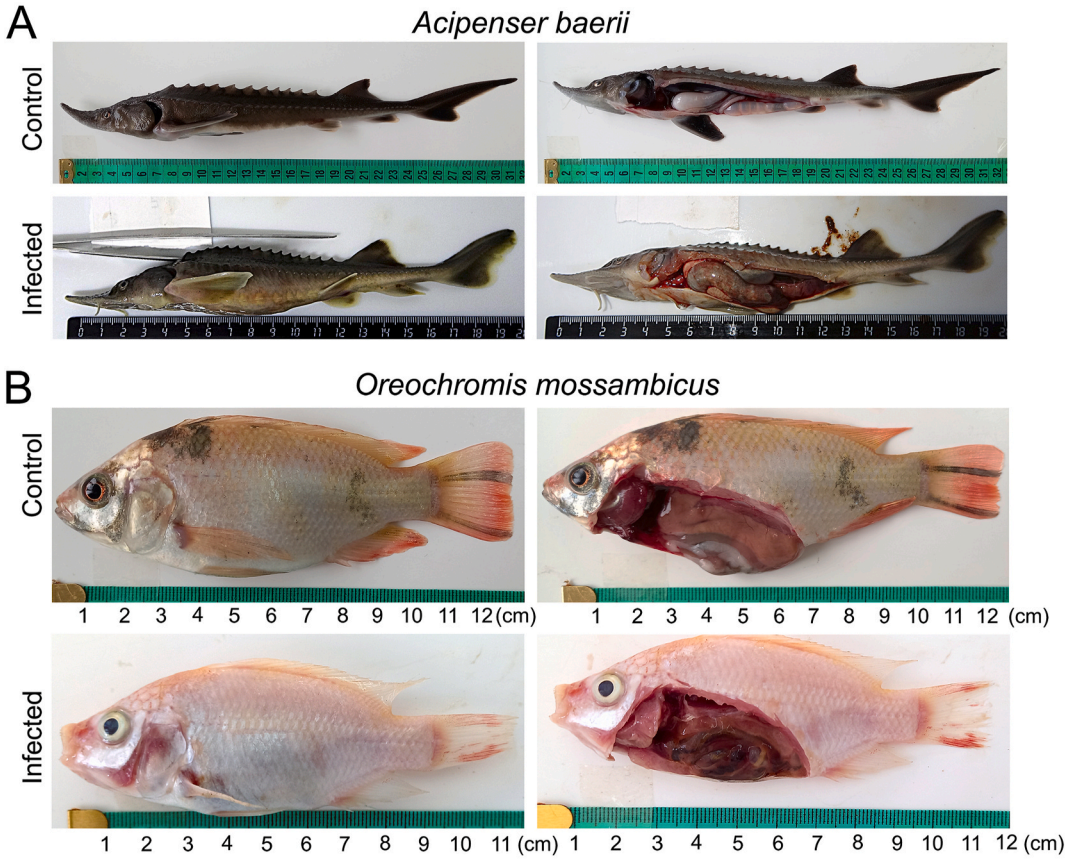

Fig. 10. Clinical features of *O. mossambicus* and *A. baerii* infected with *A. salmonicida*. (Infected) Depigmentation of the fish body, hemorrhages at the base of fins, and inflammation of the anus. (Infected) Necrosis of parenchymal organs, ascites, and gill ischemia.

