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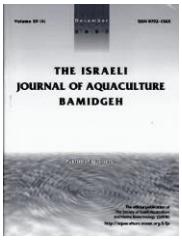

Characterization and Pathology of *Aeromonas veronii* Biovar Sobria from Diseased Sheatfish *Silurus glanis* in China

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Keywords: *Silurus glanis*; *Aeromonas veronii*; histopathology; virulent genes

Abstract

Aeromonas spp., is an important opportunistic pathogen responsible for great economic losses in aquaculture. In this paper, we report our study of an *Aeromonas* infection that occurred in cultured sheatfish *Silurus glanis* in May 2016 in a farm in Tianjin city. Typical external symptoms included petechial skin hemorrhages, ascitic distension of the abdomen, redness and swelling of the anus. The isolate SAV-165 from the diseased fish was identified as *A. veronii* biovar sobria by biochemical properties and molecular techniques. Experimental challenge showed that a 50% lethal dose (LD50) of SAV-165 was 1.59×10^6 CFU/ml in zebrafish *Danio rerio*. The antimicrobial susceptibility test indicated that the isolate was susceptible to aminoglycosides and chloramphenicol, but resistant to penicillins and sulphonamides. Histopathological examination showed fatty changes and swelling of hepatocytes in the liver, congestion in the submucosa of intestine and the renal veins of the kidney. This is the first report of *A. veronii* biovar sobria infection in *S. glanis* in China.

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Introduction

Aeromonas veronii is one of the most common Gram-negative pathogens of cultured freshwater fish causing bacterial infection and is responsible for great economic losses in aquaculture worldwide (Janda and Abbott, 2010; Chen et al., 2012). The two distinct biotypes of *A. veronii* are biovar sobria, and biovar veronii (Janda and Abbott, 1998). *A. veronii* biovar sobria is the more dominant pathogen of the two in fish and is usually associated with dermal ulceration. *A. veronii* biovar sobria was found to be associated with ulcers that develop on the body of the fish (Rahman et al. 2002). The pathogen *A. veronii* was reported to result in skin ulcerative syndrome of the farmed Channel catfish (Liu et al. 2016) and many other fish. However, no information is available on infection of *A. veronii* in *Silurus glanis*.

Sheatfish is an important local freshwater fish species in northern China (Zhao et al., 2015). In May 2016, a bacterial infection occurred in a Sheatfish farm in the Ninghe district of Tianjin City, in northern of China. All the diseased fish exhibited reduced feeding activity and typical signs including ulcers and petechial hemorrhage on the skin, congestion and hemorrhage at the fin base, distension of the abdomen, and redness and swelling of the anus. There was also severe accumulation of blood-stained peritoneal fluid in moribund fish

In this study, a Gram-negative bacterium was isolated from the liver of diseased sheatfish and was identified as *A. veronii* biovar sobria by morphological observations, biochemical tests, analysis of 16S rDNA phylogenies, gyrB gene, and several other virulence factors. In addition, experimental challenge, antimicrobial susceptibility, and histopathology examination is also described here. To the best of our knowledge, this is the first report on the isolation and characterization of *A. veronii* biovar sobria from *S. glanis*.

Materials and Methods

Fish

In May 2016, mass mortalities exceeding 80% of sheatfish *Silurus glanis* occurred in a fish farm in the Ninghe district of Tianjin City, north of China. Moribund *sheatfish* (mean weight 1000 g, mean length 45cm) were collected and packed in water-filled plastic bags, supplied with oxygen and transported to our laboratory.

Bacterial isolation and Identification of biochemical characteristics

Liver samples were taken aseptically and streaked on Tryptic Soya Agar (TSA) medium. The plates were incubated aerobically at 28°C for 18-24 hours. Dominant colonies from plates were re-streaked onto TSA plate at least three times to obtain pure isolates. The isolate was characterized by Gram stain and biochemical tests including motility, oxidase, lysine decarboxylase, arginine dihydrolase, ornithine decarboxylase, o-Nitrophenyl-β-D-Galactopyranoside (ONPG), etc. Biochemical test kits were purchased from Hangzhou Binhe Microorganism Reagent Co., Ltd., China. Incubation was performed at 28°C for 24 h before reading the reactions, and if negative, re-read at 48 h.

