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Aeromonas veronii, a potential pathogen of enteritis in snakehead fish *Ophiocephalus argus*

Huicong Wang[1], Ying Gu[2,3], Guilan Luo[1], Haipeng Cao[2,3]*

[1] Department of Animal Husbandry & Veterinary Medicine, Jiangsu Vocational College of Agriculture and Forestry, Jurong Jiangsu 212400, P.R. China.

[2] National Pathogen Collection Center for Aquatic Animals, Shanghai Engineering Research Center of Aquaculture, Shanghai Ocean University, Shanghai 201306, P.R. China.

[3] Key Laboratory of Freshwater Fishery Germplasm Resources, Ministry of Agriculture of P. R. China, Shanghai 201306, P.R. China.

Key Words: *Aeromonas veronii*, *Ophiocephalus argus*, Enteritis, Potential pathogen

Abstract

Enteritis is known as a major disease in snakehead fish *Ophiocephalus argus* aquaculture and has resulted in large economic losses. Yet only scarce information is available on *Aeromonas veronii* as a causal agent for enteritis in *O. argus*. In this study, a virulent strain, temporarily named HY2, was isolated from diseased snakehead fish suffering from enteritis, and was identified as *A. veronii* through molecular and phenotypic methods. In addition, the HY2 isolate showed an LD_{50} value of 2.8×10^5 CFU mL^{-1}, and was highly sensitive to aminoglycosides, macrolides, polypeptides, quinolones, sulfonamides and tetracyclines antibiotics. To the best of our knowledge, this is the first report of *A. veronii* as a potential pathogen of enteritis in snakehead fish.

* Corresponding author. Tel.: +862161900453, fax: +862161900452, e-mail: hpcao@shou.edu.cn The first two authors contributed equally to this work

Introduction

Snakehead fish *Ophiocephalus argus* is widely cultivated in many countries like China, India, Korea, Malaysia, Philippines and Thailand (Gu et al., 2019). Especially in China, with the rapid development of farming techniques, the snakehead fish has become one of the most important commercial freshwater fish species and has brought a great profit in recent years (Liu et al., 2012). Its production has increased to over 459,000 tons in 2018 (Ministry of Agriculture and Rural Affairs of China, 2019). However, under intensive culture, this industry has been seriously affected by bacterial diseases (Pessoa et al., 2020). Thus, more attention should be given to bacteriosis to make further development of this industry.

Enteritis is a major disease in fish aquaculture and usually results in a high mortality (Ying et al., 2020). Several studies have revealed that this disease can be caused by viral, bacterial and parasitic pathogens, including reovirus, *Aeromonas punctatus*, *Aeromonas hydrophila*, *Pseudomonas putida*, *Vibrio harveyi*, *Vibrio cholera*, *Enteromyxum scophthalmi* and *Myxidium leei* (Wu et al., 2000; Padrós et al., 2001; Zhang et al., 2006; Mao et al., 2010; Carla et al., 2012; Pang et al., 2017; Jiang et al., 2019; Li et al., 2019). *Aeromonas veronii* is a Gram-negative, rod-shaped, mesophilic, motile bacterium which is ubiquitous in aquatic environments and frequently cause disease outbreaks in warm farming waters (Tekedar et al., 2019). However, little information is available on *Aeromonas veronii* as a causal agent for enteritis in snakehead fish.

The aim of this study is to characterize the phenotype, taxonomic position and antibiotic susceptibility of *A. veronii* pathogen isolated from enteritis-infected snakehead fish. As far as we know, this is the first report of *A. veronii* as a potential bacterial pathogen of enteritis in snakehead fish, and the findings of this study can be used a reference for health management in snakehead fish.

Materials and Methods

Snakehead fish samples

Twenty diseased snakehead fish averaging 550.0±4.6 g suffering from enteritis were sampled from a snakehead fish greenhouse farm with a cumulative morbidity of 85% and water quality parameters of 28^0C, pH 8.20, 0.38 mg L^{-1} of ammonia, 0.13 mg L^{-1} of nitrite and 0.45 mg L^{-1} of dissolved oxygen in Sihong, Jiangsu China during October 2019. The snakehead fish were stocked at an initial rearing density of 4 juveniles per square meter and fed commercial feeds at a rate of 3% of body weight with twice per day. Diseased samples were placed in sterile bags, kept in ice and transported to the laboratory according to Hossain et al. (2020).

