International Scholars Journals

Advances in Aquaculture and Fisheries Management ISSN: 9424-2933 Vol. 7 (3), pp. 001-006, March, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Aeromonas veronii disease in the refined snakehead fish, *Ophiocephalus argus (Cantor)*

***Ferdinard Estrada, Jose V. Marcos and Dolphy Brocka**

Department of Fisheries Sciences, Faculty of Agriculture, University of Santo Tomas, Manila, Philippines.

Accepted 16 December, 2018

***Aeromonas veronii* has been identified as a causative agent of fish and human disease outbreaks. However, there is no definitive data on *A. veronii* infection in cultured snakehead fish *Ophiocephalus argus* (Cantor). In this study, a virulent strain S-L was isolated from the farmed snakehead fish suffering from epizootic ulcerative syndrome, and identified as an *A. veronii* strain using ARIS 2X system. Its taxonomic position was further determined through nucleotide blast search in NCBI website and phylogenetic analysis. The constructed phylogenetic tree using neighbor-joining method further showed that the S-L isolate was the *A. veronii* strain (GenBank accession no. JQ301790), which was also previously isolated from diseased freshwater fish. In addition, five genes encoding aerolysin, haemolysin, serine protease and two cytotonic enterotoxins were all found present in the S-L isolate which further confirmed its potential virulence. As far as we know, this is the first report of farmed snakehead fish infected with *A. veronii*.**

Key words: *Ophiocephalus argus* (Cantor), *Aeromonas veronii*, epizootic ulcerative syndrome, identification.

INTRODUCTION

Snakehead fish *Ophiocephalus argus* (Cantor) is widely distributed and cultivated in Asia, Tropical Africa (Hu and Chen, 1964). Especially in China, the snakehead fish has become an important commercial fish species with the production of above 358 thousand tons since 2009 (Zhang et al., 2011). However, *Aeromonas* infection has become a major economic problem in the snakehead fish farming. Thus, diseases caused by *Aeromonas* should be given more attention for the sustainable development of snakehead fish farming industry.

Aeromonas veronii has been described as an important fish and human pathogen (Singh et al., 2012.). It has been reported as a causative agent of outbreaks such as hemorrhagic septicemia and epizootic ulcerative syndrome (Austin and Austin, 1999; Rahman et al., 2002; Cai et al., 2012). These diseases caused by *A. veronii* are usually associated with its production of toxins such as proteases (Song et al., 2004), as well as cell-surface components such as lipopolysaccharide and bundle-forming pilus (Turska-Szewczuk et al., 2012; Hadi et al., 2012). *A. veronii* pathogens has been isolated from *Ictalurus punctatus*, *Colisa lalia*, *Misgurnus anguillicaudatus*, *Acipenser baerii*, *Astronotus ocellatus*, *Leiocassis longirostris* Günther (Nawaz et al., 2006; Hossain, 2008; Qin et al., 2008; Ma et al., 2009; Gong et al., 2010; Sreedharan et al., 2011 ; Cai et al., 2012). However, there is no definitive data on *A. veronii* infection in cultured snakehead fish.

In this paper, a virulent *A. veronii* strain was isolated from the diseased snakehead fish with epizootic ulcerative syndrome in Lingtang, Jiangsu China, and its phenotypic characterization, taxonomic position and virulence genes were also examined. As far as we know, this is the first report of farmed snakehead fish infected with *A. veronii*.

*Corresponding author. E-mail: ferdinard44@vtmw.ts

Table 1. Specific virulence gene primers for PCR amplification.

Virulence gene	Primer (5'→3')	Sequence length (bp)
aerA	Forward: CCTATGGCCTGAGCGAGAAG Reverse: CCAGTTCCAGTCCCACCACT	431
hlyA	Forward: GGCCGGTGGCCCGAAGATACGGG Reverse: GGCGGCGCCGGACGAGACGGGG	592
ahpA	Forward: ATTGGATCCCTGCCTATCGCTTCAGTTCA Reverse: GCTAAGCTTGCATCCGTGCCGTATTCC	1011
Alt	Forward: TGACCCAGTCCTGGCACGGC Reverse: GGTGATCGATCACCACCAGC	442
Ast	Forward: TCTCCATGCTTCCCTTCCACT	331

MATERIALS AND METHODS

Snakehead fish samples

As described by Ruso (1987), 36 diseased snakehead fish (380±10 g in weight) were sampled and transported to the laboratory from Lintang snakehead fish farm, Jiangsu China during June 2012.

