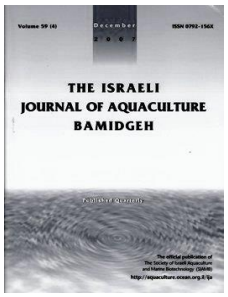
The Israeli Journal of Aquaculture - Bamidgeh, IJA_69.2017.1446, 11 pages



Isolation and Characterization of *Aeromonas veronii* from Ornamental Fish Species in China

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Keywords: *Aeromonas veronii;* ornamental fish; isolation and identification; histopathology

Abstract

Aeromonas veronii infection of fish has become a significant problem in aquaculture. In this study, pure bacterial cultures were isolated from diseased ornamental fish suffering high mortality rate in China. These fish included zebrafish (*Danio rerio)*, goldfish (*Carassius auratus)*, oscar (*Astronotus ocellatus)*, parrotfish (*Amphilophus* spp.) and crucian carp (*Cyprinus carpio koi)*. Using morphologic, physiological, and biochemical characteristics and 16S rRNA sequences analysis, nine isolates, namely strains ZG-1, ZG-2, DW-1, DW-2, KL-1, DT-116, LS-912, SJ-1 and SJ-2 were identified as *A. veronii.* A phylogenetic tree constructed on the basis of 16S rRNA sequences strongly indicated that the isolates were most closely related to the species *A. veronii* (98.43-100% similarities). The pathogenicity of the isolate was confirmed in zebrafish with median lethal dose (LD_{50}) ranging between 1.15×10^6 CFU/mL and 3.94×10^8 CFU/mL. Antibiotic susceptibility tests showed that the isolated strains were all susceptible to cephalosporins and chloramphenicol. Furthermore, histopathology of zebrafish showed leukocyte infiltration of gill filaments, necrosis of the hepatic cell and glomerulus hemorrhage. These results will provide a scientific reference for the prevention, control, and understanding of the pathogenic mechanism of *A. veronii* infection in fish.

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Introduction

Aeromonas spp. such as *A. veronii, A. hydrophila, and A. jandaei*, were isolated from diseased fish. These are commonly considered opportunistic pathogens (Sreedharan et al., 2012; Jagoda et al., 2014; Lü et al., 2016). Of all, *A. veronii* is widely distributed in aquatic environments and its high pathogenicity threatens aquaculture development and human health (Suely et al., 2010; Sreedharan et al., 2012; Jagoda et al., 2014). Recently, *A. veronii* infection in ornamental fish caused high mortality and economic losses (Martínez-Murcia et al., 2008; Lü et al., 2016; Sun et al., 2016). In ornamental fish farms the incidence of skin ulcerative disease caused by *A. veronii* is increasing characterized by internal and surface bleeding accompanied by a high mortality rate (Cai et al., 2012; Zhou et al., 2012; Sun et al., 2016).

In recent years, the ornamental fish industry has undergone rapid development and increases in China (Liu et al., 2004; Teng et al., 2007). There were reports on the isolation and characterization of *A. veronii* from diseased goldfish (Han et al., 2008; Yang, 2013; Wang et al., 2013b). Very recently, a pathogenic *A. veronii* strain CAV-134 was isolated from the ulcerative lesions and internal organs of infected goldfish in China (Lü et al., 2016). In this study, nine Gram-negative bacteria isolated from skin ulceration, dropsy, and hemorrhages of diseased ornamental fish were identified as *A. veronii* by biochemical properties and 16S rRNA sequences analysis. Pathogenicity was confirmed and the median lethal dose (LD50) was assessed in adult zebrafish by intraperitoneal injection. These studied results will provide a reference for the identification and treatment of *A. veronii* infections in ornamental fish.

Materials and Methods

Fish. From April 2013 to May 2017, diseased ornamental fish were collected from farms in Tianjin city, in China.These fish included zebrafish *Danio rerio* (ca. 0.50g), goldfish *Carassius auratus* (ca. 20g), oscar *Astronotus ocellatus* (ca. 53g), parrotfish *Amphilophus* spp. (ca. 24g) and crucian carp *Cyprinus carpio koi* (ca. 18g). Typical clinical signs were external hemorrhages and ulcers, mainly located on the head and ventral part of the body.