Confirmation of the pathogen

Each sampled diseased snakehead fish was externally disinfected with 75% alcohol and dissected according to Li et al. (2011). To verify the potential pathogens, a squash of organs (intestine, liver, kidney, muscle, gill) were made and carefully examined for parasites under the microscope as described by Feng et al. (2019). Meanwhile, virological examination and bacterial infection assay were also conducted. Two hundred and forty experimental healthy fish (64.7±1.3 g) were obtained from a snakehead fish farm in Jurong, Jiangsu China, and were acclimated in twenty-four replicate aquaria (ten fish per aquarium) supplied with 100 L of aerated filtered farming water at 28^0C for 14 days. Each treatment contained two replicates as recommended by Bela-ong et al. (2015). The virological examination was conducted by injection of bacteria-free organ filtrate from the homogenate of organs according to Perelberg et al. (2003) and Gong et al. (2010). Briefly, the homogenate of organs was made and filtered through 0.22-μm-pore-size membrane filter to remove bacteria. Two replicate treatment aquaria of ten healthy fish were injected intraperitoneally with 0.2 mL of each bacteria-free organ filtrate. Another two replicate aquaria of ten healthy fish, which were exposed to the same experimental conditions and injected intraperitoneally with 0.2 mL of normal saline, served as the control. Experimental fish were kept at 28^0C without water change. The mortality and any visible changes of the experimental fish were recorded every day for 15 days according to Huang et al. (2013). In addition, 0.1 g of liver and kidney sample of each diseased fish was cut and streaked

onto nutrient agar (NA) (Sinopharm Chemical Reagent Co., Ltd.) plates as recommended by Jiang et al. (2019). After incubation for 24 h at 28^{0}C, the dominant uniform isolates were purified by streaking and re-streaking onto NA plates. Only the isolates with dense virtually pure culture growth on NA plates were obtained according to Zhang et al. (2017). Induced infection of the pure isolates was performed according to Zhang et al. (2017) and Zhou et al. (2019). Briefly, two replicate treatment aquaria of ten healthy fish were challenged by intraperitoneal injection with 0.2 mL of each isolate at a concentration of 3.0×10^5 CFU mL^{-1} as recommended by Mo et al. (2016), which was determined by counting colony forming units after a ten-fold serial dilution in sterile saline as described by Ma et al. (2009). Another two replicate aquaria of ten healthy fish exposed to the same experimental conditions and injected intraperitoneally 0.2 mL of normal saline remained unchallenged and served as control. The experimental fish were kept at 28^{0}C and observed daily for seven days without feeding and water change. Dead fish were immediately removed to re-isolate and identify the challenge isolate as described above to confirm if the mortality was caused by the challenge isolate. mortality was caused by the challenge isolate.

Identification of the pathogen

Molecular identification

The genomic DNA was extracted from the pathogenic isolate using the TIANamp DNA Kit (Tiangen Biotech. Co., Ltd.). Its 16S rRNA and gyrB genes were amplified by PCR according to Zhu et al*.* (2017) and were sequenced by ABI 3730 XL DNA Sequencer (Applied Biosystems, USA). A homology search was performed in the National Centre for Biotechnology Information (NCBI) database for 16S rRNA and gyrB gene sequences using the Basic Local Alignment Search Tool (BLAST) program. Phylogenetic trees were constructed using neighbour-joining method.

Phenotypic identification

The pathogenic isolate was observed under transmission electron microscope (HT770, Hitachi, Japan) according to He et al. (2020), and was identified phenotypically by API 20E system recommended by Zhu et al. (2017), where the pathogenic isolate was grown on NA plates at 28^{0}C for 24h, and the bacterial suspension was then used to inoculate the API 20E test strips (Biomerieux, France) following the manufacturer's instruction. The plate was incubated at 37^{0}C and observed after 18h for checking against the API identification index and database. Information related to *A. veronii* previously reported by Dong & Cai (2001) and Yang et al. (2013) serves as a reference.

