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Studies on Etiology and Antimicrobial Susceptibility Testing of Skin Ulcer Disease in *Schizothorax prenanti*

[1]Zong-Jun Du, [1]Xiao-Li Huang, [1, 2]De-Fang Chen, [2]Kai-Yu Wang and [3]Yong-Qiang Deng
[1]Department of Aquaculture, College of Animal Science and Technology,
[2]Research Center of Fish Disease, College of Animal Veterinary Medicine,
Sichuan Agricultural University, 625014 Ya'an, Sichuan, P.R. China
[3]Sichuan Provincial Center for Animal Disease Prevention and Control,
610041 Chengdu, Sichuan, P.R. China

Abstract: Skin Ulcer Disease (SUD) is a new disease in *Schizothorax prenati*. The purpose of this study was to elucidate the etiology of SUD in recent outbreaks in Sichuan province, China. One dominant bacteria (D060501) was isolated from the diseased *Schizothorax prenanti* with typical skin ulcer. It was identified as *Aeromonas hydrophila* by morphological features, physiological and biochemical characteristics and 16S rDNA sequence. The bacterium was very sensitive to chloramphenicol and gentamicin and resistant to tetracycline, SMZ, doxycycline and carbenicillin. The virulence of the bacteria to *Schizothorax prenanti* was checked by challenged experiment.

Key words: *Aeromonas hydrophila*, *Schizothorax prenanti*, skin ulcer disease, biochemical, bacteria, China

INTRODUCTION

Skin ulcers disease are frequently recorded from a large variety of fish species. In farmed fish, skin ulcerations are usually related to septicemic conditions caused by bacteria of the genera Vibrio, Aeromonas, Pseduomonas, *Shewanella algae*, etc. (Chang and Qun, 2003; Feng *et al*., 2005). *Schizothorax prenanti* (vernacular name: Ya'an fish) is distributed in region of Changjiang, China. Due to its good meat quality and limited number of wild, this species has been artificial breeded from 1990s in Ya'an city of China. Until now, this speices has been raised on Sichuan, Yunan and Chongqing province, China. As this fish as farmed speices is shorter, its related diseases data was deficiency.

From December 2005 to April 2006, a disease characterized by the presence of serve dermal ulcers and muscle fester around the dorsal fin region broke out in several *Schizothorax prenanti* fish farms in Ya'an city and Panzhihua city which has caused a substantial economic loss to fish farmers. The diseased fish is characterized by the presence of severe, open dermal ulcers on the head, the middle of the body and the dorsal regions. The objective of this study was to elucidate the etiology of the infectious disease and to provide basci data and information for healthy aquaculture of *Schizothorax prenanti*.

MATERIALS AND METHODS

Experimental fish: Thirty sick fish (70±5 g) with typical clinical signs of dermal ulcers on one side of the dorsal fin were collected from a commercial *Schizothorax prenanti* farm in Ya'an city (Sichuan province, China) and transferred alive in plastic bags with an oxygen supply to the laboratory.

Sixty healthy fish (65±5 g) from Sichuan Agriculture University fish farm with no history of disease were kept in aquaria with aeration and fed with commerical feeds twice a day at a daily rate of 2% of their body weight. Fish were kept 1 week before bacterial challenge.

Isolation of bacteria: Diseased fish with typical symptoms were firstly cleaned by sterile physiological saline on the surface area and swabbed with 70% ethyl alcohol to prevent contamination. Then samples taken from the kidneys, livers and muscles around the perifocal areas were inoculated on Trypticase Soy Agar (TSA) plate and Blood Agar Plate (BAP), respectively. Afterwards, the plates were incubated for 24 h at 28°C and examined for the growth of bacterial colonies.

The dominant colonies were selected, streaked for purity on TSA. Pure stock isolates were stored at -80°C in steriled TSA supplemented with 15% glycerol for further research.

Corresponding Author: Kaiyu Wang, 46 Xinkang St., 625014 Ya'an, Sichuan, P.R. China

Challenge experiments: Sixty healthy fish were divided into 6 groups of 10 which included 3 experimental groups and 3 control group. Three different injected ways including via wounded skin, immersion and intramuscular injection were introduced to each group. Immersion challenge and injection challenge were basically performed as described by Geng *et al.* (2010). One group (group 1, G1) of ten stocked in fresh water containing 1.0×10^5 cfu mL^{-1} bacterial suspension after being examined without any injury on their skin. One group of 10 (G2) was repeated on the control group with sterile physiological saline in place of bacterial suspension. The 3rd group (G3) of 10 were introduced by scarifying over the dorsal fin region of each fish by using a new sterile scalpel blade then exposed to containing 1.0×10^5 cfu mL^{-1} bacterial suspension. The exposure of control group (G4) to water absent of bacterial suspension were followed. The group (G5) of 10 was injected intramuscular with 0.1 mL cell suspension at a concentration of 1.0×10^5 cfu mL^{-1}. The same volume of sterile physiological saline was injected into the 6th group (G6) as a control.