Bacterial isolation. For bacterial isolation, samples taken from livers of the moribund ornamental fish were streaked onto Luria-Bertani (LB) agar plates and incubated at 28℃ for 18-24h; bacterial colonies were restreaked at least 3 times on LB medium until a pure culture was obtained according to the method described by Lü et al. (2016).

Physiological and biochemical tests. The isolates were examined using biochemical tubes (Hang zhou Tianhe Microorganism Reagent co., Ltd., China) with reference to Bergery's Manual of Determinative Bacteriology (Bowman et al., 2005).

16S rRNA sequence analysis. Total genomic DNA was extracted according to the method described by Queipo et al. (2008); the 16S rRNA genes were amplified with universal primers 27F: 5'AGAGTTTGATCATGGCTCAG-3' and 1492R: 5'-GGTTACCTTGTTACGACTT-3'. According to the method described by Lü et al. (2011), the PCR amplification conditions were as follows: 94℃ for 5 min, 30 cycles of 94℃ for 30 s, 56℃ for 45 s, 72℃ for 1 min and final extension at 72℃ for 10 min (Lü et al., 2016). The PCR products were electrophoresis with 1% agarose gel by staining with ethidium bromide. The PCR products were sequenced by GENEWIZ (China). The BLAST search was carried out at the National Center for biotechnology information (NCBI, http://www.ncbi.nih.gov/BLAST/). Phylogenetic trees were constructed using the neighbor-joining algorithm of MEGA 6.0 software, with 1000 bootstrap replicates.

Antimicrobial susceptibility test. The antimicrobial susceptibility tests for the isolate were performed using Kirby-Bauer disc diffusion method on LB nutrient agar plates, incubation at 28℃ for 48h to measure the results of the inhibition zone record (Igbinosa et al., 2013).

Experimental challenge. The ornamental fish were experimentally infected with ZG-1, ZG-2, DW-1, DW-2, KL-1, and DT-116. The treatment experiments were conducted on 310 healthy adult zebrafish. For each isolate, fifty zebrafish were divided into five groups with ten in each group and the rest were used as the control group. Experimental groups were injected intraperitoneally at a concentration of 1.96×10^6 CFU/mL, 1.96×10^7 CFU/mL, 1.96×10^8 CFU/mL, 1.96×10^9 CFU/mL, 1.96×10^{10} CFU/mL, respectively. The dose for the infection was 10μL/fish. The control groups were injected with physiological saline. The zebrafish were observed daily for 7 days after infection; the moribund specimens were subjected to routine bacteriological examination for re-isolation of the liver.

Histopathology. According to the method described by Han et al. (2017), four organs (ie., gill, skin, liver, kidney) were collected from moribund zebrafish tissues during the experimental treatment and were preserved in Bonn's liquid for histological study. Tissues were dehydrated in a tert-Butanol series. Serial sections of paraffin-embedded tissues of 5μm thicknesses were cut using a microtome (Thermo, American) and stained with hematoxylin and eosin (H&E) and examined under a light microscope.

Results

Morphologic characteristics of the isolates. Nine isolates (ie., ZG-1, ZG-2, DW-1, DW-2, KL-1, DT-116, LS-912, SJ-1 and SJ-2) were taken from the ornamental fish; all were Gram-negative and rod-shaped bacteria. A representative strain ZG-2 is shown in Figure 1. The pure culture produced circular, convex, and light yellow medium sized colonies on LB agar-plates after incubation at 28℃ for 18-24h.