Antimicrobial susceptibility of the isolate was determined by the Kirby Bauer disc diffusion method (CLSI, 2006) using Mueller-Hinton agar and 30 different antibiotic disks. All antibiotics were obtained from Hangzhou Binhe Microorganism Reagent Co., Ltd, China. The plates were incubated at 28°C for 24 hours.

Extraction of bacterial DNAs and PCR amplification

Bacterial genomic DNAs were extracted as described by Baeck *et al.* (2006) and stored in -20°C until used. The PCR primers used for amplification of 16S rDNA and genes encoding DNA gyrase subunit B (gyrB), hemolysin (hlyIII, hlyD), aerolysin (aerA), type II secretion protein K (exeK) and outer membrane protein (ompW) were designed with reference to the sequences deposited in GenBank under accession numbers (CP012504). All primers were synthesized commercially by Genewiz, China. The primers and annealing temperature are shown in Table 1. Five microliters of PCR product were analyzed in 1% agarose gel electrophoresis with ethidium bromide staining and then visualized and photographed under UV illumination.

The amplified products of 16S rDNA, gyrB and ompW genes were purified and cloned into the pMD18-T vector (Takara, Dalian, China), then transformed into E.coli DH5α competent cells. The positive clones were sequenced by Genewiz, China. The BLAST search was done at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Alignment was performed using CLUSTAL W method in MEGA 6.0 software. Phylogenetic trees were generated using the neighbor-joining (N-J) method of MEGA 6.0 program, with 1000 bootstrap replicates.

Table 1. Primers used in PCR assay with gene sequences and expected amplified fragments

Primer	*Nucleotide Sequences (5′-3′)*	*Size/bp*	*T_m (°C)*
16S ribosomal DNA (16s rDNA)	27F: 5'-AGAGTTTGATCCTGGCTCAG-3' 1492R: 5'-GGTTACCTTGTTACGACTT-3'	1504	55
gyrase subunit B (gyrB)	F: 5'-GGTTCTGTCTCTGTGCGTGA-3' R: 5'-CACGTTCGATGATTTCCGGC-3'	1372	55
hemolysin III family protein (hlyIII)	F: 5'-GAAATGGCGCTGGGTAGAGT-3' R: 5'-TTGTCAGCAAACGCTGATGC-3'	766	54
hemolysin D (hlyD)	F: 5'-GTCGATAAAGCCAAGCAGCG-3' R: 5'-CTGATCGTCTGGTTGCTGGT-3'	885	59
aerolysin (aerA)	F: 5'- CAGCACACGACCCAGGTTAT-3' R: 5'-CGTCTTGTCCACGACCATGA-3'	740	55
type II secretion protein K (exeK)	F: 5'-GCTGATCCTCTCGGTGATGG-3' R: 5'-CCGCAGATGCGCTGTTTATT-3'	823	55
outer membrane protein (ompW)	F: 5'-TCCTCTGATGATTGCCGCTG-3' R: 5'-GGTAGCCAACACCCACCATA-3'	575	57

Experimental challenge

To confirm the pathogenicity of the isolate SAV-165, a challenge infection was carried out with healthy zebrafish and *S. glanis*. Experimental fish were raised in water temperature adjusted to 28°C for two weeks prior to challenge. Fish were fed with commercial pellets twice a day. Twelve sheatfish (mean weight 40 g) were divided into two groups, one group injected intraperitoneally with 0.2 ml of a bacterial solution at 5×10^9 CFU/ml. For 50% lethal dose (LD_{50}), thirty *D. rerio* (mean weight 0.50 g) were randomly divided into six groups, and the infected zebrafish were challenged intraperitoneally with 10 µl of a bacterial suspension at 2.17×10^4, 10^5, 10^6, 10^7 and 10^8 CFU/ml. All control groups injected with the same dose of sterile 0.85% saline solution, were observed daily for 7 days post-challenge and their mortality rates recorded. Samples from moribund fish were subjected to routine bacteriological examination for re-isolation.