Bacterial virulence assay

Bacterial virulence assay was carried out by experimentally infecting healthy snakehead fish and was conducted in strict accordance with the Regulations on Experimental Animals Administration of China (Publication No. 676). One hundred healthy snakehead fish averaging 65.5±1.1 g were obtained from a snakehead fish farm in Jurong, Jiangsu China. The experimental fish were acclimated in ten replicate aquaria (ten fish per aquarium) supplied with 100 L of aerated filtered farming water at 28^{0}C for 14 days. Each treatment contained two replicates as recommended by Bela-ong et al. (2015). Prior to the bacterial virulence assay the suspension of the pathogenic isolate was prepared according to Zhang et al. (2017) and its cell density was determined by counting colony forming units after a ten-fold serial dilution in sterile saline as described by Ma et al. (2009). Two replicate aquaria of ten healthy fish were challenged by intraperitoneal injection (Mo et al., 2016) with 0.2 mL of the pathogenic isolate at a concentration of 3.0×10^4 CFU mL^{-1} to 3.0×10^7 CFU mL^{-1}. Another two replicate aquaria of ten healthy fish exposed to the same experimental conditions and injected intraperitoneally 0.2 mL of normal saline remained unchallenged and served as control. The experimental fish were kept at 28^{0}C and were observed daily for seven days without feeding and water change. Any dead fish were immediately removed and sampled to re-isolate and confirm if the mortality was caused specifically by the challenge isolate according to Kozińska et al. (2002). Briefly, dead fish

were sampled to re-isolate the challenge isolate which was confirmed phenotypically and molecularly as described above. The mean lethal dose (LD_{50}) value is calculated using the linear regression method as recommended by Spielmann et al. (1999).

Antibiotic susceptibility assay

The antibiotic susceptibility of the pathogenic isolate was examined on NA plates using the Kirby-Bauer disk diffusion method as recommended by Fang et al. (2019). Fourteen antibiotic discs were provided by Hangzhou Binhe Microorganism Reagent Co., Ltd.. The zones of inhibition against the pathogenic isolate were measured after a 24h incubation period at 28^0C, and its susceptibility to antibiotics was assessed according to the manufacturer's guidelines.

Results

Identification of the pathogen

No parasites were found in the diseased snakehead fish, and all of the experimental fish challenged with the bacteria-free organ filtrate survived with no visible changes (data not shown), indicating that this disease was not caused by parasites or viruses. A total of five dominant isolates (temporarily numbered from HY1 to HY5) were recovered from diseased fish, and only isolate HY2, which could be isolated from liver and kidney of all the sampled fish, was confirmed as the pathogen for this disease according to Koch's postulate: (i) The HY2 isolate could be isolated from diseased snakehead fish. (ii) The death of the experimental fish was increased gradually over time after the challenge with isolate HY2. 25%-100% of the challenged fish died at an LD_{50} value of 2.8×10^5 CFU mL^{-1} (**Table 1**) and exhibited enteritis signs similar to that seen in the originally diseased fish (**Figure 1**). No clinical signs or mortality were noted in the control fish. (iii) The HY2 isolate could be re-isolated from experimentally dead fish, which was determined through phenotypic and molecular identification. These findings demonstrated that isolate HY2 was the causative agent of this disease.

Table 1. Cumulative mortality of experimental snakehead fish infected by isolate HY2.

Group	Concentration (CFU mL^{-1})	Fish no.	Dead fish no. on day after challenge							Average cumulative mortality (%)	LD_{50} value (CFU mL^{-1})
			1	2	3	4	5	6	7		
Control	0	10	0	0	0	0	0	0	0	0	2.8×10^5
		10	0	0	0	0	0	0	0		
Treatment 1	3.0×10^4	10	0	2	0	0	0	0	0	25	
		10	1	1	1	0	0	0	0		
Treatment 2	3.0×10^5	10	2	1	0	1	0	0	0	40	
		10	1	1	1	0	1	0	0		
Treatment 3	3.0×10^6	10	2	3	1	1	0	1	0	85	
		10	3	1	2	2	0	1	0		
Treatment 4	3.0×10^7	10	5	3	2	0	0	0	0	100	
		10	8	1	1	0	0	0	0		

Figure 2. Transmission electron microscopy image of isolate HY2.
Arrow shows the rod-shaped cell.

