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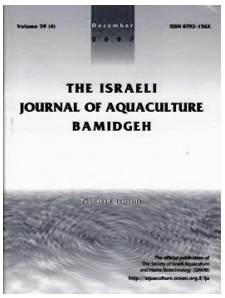

Aeromonas veronii Infection in Cultured Channel Catfish, *Ictalurus punctatus*, in Southwest China

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Key words: *Aeromonas veronii,* characterization, antimicrobial resistance, channel catfish

Abstract

Aeromonas spp. are ubiquitous inhabitants of aquatic ecosystems, and are increasingly being reported as important pathogens for aquatic and terrestrial animals, as well as humans. A common bacterial disease caused by *A.veronii* has appeared in cultured channel catfish producing symptoms of erythema, hemorrhages, skin ulcers and high mortality (> 30%) in China, causing great economic loss. From 2007 to 2013, diseased channel catfish, *Ictalurus punctatus*, displaying symptoms of septicemia, ulceration, or abdominal dropsy were collected from Southwestern China. The goals of this study were to identify the bacterial strains isolated from diseased fish and to determine the susceptibility of the pathogenic strains to many currently available antimicrobial agents. 18 bacterial isolates were obtained from diseased fish. Based on phenotypic characteristics and 16S rRNA gene sequence analysis, all isolates were identified as *A. veronii*. These results indicate that the microbiological risk posed by *A. veronii* is considerable for channel catfish cultured in Southwestern China. Susceptibility of the isolates to antibiotics was tested using the agar dilution method. All 18 *A. veronii* isolates were sensitive to florfenicol, norfloxacin and chloramphenicol, and were resistant to cefradine, clindamycin, midecamycin, penbritin, and amoxicillin. There were sensitivity diversities of the 18 *A. veronii* isolates to other test antibiotics such as sulfamethoxazole, deoxycycline, gentamycin, tobramycin, and more. This in vitro study provides enough data to recommend the use of these antibiotics for treating infectious diseases caused by *A. veronii*.

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Introduction

Aeromonas spp. are ubiquitous inhabitants of aquatic ecosystems and have been identified as important pathogens for aquatic and terrestrial animals, as well as humans (Austin and Austin, 2007; Janda and Abbott, 2010). *Aeromonas spp*. currently includes 24 known species. Among these, *A. hydrophila*, *A. salmonicida*, *A. veronii*, *A. caviae*, *A. bestiarum,* and *A. jandaei* have been described as primary or secondary pathogens of fish throughout the world (Mathur, et al., 2005, Wahli, et al., 2005, Sreedharan, et al., 2011, Cai, et al., 2012). Motile aeromonads are responsible for motile aeromonad septicemia (MAS) and bacterial hemorrhagic septicemia (BHS) and are also associated with epizootic ulcerative syndrome (EUS) in numerous freshwater and marine species (Austin and Austin, 2007; Martínez-Murcia et al., 2008). *A. veronii* was originally described as a novel species in the genus *Aeromonas,* previously referred to by the Center for Disease Control as Enteric Group 77 (Hickman-Brenner et al., 1987). It has been reported as a causative agent of outbreaks in fish and humans with hemorrhagic septicemia and epizootic ulcerative syndrome (Rahman, et al., 2002, Mencacci, et al., 2003, Cai, et al., 2012).

Channel catfish, *Ictalurus punctatus*, is the most common catfish species found in North America. It belongs to the family *Ictaluridae*, order Siluriforme. The popularity of the channel catfish as edible fish has contributed to the rapid culture of this species. Since channel catfish were introduced to China in 1984, they have been widely cultured in most provinces of China, including Hunan, Hubei, Jiangxi, Anhui, Jiangsu, Sichuan, Guizhou, Chongqing, and Guangdong. However, as channel catfish culture developed, an ever increasing number of diseases appeared and seriously hampered its culture (Geng et al., 2010). In farms in China, a common bacterial disease has developed in cultured channel catfish resulting in erythema, hemorrhages, ulcers, and abdominal distention with high mortality (> 30%), causing great economic loss. The goals of this study were to identify the bacterial strains isolated from diseased fish collected between 2007 and 2013 in southwest China, and to determine the susceptibility of the pathogenic strains to many currently available antimicrobial agents.

Materials and Methods

Sample Collection and Necropsy Examination. Diseased and moribund channel catfish with typical clinical signs were collected from farm ponds and net cages in Sichuan, Guizhou, and Yunnan Province, for clinical examination and bacterial isolation. Live specimens were transferred to the laboratory in plastic bags equipped with an oxygen supply. The diseased fish were first sanitized with 70% alcohol and then dissected in the laboratory.