Histopathology

The experiment was carried out as described by Raji and Norouzi (2010) with a slight modification. Small sections of livers, kidneys, intestines, and gills excised from the diseased fishes were fixed in Bouin's fluid for 8 hours and then transferred to 70% ethanol. The samples were dehydrated in the tertiary Butyl alcohol from 10% to 100% concentration and embedded in paraffin. Five µm sections were stained with Ehrlich's

hematoxylin-eosin (H&E) for histopathological analyses by the light microscope (Leica Application Suite V4, Germany).

Results

Morphological and biochemical characteristics of the isolate

A Gram-negative short rod-shaped bacterium was isolated from the diseased sheatfish and named SAV-165 (Fig. 1A). The colonies of strain SAV-165 appeared as small, round, translucent, convex and moist on TSA plate after 18 hours incubation at 28°C (Fig. 1B). As shown in Table 2, the isolate had a positive reaction on oxidase and indole but was negative on malonate. Acid production was from glucose, galactose, maltose, trehalose, mannose, and mannitol. Biochemical differentiation of strain SAV-165 from both biotypes of type strain *A. veronii* are shown in Table 3. Strain SAV-165 was found to be positive to lysine decarboxylase and arginine dihydrolase but negative to ornithine decarboxylase and esculin. Additionally, the antimicrobial susceptibility test results (Table 4) revealed that the strain SAV-165 was resistant to penicillin, macrolides and sulphonamides, while susceptible to aminoglycosides, chloramphenicol and cephalosporins (except cephalexin).

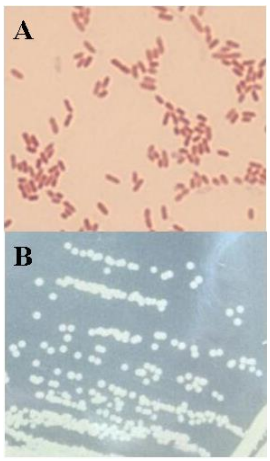

Figure 1 The Gram-negative short rod-shaped cells (A) and bacterial colonies (B) of the strain SAV-165.

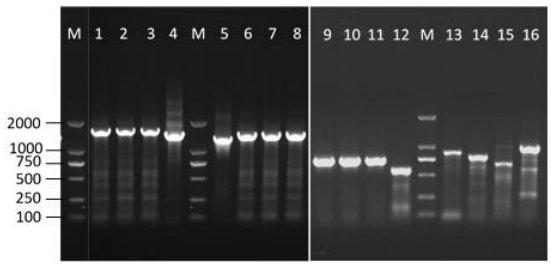

Figure 2 Agarose gel electrophoresis of PCR products of seven target genes from the isolate SAV-165

M: 2000-bp molecular size marker; Lane 1-3: PCR product of pMD18T- 16S rDNA gene; Lane 4:PCR product of 16s rDNA gene; Lane 5: PCR product of gyrB gene; Lane 6-8; PCR product of pMD18T- gyrB gene; Lane 9-11: PCR product of pMD18T- ompW gene; Lane 12: PCR product of ompW gene; Lane 13:PCR product of exeK gene; Lane 14:PCR product of aerA gene; Lane 15: PCR product of hlyIII gene; Lane 16: PCR product of hlyD gene

