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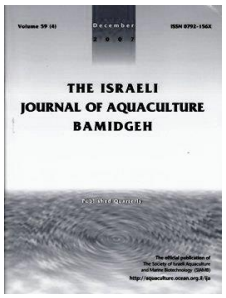

Aeromonas veronii, Associated with Skin Ulcerative Syndrome, Isolated from the Goldfish (*Carassius auratus*) in China

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Key words: *Carassius auratus*, *Aeromonas veronii*, 16S rRNA, gyrB, antimicrobial susceptibility

Abstract

Aeromonas infections are the most common bacterial disease in cultured fish. In April 2013, an epizootic ulcerative syndrome occurred on a goldfish farm in Xuzhou, central China. A gram-negative bacterium was isolated from the ulcerative lesions and internal organs of infected dragon-eye goldfish (*Carassius auratus*), tentatively named strain CAV-134. The results showed that the isolate was identified as *Aeromonas veronii* by physiological and biochemical characteristics, furthermore it was confirmed by *16S rRNA*, *gyrB*, *mu*, *asl* and *aha1* genes sequencing analysis. The pathogenicity of the isolate was confirmed in crucian carp and produced an LD_{50} of 1.99×10^6 CFU/ml. Antimicrobial susceptibility pattern of strain CAV-134 showed it was susceptible to most antimicrobial agents tested but resistant to ampicillin, amoxicillin, carbenicillin, vancomycin, teicoplanin, lincomycin and clindamycin. This is the report on the pathogenic *A. veronii* isolated from the skin ulcerative syndrome of dragon-eye goldfish.

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Introduction

Aeromonas spp. are widely distributed in various aquatic ecosystems and may be pathogenic to fish and humans (Janda and Abbott, 1998; Yadav and Kumar, 2000; Evangelista-Barreto et al., 2010). In aquaculture, *Aeromonas* sp. infection is of significant concern as it has caused high morbidity, mortality and economic losses (Wahli et al., 2005; Martínez-Murcia et al., 2008). *Aeromonas hydrophila*, *Aeromonas sobria,* and *Aeromonas salmonicida,* have been isolated from diseased goldfish (Harikrishnan et al., 2010; Mali et al., 2007; Dror et al., 2006). *A. veronii* has been found to be an important pathogen of humans (Guerra et al., 2007; Cui et al., 2007; Sánchez-Céspedes et al., 2009). *Aeromonas* sp. can cause several complications such as ulceration, abdominal distention (dropsy), exophthalmia, and tail rot (Rahman et al, 2002; Shome et al., 2005; Shao et al., 2004). Pathogenic *A. veronii* has been isolated from African catfish *Clarias gariepinus*, Israeli carp *Cyprinus carpio,* and Chinese longsnout catfish *Leiocassis longirostris Günther,* (Rahman et al, 2002; Yu et al., 2010; Cai et al., 2012). The incidence of skin ulcerative disease caused by *A. veronii* is increasing on fish farms in China (Cai et al., 2012; Zhou et al., 2012). There are few reports on the isolation and characterization of *A. veronii* from diseased goldfish (Han et al., 2008; Yang, 2013; Wang et al., 2013b).

Black dragon-eye goldfish (*Carassius auratus*), a typical variety with large protruding eyeballs is an ornamental fish of high economic value in China (Ma et al., 2008; Li and Lu, 2013). In April 2013, an ulcerative syndrome epizootic occurred on a cultured goldfish farm in Xuzhou, central China. All diseased dragon-eye goldfish presented with clinical signs of ulceration, exophthalmia and abdominal distention (dropsy).