Bacterial isolation. For bacterial examination, samples from liver, spleen, and kidney of diseased fish were taken using disposable loops and then directly streaked onto tryptone soy agar supplemented with 0.5% NaCl (TSA; Difco, U.S.A) under aerobic conditions and held at 28^0C for 24 h. The dominant isolates were purified by streaking and restreaking on the same agar plates. Pure stock cultures were saved on TSA slants at 4^0C for further analysis and identification, and maintained frozen at -40^0C in a Tryptic Soy broth (TSB; Difco, U.S.A.) with 20% (v/v) sterile glycerol.

Bacterial identification. After incubation for 24 h at 28^0C on TSA, the colonies were characterized using Gram-staining, oxidase, catalase, motility tests, and standard biochemical tests, according to standard methods. Meanwhile, growth in 0.5, 1.0, 1.5, 2.0, and 3.0% NaCl were tested in TSA broth; hemolytic activity was performed on the TSA supplemented with 5% sheep blood. 16S rDNA sequence analysis of all isolates was performed for further identification. The 16S rDNA was amplified by PCR. Briefly, isolated strains were incubated at 28^0C for 24 h. Colonies were suspended in 100µl of sterile deionized water. DNA was extracted using the Bacteria Genome DNA Extraction Kit (TaKaRa, Dalian, China) and stored at -80^0C until used. The universal primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGCTACCTTGTTACGACTT-3'), which specifically amplify the 16S ribosomal gene of bacteria (Jensen et al., 2002). Primers were used to yield an expected approximately 1.5-kb segment of the 16S rRNA gene. The PCR products were purified using the Gel DNA Purification Kit (TaKaRa, Dalian, China). Sequencing of PCR products was performed using a 3730 DNA sequencer (Shanghai Invitrogen Biotechnology Co., Ltd., Shanghai, China). The obtained 16S rDNA sequences were aligned to related sequences of bacteria in GenBank using the BLAST

program. The aligned 16S rDNA sequences of the related species were retrieved from the National Center for Biotechnology Institution's (NCBI) nucleotide database. Phylogenetic and distance analysis of the aligned sequences was performed using the program MEGA 5.0.

Susceptibility testing of isolates to anti-microbial drugs. The antibiotic susceptibility of isolates was determined via the disc diffusion method and by the criteria specified by the National committee for Clinical Laboratory Standards (Wayne, 2002). 5 ml of TSB was inoculated with one loop of culture. The suspension obtained was uniformly spread onto the surface of dry Mueller-Hinton agar (Oxoid, Basingstoke, UK) plates using broth-impregnated swabs. The inoculum concentration was approximately 1.0×10^8 CFU/ml. Discs (Hangzhou Taihe Microbiological Reagent, Hangzhou, China) of 18 antimicrobial agents were used. The plates were incubated with antibiotic-impregnated disks at 28^0C for 24 h, and the inhibition of the bacteria by the antimicrobial drugs was scored by measuring the inhibition zone diameter. The sensitivity and resistance of each isolate were determined following the manufacturer's instructions. Escherichia coli (ATCC 25922) was used as quality control strain, and the quality control result was within the recommended quality ranges.

Results

*Clinical Signs and Gross Lesions***.** At the initial stage of the disease, the cultivated fish had a low mortality rate. Diseased fish were weak and swam alone near the surface of the water Body surface color darkened, and the ailing fish were lethargic and exhibited loss of appetite. At the stage of severe infection fish stopped eating and died. The most obvious clinical symptoms are divided into BHS and EUS types.

The most obvious clinical symptoms of BHS (Fig.1) were severe erythema, hemorrhages, and necrosis in the mouth, upper jaw, and lower jaw. Fins, including dorsal, ventral, and anal fins showed rot and hemorrhaging. The spleen was enlarged and there were hemorrhages and kidney bleeding, swelling, and necrosis; both organs were soft. Hemorrhage and necrosis of the liver was also obvious. The intestinal wall was congested and bleeding.

Fig.1. Clinical signs of BHS of the diseased channel catfish.
(a) hemorrhages in the mouth and lower jaw;
(b) ulcers with muscle necrosis in the mouth and lower jaw ;
(c) hemorrhages and rot at the base of proctal fin and tail fin;
(d) enlargement and hemorrhages in spleen;
(e) swelling and hemorrhages in kidney;
(f) erythemas and hemorrhages in the alimentary tract.