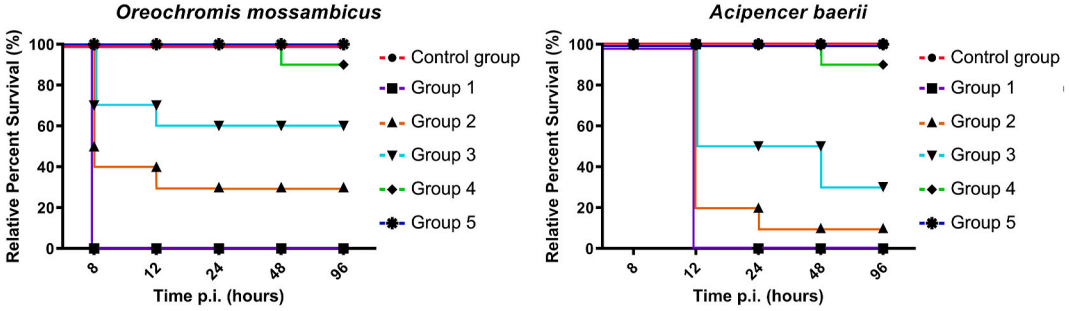

Fig. 11. Relative percentage of survival of *O. mossambicus* and *A. baerii* from infection at different doses of *A. salmonicida* AB001 within 96 h postinfection (p.i.). The animals were divided into six groups: (●) Control group, fish injected with PBS without infection; (■) Group 1, fish infected with the bacteria (10^{10} CFU/mL); (▲) Group 2, fish infected with the bacteria (10^{9} CFU/mL); (▼) Group 3, fish infected with the bacteria (10^{8} CFU/mL); (◆) Group 4, fish infected with the bacteria (10^{7} CFU/mL); (*) Group 5, fish infected with the bacteria (10^{6} CFU/mL).

for *A. baerii*. The bacterium was isolated again from infected fish, and these isolates showed the same biochemical and morphological features as did strain AB001 (data not shown).

In the gills of the infected fish, blood stasis in the central venous sinus, infiltration of the primary gill epithelium by mononuclear leukocytes as well as destruction and necrosis of secondary gill filaments were observed (Fig. 12A and B). Besides, hyperplasia of the primary gill epithelium, an increase in the number of rodlet cells (Fig. 12C), and complete loss of secondary gill filaments were registered in the gills. In the intestine, there was infiltration of the intestinal mucosa by mononuclear leukocytes and local foci of necrosis of intestinal epithelium (Fig. 12D and E). In the liver, hemorrhages, and necrosis in the subcapsular zone of the organ (Fig. 12F and G) and edema of the parenchyma adjacent to the pancreatic areas (Fig. 12H) were evident. In the kidneys, foci of infiltration of the parenchyma by leukocytes as well as necrosis of the tubular epithelium were observed (Fig. 12I and J). In the spleen, extensive necrosis