Syndrome and mortality were recorded daily for 1 week after injection of each group. The experimental groups and control groups were strictly separated to prevent any possible cross infection. In addition, pathogen was isolated from experimentally diseased fish and cultured on the TSA plate for identification though routine traditional biochemical tests.

Phenotypic characterization: Morphological features and growth of the bacterial colonies on TSA plate and BAP were observed and recorded. Gram staining and standard biochemical was tested using conventional plate and tube tests (Hangzhou Tianhe Microbiological Reagent Co., Ltd., China) (Shen *et al.*, 2005). All tests were performed referring to standard methods and compared with type stain (Holt *et al.*, 1994).

16S rDNA sequencing and phylogenetic analysis: Total DNA template was extracted using a commercial bacterial Genome DNA Extraction Kit (Tiangen, China). The *16S rRNA* gene was amplified by PCR with a commercial 16S rDNA bacterial identification PCR Kit (TaKaRa, Japan). The expected PCR amplicons were separated electrophoretically in a 0.8% agarose gel and purified using a TIANGEN PCR purification kit (Tiangen, China). The sequence of 16S rDNA with the almost full-length insert was sequenced by Takara Biotechnology (Dalian) Co., Ltd. Sequence was aligned and then compared with the BLAST database in the National Center for Biotechnology Information (NCBI). The aimed 16s rDNA was aligned with these retrieved sequences by using multiple sequence alignment in DNA star software and the phylogenetic tree was generated by using maximum-likelihood method with Bootstrap trials 1000. Distance matrices and homology were calculated according to the Jukes-Cantor model.

Antimicrobial susceptibility test: The antibiotic susceptibility of isolated strain was determined by the Disc diffusion method and fourteen antibiotic disks (Hangzhou Taihe Microbiologcial Reagent, China). The sensitivity and resistance of each isolate were determined as per the manufacutrer's instructions and by criteria of the Shryock and NCCLS (2002).

RESULTS

Isolation of the bateria: Bacteria obtained from the sample tissues (muscle, kidney and liver) of the naturally diseased fish all demonstrated an identical gram reaction result and were identified as gram-negative rods. In addition, bacterial colony grow on the TSA plate was described as shiny, white or grey-white, round shape with smooth edge. Furthermore, haemolysis around the colonies on the blood agar plate was detected. The bacterial strain was named D060501 for further research.

The experiment on healthy fish: The results showed that only exposure via wounded skin (G3) and intramuscular injection (G5) can induce the syndrome to the fish and the progression time of the disease observed in these two methods was almost the same which was explained as below: Syndrome of slow movement, fast breathing rate, scale dropping and increased emission of mucus was firstly seen 24 h post exposure (p.e.), the syndrome worsened with the ulcer showed on the skin 36 h p.e., the ulcer area enlarged with hyperemia on the ulcer edge 48 h p.e.

A dissect examination of the diseased fish found a slightly swell of the kidney and inflammation of the intestine whereas the other organs remained healthy. Under aseptic condition, pathogen isolated from the experimentally diseased fish was proved to be identical with the pathogen isolated from naturally diseased fish through a series of traditional biochemical tests. The control groups (G2, 4 and 6) had no obvious clinical lesions.

Identification of the isolates D060501: D060501 was identified as a motile gram-negative rod without capsule and spore. Under the scope, its often seen individually or pared when assembled as a colony on TSA plate, the bacteria displayed a shiny grey or white color and was in

Table 1: Physiological and biochemical characteristics of the D060501 isolate

Items	D060501	*Aeromonas hydrophila*	Items	D060501	*Aeromonas hydrophila*
Glucosa produce acid	+	+	Arginine dihydrolase	+	+
Lipase	+	+	Potassuim cyanide	+	+
Oxidase	+	+	V-P test	+	+
Glucose produce gas	+	+	Maltose	+	+
Urea	-	-	H_2S production	+	+
Arabinose	+	+	DNA enzyme	-	-
Mobility	+	+	Gelatinase	+	+

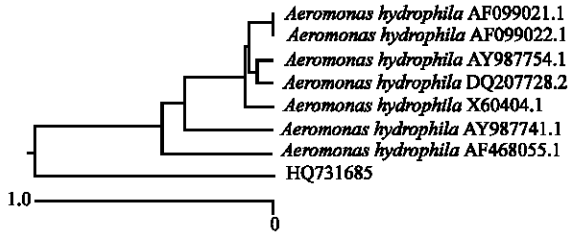

Fig. 1: The phylogenetic tree of the D060501 isolate and related *Aeromonas hydrophila* based on the full-length 16S rDNA sequence

a round form with smooth edge. Examination of colonies on the agar plate supplemented with rabbit blood detected β ring of Haemolysis.

In the physiological and biochemical tests, D060501 showed positive results on lipase, arginine dihydrolase and oxidase reaction, maltose, H_2S production, potassuim cyanide, V-P test while demonstrated negative outcome in DNA enzyme and urea reaction. In addition, D 060501 was found to be able to fermented glucose to produce gas and utilize arabinose to make acid. The detailed information of the physiological and biochemical results were shown in Table 1.