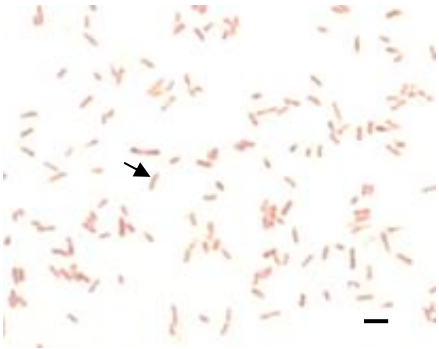

Figure 1. Strain ZG-2 was stained as Gram-negative short rod-shaped cells (arrow). Bar scale = 2 μm

Physiological and biochemical properties. The physiological and biochemical properties indicated that the eight isolates, ZG-1, ZG-2, DW-1, KL-1, DT-116, LS-912, SJ-1 and SJ-2 were negative for ornithine decarboxylase, but DW-2 was positive. The isolates ZG-1, ZG-2, DT-116, LS-912, SJ-1 and SJ-2 were positive for esculin. ZG-1 and ZG-2 were the positive for salicin, but DT-116, LS-912, SJ-1 and SJ-2 were negative. The physiological and biochemical characteristics of the nine isolates are listed in Table 1.

Table 1. The physiological and biochemical characteristics of the isolates from the ornamental fish

Characteristics	*ZG-1*	*ZG-2*	*DW-1*	*DW-2*	*KL-1*	*DT-116*	*LS-912*	*SJ-1*	*SJ-2*	*CAV-134*[a]
Gram reaction	-	-	-	-	-	-	-	-	-	-
Motility	+	+	-	+	-	+	+	/	/	+
Oxidase	+	+	+	+	+	+	+	+	+	-
O-F test	+	+	+	+	+	+	+	/	/	/
Indole	/	/	/	/	/	/	-	/	/	+
Urea	-	-	-	+	/	-	-	+	-	+
ONPG	+	+	/	/	-	+	-	/	/	+
H2S production	+	-	-	-	-	-	-	+	+	-
Glucose(gas)	-	-	+	+	+	+	-	/	/	+
Malonate	-	-	/	/	/	-	-	/	/	-
KCN	+	+	/	/	/	-	-	/	/	+
Nitrate reductase	+	+	/	+	+	+	+	-	-	+
Lysine decarboxylase	+	-	-	+	+	-	+	/	/	+
Ornithine decarboxylase	-	-	+	-	-	-	-	-	-	-
Phenylalanine deaminase	-	-	-	+	+	-	-	/	/	-
Dnase	+	+	-	/	/	+	+	+	+	+
Mucate	-	-	/	/	/	-	-	/	/	/
Esculin	+	+	/	/	/	+	+	+	+	-
Salicin	+	+	/	/	/	-	-	-	-	-
Amygdalin	-	-	/	/	/	-	-	/	/	-
Gluconate	-	-	/	+	-	-	-	-	-	+
Lactose	-	-	/	/	/	-	-	/	/	-
Trehalose	+	+	/	/	/	+	+	/	/	/
Xylose	-	-	-	-	-	-	-	/	/	-
Arabinose	-	-	/	/	/	-	-	/	/	-
Rhamnose	-	+	/	/	/	-	-	/	/	-
Maltose	+	+	/	/	/	+	+	/	/	/
Raffinose	-	+	+	-	-	-	-	-	-	-
Melibiose	-	+	/	/	/	-	-	/	/	/
Mannose	+	+	/	/	/	+	+	+	+	/
Cellobiose	+	+	/	/	/	-	+	/	/	/
Xylitol	-	-	-	/	/	-	-	/	/	-
Fructose	+	+	/	/	/	+	+	/	/	/
Sorbose	-	+	/	/	/	-	-	-	-	/
Melezitose	-	-	/	/	/	-	-	/	/	/
Turanose	-	-	/	/	/	-	-	/	/	/
Adonitol	-	-	-	-	-	-	/	-	-	/
Dulcitol	-	-	/	/	/	-	-	/	/	/
Erythritol	-	-	/	/	/	-	-	/	/	/
Inositol	-	-	/	/	/	-	-	-	-	-
Mannitol	-	-	/	/	/	+	-	+	+	+
Sorbitol	+	+	-	-	-	-	-	-	-	-

Note: + means positive; - means negative

[a]Data of strain CAV-134 was reported by our group (Lü et al. 2016)