Isolation of bacteria

Each sampled moribund snakehead fish, judged as still ventilating but unable to hold position or remain upright (Hruska et al., 2010), was disinfected externally with 75% alcohol and dissected in the laboratory. 0.2 g of the internal organ samples such as livers, and kidneys were cut to isolate and purify the bacteria according to Song et al. (2011).

Identification of bacteria

Phenotypic identification using ARIS 2X system

The isolate was biochemically identified using ARIS 2X system as recommended by Pattern et al. (1995). Briefly, the isolate was grown on nutrient agar (NA) plates (Sinopharm Chemical Reagent Co., Ltd.) at 28°C for 24 h, and then the bacterial suspension was used to inoculate the GNID plate (Sensititre, USA) following the manufacturer's instruction. The plate was incubated at 37°C and observed after 18 h for checking against the ARIS identification index and database. The type strain ATCC35624 of *A. veronii*, the type strain ATCC7966 of *A. hydrophila* and the type strain ATCC43979 of *A. sobria* were used as the control.

Molecular identification

The genomic DNA extract, 16S rRNA genes' PCR amplification and sequencing of the isolate were performed according to Cao et al. (2010). Its partial 16S rRNA sequence was assembled using MegAlign, Editseq and Seqman software with a Power Macintosh computer. Search was done against the National Centre for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) program. The phylogenetic tree from partial 16S rRNA sequence of the isolate and its homologous sequences was further constructed using neighbor-joining method.

Bacterial virulence assay

After evaluation by a careful exam of physical appearance and behavior as well as internal organs such as liver and kidney for bacterial pathogens as recommended by Zheng et al. (2012), 40 healthy snakehead fish (360±8 g in weight) were obtained from Shuixiang Aquaculture Co., Ltd. in Hangzhou, Zhejiang China, and were respectively maintained in 4 aquaria (10 fish per aquaria) supplied with 100 L de-chlorinated tap water at 28°C for 14 days. Prior to the bacterial virulence assay, the isolate' live cells were respectively prepared as recommended by Cao et al. (2010), and its cell density was determined using the dilution and spread plate technique. 10 healthy fish were respectively challenged by being immersed in 100 L of water containing the isolate's live cells at a final cell density of 5.0×10^6 cfu/ml as described by Ma et al. (2009) and Murray et al. (1992). Another 10 healthy fish were unchallenged as the control. The experimental snakehead fish were kept at 28°C and observed daily for 10 days. Dead fish were immediately removed for pathogen isolation according to Bucke (1989), and the signs and mortalities were recorded. Each experiment was conducted in two parallel.

Virulence gene assay

The genomic DNA was extracted from the pure cultures of the isolate using a genomic DNA extraction kit following instructions of the manufacturer (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.). Five virulence genes encoding aerolysin (*aerA*), haemolysin (*hlyA*), serine protease (*ahpA*), and two cytotonic enterotoxins (*alt* and *ast*) were respectively amplified by PCR using specific *aerA*, *hly*, *ahp*, *alt* and *ast* gene primers as recommended by Nawaz et al. (2010) and Zhu et al. (2006). *A. veronii* strain NQ, previously identified as the pathogen of diseased *Misgurnus anguillicaudatus* (Qin et al., 2008), and *Escherichia coli* DH5α were respectively taken as the positive and negative control bacteria. The specific primers for virulence gene amplification are listed in Table 1. The PCR products were electrophoresed on 1% agarose gel and visualized via ultraviolet trans-illumination.

