The Israeli Journal of Aquaculture – Bamidgeh • ISSN 0792-156X • IJA.74.2022.1605429, 11 pages
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Aeromonas allosaccharophila, an emerging opportunist pathogen for farmed hybrid sturgeon (*Acipenser baerii* × *Acipenser schrencki*), in southwest China

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Keywords: *Aeromonas allosaccharophila*, hybrid sturgeon, 16S rRNA, pathologic lesions

Abstract

Aeromonas allosaccharophila belongs one of the causative agent of *Aeromonas* disease in teleosts. Here, we reported on multiple outbreaks of *A. allosaccharophila* with mortality up to 80% in farmed hybrid sturgeon in Sichuan, China, from 2019 to 2021. This was the first study to *A. allosaccharophila* from diseased hybrid sturgeons by phenotypic characterization and 16S rRNA analysis. The typical clinical signs of these fish were intestinal segments empty and often with yellow mucus or swelling. Histopathological observation of diseased fish infected with *A. allosaccharophila* showed the intestinal villus structure disappeared and the intestinal mucosa epithelium was necrotic and shed. Experimental infection indicated that *A. allosaccharophila* was the pathogen responsible for the mortalities. Antibiotic sensitivity test results showed that *A. allosaccharophila* was resistant to ampicillin, neomycin, tetracycline, and penicillin, while sensitive to sulfamethoxazole, streptomycin, florfenicol, enrofloxacin, and cefoxitin. Our findings provide references for the disease diagnosis and treatment.

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Introduction

Sturgeon belongs to the family *Acipenser*, order *Acipenseriformes*, which consists of 27 extant sturgeon species, is one of the primitive lineages of recent vertebrates (Luo et al., 2019), and broadly distributes in Europe, Asia, and North America (Birstein and DeSalle., 1998). Caviar production, the part of the economic importance of the *Acipenseridae* family, retails from 1000 to 5000 US$ kg^{-1} (Benedetto Sicuro., 2018), which promoted the development of the cultured sturgeon industry. Until 2017, a total of 2329 commercial sturgeon farms were recorded globally, and 54% of these farms were located in China, which country contributed about 79,638 t to the overall production in 2017 (Bronzi et al., 2019).

However, along with the continuous expansion of the breeding scale and density, more and more diseases raised appeared and seriously limited the development of sturgeon farming. Over the past years, a very serious epidemic disease broke out in sturgeon farms in Sichuan Province, which posed about range from 630.10 to 1,890.30 tonnes in loss in total farmed commodity sturgeon (6,301.00 tonnes) in 2020, but the pathogen was unknown. It spread rapidly and became the most prevalent disease in sturgeon farms in this province. Diseased fish infected with pathogens significantly exhibited anorexia with ascites and enteritis, swimming and whirling abnormally, especially empty in intestinal segments and often with yellow mucus or swelling. These clinical signs appear in over 80% of diseased fish. The opportunistic bacterial pathogen *Aeromonas allosaccharophila* was frequently isolated from hybrid sturgeon in our laboratory.

Since the first discovery and isolation of *A. allosaccharophila* from a woman with diarrhea was reported in the 1980s by Lynch (Lynch et al., 1985), and then it was found in immunocompromised patients (Janda and Abbott., 2010), and considered to be the cause of gastroenteritis in human and decomposition of muscle in pig carcasses (Mann et al., 2016). Several studies have found that *A. allosaccharophila* can cause mortality in elvers (*Anguilla anguilla*) (Martinez-Murcia et al., 1992), rainbow trout (*Oncorhynchus mykiss*) (Vicente et al., 2017), and the crayfish (*Procambarus clarkia*) (Zhang et al., 2018). However, whether it could infect more aquatic species is still unclear.

In March 2021, a disease outbreak occurred in cultured hybrid sturgeon fry (*Acipenser baerii* × *Acipenser schrencki*) in Sichuan Province, China, which caused 800,000 out of one million fish to die, with final mortality reaching up to 80%. The present study was conducted to isolate the pathogen from diseased fish and characterize it and determine the pathogen's antibiotic resistance to provide references for the disease diagnosis and treatment.