In this study, a Gram-negative bacterium, isolated from the diseased dragon-eye goldfish, was identified as *A. veronii* by *16S rRNA*, three housekeeping gene gyrase B (*gyrB*), Mu-like prophage FluMu I protein (*mu*) and argininosuccinate lyase (*asl*) and one virulent gene major adhesin Aha1 (*aha1*) sequences analysis. Pathogenicity was confirmed in crucian carp and the value of 50% lethal dose (LD_{50}) was assessed in this study. To our knowledge, this is the first description of isolation and characterization of *A. veronii* from the cultured dragon-eye goldfish (*C. auratus*).

Materials and Methods

Isolation and biochemical identification of bacterial isolate. An ulcerative disease epizootic occurred in April 2013 in a cultured goldfish (*C. auratus*) farm at Xuzhou City, Jiangsu Province, China. The diseased dragon-eye goldfish (length 10±2cm; weight 20±5g) exhibited clinical signs of skin ulcers and petechial hemorrhages. Samples taken from skin and liver lesions of moribund dragon-eye goldfish were streaked onto Luria-Bertani (LB) agar plates and incubated at 28^0C for 24h. Colonies from plates were re-streaked at least 3 times on LB medium until a pure culture was obtained. The isolate was examined with biochemical tubes including oxidase, ornithine decarboxylase, lysine decarboxylase, arginine dihydrolase and urease; production of indole and H_2S; reactions for Methyl red and Voges-Proskauer; nitrate reductase, gluconate and DNase; hydrolyzation of starch and esculin; acid production from sorbitol and mannitol, lactose, glucose, sucrose, xylose, etc. The conventional tubes were incubated for 48h before reading the reactions (Bowman, 2005).

Molecular identification of CAV-134 by 16S rRNA gene, housekeeping genes and virulence gene sequences analysis. Total genomic DNA of the isolate strain was extracted using the UNIQ-10 column genomic DNA extraction kit (Sangon, China) according to the instructions of the manufacturer. The PCR system contained 1μl DNA template, 1μl both primers and 12.5μl 2×PCR MasterMix, in a final volume made up to 25μl with sterile double-distilled water. Characteristics of primers used for PCR amplification of *16S rRNA*,

gyrB, *mu*, *asl* and *aha1* genes are summarized in Table 1 and *mu*, *asl*, *aha1* genes primers are specifically designed according to *Aeromonas veronii* B565 strain (accession no. CP002607). The *16S rRNA*, *gyrB* and *aha1* genes were performed under the following conditions: an initial denaturation step at 94^0C for 5min; 30 cycles of 95^0C for 45s, annealing for 30s and 72^0C for 1min30s; a final extension step at 72^0C for 10 min. The reaction mixture of *mu* and *asl* genes was subjected to another PCR regimen: denaturation at 94^0C for 5min; 30 cycles of 95^0C for 45s, annealing for 30s and 72^0C for 1min; a final extension step at 72^0C for 10 min. The PCR products were evaluated by eletrophoresis in 1% agarose gel by staining with ethidium bromide. The PCR products were sequenced by Sangon (China). The BLAST search was done at the National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic tree based on 16S rRNA gene was constructed using the neighbor-joining algorithm of MEGA 5.1 software, with 1000 bootstrap replicates.

Table 1. Primers used for PCR amplification of *16S rRNA*, *gyrB*, *mu*, *asl* and *aha1* genes in this study.

Genes	Primers Sequences (5'-3')	Size/ bp	Temperature/ ℃
16S rRNA	27-F: AGAGTTTGATCATGGCTCAG 1492-R: TACGGTTACCTTGTTACGACTT	1506	55
gyrB	gyrB-F: ACAACTCCTACAAGGTCTCCG gyrB-R: TCAGCAGCAGGGTACGGATGT	1215	55
mu	mu-F: CGGGCGAGAATAATGAATG mu-R: GCAGCCTTGGTGTTGGAC	1077	48
asl	asl-F: AAAATGGCTCCTTCCCTGAT asl-R: GTGTTTTTGCTCAGTTGGCG	561	58
aha1	Aha-F : AACGAGCCGAATAATCTA Aha-R : CAGGGAATAACAACGACT	1609	44