RESULTS

Identification of the isolate

95% of the challenged fish acutely died with the same

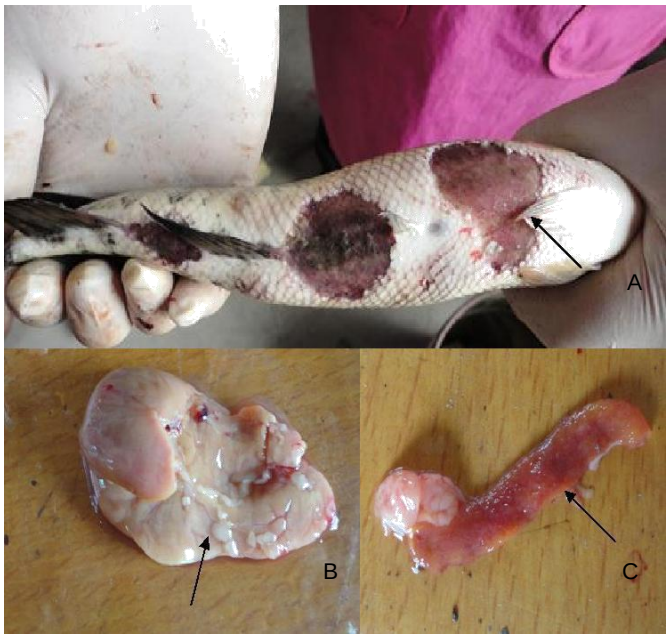

Figure 1. The pathological symptoms of naturally infected snakehead fish**. A.** Arrow indicated the ulcerative skin. **B.** Arrow indicated the faint yellow liver; C: arrow indicated the hyperaemia of the intestine wall.

clinical ulcerative syndrome signs similar to the originally infected fish (Figure 1), and no acute mortality or visible changes were observed in the control snakehead fish (Table 2). The ARIS 2X system identified the S-L isolate as an *A. veronii* strain (Table 3), and showed an identity of 96.9% with the Type strain ATCC35624 in phenotypic characterization. In addition, the partial 16S rRNA sequence (ca. 1.4 kb) of the S-L isolate was submitted to GenBank database with the accession no. JX262992. Similarities between its 16S rRNA sequence and those of *A. veronii* strains in the GenBank database were 99 to 100%, and the constructed phylogenetic tree using neighbor-joining method further demonstrated the S-L isolate as the *A. veronii* strain (GenBank accession no. JQ301790) (Figure 2). This proved the initial identification.

Virulence genes of the isolate

The specific PCR amplification of virulence genes is shown in Figure 3. The specific virulence gene (*aerA*, *hlyA*, *ahpA*, *alt* and *ast*) fragments were obtained with the S-L isolate using a pair of *aerA*-specific primers, *hlyA*-specific primers, *ahpA*-specific primers, *alt*-specific primers and *ast*-specific primers, respectively. This was in accordance with that with the positive control *A. veronii* strain NQ. No fragments were present with the negative control strain DH5α (Data not shown). The result demonstrates that the virulence genes (*aerA*, *hlyA*, *ahpA*, *alt* and *ast*) were all present in the S-L isolate.

DISCUSSION

Epizootic ulcerative syndrome is one of the most destructive diseases affecting over 100 species of wild and cultured freshwater as well as estuarine finfish since 1971 (Saikia and Kamilya, 2012). Thus, more attention should be paid to its novel pathogen. So far, several virulent bacteria such as *A. salmonicida*, and *A. hydrophila* have been reported to cause epizootic ulcerative syndrome of *Cyprinus carpio*, *Clarias batrachus*, *Carassius carassius*, and *Glossogobius giurus* (Evenberg et al., 1986; Llobrera and Gacutan, 1987). However, no relevant information is available about the *A. veronii* infection in snakehead fish. In this study, we isolated a virulent strain of *A. veronii* from the cultured snakehead fish with epizootic ulcerative syndrome, and assayed its phenotypic characteristics, taxonomic position, and virulence genes. As far as we know, this is the first report of farmed snakehead fish infected with *A. veronii*.

Previous reports showed that the occurrence of genes encoding haemolysin (*hlyA*), aerolysin (*aerA*), serine protease (*ahpA*), and cytotonic enterotoxins (*alt* and *ast*) may contribute to the virulence of *Aeromonas* pathogens (Wong et al., 1998; Sha et al., 2002). *Aeromonas* isolates with *hlyA, aerA* and *ahpA* genes were confirmed as strong virulent strains (Zhu et al., 2006). In the present study, the S-L isolate was highly virulent to healthy snakehead fish (Table 2) and contained the five virulence genes (Figure 3). This was in accordance with previous findings (Nawaz et al., 2010). Apart from pathogenicity of the S-L isolate, there might be other causes for the incidence of epizootic ulcerative syndrome such as high densities of fish, lack of food disinfection and water degradation (Cao et al., 2010). These should also raise concerns.

