IRSTI 34.15.27 https://doi.org/10.26577/IJBCh2024v17.i2.4

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(Received 20 March 2024; received in revised form 15 June 2024; accepted 30 June 2024)

Biochemical and molecular genetic identification of the bacterial pathogen – *Aeromonas bestiarum* from a diseased Siberian sturgeon (*Acipenser baerii*)

Abstract. The article presents the results of isolation and identification of the bacterium *Aeromonas bestiarum* from diseased individuals of Siberian sturgeon (*Acipenser baerii*) reared in recirculating aquaculture system (RAS). As a result of biochemical studies, the isolated strain *A. bestiarum* AB002 is characterized as: a Gram-negative, non-motile oxidase-positive bacillus capable of growing in a wide temperature range from 13 to 42 ºC. In addition, strain AB002 is characterized by the hydrolysis of gelatin and esculin, forming H_2S and indole, and exhibits arginine dihydrolase activity. The analysis of bacterial resistance to antibiotics revealed that strain AB002 is resistant to multiple groups of antibiotics, including Penicillins (Oxacillin, Penicillin G, Ampicillin, Amoxicillin); Cephalosporins (Cefazolin); Macrolides (Erythromycin); Lincomycins (Lincomycin); Rifamycins (Rifampicin); Coumarins (Novobiocin). The analysis of virulence factors revealed that the pathogenic strain *A. bestiarum* AB002 is characterized by the presence of 6 virulence genes out of 10 studied, among which lipase (*pla*), cytotonic enterotoxin (*alt*), serine protease (*ahe2*), DNAase (*nucl*), cholesterol acyltransferase (*gcaT*), aerolysin (*aerA*) were identified.
Key words: *Acipenser baerii*, *Aeromonas bestiarum*, biochemical characteristics, 16S rRNA gene, *gyr*B gene.

Introduction

Bacteria of the genus *Aeromonas* are ubiquitous and represent a large community in the ecosystem [1]. The bacteria of the genus *Aeromonas* have a wide distribution in water and aquatic environments. Their ability to move across the ecosystem is facilitated by the presence of flagella [2]. Similar to numerous Gram-negative bacteria, the *Aeromonas* genus is capable of causing disease in animals [3], and certain members of the genus can potentially cause disease in humans under specific conditions [4, 5]. In addition, sturgeon fish are no exception and are also susceptible to diseases caused by bacteria of the *Aeromonas* genus [6]. The most common pathogenic representatives of bacteria of the genus *Aeromonas* are *A. hydrophila*, *A. salmonicida*, *A. veronii*, which can cause death of fish in aquaculture conditions [7-9]. The main virulence factors in bacteria of the genus *Aeromonas* include: haemolysin, aerolysin, elastase, and cytolytic enterotoxins [10]. Through the presence of virulence factors, bacteria of the genus *Aeromonas* cause numerous hemorrhages on the body, boils, branchial ischemia, and catarrhal and hemorrhagic inflammation of internal organs in fish [11, 12]. At the same time, many members of the genus *Aeromonas* have recently shown multiple resistance to the antibiotics used against them, which causes a serious risk. *Aeromonas bestiarum* is one of the representatives of pathogenic bacteria of the genus *Aeromonas*. As a result of *A. bestiarum* infection, the following clinical signs are observed in fish: necrosis of the fins, numerous hemorrhages on the body [13, 14]. However, information on the representative of *A. bestiarum* is very limited. In this regard, the conducted studies provide a comprehensive understanding of the biology of *A. bestiarum*. The results include detailed information on the biochemical and physiological characteristics of the bacterium, as well as analyses of the presence of virulence and antibiotic resistance genes. These findings will be crucial for enhancing measures to protect and prevent diseases caused by this bacterium.

Materials and methods

Biological specimens were obtained from the ulcers and internal organs of diseased Siberian sturgeons (*A. baerii*) reared under industrial aquaculture conditions. The obtained biological materials were inoculated into both liquid and solid nutrient media, specifically Lisogenic Broth (LB) and LB agar, respectively. Bacterial colonies were grown at a temperature of 37 ºC in a thermostat within 16 hours. Isolated colonies were selected for further research. The morphological properties of bacterial colonies were determined. Biochemical identification was performed according to Bergey's manual [15] using the following biochemical tests: oxidase test, methyl red test, oxidative-fermentative (OF) test, Voges-Proskauer reaction, amino acid decarboxylation and hydrolysis tests, gelatin and esculin hydrolysis test, formation of acids from carbohydrates [15-17]. Bacterial DNA was isolated using the boiling method [18] and the Easy Pure Bacteria Genomic DNA Kit (Trans Gen Biotech, China). Molecular genetic identification of the bacterium was performed using the following 16S rRNA gene primers: 27F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R: 5'-GGCTACCTTGTTACGACTT-3' [19]. Primers used for the *gyr*B gene, *gyr*B-F: 5'-TCCGGGGCGGTCTGCACGGCGT-3' and *gyr*B-R: 5'-TTGTCCGGGGGTTGTTGTACTCGTC-3' [20]. The nucleotide sequence was determined using the Sanger method. The obtained sequences of the 16S rRNA and *gyr*B genes of the *A. bestiarum* strain were used to construct phylogenetic trees. Sequence searches were performed using BLAST through the NCBI website. Phylogenetic trees were constructed using the neighbour-joining method in MEGA XI software according to Han et al. (2017) [21]. The primers of the following genes were used to analyse virulence factors: haemolysin (*hlyA*), aerolysin (*aerB* and *aerA*), cytotonic enterotoxins (*alt* and *ast*), elastase (*ahpB*), cholesterol acyltransferase (*gcaT*), lipase (*pla*), DNAase (*nucl*), serine protease (*ahe2*) presented in Table 1.