Table 2 Biochemical properties of SAV-165 isolated from diseased sheatfish

Tests	*SAV-165*	*A. veronii biovar sobria*[a]	*A. veronii biovar veronii*	*Tests*	*SAV-165*	*A. veronii biovar*	*A. veronii biovar veronii*[a]
Motility	+	+	+	Acids from			
Indole	+	+	+	Glucose	+	nd	nd
Oxidase	+	+	+	Lactose	-	d	d
Dnase	-	d	d	Xylose	-	-	-
Urease	-	-	-	Cellobiose	-	d	d
ONPG	+	+	+	Galactose	+	nd	nd
Citrate	+	d	+	Maltose	+	+	+
Malonate	-	-	-	Trehalose	+	+	+
KCN growth	-	d	-	Raffinose	-	-	-
Phenylalanine deaminase	-	+	+	Melibiose	-	-	-
O-F	F	F	F	Mannose	+	+	+
Glucose(gas)	+	d	d	Rhamnose	-	-	-
H_2S production	-	d	d	Adonitol	-	-	-
Nitrate reductase	+	+	+	Dulcitol	-	-	-
Mucate	-	-	-	Erythritol	-	-	-
Gluconate	+	d	d	Inositol	-	-	-
Methyl red	-	nd	nd	Sorbitol	-	-	-
Salicin	-	-	+	Amygdalin	-	-	-

Note: '+' is positive, '-' is negative, '±' is weak positive, 'd' is 11-89% positive, 'nd' is not determined

Table 3 Biochemical differentiation of strain SAV-165 from two biotypes of *A. veronii*.

Tests	*SAV-165*	*A. veronii biovar sobria*[a]	*A. veronii biovar veronii*[a]
Lysine decarboxylase	+	+	+
Ornithine decarboxylase	-	-	+
Arginine dihydrolase	+	+	-
Voges-Proskauer	+	+	+
Esculin	-	-	+
Arabinose	-	-	-
Mannitol	+	+	+

Note: '+' means positive; '-' means negative;
[a] Data are from Bergey's Manual of Systematic Bacteriology (Martin-Carnahan & Joseph, 2005)

Table 4 Antimicrobial susceptibility tests of the strain SAV-165

Categories	*Antimicrobial agents*	*SAV-165*	*Categories*	*Antimicrobial agents*	*SAV-165*
Penicillins	Amoxicillin	7/R	Quinolones	Nalidixic Acid	13/R
	Ampicillin	7/R		Norfloxacin	18/S
Carbapenems	Meropenem	14/I		Enoxacin	15/I
	Imipenem	12/R		Levofloxacin	24/S
Cephalosporins	Cefixime	25/S		Enrofloxacin	21/S
	Cefoperazone	28/S	Sulphonamides	Trimethoprim	7/R
	Cefotaxime	32/S		Sulfamethoxazole	7/R
	Cephalexin	9/R		Trimethoprim/sulfamethoxazole	7/R
Macrolides	Azithromycin	11/R	Chloramphenicol	Chloromycetin	30/S
	erythromycin	7/R		Florfenicol	34/S
Aminoglycosides	Amikacin	21/S	Others	Nitrofurantoin	15/I
	Gentamycin	19/S		Rifampicin	16/R
	Kanamycin	20/S		Vancomycin	11/I
	Streptomycin	19/S		Clindamycin	9/R
	Neomycin	19/S		Tetracycline	10/R

Symbols: 'S' means susceptible; 'I' means intermediate susceptible; 'R' means resistant; 'N' means not determined. Numbers is Inhibition zone (mm)