Antimicrobial susceptibility test. Susceptibility of the isolate CAV-134 to selected antimicrobials was tested on Mueller-Hinton agar plates using the Kirby-Bauer disc diffusion method. The antimicrobial agents (Hangzhou Microbial Reagent Co., Ltd, China) included ampicillin (10), amoxicillin (10), carbenicillin (100), meropenem (10), imipenem (10), cefamandole (30), cefixime (5), cefotaxime (30), cephalothin (30), cephalexin (30), cefoperazone (75), piperacillin (100), amikacin (30), gentamicin (10), kanamycin (30), netilmicin (30), neomycin (30), streptomycin (10), tetracycline (30), chloramphenicol (30), nitrofurantoin (300), norfloxacin (10), ofloxacin (5), pefloxacin (10), enrofloxacin (5), enoxacin (10), sulfamethoxazole/trimethoprim (23.75/1.25), sulphafurazole (300), trimethoprim (5), azithromycin (15), erythromycin (15), teicoplanin (30), vancomycin (30), rifampicin (5), clindamycin (2), lincomycin (2). The number in brackets indicates the dose of each antibiotic (μg per disc). The results were recorded after overnight incubation .

Pathogenicity in vivo. The pathogenicity of the isolate CAV-134 in vivo was tested on red crucian carp as model. For 50% lethal dose (LD_{50}) determination, five groups of six crucian carp (weight 20±5g) each were injected intraperitoneally with 0.2 ml of a saline suspension of the pathogen CAV-134 at 10^3, 10^4, 10^5, 10^6 and 10^7 CFU/ml. The control group was injected with 0.2 ml of saline. Morbidity and death of the fish was monitored daily for 7 days. The moribund specimens were subjected to routine bacteriological examination for re-isolation of the organism.

Results

Phenotypic and biochemical characterization of the bacterial isolate CAV-134. A bacterium was isolated from skin and liver lesions samples of moribund dragon-eye

goldfish, tentatively named strain CAV-134. The pure culture appeared circular, convex, and opaque as seen on LB agar-plates after 24h incubation. Results showed Gram-negative short rods (Figure 1), motile and fermentative with gas production from glucose. Biochemical tests showed that the isolate CAV-134 had *A. veronii* characteristics, positive for lysine decarboxylase and arginine dihydrolase, but negative for ornithine decarboxylase, esculin hydrolysis (Table 2). Isolate CAV-134 acidified glucose, sucrose and mannitol, but could not produce acid from arabinose, lactose, sorbitol or inositol.

Figure 1. The strain CAV-134 was stained as Gram-negative short rod shaped cells.

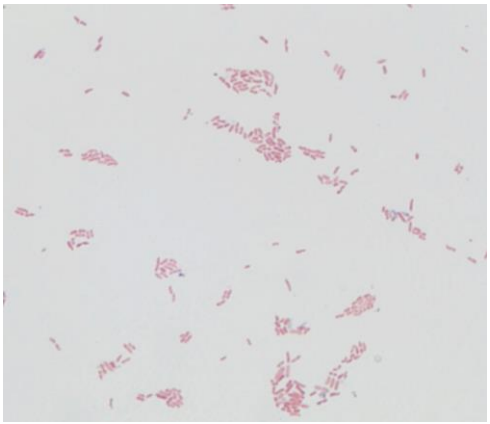

Table 2. Morphological and biochemical properties of the strain CAV-134 isolated from the diseased dragon-eye goldfish.