16S rRNA sequence analysis

The 16S rRNA gene sequences of the nine isolates, namely ZG-1, ZG-2, DW-1, DW-2, KL-1, DT-116, LS-912, SJ-1 and SJ-2 were respectively 1402 bp, 1400 bp, 1386 bp, 1426 bp, 1507 bp, 1389 bp, 1426 bp, 1410 bp and 1391 bp in length. These were submitted to the GenBank with the accession numbers of MG063196 to MG063204, respectively. Agarose gel electrophoresis of PCR products of the 16S rRNA genes from the four isolates are shown in Figure 2. The 16S rRNA sequences of the isolates were analyzed by BLAST analysis, and the 16S rRNA genes of ZG-1, ZG-2, DW-1, DW-2, KL-1, DT-116, LS-912, SJ-1 and SJ-2 showed high similarities (98.43-100%) to those of both type strain *A. veronii* ATCC 35624^T(X74684) and ATCC 9071^T(AF410949). A phylogenetic tree constructed on the basis of 16S rRNA sequences strongly indicated that the nine isolates were most closely related to the species *A. veronii,* and were grouped into the cluster II (Figure 3). All nine isolates were identified as *A. veronii* by using morphologic, physiological, and biochemical characteristics and 16S rRNA sequences analysis.

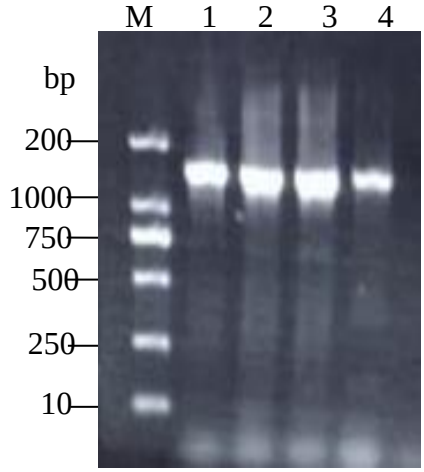

Figure 2. The electrophoresis result of PCR amplification of 16S rRNA gene from the strain ZG-1, ZG-2, DW-1 and DW-2 (lines 1, 2, 3 and 4). M is DL2000 DNA Marker

Table 2. Antimicrobial susceptibility patterns of the isolates

Antibiotics	*ZG-1*	*ZG-2*	*DW-1*	*DW-2*	*KL-1*	*DT-116*	*LS-912*	*SJ-1*	*SJ-2*	*CAV-134*[a]
Amoxicillin	0/R	0/R	8/R	8/R	3/R	0/R	10/R	15/I	5/R	0/R
Ampicillin	0/R	0/R	9/R	7/R	3/R	0/R	0/R	0/R	25/S	0/R