Materials and Methods

Sample fish and necropsy examination

In March 2021, a disease outbreak occurred in Chongzhou, Sichuan Province, China, hybrid sturgeon farms. It resulted in the death of 800,000 out of 1,000,000 fish. Most of the affected fish were fry. Ten moribund hybrid sturgeon fry (ranging from 0.3 to 0.6 g) were necropsied. All sampled fish were examined externally and internally. The gills and body surface were examined microscopically for parasites and bacteria to detect the causal pathogenic agents preliminarily.

Bacterial isolation and identification

For bacterial isolation, the diseased fish were first sanitized with 75% alcohol and dissected on sterile workstations in the laboratory. Samples obtained from the liver of each fish were directly streaked onto brain heart infusion agar (BHIA; Difico, Detroit, MI, USA). After incubation for 24–48 h at 28° C, the single dominant colonies were selected and re-streaked on the same type of agar plates. Colonies were characterized using Gram-staining. Meanwhile, a hemolysis test for isolates was performed on Columbia Blood Agar Base at 28° C.

The biochemical characteristics of the isolates were analyzed using trace biochemical reaction tubes (Hangzhou City, Zhejiang Province, China). The bacterial isolates were identified with 16S rRNA sequencing (primers: F: 5'-AGAGTTTGATCCTGGCTCAG-3' and R: 5'-GGCTACCTTGTTACGACTT-3') (Huang et al., 2020). And the primer used in the PCR amplification was synthesized by Sangon BioTech-nologies (Shanghai, China). The PCR conditions were set up as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 10 s, annealing at 55°C for 10 s, extension at 72°C for 20 s, and a final extension at 72°C for 5 min. PCR products were detected with 1% agarose gel electrophoresis followed by ethidium bromide staining and checked with UV-induced fluorescence, and the products were sequenced at Sangon BioTechnologies (Shanghai, China). Then amplified sequences were compared with the GenBank database (https://www.ncbi.nlm.nih.gov/) with a BLAST analysis. Homology analysis was performed with MEGA.X and a phylogenetic analysis with the neighbor-joining method.

Histopathological examination

The sampled fish was used to euthanize by MS-222, and the whole fish was promptly fixed in 10% neutral-buffered formalin for histopathological examination after a complete necropsy. After decalcification, tissues were trimmed into cassettes, dehydrated in graded ethanol solutions, cleared in xylene, and embedded in paraffin wax. Before microscopic analysis, sections (4 μm) were prepared for hematoxylin and eosin (H&E) staining. Images were recorded on a microscope of 11.2 color Mosaic (Diagnostic) camera mounted on a Nikon Eclipse 50i (Nikon, Tokyo, Japan).

Experimental infection

To evaluate the pathogenicity of the two isolates (*Aerall01* and *Aerall02*) obtained from the diseased hybrid sturgeons, experimental infection was conducted in the exact specification healthy hybrid sturgeons. A total of 45 hybrid sturgeons from another farm without disease were acclimated in our laboratory for two weeks before experimentation. They were randomly divided into three treatment groups (n=15 per group). The virulence tests were conducted by immersing with 5 L bacterial suspensions with a total number of colonies of approximately 10^7 and 10^6 CFU/ml for 2 h, respectively. The control groups were immersed in the same volume of diluted broth medium. Fish mortality was recorded daily for 14 days post-challenge. All animal challenges were conducted following IACUC-approved protocols of Sichuan Agricultural University.

PCR for virulence-related genes detection

5 common genes encoding virulence factors were determined using PCR in the isolates. And the five virulence genes were detected with primers (**Table 1**) described by Solaiman and Micallef (2021).

Table 1 Primer pairs used to detect virulence genes of isolated strains.