Identification of the pathogen

Isolate HY2 was rod-shaped (**Figure 2**). Its near complete 16S rRNA and gyrB gene sequences were submitted to GenBank database with the accession nos. MT875223 and MT894140. The phylogenetic trees (**Figures 3 and 4**) indicate that the HY2 isolate is identified as an *A. veronii* strain. Besides, isolate HY2 was also confirmed by the phenotypic features (Table 2) as *A. veronii* with 100% identity compared to the reference strain. Thus, isolate HY2 was identified as *A. veronii*.

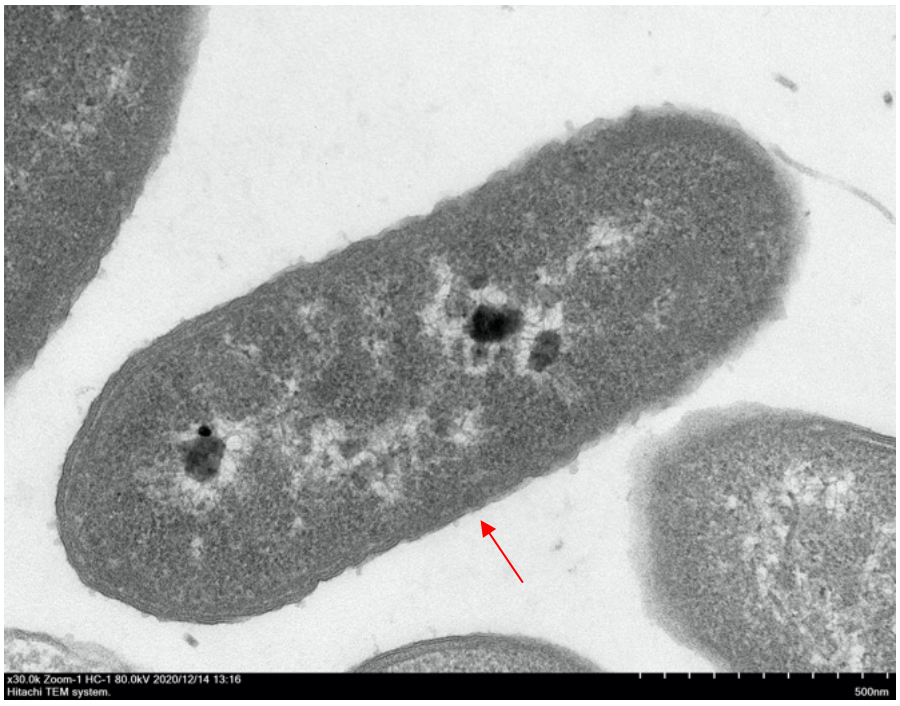

Figure 2. Transmission electron microscopy image of isolate HY2. Arrow shows the rod-shaped cell.