Cefixime	/	/	27/S	28/S	21/S	35/S	/	22/S	30/R	37/S
Cefoperazone	34/S	32/S	21/S	30/S	26/S	24/S	31/S	30/S	23/S	22/S
Cefotaxime	47/S	35/S	38/S	39/S	25/S	20/S	38/S	40/S	40/S	38/S
Cephalexin	26/S	0/R	20/S	24/S	37/S	19/S	19/S	15/I	17/I	21/S
Meropenem	22/S	15/I	12/R	18/S	20/S	15/I	10/R	30/S	14/I	15/S
Imipenem	19/S	14/I	10/I	15/I	11/R	14/I	9/R	30/S	14/I	15/S
Vancomycin	11/R	0/R	12/R	15/I	10/I	0/R	0/R	/	/	0/R
Amikacin	20/S	22/S	18/S	25/S	12/R	15/I	16/I	13/R	16/I	21/S
Gentamycin	0/R	0/R	19/S	18/S	21/S	14/I	16/S	15/I	17/R	20/S
Kanamycin	21/S	14/I	21/S	24/S	19/S	13/R	20/S	14/I	14/I	18/S
Streptomycin	25/S	0/R	21/S	27/S	14/I	10/R	17/S	12/I	18/S	16/S
Neomycin	25/S	24/S	22/S	20/S	26/S	18/S	23/S	/	/	18/S
Azithromycin	19/I	14/R	22/S	29/S	22/S	21/S	23/S	0/R	22/S	18/S
Clindamycin	0/R	0/R	15/I	15/I	32/S	0/R	0/R	/	/	0/R
Erythromycin	23/S	0/R	20/S	23/S	/	20/S	15/I	10/I	13/I	18/S
Tetracycline	0/R	16/I	8/R	27/S	12/R	0/R	10/R	/	/	15/S
Levofloxacin	24/S	21/S	22/S	29/S	34/S	23/S	18/R	25/S	22/S	/
Nalidixic Acid	11/I	12/I	12/R	29/S	14/I	22/S	0/R	0/R	11/I	21/S
Norfloxacin	/	/	18/S	29/S	31/S	18/S	13/I	20/S	15/S	21/S
Enrofloxacin	21/I	18/I	24/S	22/S	33/S	18/I	28/S	22/S	22/S	22/S
Chloramphenicol	36/S	25/S	29/S	34/S	34/S	32/S	30/S	25/S	25/S	33/S
Macrodantin	/	/	21/S	21/S	20/S	16/I	/	/	/	/
Rifampicin	13/R	19/I	16/I	15/I	/	21/S	15/R	/	/	18/S
Sulfisoxazole	19/S	21/S	23/S	29/S	/	21/S	0/R	0/R	0/R	/
sulfamethoxazole	23/S	0/R	12/I	32/S	/	23/S	0/R	0/R	0/R	26/S
Florfenicol	**34/S**	39/S	28/S	33/S	37/S	36/S	25/S	25/S	30/S	21/S
Trimethoprim	23/S	0/R	18/S	29/S	/	12/R	0/R	/	/	/