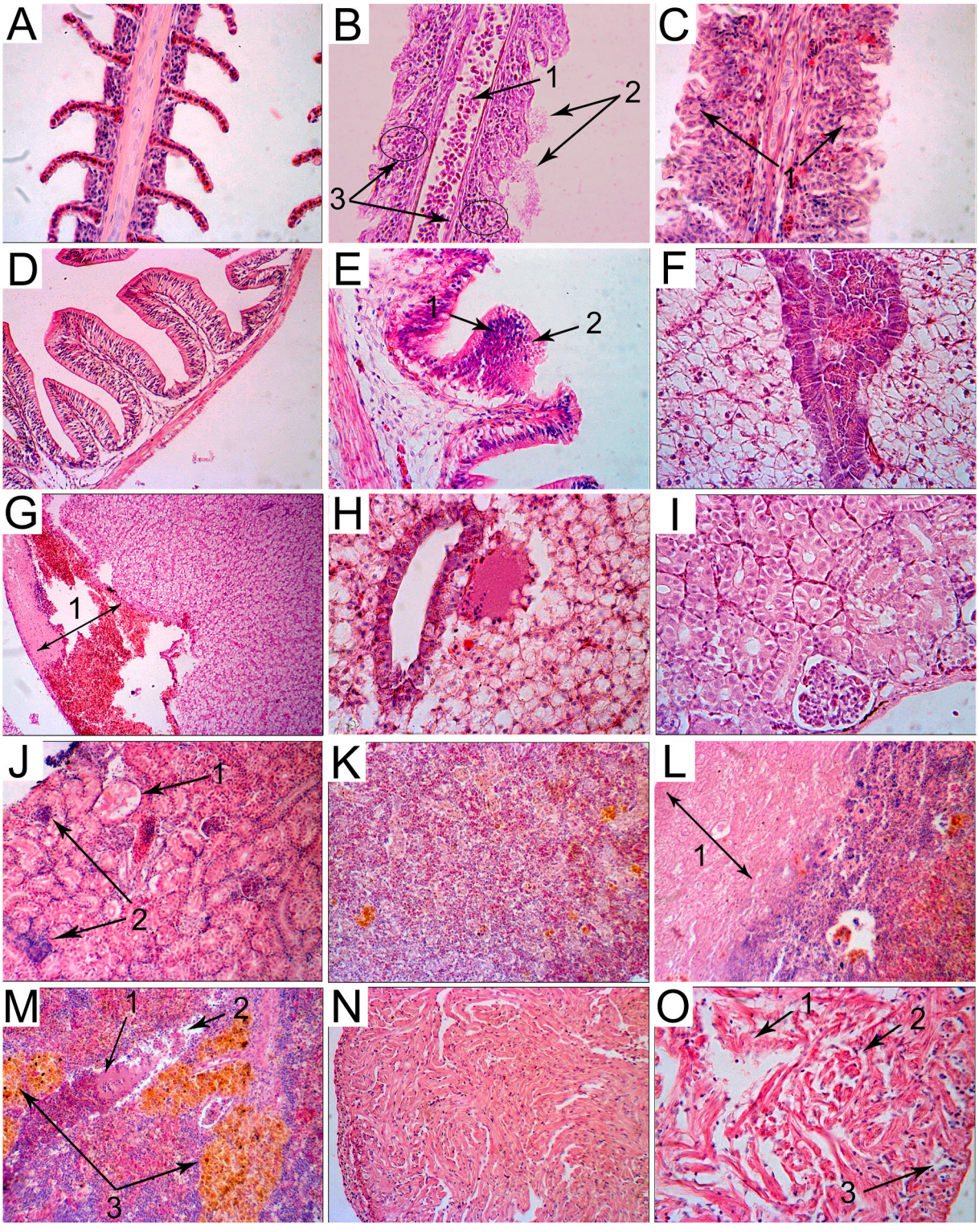

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Fig. 12. Histopathological changes in *O. mossambicus* subjected to experimental infection with *A. salmonicida* AB001. (A) Gills of control fish (×400). (B) Gills of an infected fish (×400). 1: Blood stasis in the central venous sinus; 2: necrosis of the apical epithelium of secondary gill filaments and their destruction; 3: infiltration by mononuclear leukocytes. (C) Hyperplasia of the primary gill epithelium (×400). 1: Rodlet cells (D) An intestine of control fish (×400). (E) An intestine of an infected fish (×400). 1: Infiltration by mononuclear leukocytes; 2: enterocyte necrosis (F) A liver of a control fish (×200). (G) Liver of an infected fish (×100). 1: Hemorrhage and necrosis of the subcapsular zone. (H) Liver of an infected fish (×400). Edema and necrosis of the parenchyma adjacent to the pancreatic area (×400). (I) A kidney of a control fish (×400). (J) Kidney of an infected fish (×400). 1: Renal tubular necrosis; 2: inflammatory infiltration. (K) A spleen of a control fish (×200). (L) A spleen of infected fish (×200). 1: Necrosis of the marginal zone of the spleen. (M) A spleen of infected fish (×200). 1: Hemolysis of red pulp erythrocytes; 2: necrosis in the parenchyma; 3: hemosiderin deposits in melanomacrophage aggregates. (N) A heart of a control fish (×100). (O) A heart of an infected fish (×200). 1: Myocardial lysis; 2: myocardial fragmentation; 3: mononuclear leukocyte infiltration of the pericardium. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

was noted along the organ's marginal zone (Fig. 12K and L); there was also a noticeable increase in melanomacrophage centers (aggregates) due to an increase in the hemosiderin amount, hemolysis of red pulp erythrocytes, and necrosis of the organ parenchyma (Fig. 12M). In the heart fragmentation, curvature and lysis of cardiac muscle fibers as well mononuclear leukocyte infiltration were detected (Fig. 12N and O).