The EUS (Fig.2) were characterized by fade spots, hemorrhaging, and ulcers on the body surface. Initially, some regularly shaped small lesions (1-6mm in diameter) with clear boundaries were seen on the skin of diseased fish. At later stages of infection the skin lost its pigmentation and subcutaneous muscle became ulcerated. Lesions, containing large amounts of necrotized muscle tissue, emitted a putrid odor. Hemorrhaging was also observed in upper jaw, lower jaw, and on the head. The abdomen become distended and filled with bloody or yellow ascites. The anus turned red and swollen. Necropsy revealed that these affected fish had pale gills, swollen and mottled livers, and swollen spleens. There was congestion and hemorrhaging in the gastrointestinal tracts without food. These fish died within a week after infection.

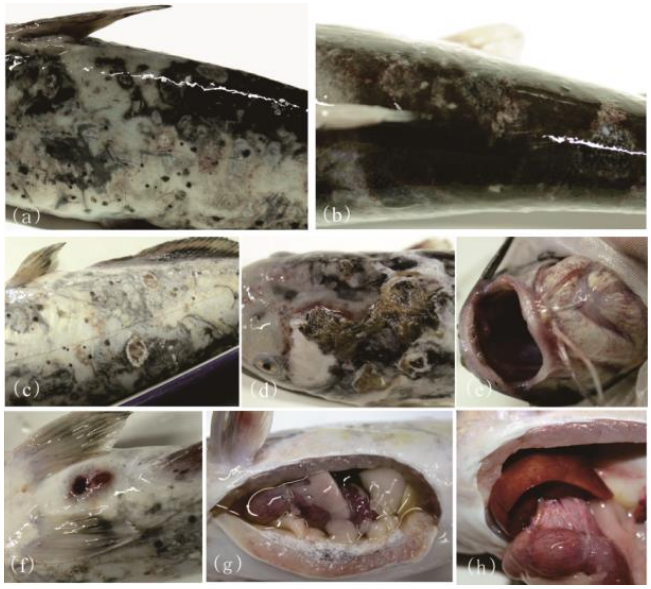

Fig. 2. Clinical signs of EUS of the diseased channel catfish. (a) fade spots on the body side; (b) fade necrosis foci on the back; (c) ulcers with clear boundaries on the skin; (d) necrosis on the head; (e) hemorrhage in the mouth; (f) the anus became red and swollen; (g) abdominal distension with yellow ascites; (h) swelling and hemorrhage in the liver; congestion and hemorrhage in the intestine.

Bacterial isolation and identification. Large numbers of colonies grew on each plate of isolations from spleen, kidney, and liver, from all the diseased fish. However, after incubation for 24 h on TSA, only one type of colony was dominant on each plate: colonies were round or elliptical, colorless, transparent, smooth with a diameter of 1-3 mm. The colonies were checked by Gram staining under a microscope and showed Gram-negative rods bacilli bacteria, arranged, in single or pairs (0.5~1.0um ×1.0~2.5um). 18 bacterial strains were isolated from different farms and numbered XJ0708-1, MS0709-1, DY0711-1, MS0711-1, MS0711-2, FF102, MS0804-1, MY0804-1, MY0804-2, MY0804-3, DY0807-1, DY0807-2, FF136, JY0808-1, MS0809-1, MS0809-2, LD-2013 and LD-2013-1. All isolates had the same biochemical and phenotypic characteristics and were consistent with the *A. veronii* type strain ATCC35604 (Table 1).

Table1. Main Phenotypic characteristics of isolates compared with published description of *A. veronii* *.

Phenotypic characteristics	*Isolates*	*A. veronii**	*Phenotypic characteristics*	*Isolates*	*A. veronii**
Motility	+	+	Nitrate reduction	-	-
Oxidation/Fermentation	F	F	Esculin hydrolysis	+	+
Oxidase	+	+	KCN, growth in	-	-
DNAase(25℃)	+	+	Indole	+	+
Lipase	+	+	Gas from D-glucose	+	+
Urease	-	-	Acid production from		
Phenylalanine deaminase	-	+	D-glucose	+	+
Lysine decarboxylase	+	+	Sorbitol	-	-
Arginine dihydrolase	-	-	Trehalose	+	+
Ornithine decarboxylase	+	+	Adonitol	-	-
β-hemolysis on sheep blood	+	+	Mannitol	+	+
Critate	-	+	Cellobiose	-	+
Malonate	-	-	Dulcose	-	-
Methyl red	+	+	Xylose	-	-
Voges-Proskauer	+	+	Melibiose	-	-
ONPG	+	+	Mannose	+	+
H_2S production	-	-	Sucrose	+	+
Gelatine hydrolysis	+	+	Rhamnose	-	-
Sensitivity to 0/129	-	-	Arabinose	-	-
Growth in nutrient broth plus NaCl at			Raffinose	-	-
0.50%	+	+	Arabitol	-	-
1 .0%	+	+	Erythritol	-	-
1.50%	-	NA	Glycerol	-	-
2.00%	-	NA	Inositol	-	-
3.00%	-	NA	Lactose	-	-
			Salicin	+	+