The excellent performance and accuracy of ARIS 2X system allows its good use in routine identification of gram-negative bacteria in the clinical laboratory (Fritsche et al., 2011). For example, Dickenson et al. (2006) accurately identified over 330 gram-negative isolates at the species level using ARIS 2X system. Thus, in the present study, ARIS 2X system was employed to correctly identify the S-L isolate as an *A. veronii* strain (Table 3). Additionally, in order to make a better understanding of its taxonomic position, molecular phylogenetic study was conducted as recommended by Lee et al. (2000). The identification result from phylogenetic analysis (Figure 2) was also consistent with that found through the ARIS 2X system. This agrees with the previous finding obtained by Clapini et al. (2002).

In conclusion, the present study for the first time reported *A. veronii* infection in the farmed snakehead fish. The virulence of the isolate supported this infection

Table 2. Virulence of the S-L isolate to snakehead fish.

Group	Concentration（cfu/ml）	Fish number	Dead fish number per day										Average mortality (%)
			1	2	3	4	5	6	7	8	9	10	
Control	0	10	0	0	0	0	0	0	0	0	0	0	0
S-L	5.0×10^{6}	10	0	0	0	0	0	0	0	0	0	0	95
		10	0	0	0	0	0	0	2	4	2	1	
		10	0	0	0	0	0	1	3	2	2	2	

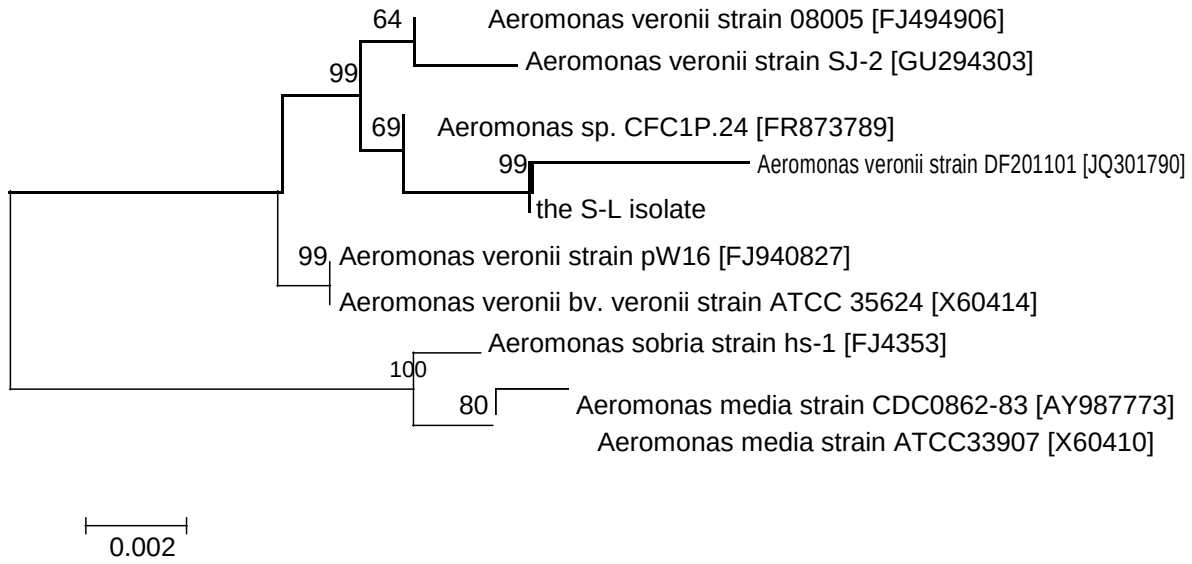

Figure 2. The phylogenetic analysis of the S-L isolate constructed using neighbor-joining method.