Note: S: susceptible; I: intermediate susceptible; R: resistant

[a]Data of strain CAV-134 was reported by our group (Lü et al. 2016)

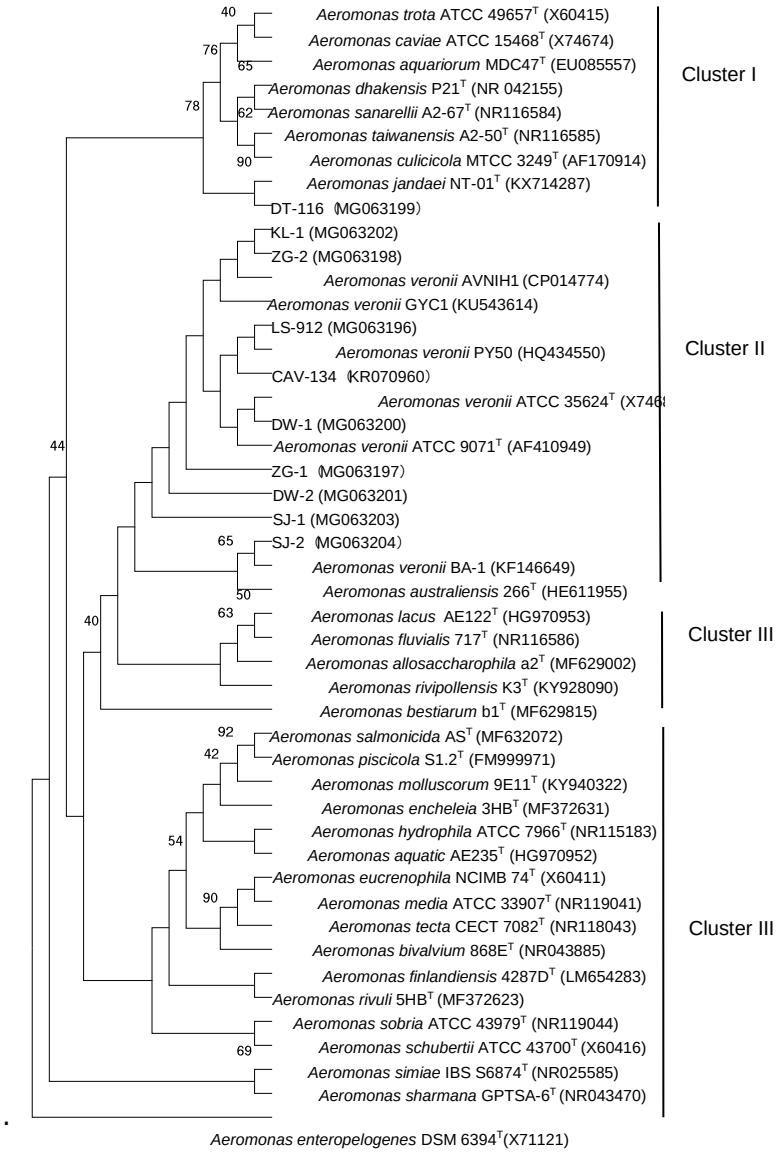

Figure 3. Phylogenetic tree analysis of Aeromonas species based on 16S rRNA nucleotide sequences. The sequences of 16S rRNA strain AVNIH1, GYC1, PY50 and BA-1 were from the references by Hughes et al. (2016), Sun et al. (2016), Cai et al. (2010) and Chi et al. (2014), respectively. The strain CAV-134 was reported by our group (Lü et al., 2016).

Antimicrobial susceptibility patterns. Antibiotics susceptibility test showed that the strains ZG-1, ZG-2, DW-1, DW-2, KL-1, DT-116, LS-912, SJ-1 and SJ-2 were all susceptible to cephalosporins (ie. cefixime, cefoperazone, cefotaxime, and cephalexin), quinolones (except for nlidixic cid), and chloramphenicol (ie. chloramphenicol, florfenicol); but all (except for SJ1 and SJ2) were resistant to penicillins (ie. amoxicillin, ampicillin), as shown in Table2.

Infection experiments. The zebrafish were infected with the isolates ZG-1, ZG-2, DW-1, DW-2, KL-1 and DT-116 after which identical symptoms were observed (see Figure 4). There were no clinical symptoms or mortalities in the control group. Bacteria with the same characteristics as *A. veronii* were re-isolated from all infected fish. The LD_{50} values for ZG-1, ZG-2, DW-1, DW-2, KL-1, and DT-116 were 1.15×10^6 CFU/mL, 1.27×10^6 CFU/mL, 1.17×10^6 CFU/mL, 3.94×10^8 CFU/mL, 1.40×10^7 CFU/mL and 1.18×10^6 CFU/mL, respectively.

Figure 4. Clinic signs of ornamental fish infected with *Aeromonas veronii* (A, D, G, I: goldfish *Carassius auratus*; B: crucian carp *Cyprinus carpio koi*; C: oscar *Astronotus ocellatus*; E, H: zebrafish *Danio rerio*; F: parrotfish *Amphilophus* A, B, D, G and I: arrows indicate typical skin ulceration; C: arrow indicate bleeding of internal organs; E, H: arrows indicate abdominal distension. and bleeding; F: hemorrhage near its pectoral fin.

Histological manifestation. Histopathology of zebrafish infected with *A. veronii* revealed gill lamellae damage, mucus cell proliferation, and leukocyte infiltration of gill filaments, separation of superficial and deep layer in skin, congestion of central veins and necrosis of the hepatic cell, glomerulus hemorrhage, and glomerular capsule filled with blood cells in the kidney. The histopathology of control and infected zebrafish are shown in Figure 5.