+: Positive. -: Negative. F: Fermentative.D:60% of isolates were positive. NA: No data available.

The nearly full-length 16S rRNA gene sequences (about 1.5 kb) of the isolates analyzed were amplified and compared with the related 16S rRNA sequences of bacteria in GenBank. The 16S rRNA gene sequences of four strains (FF102, FF136, LD-2013 and LD-2013-1) were submitted to the GenBank database and the GenBank accession numbers were GQ180116, GQ280902, KF761317 and KF761318, respectively. A phylogenetic tree was constructed based on 16S rDNA sequences of isolated strains and other homologous sequences (Fig.3). The strains investigated in this study, together with *A. veronii* (AF099023), *A. veronii* (AY987729), *A. veronii* (FJ940827), *A. veronii* (EU770307) *et al*., formed a tight cluster with 99%-100% sequence similarity. On the basis of the above information, all isolated strains were identified as *A. veronii.*

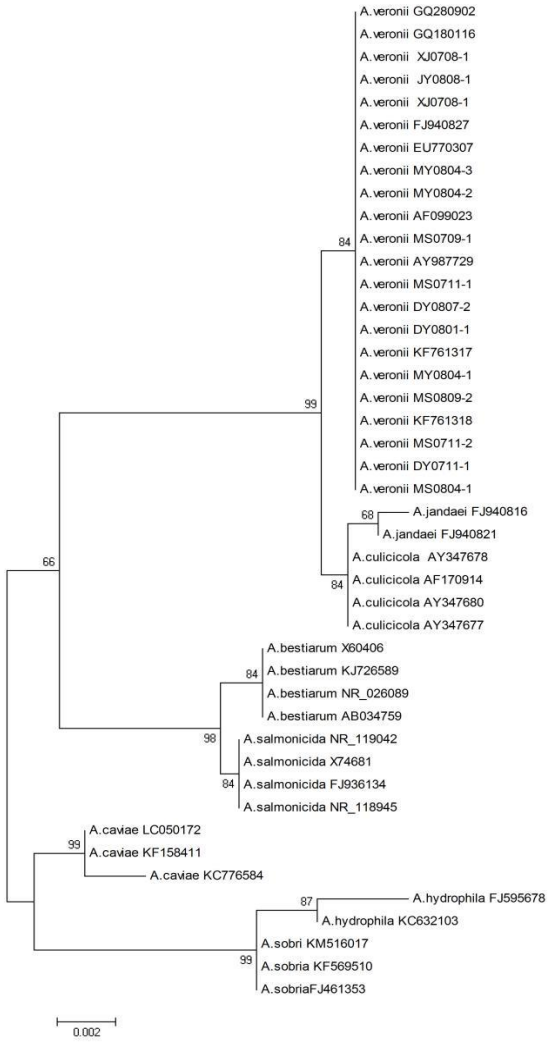

Fig.3. The phylogenetic tree of *A. veronii*, based on 16S rDNA sequences, was generated using the neighbor-joining method; Maximum Composite Likelihood; 1000 bootstrap replicates. The bootstrap values (%) were shown besides the clades, accession numbers were written besides the name of strains, and scale bars represented distance values.

Susceptibility testing of isolates to anti-microbial drugs. The susceptibility levels of the 18 *A. veronii* isolates to antimicrobial agents are shown in Table 2.

Table2. The sensitivity of 18 A. veronii isolates to antibiotics.