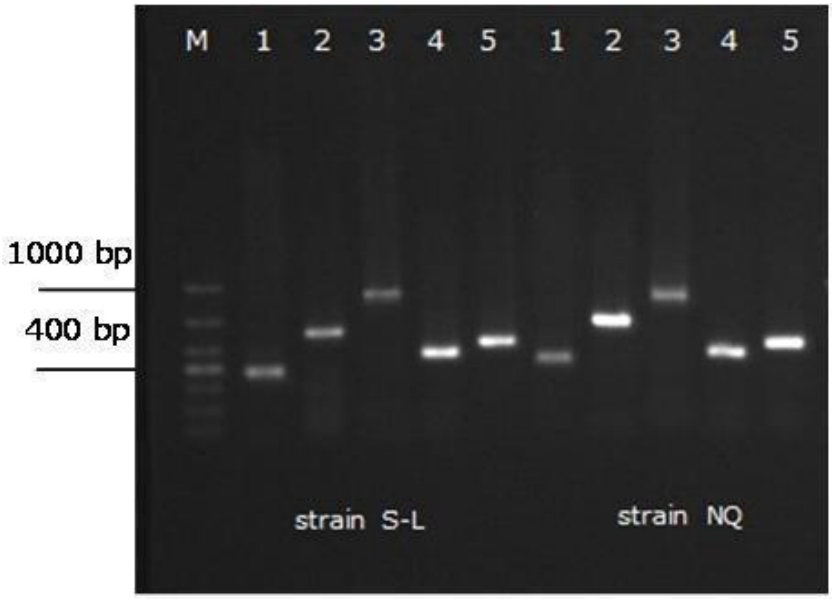

Figure 3. The PCR amplification of virulence genes in the S-L isolate. Lane M, DNA marker; lane 1, *aerA* gene; lane 2, *hlyA* gene; lane 3, *ahpA* gene; lane 4, *Ast* gene; lane 5, *Alt* gene.

Table 3. Phenotypic characterization of the S-L isolate in comparison with the type strain ATCC35624 of *A. veronii*, the type strain ATCC7966 of *A. hydrophila* and the type strain ATCC43979 of *A. sobria*.

Test item	Reaction			
	S-L	ATCC35624	ATCC7966	ATCC43979
7AMC-Lysine	R-	R-	R-	R-
Xylose	R-	R-	R-	R-
4MU-Phostate	R+	R+	R+	R+
Maltose	R+	R+	R+	R+
7AMC-Proline	R+	R+	R+	R+
Arabinose	R-	R-	R+	R-
7AMC-γ-Glutamine	R-	R+	R+	R+
Malonate	R-	R-	R-	R-
Urea	R-	R-	R-	R-
4MU-2-Acetamido-2-Deoxyglucopyranoside	R+	R+	R+	R+
Trehalose	R+	R+	R+	R+
4MU-α-D-Glucopyranoside	R-	R-	R-	R-
Fructose	R+	R+	R+	R+
Lysine	R+	R+	R+	R+
Arginine	R-	R-	R-	R-
Pyruvate	R-	R-	R-	R-
Ornithine	R+	R+	R+	R+
Sucrose	R-	R+	R+	R-
4MU-Bis-Phosphate	R-	R-	R-	R-
Inositol	R-	R-	R+	R-
Esculin	R-	R+	R-	R-
Tryptophan deaminase	R-	R-	R-	R-
4MU-α-D-Galactopyranoside	R-	R-	R-	R-
Citrate	R-	R-	R+	R-
Sorbitol	R-	R-	R-	R-
4MU-Glucuronide	R-	R-	R-	R-
Mannitol	R+	R+	R+	R+
4MU-β-D-Galactopyranoside	R-	R-	R-	R+
Arabitol	R-	R-	R-	R-
Raffinose	R-	R-	R-	R-
Cellobiose	R-	R+	R-	R+
Agmatine	R-	R-	R-	R-

R^+,positive reaction; R^-, negative reaction.

as an emerging threat in snakehead fish farming and food safety.

ACKNOWLEDGMENTS

This work was financially supported by the National 863 Program (No. 2011AA10A216), the Special Fund for the Agroscientific Research in the Public Interest (No. 201203085), the Earmarked Fund for China Agriculture Research System (No. CARS-46), and Lianyungang Key Technology Program for Agriculture, P.R. China.