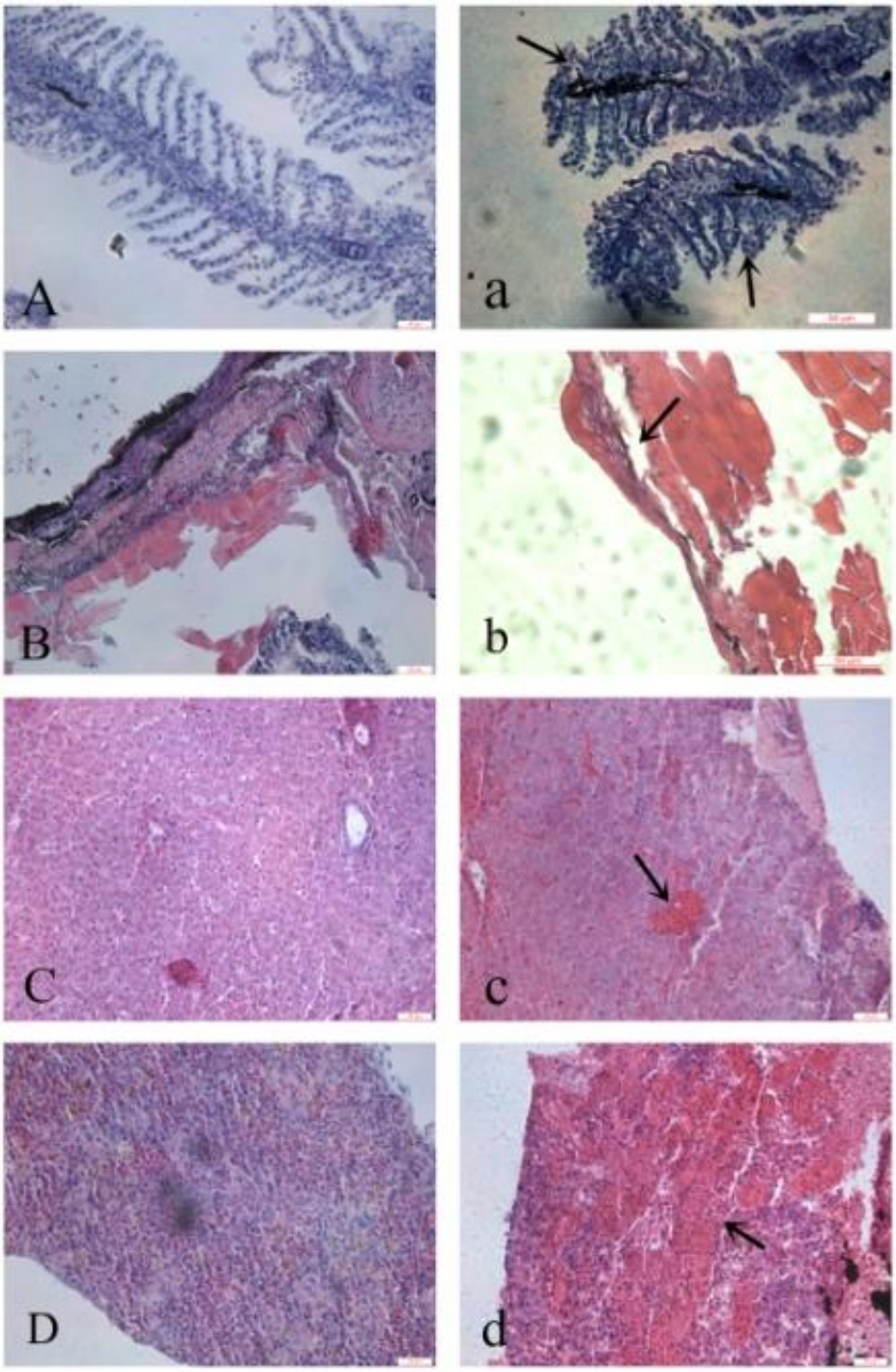

Figure 5. Histological changes of the zebrafish infected with *Aeromonas veronii*. (A), (B), (C), (D) were gill, skin, liver, and kidney of control fish; (a) gill showing leukocyte infiltration of gill filaments (arrows); (b) skin showing separation of boundary layer in skin (arrow); (c) liver showing congestion of central veins (arrow); (d) kidney showing glomerulus hemorrhage (arrow).