Antimicrobial agents	*Disk content (μg/ Tablet)*	*Percent of Isolates (total 18) (%)*		
		Susceptible	*Intermediate*	*Resistant*
Florfenicol	30	100	0	0
Norfloxacin	10	100	0	0
Ciprofloxacin	5	88.9	11.1	0
Cefradine	30	0	0	100
Amikacin	30	38.9	0	61.1
Gentamycin	10	55.6	5.5	38.9
Kanamycin	30	50	11.1	38.9
Neomycin	30	22.2	27.8	50
Tetracycline	30	83.3	5.6	11.1
Deoxycycline	30	66.7	27.7	5.6
Azithromycin	15	61.1	16.7	22.2
Sulfamethoxazole	300	16.7	16.6	66.7
Minocin	30	11.1	27.8	61.1
Tobramycin	10	44.4	11.2	44.4
Clindamycin	2	0	0	100
Chloramphenicol	30	100	0	0
Polymyxin B	300	0	100	0
Midecamycin	30	0	0	100
Penbritin	10	0	0	100
Amoxicillin	10	0	0	100

All 18 *A. veronii* isolates were sensitive to florfenicol, norfloxacin and chloramphenicol; and resistant to cefradine, clindamycin, midecamycin, penbritin and amoxicillin. But there were sensitive diversities of the 18 *A. veronii* isolates to other test antibiotics such as minocin, sulfamethoxazole, deoxycycline, gentamycin, kanamycin, tobramycin and more.

Discussion

A. veronii was originally described as a novel species in the genus *Aeromonas,* previously referred to by the Center for Disease Control as Enteric Group 77 (Hickman-Brenner et al., 1987). Recently, reports have shown that *A. veronii* can cause diverse diseases in different aquatic animals including tilapia *Oreochromis niloticu,* (Li et al., 2011), common carp *Cyprinus carpio,* (Gong et al., 2010), oscar cichlid, *Astronotus ocellatus,*(Sreedharan et al., 2011), sturgeon, *Acipenser baerii* (Ma et al., 2009), snakehead fish *Ophiocephalus argus,* (Zheng et al., 2012), sea bass, *Dicentrarchus labrax,* (Uzun and Ogut, 2015), lake whitefish *Coregonus clupeaformis,* (Loch and Faisal, 2010), gourami *Colisa lalia,* (Hossain, 2008) and Crab *Eriocheir sinensis,*(Fang et al., 2008). Our results indicate that the microbiological risk posed by *A. veronii* should be considered for channel catfish that are cultured in Southwestern China.

Previous studies have shown that *A. veronii* exhibits high-level resistance to a variety of antibiotics, including β-lactams (Vila et al., 2002) and tetracycline (Jacobs and Chenia, 2007). This can be attributed to the horizontal transfer of mobile genetic elements such as viz plasmids and class 1 integrons (Jacobs and Chenia, 2007). In our study, only florfenicol, norfloxacin, and chloramphenicol, were sensitive to all *A. veronii* isolates from channel catfish. The results of susceptibility tests indicated the prevalence of resistance to multiple agents in the populations of *A. veronii* associated with cultured channel catfish. The multiple–antibiotic resistance of the isolates increases the potential loss of fish. Although the most common strategy to fight bacterial disease in aquaculture is the use of antibiotics, such usage can encourage the development of drug resistance in fish pathogens, which can be transferred to the environment, and eventually become pathogenic to humans (Giraud et al., 2004; Tom et al., 2011; Heuer et al.,2009). Some antibiotics, such as levofloxacin, ciprofloxacin, and chloramphenicol, have been banned in Chinese aquaculture due to these adverse effects (Zou et al., 2002), therefore other treatments are needed to control the disease.

Aeromonas spp. is considered to be an opportunistic agent provoking clinical signs in stressed fish or fish affected by concurrent infections (Woo and Bruno, 2011). The major predisposing stress factors include overcrowding, temperature shock, low oxygen level, high ammonia, and other adverse water quality conditions (Eissa et al., 1994; Esch and Hazen, 1980; Walters and Plumb, 1980; Woo and Bruno, 2011). Increased water temperature, decreased dissolved oxygen level, or increased ammonia and carbon dioxide concentrations have been shown to promote stress in fish and trigger motile

aeromonad infections. Channel catfish in China are cultured under severely stressful conditions, especially overcrowding (above 100 kg m-3). These sub-optimal husbandry conditions in the ponds may have triggered infection in the observed fish. Improving water quality is therefore vital. In addition, improvement of innate defenses and resistance against diseases in fish is increasingly important in aquaculture. For example, adding the β-1,3-glucan and chitosan to the diet could significant enhance the immune responses of fish and improve resistance to *A. veronii* infection (Lin et al., 2011). Apart from use of drugs, effective management is needed to avoid infections and subsequent epizootics caused by members of the genus *Aeromonas*.

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