Characteristics	*CAV-134*	*Characteristics*	*CAV-134*
Gram stain	-	Gluconate	+
Motility	+	Citrate	±
O/129	-	Indole	+
Oxidase	-	Starch	+
Esculin	-	Acids from	
Ornithine decarboxylase	-	Lactose	-
Lysine decarboxylase	+	Glucose	+
Arginine dihydrolase	+	Arabinose	-
Methyl red	-	Sucrose	+
Voges-Proskauer	+	Rhamnose	-
Glucose (gas)	+	Raffinose	-
ONPG	+	Xylose	-
KCN growth	+	Salicin	-
Phenylalanine deaminase	-	Sorbitol	-
Nitrate reductase	+	Xylitol	-
H_2S production	-	Inositol	-
DNase	+	Mannitol	+
Urease	+	Growth at 37℃	+

+: positive.
−: negative.
±: weak reaction.

16S rRNA gene, three housekeeping genes and virulence gene sequences analysis

The almost complete *16S rRNA* gene sequence (1506 bp) of the isolate CAV-134 was amplified from the genomic DNA using PCR (Figure 2). The BLAST results of the sequence obtained matched 99.93% with *Aeromonas veronii* B565 strain (NR102789) and 99.80% with *Aeromonas veronii* ATCC 35624 (X60414). The phylogenetic tree based on *16S rRNA* gene sequences clustered the isolate CAV-134 with *A. veronii* B565 strain

(NR102789) (Figure 3). Three housekeeping genes i.e. the *gyrB* gene (1215 bp in length), *mu* gene (1077 bp in length) and *asl* gene (561 bp in length) sequences of strain CAV-134 also exhibited highest identities (96.13%, 96.27% and 93.37%) with *Aeromonas veronii* B565 strain (CP002607). The aha1 gene of strain CAV-134 showed 76.56% identities to *A. veronii* B565 strain virulence gene major adhesion *Aha1* (CP002607). The phylogenetic tree derived from gyrB-mu-asl-aha1 concatenated sequences (data not shown) was similar to the *16S rRNA* tree analysis.

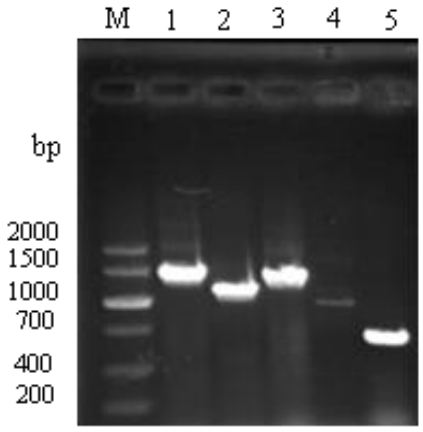

Figure 2. Agarose gel electrophoresis of PCR products of the *16S rRNA*, *gyrB*, *aha1*, *mu* and *asl* genes from the isolate CAV-134.
M: Marker; 1: *16S rRNA*; 2: *gyrB*; 3: *aha1*; 4: *mu*; 5: *asl*.

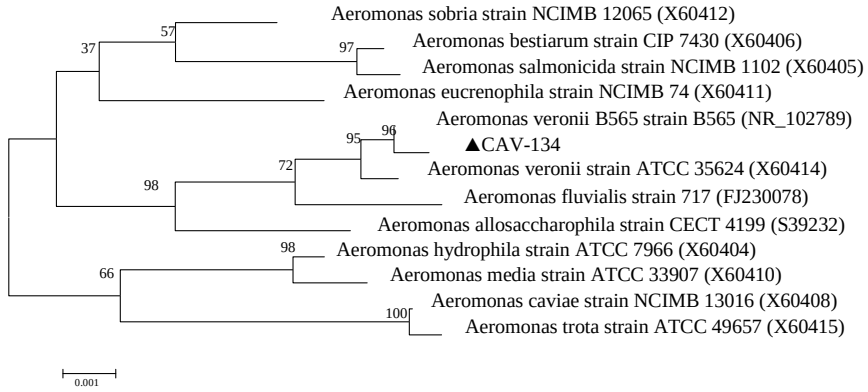

Figure 3. Neighbor-joining phylogenetic trees of CAV-134 strain based on *16S rRNA* sequences. The numbers next to the branches indicate percentage values for 1000 bootstrap replicates. The scale bar represents 0.001 substitutions per site.