Discussion

Aeromonas veronii is a Gram-negative, rod-shaped bacterium found in fresh water, sewage, soil, and food (Kirov et al., 2010; Igbinosa et al., 2013; Zhang et al., 2014). In humans, *A. veronii* often cause diarrhea, pneumonia, and hemolytic disease (Janda et al., 2010; Hassan et al., 2011). In recent years, it was reported that *A. veronii* were associated with skin ulcerative syndrome (SUS) in channel catfish *ictalurus lunetas* (Huang et al.,

2012), *Ictalurus* punctatus (Liu et al., 2015), goldfish *Carassius auratus* (Lü et al. 2016), and crucian carp *Cyprinus carpio koi,* and Siamese fighting fish *Betta splendens* (Jagoda et al., 2014), and were also considered epizootic causative agents of skin ulceration, dropsy, fin rot, hemorrhages, and septicemia. *A. veronii* were isolated from diseased zebrafish, caused exophthalmia, dropsy and bleeding symptoms (Kar et al. 2016). The 16S rRNA gene sequence is commonly used in analysis of the phylogenetic tree of bacterial genera (Cai et al., 2012; Lü et al., 2016). However, difficulties often arise when using 16S rRNA sequences for species identification for *Aeromonas* spp. due to its smaller discriminatory power (Yánez et al., 2003; Sun et al., 2016). In this study, nine *A. veronii* strains were isolated from diseased ornamental fish and a phylogenetic tree was constructed on the basis of 16S rRNA sequences. These strongly indicated that the nine isolates from the diseased ornamental fish were most closely related to *A. veronii,* and were grouped into cluster II that included the strains ATCC 35624^{T} and ATCC 9071^{T}, CAV-134 from goldfish (Lü et al., 2016), PY50 from Chinese long-snout catfish (Cai et al., 2012), and BA-1from common carp (Chi et al., 2014), and AVNIH1 from the patients (Hughes et al., 2016), suggesting that the *A. veronii* strains may be a potential zoonotic pathogen. Additionally, *A. veronii* are divided into biovars of *A. veronii bv. veronii* and *A. veronii bv. sobria* based on different biochemical for ornithine decarboxylase, esculinand and salicin (Bowman, 2005; Lorén et al., 2010; Zhu et al., 2016), but were very similar based on the phylogenetic analysis of 16S rRNA sequences (Küpfer et al., 2006; Sun et al., 2016). However, nine isolates were characterized for further future determination of biotype.

In this study, our results with histopathology of zebrafish infected by *A. veronii* is an important beginning for demonstrating the pathogenic mechanism in ornamental fish. Previous reports demonstrated that *A. veronii* was the common pathogen in fish (Han et al., 2008; Cai et al., 2012; Abolghait et al., 2013). In this study, isolated *A. veronii* were strongly virulent to ornamental fish which displayed skin ulcerative syndrome, leukocyte infiltration of gill filaments, necrosis of the hepatic cell, and glomerulus hemorrhage. Similar alterations have also been reported in fish infected by *Aeromonas* spp (Chen et al., 2012; Eathel Poline et al., 2014; Dong et al., 2017). However, limited literature is available about the histological observation of fish infected by *A. veronii*. (Dong et al., 2017).

The antibiotic susceptibility will provide information for clinical treatment and infection prevention caused by *A. veronii* in fish (Janda et al., 1985; Cai et al., 2012; sun et al., 2016; Lü et al., 2016). Our data revealed that the nine isolates were all susceptible to cephalosporins, which is in accordance with the results of *A. veronii* isolated from ornamental fish (Joseph et al., 1991; Yu et al., 2010; Sreedharan et al., 2011; Cai et al., 2012). However, they were different for *A. veronii* isolated grass carp *Ctenopharyngodon idellus* susceptible to kanamycin, sulfamethoxazole, and chloromycetin, which may be associated with the bacterial origin, aquatic environment, and clinical medication (Martínezmurcia et al., 2008; Wei et al., 2013).

In this study, we confirmed that representative strains of *A. veronii* are highly pathogenic to zebrafish, and that *A. veronii* is the pathogen that caused the outbreaks of ulcerative syndrome in the tested ornamental fish. These data can provide a scientific reference for characterization of *A. veronii* and future prevention of this disease in ornamental fish.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (No. 31272692), Key Scientific Research Project Universities and Colleges in Henan province (No. 17A240001), Team of Provincial Science and Technology Innovation of Henan High Education (No. 15IRTSTHN018), Natural Science Foundation of Tianjin city (No. 16JCZDJC33500, 15JCZDJC34000) and Innovation Team of Tianjin Fisheries Research System (No. ITTFRS2017009).

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