Function/protein	*Gene*	*Size(bp)*	*Primer*	*Sequences (5´-3´)*
Aerolysin/haemolysin	aerA	309	Forward	CAAGAACAAGTTCAAGTGGCCA
			Reverse	ACGAAGGTGTGGTTCCAGT
Cytotoxic Heat-labile Enterotoxin	act	232	Forward	GAGAAGGTGACCACCAAGAACA
			Reverse	AACTGACATCGGCCTTGAACTC
Cytotonic Heat-labile Enterotoxin	alt	361	Forward	TGCTGGGCCTGCGTCTGGCGG
			Reverse	AGGAACTCGTTGACGAAGCAGG
Cytotonic Heat-labile Enterotoxin	ast	536	Forward	GACTTCAATCGCTTCCTCAACG
			Reverse	AGGAACTCGTTGACGAAGCAGG
Polar flagella	flaA	608	Forward	TCCAACCGTYTGACCTC
			Reverse	GMYTGGTTGCGRATGGT

Susceptibility testing of isolates to anti-microbial drugs

The susceptibilities of isolates to 14 antimicrobial agents were investigated using the disc diffusion method according to the Clinical Laboratory Standards Institute guidelines. Briefly, single bacteria colonies were expanded and cultured at 28° C for 24h firstly. Each

LB plate was evenly coated with 200 µl of bacterial suspension, covered by the drug disc (Hangzhou Microbial Research Co., Ltd.). After incubation at 28 ℃ for 24h, the zones of inhibition were observed and measured. The sensitivity and resistance of each isolate were determined.

Results

Clinical symptoms and necropsy findings

In 2021, the sturgeon farms where we collected samples had an 80% mortality rate. The water temperature was about 17℃, other water quality indicators such as ammonia nitrogen content and dissolved oxygen were normal. Diseased fish exhibited anorexia, abnormal swimming, and whirling, but no other obvious clinical signs. Necropsy revealed gross lesions including ischemia whitening of gill filaments and intestinal segments empty and often with yellow mucus or swelling (**Figure 1**). Fish in several other ponds showed similar clinical signs and pathology.

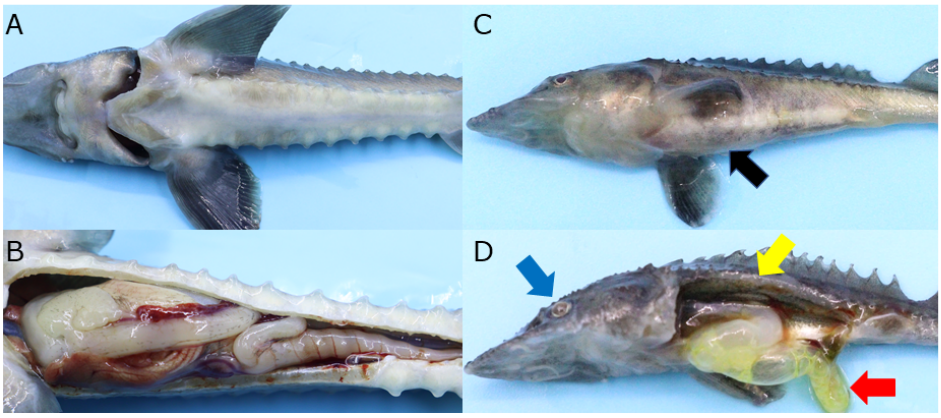

Figure 1 Gross pathology of healthy (**A**-**B**) and diseased (**C**-**D**) hybrid sturgeons. Swollen belly of sturgeons (black arrow); eyes bulging out (blue arrow); curved spine (yellow arrow); intestines containing large amounts of gas and yellow mucus (red arrow).

Histopathological diagnoses

Histopathological examination revealed that the gill, brain and gastrointestinal tract were the sites of major lesions. The epithelial cells of the second lamellae of gill were swollen and fused, and the basal cell layer surface was hyperplastic (**Figure 2A**). There was hemorrhage in the meninges and inflammatory cell infiltration in the brain (**Figure 2B**). The mucosal and muscular layers of the stomach were separated and submucosal was mildly edemic (**Figure 2C**). The intestinal wall was thinned, and the mucosa was shortened with some necrotic in the mucosal epithelium. The lamina propria was infiltrated with leukocytes (**Figure 2D**).