Sequencing and phylogenetic analysis

The 16S rDNA and gyrB gene sequences of the strain SAV-165 were 1504 bp and 1372 bp in length (Fig. 2). These were submitted to GenBank with an accession number of MF784485 and MF784486. The phylogenetic analysis of the isolate SAV-165 via BLAST revealed that SAV-165 shared 99.59% identity with *A. veronii* biovar sobria ATCC 9071^T (AF410949). The gyrB gene sequence exhibited 99.18% identity to *A. veronii* biovar sobria ATCC 9071^T (FN796747). The results based on 16s rDNA gene showed the target strain SAV-165 clustered with *A. veronii* biovar sobria ATCC 9071^T (AF410949) and *A. veronii* biovar veronii ATCC 35624^T (X74684) (Fig. 3A). By contrast, the phylogenetic tree based on gyrB gene sequence only clustered the target strain SAV-165 with *A. veronii* biovar sobria ATCC 9071^T (FN796747). The virulence-associated genes encoding hemolysin (hlyIII, hlyD), aerolysin (aerA), type II secretion protein K (exeK) and outer membrane protein (ompW) were also amplified and demonstrated all positive PCR results for the genes sequenced from *A. veronii* (Fig. 2). All the expected fragments were obtained, namely 575-bp of ompW gene product, 885-bp hlyD, 766-bp hlyIII, 740-bp aerA, and 823-bp exeK in length, and they were all submitted to GenBank with accession numbers of MF784487 to MF784491. In addition, analysis of the deduced amino acid sequence of aerA, hlyIII, hlyD, exeK, ompW for homology to known gene sequences in databases via BLAST was performed and the results showed that the strain SAV-165 shared high amino acid sequence identities (99.19%, 99.53%, 98.98%, 99.64% and 99.48%) to aerA, hlyIII, hlyD, exeK and ompW from *A. veronii*. The phylogenetic tree constructed from ompW amino acid sequences clustered the isolate SAV-165 with *A. veronii* (WP 005340187) (Fig. 3C).

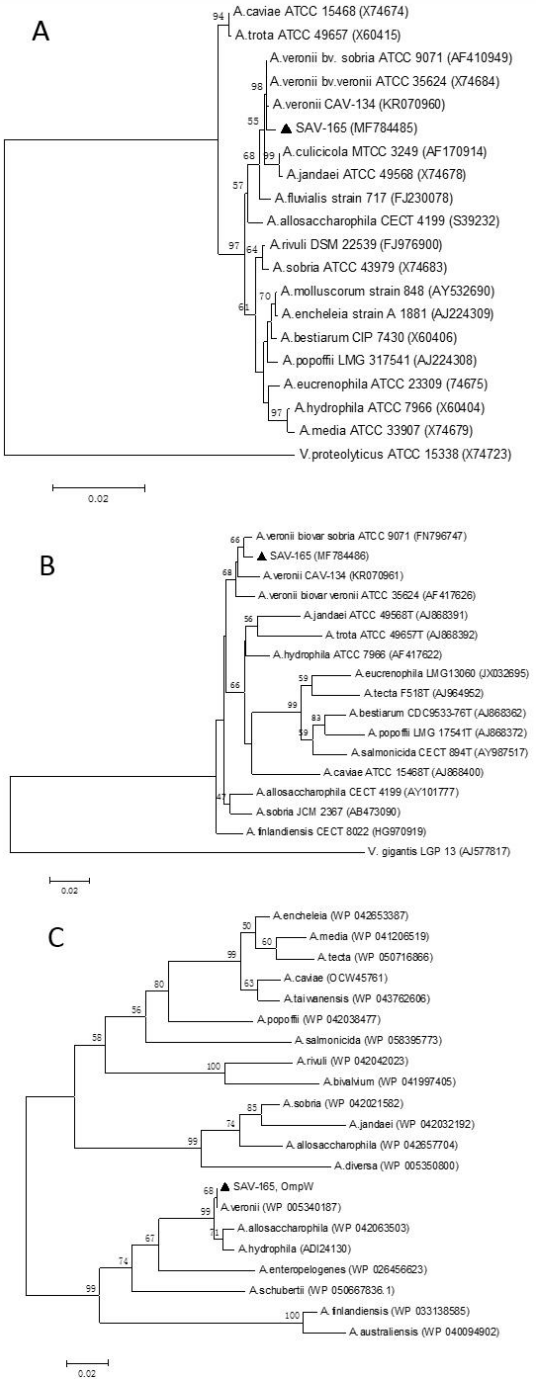

Figure 3 Phylogenetic tree analysis of the Aeromonas genus based on 16S rDNA, gyrB gene sequence and OmpW amino acid sequences translated from the nucleotide sequences of the strain SAV-165. The tree was generated using the neighbor-joining method and bootstrap values (%) of 1000 replicates were shown next to each branch. Accession numbers are shown in brackets. A: Phylogenetic tree based on 16S rDNA; B: Phylogenetic tree based on gyrB gene; C: Phylogenetic tree based on OmpW amino acid sequences