Table 1 – Sequences of primers used to determine the presence of virulence genes

Primers	DNA sequence (5'-3')	Amplicon (b.p.)	Source
AH-aerAF	CAAGAACAAGTTCAAGTGGCCA	309	[22]
AH-aerAR	ACGAAGGTGTGGTTCCAGT		
hlyA-F	GGCCGGTGGCCCGAAGATACGGG	595	[23]
hlyA-R	GGCGGCGCCGGACGAGACGGG		
Aer-F	CCGGAAGATGAACCAGAATAAGAG	451	[24]
Aer-R	CTTGTCGCCACATACCTCCTGGCC		
Ast-F	TCTCCATGCTTCCCTTCCACT	331	[25]
Ast-R	GTGTAGGGATTGAAGAAGCCG		
Pla-F	ATCTTCTCCGACTGGTTCGG	382	[25]
Pla-R	CCGTGCCAGGACTGGGTCTT		
AhpB-F	ACACGGTCAAGGAGATCAAC	513	[25]
AhpB-R	CGCTGGTGTTGGCCAGCAGG		
Alt-F	TGACCCAGTCCTGG	442	[26]
Alt-R	GGTGATCGATCACC		
Ahe2-F	ACGGGGTGCGTTCTTCCTACTCCAG	211	[27]
Ahe2-R	CCGTTCATCACGCCGTTATAGTCG		
ExN-F	CAGGATCTGAACCGCCTCTATCAGG	504	[27]
ExN-R	GTCCCAAGCTTCGAACAGTTTACGC		
gcaT-F	CTCCTGGAATCCCAAGTATCAG	237	[28]
gcaT-R	GGCAGGTTGAACAGCAGTATCT		

The following 19 antibiotics (Condalab, Spain) were used to determine antibiotic resistance: oxacillin (1 μg), rifampicin (5 μg), enrofloxacin (5 μg), ampicillin (10 μg), amoxicillin (10 μg), penicillin G (10 μg), norfloxacin (10 μg), gentamicin (10 μg), streptomycin (10 μg), chloramphenicol (10 μg), lincomycin (10 μg), erythromycin (15 μg), trimethoprim + sulfamethoxazole (25 μg), cefazolin (30 μg), tetracycline (30 μg), oxytetracycline (30 μg), novobiocin (30 μg), florfenicol (30 μg), nitrofurantoin (300 μg). The antibiotic resistance of microorganisms was assessed using disc diffusion method according to relevant guidelines [29, 30].

Results and discussion

The isolated bacterial colonies were characterized as small, rounded with even edges, translucent beige in color, up to 1.5 mm in size, with a smooth, soft slightly slimy surface. Bacterial colonies appear as bulging masses on the surface of a dense nutrient medium. The isolate AB002 is a Gram-negative, non-motile, oxidase-positive bacillus (Table 2).

Table 2 – Results of biochemical and physiological characteristics of *A. bestiarum* AB002

№	Characteristics	AB002	№	Characteristics	AB002
1	Gram stain	-	14	Arginine dihydrolase	+
2	Morphology	rod	15	ONPG	+
3	Motility	-	Acid formation from:		
4	Oxidase	+	16	Sucrose	+
5	Methyl red	+	17	Trehalose	+
6	Voges-Proskauer test	+	18	D-xylose	-
7	O/F test	F	19	Lactose	-
8	Hydrolysis of gelatine	+	Growth under conditions:		
9	Hydrolysis of esculin	+	20	0-4% NaCl	+
10	H_2S formation	+	21	5% NaCl	-
11	Indole formation	+	22	13, 27, 32, 37, 42°C	+
12	Lysine decarboxylase	-	23	pH 3.0	-
13	Ornithine decarboxylase	-	24	pH 5.0-9.0	+
Note: «+» – positive, «–» – negative, « F» – fermentative					

The results of the studies demonstrated that strain AB002 was capable to grow in a wide range of NaCl concentrations from 0 to 4% and at temperatures from 13-42 ºC and pH values from 5.0 to 9.0. It exhibited a positive reaction in methyl red test, Voges-Proskauer test, and was also capable of hydrolyzing gelatin and esculin, forming H_2S and indole. A negative reaction was observed in the lysine decarboxylase and ornithine decarboxylase tests and in the D-xylose and lactose tests.