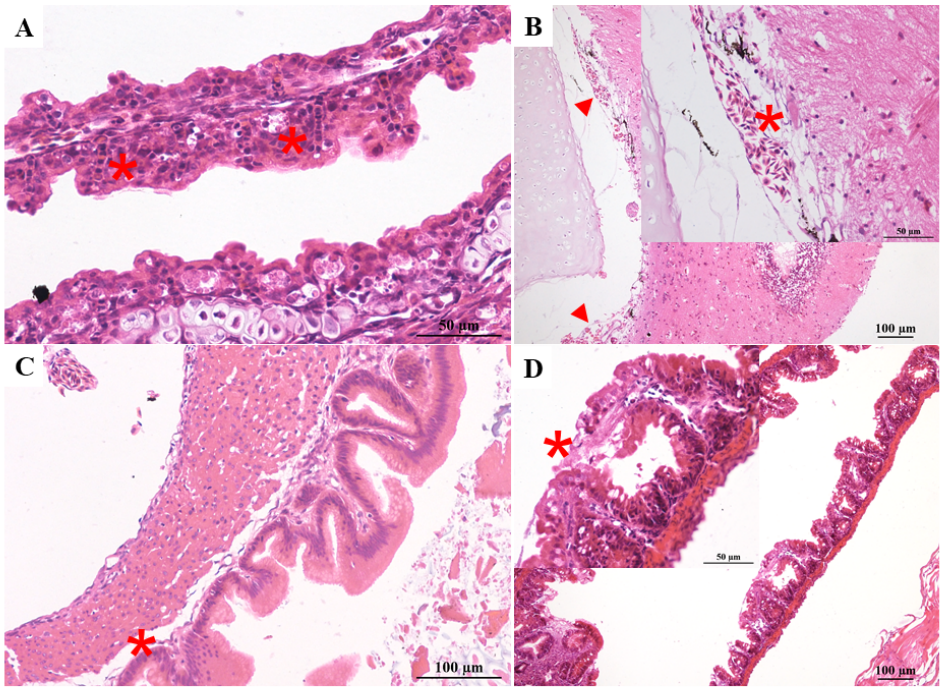

Figure 2 Pathologic assessment of some lesion organs of hybrid sturgeon. **A** The epithelial cells of gill lamellae and basal cell layer proliferated severely and fused together to form plates (asterisk). **B** Meningeal hemorrhage and inflammatory cell infiltration (asterisk and triangle). **C** Submucosa of the stomach was mild edema (asterisk). **D** The intestinal wall was thinned, and the mucosa was shortened with some necrosis in the mucosal epithelium, and the lamina propria was infiltrated with leukocytes (asterisk).

Bacterial isolation and identification

After 48h of LB culture at 28°C, two strains of bacteria were isolated and numbered *Aerall*01 and *Aerall*02. The colonies were round, neatly bordered and transparent, and the bacteria were gram-negative short bacilli. After incubation at 28 °C for 48 h on Columbia Blood Agar Base, no hemolysis of individual colonies could be seen. The metabolic response of bacteria is represented by a microbiochemical reaction tube (Hangzhou Microbial Research Co., Ltd.) (**Table 2**). Basic physiological and biochemical characterization of these isolates indicated characteristics of *Aeromonas*.

Table 2 Biochemical characteristics of the isolated strain.

Test	*Reactions*		
	Aerall01	*Aerall02*	*SJ-2 (Hu et al., 2018)*
Hemolysis	-	-	N
Lysine decarboxylase	+	+	+
Arginine dihydrolase	+	+	N
Oxidase	-	-	+
Urease	-	-	+
β-galactoside (ONPG)	+	+	N
O-F	+	+	N
Malonate	-	-	N
D-Sorbitol	-	-	-
D-glucose	+	+	+
Nitrate	+	+	+

"+" indicates a positive reaction.
"-" indicates a negative reaction.
"N" indicates Not measured.

The nearly full-length 16S rRNA gene sequences (about 1.5 kb) of the two isolates analyzed were amplified and compared with the related 16S rRNA sequences of bacteria in GenBank. The 16S rRNA gene sequences of the two strains (*Aerall01, Aerall02*) were submitted to the GenBank database with the accession numbers OK513093, OK513094, respectively. A phylogenetic tree was constructed based on the 16S rDNA sequences of the strains and the homologous sequences of other strains (**Figure 3**). The two strains observed in this study, together with *A. allosaccharophila* (GenBank accession number: GQ359956.1; MG428879.1), formed a tight cluster with more than 99% sequence similarities. The two dominant strains were confirmed as *A. allosaccharophila* by morphological, physiological, and 16S rRNA sequence analysis results.