4. Discussion

A. salmonicida is a water-borne gram-negative facultative anaerobic bacterium that is widespread in marine and freshwater environments. Historically, *A. salmonicida* has been considered a leading pathogen that causes furunculosis in wild and cultured salmonids. Nonetheless, as time has passed, the obvious host range of the pathogen has steadily widened and now encompasses various nonsalmon species [10]. *A. salmonicida* strains are categorized as atypical or typical judging by their origin of isolation and phenotype. Strains of *A. salmonicida* isolated from salmonids are categorized as "typical," whereas strains isolated from environmental samples or nonsalmonid hosts are regarded as "atypical" strains [53,54]. Both atypical and typical *A. salmonicida* infections have been recorded worldwide, with the exception of Kazakhstan [55]. In the present paper, an atypical strain of *A. salmonicida* isolated from an ulcer on diseased *A. baerii* is described and analyzed. Infection with *A. salmonicida* may have various clinical signs in different species of fish, although skin ulcers and hemorrhages are recurring features [56]. In the current work, the main clinical manifestations of aeromonad infection were stand-alone skin ulcers deeply penetrating into muscles along the edges on abdominal, dorsal, or tail sections of the fish as well as petechial hemorrhages in the abdominal part of the body (Fig. 1). Although AB001 is most similar to *A. salmonicida* subsp. *salmonicida* in morphological and biochemical characteristics (Table 2), AB001 differs because it does not produce a brown diffusible pigment, is motile, and grows at ≥37 °C and at temperatures as low as those of psychrophilic strains. It is worth mentioning that the species *A. salmonicida* is subdivided into officially recognized five subspecies: *achromogenes*, *smithia*, *salmonicida*, *masoucida*, and *pectinolytica* [13]. Strains of subspecies *salmonicida*, *smithia*, *achromogenes*, and *masoucida* can infect diverse fish species and are unable to grow at temperatures higher than ~25 °C [10], whereas the subspecies *pectinolytica* has no known host and can grow at 37 °C [14]. Moreover, until very recently, mesophilic *A. salmonicida* strains have not been regarded as pathogenic, but lately, mesophilic pathogenic strains have been isolated from diseased fish [17], leeches [57], human patients [15], and birds [16]. To the best of our knowledge, the current article is the first to report an infection by a mesophilic *A. salmonicida* strain in *A. baerii* cultivated in Kazakhstan. The AB001 strain from diseased *A. baerii* was characterized based on sequences of the 16S rRNA gene, which revealed that this bacterium is most strongly related to *A. salmonicida*, with identity of over 99.3% (Fig. 6). Previously, it has been reported that housekeeping genes are more suitable molecular markers than gene 16S rRNA is for investigation of taxonomic and phylogenetic relations at a species level [21]. As displayed in Fig. 6 and 6, sequences of two housekeeping genes (*gyr*B and *rpo*D) of the AB001 strain are most closely related (albeit not identical) to those of all four *A. salmonicida* subspecies, including *A. salmonicida* subsp. *pectinolytica*. As mentioned above, the lack of motility has been considered one of the reliable traits for the differentiation of representatives of *A. salmonicida* from other aeromonads [10]. Of note, strain AB001 exhibited marked motility when observed under a light microscope. In addition, swimming (polar flagellum) motility assays revealed that the motility of AB001 is regulated by temperature. AB001 cells manifested pronounced motility when grown at 20 and 30 °C, whereas the motility is significantly slowed down at 15 and 37 °C (Fig. 4).