REFERENCES

Austin B, Austin DA (1999). Bacterial fish pathogens: disease in farmed and wild fish, 3rd edition. Springer Verlag, New York, pp. 263-287.

Bucke D (1989). Histology. In Austin et al. (eds) Methods for the Microbiological Examination of Fish and Shellfish, Wiley, New York, pp 69-97.

Cai S, Wu Z, Jian J, Lu Y, Tang J (2012). Characterization of pathogenic *Aeromonas veronii* bv. *veronii* associated with ulcerative syndrome from Chinese longsnout catfish (*Leiocassis longirostris*

Günther). Braz. J. Microbiol. 43(1):382-388.

Cao HP, He S, Lu L, Hou L (2010). Characterization and phylogenetic analysis of the bitrichous pathogenic *Aeromonas hydrophila* isolated from diseased Siberian sturgen (*Acipenser baerii*). Isr. J. Aquacult.Bamid. 62:182-189.

Clapini A, Dei R, Sacco C, Ventura S, Viti C, Giovannetti L (2002). Phenotypic and genotypic characterisation of *Aeromonas* isolates. Ann. Microbiol. 52:339-352.

Dickenson RA, Chapin KC (2006). Comparative evaluation of the Sensititre® ARIS 2X® and the BD Phoenix automated identification and antimicrobial susceptibility test systems. In proceedings of American Society for Microbiology Annual Meeting held at Orlando, Florida. Poster C-121.

Evenberg D, De Graaff P, Fleuren W, Van Muiswinkel WB (1986). Blood changes in carp (*Cyprinus carpio*) induced by ulcerative *Aeromonas salmonicida* infections. Vet. Immunol. Immunopathol. 12:321-330.

Fritsche TR, Swoboda SE, Olson BJ, Moore FM, Meece JK, Novicki TJ (2011). Evaluation of the Sensititre® ARIS 2X and Vitek® 2 automated systems for identification of bacterial pathogens recovered from veterinary specimens. In proceedings of American Society for Microbiology Annual Meeting held at Orlando, Florida. Paper p. 3229.

Gong Q, Gao S, Shan X, Guo W, Meng Q, Wang W, Qian A (2010). Isolation and identification of pathogenic *Aeromonas veronii* from *Cyprinus carpio*. Chin. J. Prev. Vet. Med. 32(12):981-983.

Hadi N, Yang Q, Barnett TC, Tabei SM, kirov SM, Shaw JG (2012). Bundle-forming pilus locus of *Aeromonas veronii* bv. *sobria*. Infect. Immun. 80(40):1351-1360.

Hossain M (2008). Isolation of pathogenic bacteria from the skin ulcerous symptomatic gourami (*Colisa lalia*) through 16S rDNA analysis. Univ. J. Zool. Rajshahi. Univ. 27:21-24.

Hruska KA, Hinch SG, Heale MC, Patterson DA, Larsson S, Farrell AP (2010). Influences of sex and activity level on physiological changes in individual adult sockeye salmon during rapid senescence. Physiol. Biochem. Zool. 83(4):663-676.

Hu H, Chen S (1964). Reproductive behavior and embryonic development of snakehead fish *Ophiocephalus argus* (Cantor). B. Biol. 2:17-19.

Lee SD, Kim ES, Hah YC (2000). Phylogenetic analysis of the genera *Pseudonocardia* and *Actinobispora* based on 16S ribosomal DNA sequences. FEMS Microbiol. Lett. 182:125-129.

Llobrera A, Gacutan RQ (1987). *Aeromonas hydrophila* associated with ulcerative disease epizootic in Laguna de Bay, Philippines. Aquaculture 67:273-284.

Ma Z, Yang H, Li T, Luo L, Gao J (2009). Isolation and identification of pathogenic *Aeromonas veronii* isolated from infected siberian sturgen (*Acipenser baerii*). Acta Microbiol. Sin. 49(10):1289-1294.

Murray CB, Evelyn TPT, Beacham TD, Barner LW, Ketcheson JE, Prosperi-Porta L (1992). Experimental induction of bacterial kidney disease in Chinook salmon by immersion and cohabitation challenges. Dis. Aquat. Organ. 12:91-96.