Pathogenicity in vivo

All the sheatfish challenged with the strain SAV-165 were dead in 96 h. The LD_{50} values of SAV-165 infected the zebrafish was as expected 1.59×10^6 CFU/ml. In the diseased sheatfish, typical clinical and microscopic lesions produced by the experimental challenge were similar to those observed in natural infections including skin ulcers and

necrosis, congestion of the fins, ascitic distension of the abdomen (Fig. 4A) and redness and swelling of the anus (Fig. 4D). The liver was extremely swollen and had a pale-yellow appearance (Fig. 4E). Also, the stomach was swollen and congested (Fig. 4E), the intestine appeared severely congested and hemorrhagic (Fig. 4C). In experimentally infected zebrafish, skin congestion was observed (Fig. 4B). No deaths or clinical signs were observed in the control groups.

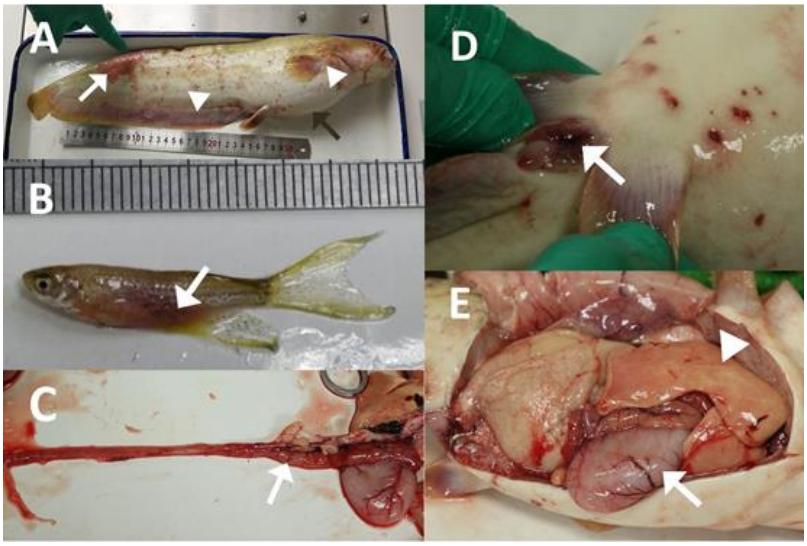

Figure 4 Clinical signs of naturally infected sheatfish *Silurus glanis* and experimentally infected zebrafish *Danio rerio*. A: White arrow indicates skin ulceration and hemorrhage in sheatfish; arrowheads indicate congestion on fin bases; black arrow indicates abdominal distension; B: White arrow indicates congestion and hemorrhage in the skin of the infected D. rerio; C: White arrow indicates severe hemorrhage in the intestine of sheatfish; D: White arrow indicates redness and swelling of the anus in sheatfish; E: Arrowhead and White arrow indicates the liver and stomach swollen and congested of sheatfish respectively

Histopathology

Histopathology from naturally infected fish showed fatty changes and swelling of the hepatocytes in the livers (Fig. 5A). The main histopathology found in the intestines increased the numbers of mucous cells (Fig. 5B). There was also frequent cell vacuolation and congestion in the sub-mucosal layer. The most important alterations found in the kidneys were blood congestion in renal veins, and enlargement of the Bowman's space (Fig. 5C). In the gills of *S. glanis*, lamellar disruption was observed. Another significant gill lesion was lamellar fusion which resulted from hyperplasia of the lamellar epithelium (Fig. 5D).