Antimicrobial susceptibility test. The results of antimicrobial susceptibility tests showed that the isolate CAV-134 is susceptible to topiperacillin, carbapenems, cephalosporins, aminoglycosides, quinolones, sulphonamides and macrolides, but is resistant to ampicillin, amoxicillin, carbenicillin, vancomycin, teicoplanin and lincosamide (lincomycin, clindamycin) (Table 3).

Table 3. Antimicrobial susceptibility pattern of the isolate CAV-134.

Categories	*Antimicrobial agents*	*Inhibition zone (mm)*
Penicillins	Ampicillin	0/R
	Amoxicillin	0/R
	Carbenicillin	0/R
	Piperacillin	19/S
Carbapenems	Meropenem	15/S
	Imipenem	15/S
Cephalosporins	Cephalothin	29/S
	Cephalexin	21/S
	Cefamandole	30/S
	Cefixime	37/S
	Cefoperazone	22/S
	Cefotaxime	38/S
Aminoglycosides	Amikacin	21/S
	Gentamycin	20/S
	Kanamycin	18/S
	Netilmicin	29/S
	Neomycin	18/S
	Streptomycin	16/S
Quinolones	Nalidixic Acid	21/S
	Norfloxacin	21/S
	Levofloxacin	21/S
	Pefloxacin	22/S
	Enrofloxacin	22/S
	Enoxacin	19/S
Sulphonamides	Sulphafurazole	17/S
	Sulfamethoxazole/trimethoprim	21/S
	Trimethoprim	26/S
Macrolides	Azithromycin	18/S
	Erythromycin	18/S
Others	Chloramphenicol	33/S
	Nitrofurantoin	22/S
	Tetracycline	15/S
	Rifampicin	18/S
	Vancomycin	0/R
	Teicoplanin	0/R
	Clindamycin	0/R
	Lincomycin	0/R

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

Pathogenicity in vivo. Isolate CAV-134 was confirmed as the pathogen of dragon-eye goldfish through challenge experiments. Results indicated that the LD_{50} value in crucian carp was 1.99×10^6 CFU/ml. The clinical signs, lesions and microscopic signs produced by experimental inoculation were similar to those observed in natural goldfish infections. Moribund crucian carp exhibited sluggish behavior, ulcers on the skin, abdominal distention (dropsy), and petechial hemorrhage (Figure 4). No clinical signs appeared in the control group.

Figure 4. Clinical signs of skin ulceration of dragon-eye goldfish and crucian carp infected with CAV-134 strain. Arrows indicate typical skin ulceration. A: natural diseased dragon-eye goldfish; B: infected crucian carp.

Discussion

Aeromonas spp. are widely distributed in various aquatic ecosystems such as freshwater, coastal water, and sewage (Evangelista-Barreto et al., 2010; Monfort and Baleux, 1990). *A. veronii* infections are the most common bacterial disease in cultured fish in China, and have been considered an epizootic causative agent of ulceration, dropsy, and septicemia (Yu et al., 2010; Kang et al., 2014). In our study, one *A. veronii* strain was isolated from skin ulceration and liver lesions of diseased dragon-eye goldfish (*Carassius auratus*). It was confirmed to be virulent to crucian carp with an LD_{50} value of 1.99×10^6 CFU/ml. The infected crucian carp had skin ulcerations. The LD_{50} value of *A. veronii* CAV-134 is very close to *A. veronii* strain RY001 isolated from goldfish (1.6×10^6 CFU/ml) (Han et al., 2008). In another study the LD_{50} value of the isolate *A. veronii* strain PY50 from ulcerative syndrome Chinese longsnout catfish was found to be lower (3.47×10^4 CFU/ml) (Cai et al. 2012). Several virulence genes such as aerolysin, cytotoxic enterotoxin, adhesion, etc. have been amplified from virulent *A. veronii* isolates and the infected fish died within a week (Zhou et al., 2012; Kang et al., 2014). The virulence of the isolate *A. veronii* CAV-134 could be associated with the virulence gene Aha1 (major adhesion). *A. veronii* infection is a growing problem in cultured goldfish causing major economic losses in China (Longyant et al., 2010; Yang, 2013; Song et al., 2009; Hu et al., 2008; Xu et al., 2006).