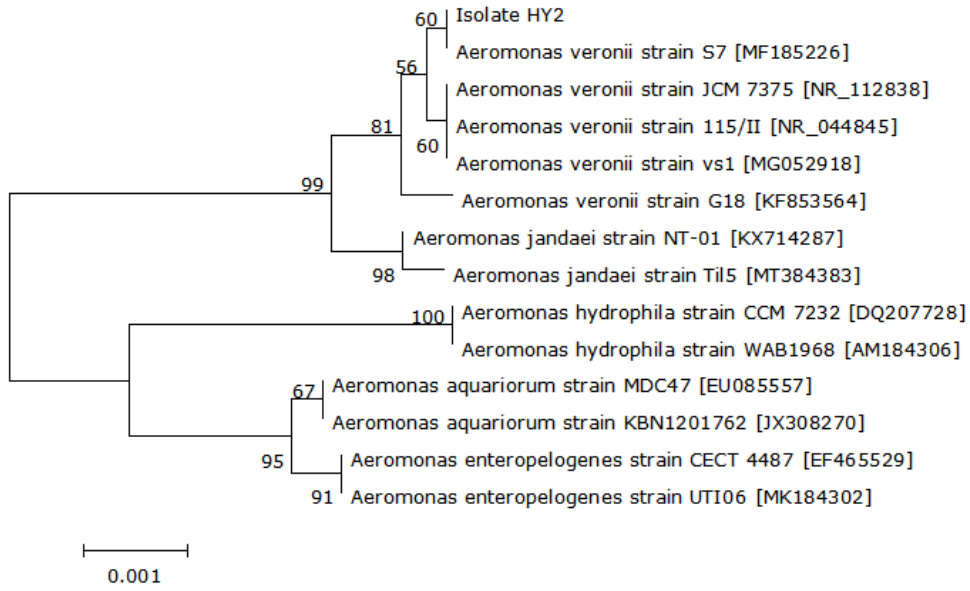

Figure 3. A 16S rRNA gene tree of 13 known bacteria and the HY2 isolate constructed using the neighbor-joining method. The bootstrap values (%) are shown besides the clades, accession numbers are indicated beside the names of strains, and scale bars represent distance values.

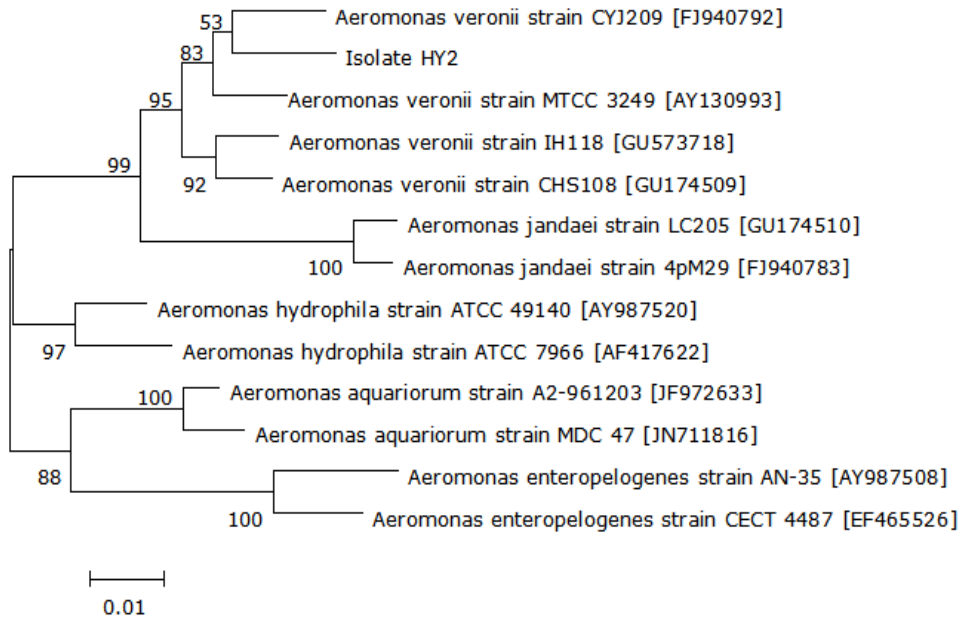

Figure 4. A gyrB gene tree of 12 known bacteria and the HY2 isolate constructed using the neighbor-joining method. The bootstrap values (%) are shown besides the clades, accession numbers are indicated beside the names of strains, and scale bars represent distance values.

Antibiotic susceptibility of the pathogen

The antibiotic susceptibility of isolate HY2 is shown in **Table 3**. The data indicate that isolate HY2 is sensitive to amikacin, cotrimoxazole, enrofloxacin, gentamicin, kanamycin, levofloxacin, midecamycin, norfloxacin, ofloxacin, polymyxin B, streptomycin, tetracycline, vancomycin, and exhibit resistance to ampicillin. This suggests that isolate HY2 can be potentially controlled by aminoglycosides, macrolides, polypeptides, quinolones, sulfonamides, tetracyclines antibiotics.

Table 3. Susceptibility of isolate HY2 to antibiotics.