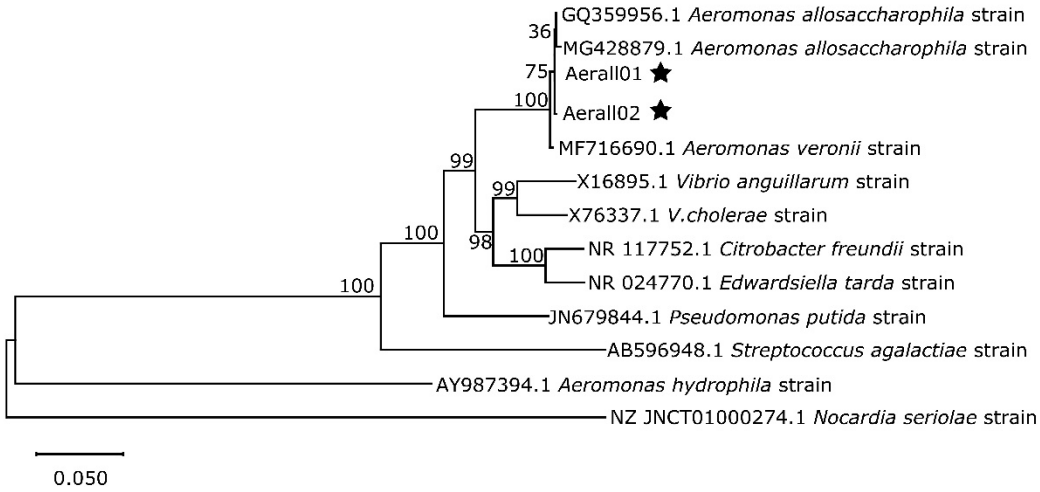

Figure 3 Phylogenetic tree generated with MEGA X based on an alignment of the 16S rRNA gene sequences of two isolated strains (*Aerall*01 and *Aerall*02, asterisk) and related species. The 16S rRNA gene sequence of related species originate from NCBI (https://www.ncbi.nlm.nih.gov/)

Experimental infection

The pathogenicity of the isolates for juvenile hybrid sturgeons was evaluated through immersion. The result of the challenge test showed that the isolates had a lethal effect on the juvenile hybrid sturgeon. The two-week cumulative mortality of infective juvenile fish with concentrations of 10^7, 10^6 CFU/ mL was 100% and 66.7%, respectively (**Figure 4**). Infected fish usually died within a week of immersion with acute symptoms, especially in the high concentration group. Affected fish showed the same symptoms of the disease as naturally infected juvenile hybrid sturgeon. No clinical signs of abnormalities and mortality were observed in the control group. And the bacterial strain was reisolated from the liver of dying fish and identified as *A. allosaccharophila* by 16S rRNA sequencing.

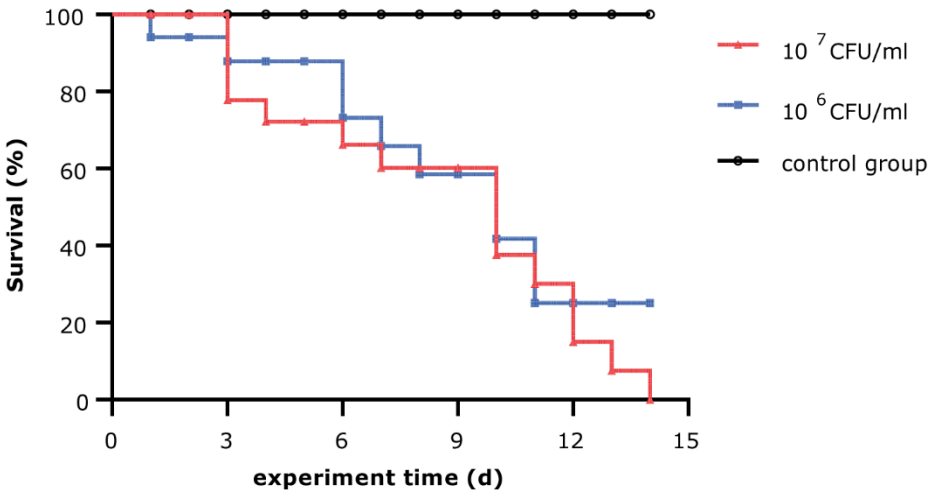

Figure 4. Cumulative mortality of artificially infected fish.