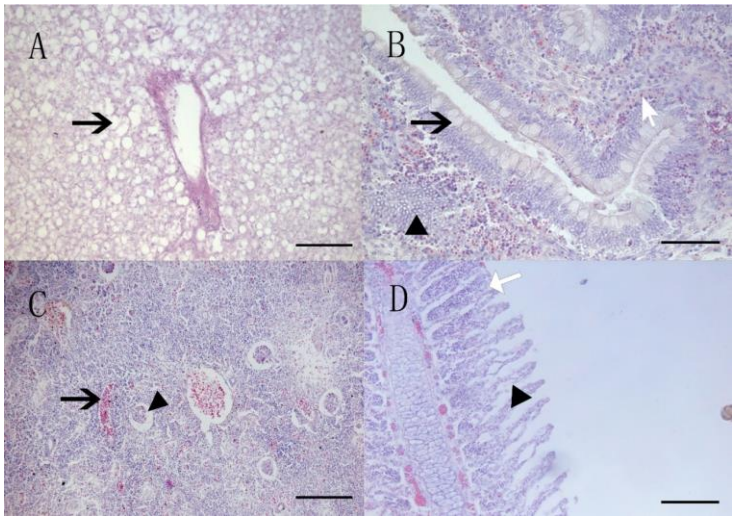

Figure 5 Examination of sections stained with hematoxylin and eosin. (A) liver of sheatfish infected with isolate SAV-165, showing the fatty change and swelling of hepatocytes (black arrow), scale bar 100 µm; (B) intestine showing the increased numbers of mucus cells (black arrow), congestion in the sub-mucosal layer (white arrow) and cell vacuolation (arrowhead), scale bar 50 µm;
(C) kidney showing blood congestion in a renal vein (black arrow), the enlargement of Bowman's space(arrowhead), scale bar 200 µm;
(D) gill showing lamellar disorganization (arrowhead) and lamellar fusion (white arrow), scale bar 50 µm.

Discussion

Waterborne *Aeromonas spp,* are ubiquitous in various aquatic ecosystems and can be food contaminants (Hänninen and Siitonen, 1995; Sen and Rodgers, 2004). These bacteria are regarded as important opportunistic pathogens in fish which are associated with motile Aeromonas septicaemia, leading to severe economic losses (Jagoda *et al.*, 2014; Lü *et al.*, 2016). It has been also regarded as an etiologic agent to reptiles and even human beings (Hänninen and Siitonen, 1995). In our study, an *A. veronii* biovar sobria SAV-165 strain was isolated from liver lesions of infected sheatfish. Its pathogenicity was confirmed by infecting zebrafish with LD_{50} of 1.59×10^6 CFU/ml. Moreover, bacteria with the same properties as the isolate SAV-165 were re-isolated as pure colonies from moribund zebrafish and sheatfish respectively. In accordance, Lü *et al.* (2016) showed that LD_{50} value of *A. veronii* in crucian carp *C. carassius* (1.99×10^6 CFU/ml) is similar to that of the strain SAV-165 infected zebrafish. Five *A. veronii* biovar sobria isolates from gilthead sea breams *Sparus aurata* exhibited a 50% lethal dose (LD50) of 5.0×10^3 to 5.2×10^9 CFU/fish (Gashgari and Selim, 2015). *A. veronii* MCCB 137 isolated from the oscar *Astronotus ocellatus* exhibited a 50% lethal dose (LD_{50}) of $10^{5.071}$ CFU/ml in *C. auratus* (Sreedharan *et al.,* 2011). The difference in the pathogenicity may be species specific, or due to environmental conditions and immunocompetence of the host.