It should be noted that suppression of swimming motility takes place at optimum growth temperature of *A. salmonicida* just like in many bacteria. This effect has been researched in animal and plant pathogens and is evidently explained by the inhibition of expression of flagellum components necessary for swimming motility [58–62]. Previously, eight atypical motile isolates of *A. salmonicida* have been extracted from ulcers (but not from kidney tissue) of goldfish, carp, and roach [63]. Moreover, these isolates did not dissociate into different types of colonies but grew at 37 °C. Besides, two flagellin genes (*fla*A and *fla*B, which code for polar flagella) in *A. salmonicida* have been isolated and characterized [64]. However, the role of temperature in the form of a stimulus for shifts in flagellar components' gene expression in *A. salmonicida* has not been examined. Consequently, the precise role of temperature in motility control of *A. salmonicida* remains to be evaluated.

It is now well known that the flagellum in bacteria is the most crucial organelle for the motility of bacteria and performs a key function in numerous bacterial properties, including virulence [22]. To examine the presence a motility-associated flagellum gene, the full-length *fla*A gene of these strains was sequenced, and then a phylogeny was constructed (Fig. 8). The results of our BLASTN search in database GenBank confirmed that the obtained sequence belongs to the flagellin gene of *A. salmonicida* and uncovered strong homology with the *fla*A gene. Our phylogenetic analysis on the basis of the *fla*A gene sequence identified strain AB001 as *A. salmonicida*

subsp. *pectinolytica* (Fig. 8). On the other hand, AB001 is undoubtedly not *A. salmonicida* subsp. *pectinolytica* because AB001 is motile and does not produce a brown diffusible pigment. Although we used several molecular markers in the phylogenetic analysis, the exact subspecies identity of AB001 remains to be resolved. It is possible that whole-genome sequencing will help to determine the subspecies identity of the AB001 strain, which is the aim of our future studies. Other authors have used the *vap*A (virulence array protein) gene to identify a subspecies of *A. salmonicida* [23]. By contrast, in our work, we failed to detect the sequence of the *vap*A gene in strain AB001, consistently with other reports about *A. salmonicida* subsp. *pectinolytica* [65]. The A layer protein is an important virulence factor of *A. salmonicida* and facilitates virulence by promoting bacterial attachment to host cells and by protecting the pathogen from the host's proteases [66]. Some researchers attribute the lack of virulence in some strains of *A. salmonicida* to a loss of the A-layer virulence factor [67–69].