Analysis of virulence genes of isolates

We screened the Aerolysin (aerA), Cytotoxic Heat-labile Enterotoxin (act), Cytotonic Heat-labile Enterotoxin (alt), Cytotonic Heat-labile Enterotoxin (ast) and Polar flagella (flaA) genes, which were typical virulence genes in Aeromonas spp. And act, alt, flaA, ast were found in A. allosaccharophila but not aerA (**Figure 5**).

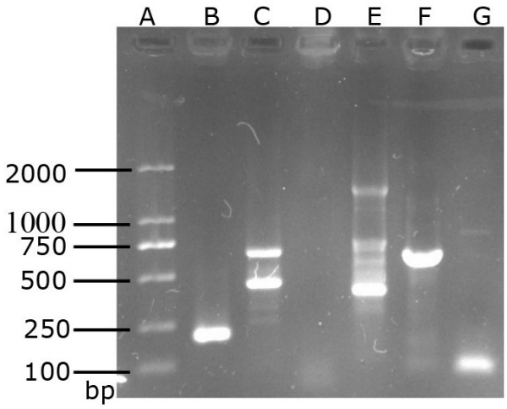

Figure 5 Agarose gel photograph of the PCR amplification using specific primer. Lanes: A: 2000 bp molecular marker; B: other samples; C: act; D: aerA; E: alt; F: flaA; G: ast.

Susceptibility testing of isolates to the antimicrobial agents

Resistance and sensitivity were determined according to the Institute for Clinical and Laboratory Standards for Animal Isolates. Both *A. allosaccharophila* isolates were resistant to penicillin, ampicillin, neomycin, and tetracycline, while sensitive to sulfamethoxazole, florfenicol, ciprofloxacin and cefoxitin (**Table 3**).

Table 3. Susceptibility of isolates to antimicrobial agents.

Antimicrobial agents	*Zone Diameter (mm)*			
	Interpretive Criteria			*Result(mm)*
	sensitive	*intermediate*	*resistant*	
Penicillin	≥ 20	-	≤ 19	6.00/R
Sulfamethoxazole	≥ 16	11-15	≤ 10	21.73/S
Ciprofloxacin	≥ 21	16–20	≤ 15	22.86/S
Florfenicol	≥ 18	13-17	≤ 12	21.01/S
Tetracycline	≥ 19	15-18	≤ 14	7.30/R
Streptomycin	≥ 15	12–14	≤ 11	16.48/S
Norfloxacin	≥ 17	13-16	≤ 12	17.61/S
Ofloxacin	≥ 16	13-15	≤ 12	19.17/S
neomycin	≥ 17	13-16	≤ 12	11.20/R
levofloxacin	≥ 17	14–16	≤ 13	19.07/S
Gentamicin	≥ 15	13–14	≤ 12	13.92/I
cefoxitin	≥ 15	12–14	≤ 11	22.99/S
Enrofloxacin	≥ 21	16-20	≤ 15	23.34/S
Ampicillin	≥ 15	12–14	≤ 11	6.00/R

" S": sensitive. "I": intermediate. "R": resistant.

Discussion

In March 2021, we isolated two dominant strains from diseased sturgeon liver and successfully confirmed the pathogens responsible as *A. allosaccharophila* by comprehensive analysis of morphological observation, biochemical reaction, and 16S rRNA gene sequencing. *A. allosaccharophila* belongs to the genus *Aeromonas*. As far as we know, *A. allosaccharophila* is not more general than *A. veronii*, *A. hydrophila,* and other *Aeromonas* species (Joseph and Carnahan., 1994). And few studies have been conducted on *A. allosaccharophila* pathology, one of the reasons is that *A. allosaccharophila* was classified as clinically non-significant in 2010 based on its rare association with human disease, in contrast with several other *Aeromonas* spp. (Eb Meyer et al., 2019). However, in this study, death of hybrid sturgeon caused by *A. allosaccharophila* in Sichuan, southwest China, whose mortality rate was as high as 80% in one of the farms, and experimental infection trials using hybrid sturgeon fingerlings clearly demonstrated the strong pathogenicity of *A. allosaccharophila*, whose death rate in these fish was 100%. It was so serious that caused incalculable economic losses to the local aquaculture industry.