Phylogenetic analysis is based on the 16S rDNA gene sequence applied by PCR technology, that is regarded as the backbone for modern microbial taxonomy (Cristóbal *et al.*, 2008). This technique distinguishes between closely related species due to the low evolution of the 16S rDNA genes (Cristóbal *et al.*, 2008; Lu *et al.*, 2017). Instead of 16S rDNA, the gyrase B (gyrB) gene is more suitable as a phylogenetic marker to distinguish *Aeromonas* at the species level for its higher expression level (Sun *et al.*, 2016; Yanez *et al.*, 2003). Therefore, in our study, the phylogenetic tree is based on 16S rDNA, and the gyrB gene was generated using the neighbor-joining algorithm but only the phylogenetic tree based on gyrB gene showed that the strain SAV-165 clearly has is related to *A. veronii* biovar sobria ATCC 9071^T (FN796747), supported by a high bootstrap value. In addition, biochemically, the strain SAV-165 was positive to lysine decarboxylase and arginine dihydrolase but negative to ornithine decarboxylase and esculin hydrolysis, which were in accordance with the characteristics of *A. veronii* biovar sobria (Martin-Carnahan and Joseph, 2005).

Aeromonas spp. are foodborne pathogens when they carry aerolysin and hemolysin genes (Nawaz *et al.*, 2010). González-Serrano *et al.* (2002) and Pollard *et al.* (1990) failed to detect the aerA gene in any of their *A. veronii* isolates. In contrast, Nawaz *et al.* (2010) and Gashgari and Selim (2015) reported that *A. veronii* isolates from catfish *Ictalurus punctatus* and *S. aurata* harbored the aerA gene and the cytotoxic enterotoxin act gene. Similarly, in our study, aerA gene was also detected in the strain SAV-165, indicating it is virulent to the host. Additionally, the hlyIII, hlyD, exeK and ompW genes were present in the strain SAV-165. These genes are closely related with the pathogenicity of *Aeromonas* (Nawaz *et al.,* 2010; Gashgari and Selim,2015). HlyIII gene has proven to encode a protein responsible for hemolysis (Baida and Kuzmin, 1996). Additionally, results of virulent genes may provide a reference for the detection of *A. veronii* and the construction of a multivalent vaccine against *A. veronii* infection.

In aquaculture, the frequent use of antibiotics are quinolones, tetracyclines, and aminoglycosides (Sun *et al.*, 2016; Weir *et al.*, 2012). The antimicrobial susceptibility test indicated that there were differences in the sensitivity and resistance patterns of the isolates of *A. veronii* from China (Lü *et al.*, 2016). The results of this study revealed the drug susceptibility profiles for aminoglycosides and cephalosporins (except cephalexin) and the resistance profile for penicillins, which was in accordance with the previous study (Lü *et al.*, 2016). Besides, aminoglycoside resistant genes, sulphonamide resistant genes and tetracycline resistant genes have been recovered from different *A. veronii* strains (Lü *et al.*, 2016; Nawaz *et al.*, 2006). The strain SAV-165 was resistant to tetracycline and nalidixic acid, similar to the results of *A. veronii* biovar sobria isolated by Gashgari and Selim (2015). These differences in the antibiotic susceptibility patterns may be

associated with the bacterial sources and usage of antibiotics in clinical practice. These results also support the idea that antibiotics are selectively effective resulting in the appearance of drug-resistant strains. Taken together, the results of our drug susceptibility test indicate that aminoglycosides and cephalosporins were highly effective against *A. veronii* in fish.

In conclusion, a pathogenic strain SAV-165 was obtained from diseased *S. glanis*, which was further confirmed as *A. veronii* biovar sobria based on phenotypic traits and 16S rDNA, gyrB and five virulent genes (ie., hlyIII, hlyD, aerA, exeK, and ompW) sequencing. To our knowledge, this is the first report on the characterization of *A. veronii* biovar sobria infection in *S. glanis*, which can provide a scientific reference to the diagnosis, prevention and treatment in fish caused by *Aeromonas* spp. infection.

Acknowledgements

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