The species that are the most reared in aquaculture belong to the genus *Oreochromis* [70,71]. Blue tilapia (*Oreochromis aureus*), Nile tilapia (*Oreochromis niloticus*), and Mozambique tilapia (*Oreochromis mossambicus*) as well as their hybrids, are successfully cultivated in Kazakhstan [72,73]. *O. mossambicus* is a staple food fish and the most widespread of tilapias. Moreover, this species has also been utilized as a model to investigate environmental factors [74] and a response to pathogens [75,76]. To elucidate the pathogenicity pattern of *A. salmonicida* strain AB001 isolated from *A. baerii*, first fish challenge experiments were performed by means of *O. mossambicus*. Experimental challenge assays here confirmed the virulence of the isolate in question against healthy *O. mossambicus*, as demonstrated by 100% mortality at 8 h postinfection for 10^{10} CFU/mL (Fig. 11). Histopathological alterations within internal organs of *O. mossambicus* are also typical for infections by *Aeromonas* spp. [33,69,77,78]. In our study, nearly 100% of *A. baerii* individuals were dead at 24 hpi for the same bacterial-cell concentration (10^{10} CFU/mL). LD_{50} was found to be 1.7×10^8 CFU/mL for *O. mossambicus* and 7.2×10^7 CFU/mL for *A. baerii*. Extensive hemorrhages in different body parts, pale gills, and necrosis of internal organs were the most obvious signs in the artificially infected *O. mossambicus* and *A. baerii*. Thus, our results indicate that the AB001 strain is highly pathogenic despite the absence of the *vap*A gene. This is an expected finding because avirulent *A. salmonicida* strains that express the A-layer as well as virulent *A. salmonicida* strains that do not express the A-layer have been reported before [79–81]. Therefore, the A-layer protein is not the only virulence factor, and it is possible that the AB001 strain uses other virulent determinants to manifest pathogenicity against *A. baerii* and *O. mossambicus*. Indeed, in the current study, we revealed that the AB001 strain has seven genes (*ahe*2, *gca*T, *pla*, *hly*A, *aer*A, *ahp*B and *alt*) of the 10 virulence genes typical for *Aeromonas* (Fig. 9). These genes respectively code for serine protease, phospholipid-cholesterol acyltransferase (GCAT), phospholipase A1, hemolysin, aerolysin, elastase, and cytotonic enterotoxin. The extracellular products such as hemolysins, aerolysins, lipopolysaccharides, proteases, and various toxins have been found in many studies on *A. salmonicida* over the years [9,82,83]. Ellis et al. [83] have demonstrated that protease and hemolysin activities promote the development of lesions in fish. It has been shown that GCAT is lethal for the Atlantic salmon (*Salmo salar* L.) in a dose of 0.045 μg of the protein per gram of body weight [84]. Besides, it has been revealed that this toxin can aggregate with lipopolysaccharide, the resulting complex being even more toxic than GCAT by itself [84]. Several virulence genes present in strain AB001 imply that they play considerable roles in the occurrence of infections, but the mechanism of toxic action of this bacterium still needs to be explored further. The AB001 strain turned out to be resistant to penicillins, cephalosporins, lincomycins, rifamycins, and coumarins. The well-pronounced drug resistance of AB001 may be explained by the extensive use of antibiotics on farms for the control of bacteria in aquaculture. It is worth noting that most of the antibiotics employed in aquaculture are also used against human diseases [85], and this state of affairs can not only lead to a failure of an antibiotic therapy but also lead to questions about safety of fish products. Therefore, new strategies are needed to combat these drug-resistant bacterial pathogens.

In sum, an *A. salmonicida* AB001 isolate was extracted from an ulcer of diseased *A. baerii*. *A. salmonicida* has not been reported previously to be a pathogen of cultured *A. baerii* in Kazakhstan. Thus, this is the first report of an *A. salmonicida* infection in farmed *A. baerii*; this pathogen has substantial virulence and causes acute infection in fish. The AB001 strain grows in a wide temperature range (13–42 °C) and has temperature-dependent motility, which is additional evidence that the mesophilic group within the species *A. salmonicida* is more complicated and diverse in comparison with the psychrophilic one. Recent studies indicate that certain mesophilic strains of *A. salmonicida* are capable of infecting mammals and humans [86,87]. Hence, it is necessary to figure out the risk of animal-to-human transmission of diseases caused by the *A. salmonicida* AB001 strain and to draw up relevant recommendations for producers and consumers.

It is known that temperature of water is the most crucial environmental factor that influences bacterial diseases in aquaculture; consequently, further research including whole-genome sequence analysis is required for evaluating the effect of temperature variation on the lifestyle of this unusual *A. salmonicida* strain.

Ethics statement

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).

Author contributions

Amangeldy Bissenbaev: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper. Serik Bakiyev: Analyzed and interpreted the data; Performed the experiments. Izat Smekenov: Analyzed and interpreted the data; Performed the experiments. Irina Zharkova, Saidina Kobegenova: Performed the experiments; Contributed reagents, materials, analysis tools or data. Nurlan Sergaliyev, Gaisa Absatirov: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data. All authors have reviewed and approved the submitted version of this

manuscript.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e17775.

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