Meanwhile, strain AB002 exhibits arginine dihydrolase activity and forms acids from sucrose and trehalose. Thus, the obtained results of biochemical characteristics of strain AB002 corresponded to bacteria of the genus *Aeromonas* [11, 12].

To determine the species identity of strain AB002 within the genus *Aeromonas*, sequencing of full-length 16S rRNA and *gyr*B genes was performed. Analysis of the PCR products by electrophoresis in 1% agarose gel showed single specific bands of about 1000 and 1500 bp in length (Figure 1).

Sequencing of full-size 16S rRNA and *gyr*B genes of the AB002 strain with subsequent phylogenetic tree construction has identified the *gyr*B gene with a high level of homology of up to 99% as a species of *Aeromonas bestiarum* (Figure 2).

According to the obtained results of antibiotic resistance studies of the isolated strain, AB002 was shown to exhibit multiple resistance to different groups of antibiotics, including β-lactams (Table 3).

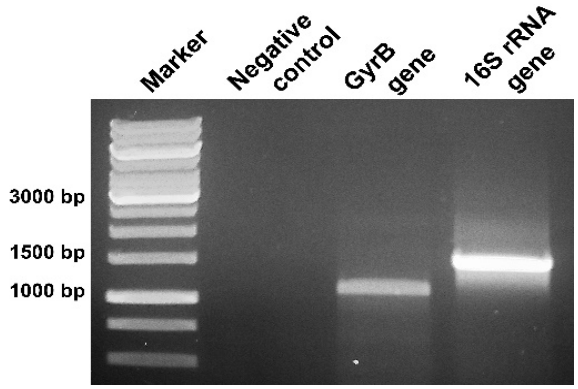

Figure 1 – Agarose gel electrophoresis of PCR products of the 16S rRNA and *gyr*B genes

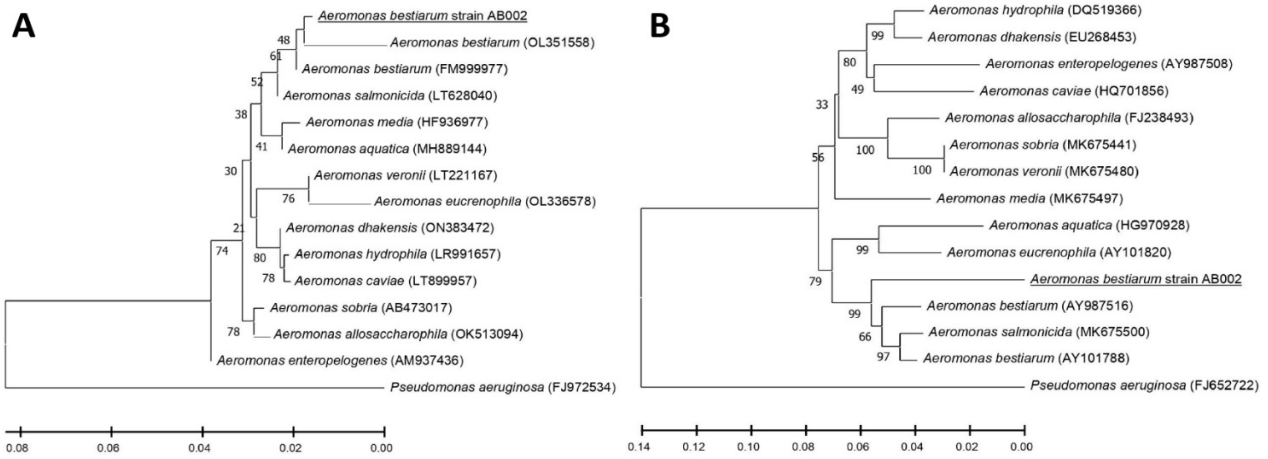

Figure 2 – Phylogenetic trees of the isolated strain *A. bestiarum* AB002 based on 16S rRNA (A) and *gyr*B (B) gene sequences with known bacteria of the genus *Aeromonas*

Table 3 – Results of antibiotic resistance analysis of the isolated AB002 strain

Group	Antibiotic	Disk Content (μg)	AB002	
			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
	Norfloxacin	10	S	28±1
Cephalosporins	Cefazolin	30	R	0
Aminoglycosides	Gentamicin	10	S	20.3±1.5
	Streptomycin	10	S	17.7±0.6
Nitrofurans	Nitrofurantoin	300	S	18±1