Antibiotics	Content (μg/disc)	Inhibition zone diameter (mm)
Amikacin	30	23.59 ± 3.36^{S}
Ampicillin	10	0 ± 0^{R}
Cotrimoxazole*	1.25/23.75	25.61 ± 2.18^{S}
Enrofloxacin*	5	31.23 ± 1.26^{S}
Gentamycin	10	20.76 ± 0.76^{S}
Kanamycin	30	20.88 ± 2.28^{S}
Levofloxacin	5	33.49 ± 1.07^{S}
Midecamycin	30	13.88 ± 1.43^{S}
Norfloxacin	10	31.41 ± 1.02^{S}
Ofloxacin	5	30.88 ± 2.77^{S}
Polymyxin B	300	12.44 ± 1.39^{S}
Streptomycin	10	21.20 ± 1.89^{S}
Tetracycline*	30	21.19 ± 0.81^{S}
Vancomycin	30	13.48 ± 0.36^{S}

Data are presented as the mean ± standard deviation. ^{S}Sensitive; ^{R}Resistant.

*Antibiotics for aquaculture use (Ministry of Agriculture of China, 2013).

Discussion

Aeromonas species are dominant flora in fish aquaculture (Zhang et al., 2006; Wang et al., 2020), and are also major bacterial fish pathogens (Lazado et al., 2018). For example, Ying et al. (2020) proved a dominant virulent isolate of *A. punctata* as the pathogen of enteritis in *Symphysodon aequifasciatus.* Yang et al. (2007) confirmed a dominant virulent isolate of *Aeromonas salmonicida* as the pathogen of skin ulceration disease in *Apostichopus japonicus*. In the present study, the dominant isolate HY2 was found to show an LD_{50} value of 2.8×10^5 CFU mL^{-1} and was confirmed as a causal agent of enteritis in snakehead fish, further indicating that the dominant pathogenic isolate is probably the causal agent of fish diseases. To our knowledge, this is the first report of an *A. veronii* pathogen of enteritis in snakehead fish.

A. veronii is widely distributed in aquatic ecosystems and possesses a variety of virulence factors, including aerolysin, cytotoxic enterotoxins, DNases, elastase, hemolysins, leucocidins, lipases, outer membrane proteins, proteases (Chen et al., 2019; Li et al., 2020). The association of *A. veronii* in aquaculture has been well documented with mortality of *Coregonus clupeaformis* (Loch et al., 2010), *Astronotus ocellatus* (Sreedharan et al., 2011), *Leiocassis longirostris* Günther (Cai et al., 2012), *Dicentrarchus labrax* (Uzun et al., 2015), *Cyprinus carpio* (Sun et al., 2016), *Oreochromis niloticus* (Hassan et al., 2017), *Carassius auratus gibelio* (Chen et al., 2019), *Eriocheir sinensis* (Zhou et al., 2019), *Ictalurus punctatus* (Tekedar et al., 2020), *Colossoma macropomum* (Pessoa et al., 2020), *Procambarus clarkii* (Hu et al., 2020) and *Neophocaena phocaenoides asiaeorientalis* (Liu et al., 2020). In our study, *A. veronii* isolate HY2 was demonstrated to cause mortality in snakehead fish, further revealing its potential threat to fish farming. Apart from the virulence of the HY2 isolate, there might be other secondary factors that induce enteritis in snakehead fish such as poor water quality, temperature changes and over intensification of stocking density (Zhu et al., 2020; Zhou et al., 2020); these should also be raised as concerns.

The development of antimicrobial resistance in *A. veronii* is a matter of concern (Tekedar et al., 2020). In our study, the HY2 isolate developed resistance to ampicillin. The same resistance has been observed in the pathogenic *A. veronii* isolated from diseased *C. auratus gibelio*, *O. niloticus* and *I. punctatus* (Xia et al., 2012; Hassan et al., 2017; Tekedar et al., 2020). The HY2 isolate in our study has exhibited sensitivity to aminoglycosides, quinolones, tetracyclines and sulfonamides drugs used in fish farming regions, suggesting that the outbreak of this disease may not have resulted from the abuse of antibiotics.

In conclusion, the present study for the first time reports an *A. veronii* isolate as a potential pathogen of enteritis in *O. argus*. The pathogenicity of the HY2 isolate supports this infection as a potential threat in snakehead fish farming.

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