Nawaz MS, Khan SA, Khan AA, Sung K, Tran Q, Kerdahi K, Steele R (2010). Detection and characterization of virulence genes and integrons in *Aeromonas veronii* isolated from catfish. Food Microbiol. 27:327-331.

Nawaz MS, Sung K, Khan SA, Khan AA, Steele RS (2006). Biochemical and molecular characterization of tetracycline-resistant *Aeromonas veronii* isolates from catfish. Appl. Environ. Microbiol. 72:6461-6466.

Pattern VH, Shin SJ, Cole J, Watson CW, Fales WH (1995). Evaluation of a commercial automated system and software for the identification of veterinary bacterial isolates. J. Vet. Diagn. Invest. 7(4):506-508.

Qin L, Xu J, Zhang X (2008). Infection with *Aeromonas veronii* biovar. sobria in *Misgurnus anguillicaudatus*. Chin. J. Zool. 24(12):1100-1102.

Rahman M, Colque-Navarro P, Kühn I, Huys G, Swings J, Mőllby R (2002). Identification and characterization of pathogenic *Aeromonas veronii* biovar. sobria associated with epizootic ulcerative syndrome in fish in Bangladesh. Appl. Environ. Microbiol. 68(2):650-655.

Ruso AR (1987). Role of habitat complexity in mediating predation by the gray damselfish *Abudefduf sordidus* on epiphytal amphipods. Mar. Ecol. Prog. Ser. 36:101-105.

Saikia D, Kamilya D (2012). Immune responses and protection in catla (*Catla catla*) vaccinated against epizootic ulcerative syndrome. Fish Shellfish Immun. 32:353-359.

Sha J, Kozlova EV, Chopra AK (2002). Role of various enterotoxins in *Aeromonas hydrophila*-induced gastroenteritis: generation of enterotoxin gene-deficient mutants and evaluation of their enterotoxic activity. Infect. Immun. 70:1924-1935.

Singh V, Chaudhary DK, Mani I (2012). Molecular characterization and modeling of secondary structure of 16S rRNA from *Aeromonas veronii*. Int. J. Appl. Biol. Pharm. Technol. 3(1):253-260.

Song H, Wu Y, Shen Y, Hu M, Liu Y, Lu C (2011). Identification and detection of virulence factors of *Aeromonas hydrophila* in freshwater fish from a market in Nanjing. Chin. Vet. Sci. 41(2):111-115.

Song T, Toma C, Nakasone N, Iwanaga M (2004). Aerolysin is activated by metalloprotease in *Aeromonas veronii* biovar *sobria*. J. Med. Microbiol. 53:477-482.

Sreedharan K, Philip R, Singh ISB (2011). Isolation and characterization of virulent *Aeromonas veronii* from ascetic fluid of oscar *Astronotus ocellatus* showing signs of infectious dropsy. Dis. Aquat. Organ. 94(1):29-39.

Turska-Szewczuk A, Lindner B, Pekala A, Palusińska-Szysz M, Choma A, Russa R, Holst O (2012). Structural analysis of the O-specific polysaccharide from the lipopolysaccharide of *Aeromonas veronii* bv. *sobria* strain K49. Carbohydr. Res. 353:62-68.

Wong CYF, Heuzenroeder MW, Flower RLP (1998). Inactivation of two haemolytic toxin genes in *Aeromonas hydrophila* attenuates virulence in a suckling mouse model. Microbiology 144:291-298.

Zhang X, Chen K, Zhu X (2011). Research status for the culture of *Channa* argus, *C. maculata* and their reciprocal hybrids. Guandong Agric. Sci. 22:132-134.

Zheng W, Cao H, Yang X (2012). Grass carp (*Ctenopharyngodon idellus*) infected with multiple strains of *Aeromonas hydrophila*. Afr. J. Microbiol. Res. 6(21):4512-4520.

Zhu DL, Li AH, Wang JG, Li M, Cai TZ, Hu J (2006). The correlation between the distribution pattern of virulence genes and the virulence of *Aeromonas hydrophila* strains. Acta Sci. Nat. Univ. Sunyatseni, 45:82-85.