The symptoms caused by *A. allosaccharophila* can be variable in different host species. Guo et al. (2016) reported that ulceration and swelling, abdominal cavity enlargement, anal swelling and protrusion occurred in eel when artificially infected with *A. allosaccharophila* with death rate of 100%. Zepeda-Velazquez et al. (2017) reported that the bacterium could infect the liver of rainbow trout (*Oncorhynchus mykiss*). Zhang et al. (2018) described the symptoms of diseased crayfish *Procambarus clarkii* infected with *A. allosaccharophila* as low activity, acted slowly, and more tissue fluid flowed out from carapace.

In this study, the main symptoms of the unhealthy hybrid sturgeon were anorexia, swimming along the pool or non-directional swimming. Autopsy showed visible lesions of intestine, such as empty intestinal segments often with yellow mucus or swelling, and other

no obvious lesions. But histopathological observation found multiple lesions, especially in the intestine and brain, which was supported the symptoms of non-directional swimming and enteritis in fish from the pond.

Many pathogenic factors, such as hemolysins and enterotoxins, have been implicated in the pathogenesis of *Aeromonas*. Moreover, the flagella, pili, LPS, and membrane proteins of the microbial play an important role in the process of tissue adhesion, invasion, and cell recognition, which enable them to act as disease agents (Rosalina et al., 2010). The virulence genes of *A. allosaccharophila* were detected and virulence genes including alt, act, and flaA were found. These results suggest that alt, act, and flaA may be a more important pathogenic factors of the *A. allosaccharophila* than Aerolysin. Strains possessing both alt and ast genes are regarded as true diarrheal pathogens in humans (Albert et al., 2000). Polar flagella are a typical virulence factor of gram-negative bacterial pathogens. It mediates the movement of the bacteria and helps the bacteria reach the target host tissue. In *Aeromonas*, polar flagella are expressed constitutionally and assist the bacteria swimming in the aquatic environment, while peritrichous flagella contribute to cell adhesion, invasion, and biofilm formation in vitro (Rosalina et al., 2010). Chenia and Duma et al. (2016) found that even under dystrophic conditions, *A. allosaccharophila* also has stronger biofilm formation ability. Because of the presence of these virulence factors in *A. allosaccharophila*, it is reasonable that infected juvenile hybrid sturgeons may be killed.
The results of the antibacterial drug sensitivity tests showed that *A. allosaccharophila* is sensitive to most antibiotics, while resistant to Penicillin, Tetracycline, Neomycin, Ampicillin. Although the most common strategy to fight bacterial disease in aquaculture is the use of antibiotics, the accumulation of antibiotics in fish can be harmful to the environment as well as consumers, Some antibiotics, such as levofloxacin, enrofloxacin and chloramphenicol, have been strictly banned in Chinese aquaculture because of their adverse effects (Geng et al., 2012). Thus, rapid diagnostic methods and safer antibacterial medication against the disease are required to better control the disease.

Conclusion

In this work, *A. allosaccharophila*, an emergent opportunistic pathogen, from hybrid sturgeon in southwest China was discovered, which can contribute to gastroenteritis. Mitigating the influence of pathogens on the organism requires rapid and accurate diagnosis. Classical histopathological techniques and scientific molecular biology methods can guarantee the discovery and treatment of infections caused by *A. allosaccharophila*.

Acknowledgments

This study was supported by the Opening Fund of Key Laboratory of Sichuan Province for Fishes Conservation and Utilization in the Upper Reaches of the Yangtze River (Ref.: NJTCSC09; NJSYKF-002;NJTCCJSYSYS03).

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