Continuation of the table

Group	Antibiotic	Disk Content (μg)	AB002	
			Sensitivity	Zone diameter (mm)
Tetracyclines	Tetracycline	30	S	23.3±0.6
	Oxytetracycline	30	I	21.7±0.6
Macrolides	Erythromycin	15	R	13.2±0.3
Lincomycins	Lincomycin	10	R	7.7±1.2
Rifamycins	Rifampicin	5	R	9.3±0.6
Coumarins	Novobiocin	30	R	9.2±0.3
Amphenicols	Chloramphenicol	10	S	24.3±0.6
	Florfenicol	30	S	25.3±1.5
Folic acid synthesis inhibitors	Trimethoprim + sulfamethoxazole	25	S	16.5±0.9
Note: R – resistant, I – intermediate, S – sensitive				

The strain AB002 has been found to be resistant to several antibiotic groups, including penicillins (oxacillin, 1 μg, ampicillin, 10 μg, amoxicillin, 10 μg, penicillin, 10 μg), cephalosporins (cefazolin, 30 μg), macrolides (erythromycin, 15 μg), lincomycins (lincomycin, 10 μg), rifamycins (rifampicin, 5 μg), and coumarins (novobiocin, 30 μg). The sensitivity of strain AB002 to the following antibiotics was found: enrofloxacin (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). The results showed that the strain had an intermediate sensitivity to oxytetracycline (30 μg). The analysis of antibiotic resistance revealed that strain AB002 is resistant to 9 out of 19 antibiotics tested, indicating that *A. bestiarum* is a multi-drug-resistant strain. For instance, resistance to β-lactams (penicillins, cephalosporins and carbapenems) is a common occurrence in *Aeromonas* bacteria [11, 31-33]. The resistance to a wide range of antibiotics may be attributed to their extensive use in prophylactic veterinary practices in aquaculture fish production. However, despite the multidrug resistance, the study also found that *A. bestiarum* is sensitive to several antibiotics, including quinolones, aminoglycosides, nitrofurans, amphenicols, and inhibitors of folic acid synthesis.

Assessing the pathogenicity and toxicity of pathogenic bacteria relies heavily on their virulence factors [34]. The study also examined the presence of virulence factors in the isolated strain AB002, as shown in (Figure 3).

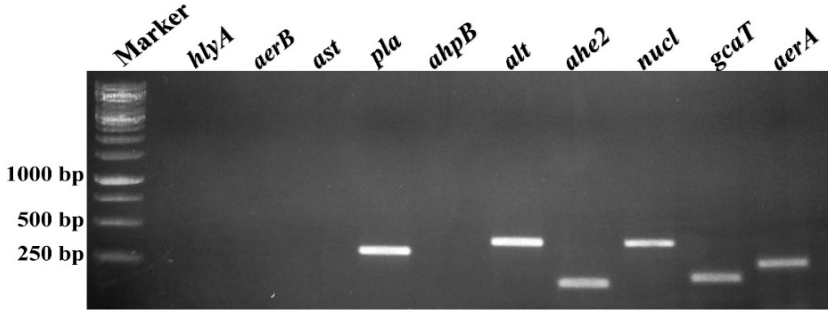

Figure 3 – Agarose gel electrophoresis of the amplicons of virulence genes of isolate AB002

The study identified several virulence factors in strain AB002, including lipase (*pla*), cytotonic enterotoxin (*alt*), serine protease (*ahe2*), DNAase (*nucl*), cholesterol acyltransferase (*gcaT*), and aerolysin (*aerA*). Notably, strain AB002 possesses a complex of serine protease and aerolysin, which is known to increase the bacterium's overall pathogenicity [35].

Conclusion

AB002 was isolated as a result of the conducted studies. The study provides the findings of the biochemical and physiological characteristics of strain AB002, which was identified as an oxidase-positive, non-motile, Gram-negative bacillus capable of growing over a wide temperature range from 13 to 42 ºC. As a result of molecular genetic analysis of the nucleotide sequences of 16S rRNA and *gyr*B genes, strain AB002 was identified as a species of *A. bestiarum*. The isolated strain AB002 is characterized as multi-drug resistant, showing resistance to 9 and 19 antibiotics tested. In addition, 6 virulence factors were detected in strain AB002 out of 10 tested, indicating that the isolated strain exhibits pathogenicity. The research results provide new insights into the biology of the pathogenic bacterium *A. bestiarum*, including its antibiotic resistance and virulence factors. These findings can be used to develop control measures against pathogenesis caused by *A. bestiarum*.

Acknowledgments

This project was supported by the Ministry of Science and Higher Education of the Republic of Kazakhstan (grant number AP23487566).

Conflict of interest

All authors are aware of the article's content and declare no conflict of interest.

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