Phylogenetic analysis based on the 16S rRNA gene is regarded as an appropriate tool for the reconstruction of phylogenetic relationships of bacterial genera and is used universally (Stackebrandt and Goebel, 1994). In this study, the *16S rRNA* sequence of the isolated strain CAV-134 showed highest identity of 99.93% with *A. veronii* B565 strain (NR_102789) and 99.80% with *A. veronii* ATCC 35624 (X60414). The *16S rRNA* phylogenetic tree constructed with the Neighbor-joining algorithm clustered the strain CAV-134 with *A. veronii* B565 strain (NR_102789). The *gyrB* gene has proved to be an excellent molecular chronometer for phylogenetic studies of the genus *Aeromonas* (Yáñez et al., 2003). The *gyrB*, as well as *mu* and *asl* gene sequence analysis presented here demonstrated the closest relationship between strain CAV-134 and *A. veronii* B565 strain (CP002607) and the complete genome was sequenced (Li et al., 2011). The phylogenetic tree derived from the *gyrB-mu-asl-aha1* gene sequences was similar to the *16S rRNA* tree analysis. Biochemically, the *A. veronii* CAV-134 is positive for lysine decarboxylase and arginine dihydrolase, but is negative for ornithine decarboxylase, esculin hydrolysis, which is in accordance with *A. veronii* (Bowman, 2005). The isolate CAV-134 was identified and confirmed as *A. veronii* by biochemical and molecular analysis.

A. veronii was resistant to ampicillin and carbenicillin but sensitive to cephalothin This may be innate (Sreedharan et al., 2011; Cai et al., 2012; Yu et al., 2010; Joseph et al., 1991). The antimicrobial susceptibility patterns of isolate CAV-134 from the diseased goldfish are similar to those in humans and were susceptible to cephalosporins, aminoglycosides, quinolones, sulphonamides and macrolides (Joseph et al., 1991). Aminoglycoside resistance genes, sulphonamide resistance genes, and tetracycline resistance genes have been recovered from different *A. veronii* strains (Wang et al., 2013b; Nawaz et al., 2006). One-daily dose of 20 mg/kg enrofloxacin for 3-5 days was found to be optimal for controlling *A. veronii* infection (Wang et al., 2013a). It is worth noting that the isolate CAV-134 was resistant to ampicillin, amoxicillin and carbenicillin, but was sensitive to piperacillin which is consistent with results of *A. veronii* isolated by Sreedharan et al. (2011). We have provided the antimicrobial susceptibility pattern of *A. veronii* CAV-134, which laid a foundation for further investigating the antimicrobial resistance mechanisms of *A. veronii* in fish.

A. veronii is usually collected from environment and freshwater fish. In this study, one *A. veronii* CAV-134 strain was isolated from diseased dragon-eye goldfish in central China. Morphological and biochemical tests, as well as phylogenetic analysis derived from *16S rRNA*, *gyrB*, *mu*, *asl* and *aha1* genes sequencing all strongly indicated that the isolate was identical to *A. veronii*. Moreover, the isolate *A. veronii* CAV-134 was confirmed as the pathogen of crucian carp with an LD_{50} value of 1.99×10^6 CFU/ml. This report on a case of skin ulcerative syndrome associated with pathogenic *A. veronii,* may provide a scientific reference for characterization of *A. veronii* and prevention of bacterial disease in ornamental dragon-eye goldfish.

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