16 S rDNA sequence and phylogenetic tree analysis: The amplified gene product of D060501 was detected to have about 1500 nucleotide by algar gel electrophoresis. According to the sequence comparison of 16s rDNA sequence of D060501 with those in the gene bank, D060501 showed high homology in its 16s rDNA sequence with Aeromonas species. In addition, the phylogentic tree analysis also demonstrated that D060501 together with other *Aeromonas hydrophila* formed a cluster and displayed the highest homology (99.1%) with *Aeromonas hydrophila* (GenBank No.: HQ73168) (Fig. 1).

Antimicrobial susceptibility test: Among the nine antibiotics, D060501 was most sensitive to gentamicin and chloramphenicol and moderately sensitive to erythormycin, cefaclor and norfloxacin while displayed resistance to tetracycline, compound sulfamethoxazol, carbenicillin and doxycycline (Table 2).

Table 2: The medicine sensitivity test results of the D060501 isolate

			*Criterion			
Antibiotics	Contents (μg/Tablet)	Diameter of Inhibition (mm)	Resistance	Medium sensitivity	Sensitivity	Results
Erythormycin	15	15	≤13	14-22	≥23	(M)
Cefaclor	5	16	≤14	15-17	≥18	(M)
Gentamicin	10	15	≤12	13-14	≥15	(S)
Norfloxacin	10	14	≤12	13-16	≥17	(M)
Chloramphenicol	30	22	≤12	13-17	≥18	(S)
Tetracycline	30	0	≤14	15-18	≥19	(R)
Compound sulfamethoxazole	75	0	≤10	11-15	≥16	(R)
Doxycycline	30	0	≤12	13-15	≥16	(R)
Carbenicillin	100	0	≤19	20-22	≥23	(R)

DISCUSSION

The present study identified the bacterial pathogen isolated in the diseased fish from the commercial fish farm in Ya'an to be *Aeromonas hydrophila* by applying both traditional biochemical tests and 16S rDNA sequences and phylogentic tree analysis. *Aeromonas hydrophila* was a common caustic pathogen in aquaculture which had caused substantial economic loss. Currently, disease caused by *Aeromonas hydrophila* on wood frog and other vrious fish (Rey *et al*., 2009; Hird *et al*., 1983) was reported. However, information of disease caused by Aeromonas hydrophila on *Schizothorax prenanti* has not been reported.

Between December 2005 and April 2006, disease characterized with skin ulcers on the dorsal fin regions broke out in several *Schizothorax prenanti* fish farms and was named ulcer disease for temporary use. Approximately 30-80% of the raised fish were infected and 90% of the diseased fish shared a similar ulcer region. Further examination under the microscope saw degeneration, necrosis, lysis of the cells which might be associated with some of the factors that can lead to fiber lysis and breakage. This current experiment was then conducted to investigate the cause and find possible treatment solution. Results showed that the diseased fish shared a high similarity with *Aeromonas hydrophilain* on morphological features and physiological and biochemical characteristics. The 16S rDNA sequences analysis and phylogenetic tree analysis further ascertained the isolates to be *Aeromonas hydrophila*. The final conclusion was made that *Aeromonas hydrophila* can be a causitic agent of *Schizothorax prenanti* skin ulcers. Reportedly *Aeromonas hydrophila* was found to cause haemorrhage

and local infection of the diseased animals with a syndrome of hyperaemis, hemorrhage of the skin and systemic hemorrhage of the organs. Virulence factors of the bacteria included toxin, extracellular protease, S protein, pilus, outer membrane protein and lipopolysacharide, etc. (Howard and Buckley, 1983, 1985; Avison *et al*., 2000; Kanatani *et al*., 1993). Fish are potentially more vulnerable to these factors when injured, stressed or suffering from weaked immune systems.

Antimicrobial susceptibility tests of this current experiment showed that the *Aeromonas hydrophila* isolated in this case was sensitive to chloromycetin and Gentamicin. According to some reports, Antimicrobial susceptibility of the *Aeromonas hydrophila* varies with different isolate strains. The isolates from the bacterial sepsis diseased crucian carp for example were sensitive to tobramycin, amikacin, neromycin, chloromycetin, gentamicin, norfloxacin and enrofloxacin baytril while isolates from vertical scale diseased fish were sensitive to chloromycetin, gentamicin, neomycin, streptomycin, kanamycin and tetracycline. Clinical treatment with several antibiotics was used in the case but with few effects. However, addition of vitamins and planktons proved to be useful. Possibility of the occurrence of the disease, thus could lay on the unbalance of nutrient in the fish food as a result of inadequate knowledge and information about *Schizothorax prenanti* fish nutrition.

CONCLUSION

In this study, the pathogen that caused the skin ulcer on the *Schizothorax prenanti* was proved to be *Aeromonas hydrophila*. Antimicrobial susceptilbity tests suggest treatment with thiamphenicol and florfenicol in place of tetracycline could be effective together with the balance of nutrients and improvement of the immune parameters.

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