Pathogenicity of *Aeromonas hydrophila* to blunt snout bream *Megalobrama amblycephala*

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Abstract- This study was carried out to confirm whether *Aeromonas hydrophila* is a main haemorrhagic pathogen in blunt snout bream *Megalobrama amblycephala* (BSB). The fish was challenged with bacterial concentrations of 1.7×10^4, 1.7×10^5, 1.7×10^6 and 1.7×10^7 cfu/fish for 7 days post infection. The results showed that mortality was bacterial-dose dependent, with 100% mortality observed at 1 day at the highest dose level (1.7×10^7 cfu/fish). For comparison, control fish exhibited cumulative mortalities of 0%. The median lethal dose (LD_{50}) was 5×10^5 cfu/fish. A total of 15 bacterial strains of *Aeromonas* were re-isolated from challenged fish and re-identified based on morphological characteristics, biochemical tests and genomic DNA gene sequencing. No bacteria were isolated from the control group. This study results indicated that *A. hydrophila* is capable of causing haemorrhagic septicaemia in BSB. Antibiotic susceptibility test with two strains D4 and HU201301 was investigated; both strains showed sensitive to most of the tested drugs.

I. INTRODUCTION

Blunt snout bream *Megalobrama amblycephala* (BSB) or Wuchang fish is an herbivorous fish species and commonly distributes in the middle portion of Yangtze River (Fishbase 2012). BSB has become a favourable freshwater aquaculture species in China (Tsao 1960; Zhou et al. 2008). During the past culture time, BSB culture industry has suffered problems related to degrading pond environments due to development of intensification which resulted in the odd of stress-induced disease (Nielsen et al. 2001). Many fish farms have been particularly infected by epidemics of *Aeromonas hydrophila* which has been reported causing haemorrhagic septicaemia clinical signs and histopathological changes, and causing a great loss in cultured BSB (Neilsen et al. 2001; He et al. 2006).

It has been known for decades that *Aeromonas* plays a causative agent role in fish diseases. The wide-spread distribution in aquatic ecology systems indicates that interactions of *Aeromonas* species with fish are continuous and unavoidable, facilitating their opportunistic pathogenicity (Ottaviani et al. 2011; Hu et al. 2012). *A. hydrophila* were recorded widely infecting freshwater fish and marine fish species associated with skin lesions, tail and fin rot, haemorrhagic septicaemia over the body and tissue destruction, epizootic ulceration and necrosis in the liver and kidney of fish (Austin and Adams 1996; Doukas et al. 1998; Janda and Abbott 2010). It was considered as a significant economic problem, particularly in China and India over the past decade (Citarasu et al. 2011). The existence and pathogenicity of *A. hydrophila* have been reported in a variety of freshwater species, comprising *Salmo gairdneri* (Peters et al. 1988), *Clarias batrachus* (Angka 1990), tilapia (Liu et al. 1999), *Carassius auratus* (Iqbal et al. 1999; Citarasu et al. 2011), *Cyprinus carpio* (Chirila et al. 2008; Citarasu et al. 2011), *Oreochromis niloticus* (Ibrahem et al. 2008), and *Channa striata* (Duc et al. 2013). Nevertheless, to date the infection information of BSB in China is still limited. In present study, the efforts were conducted by a challenge test to confirm whether *A. hydrophila* is a main haemorrhagic pathogen in BSB.

II. MATERIALS AND METHODS

A. Bacterial strain and bacterial suspension preparation

A. hydrophila D4 used in this experiment was isolated from BSB with clinical signs of haemorrhagic septicaemia cultured in Dongxihu, Hubei, China in 2012 and maintained in the College of Fisheries, Huazhong Agricultural University. The bacterium was cultured on the agar medium (including 3 g beef extract, 10 g peptone, 5 g NaCl, and 15 g agar in 1,000 mL distilled water) and incubated at 28 ^oC for 24 hours. In order to harvest bacterial cells, sterile physiological saline (0.85% NaCl) was added into the bacterial incubated test tubes, and the surface colonies were crushed by using a sterile loop to dilute bacterial suspension. The desired densities of bacteria (1.7×10^5, 1.7×10^6, 1.7×10^7 and 1.7×10^8 cfu/mL) were determined by counting on a neubauer haemocytometer and prepared by ten-fold dilution method. To confirm the inocula density of bacterial suspension, a method for plate count by serial dilution was conducted.

B. Fish for experiment

For pathogenicity test, healthy BSB weighing 27.1±8.3 g were collected in the fish farm in Huanggang, Hubei, China and transferred to the laboratory of the College of Fisheries, Huazhong Agricultural University. All tested fish were acclimatized in $4m^3$-glass tanks (maintained at 28 ^oC and pH of 7.5) for 2 week before the artificial injection experiment. During the acclimatization period, fish were fed to satiation twice a day (08:00 and 16:00) with commercial pelleted feed.

C. Pathogenicity test

Fifty fish without clinical signs by naked eye observation were used for intraperitoneal injection experiment. The experiment included 5 groups (n=10 for each), comprising the negative control (physiological saline injection) and four concentrations of bacterial suspension. Before infection experiment, tested animals

were slightly anesthetized by MS-222 (Sigma, USA). Inocula were set up as intraperitoneal injections at 0.1 mL/fish of physiological saline or desirable densities of bacterial suspension. The experiment was conducted in 50 L aquarium with aeration at 28 ^{o}C for 7 days. No feed was fed during the experiment.

The fish were monitored daily and mortality was recorded. The cumulative mortality was calculated after challenge test. Moribund fish was observed for the external and internal clinical signs. Bacteria were re-isolated and identified by biochemical tests and sequencing on genomic DNA gene. The median lethal dose (LD_{50}) was finally determined (Reed and Munch 1938).

D. Biochemical tests

Biochemical characteristics of bacterial strains (D4, HU201301, HU201302 and HU201304) were performed at 37 ^{o}C unless otherwise specified. Gram staining, motility, catalase activity, oxidase activity, carbohydrate fermentation (glucose, sucrose, lactose, arabinose, mannitol and salicine), Methyl Red/Voges-Proskauer (MR/VP), citrate utilization, esculine and starch hydrolysis, indole and hydrogen sulphide (H_2S) production and growth on *A. hydrophila* specific-medium (AHM) were investigated. Arabinose, aesculin, mannitol, salicin were carried out by microbial biochemical identification tubes (Hangzhou Microbial Reagent Co., Ltd) following to manufacture's instruction. Two strains (D4 and HU201301) were used in tolerance of to salt environment at 28 ^{o}C for 24 hours.

E. Antibiotic susceptibility test

The antibiotic susceptibility test was performed by the disk diffusion method described previously (Bauer et al. 1966). A total 20 antimicrobial agents (Hangzhou Microbial Reagent Co., Ltd) including amoxicillin (10 µg/disc), florfenicol (75 µg/disc), levofloxacin (5 µg/disc), tetracycline (30 µg/disc), neomycin (30 µg/disc), novobiocin (30 µg/disc), doxycycline (30 µg/disc), gentamicin (10 µg/disc), norfloxacin (10 µg/disc), aztreonan (30 µg/disc), fortum (30 µg/disc), kanamycin (30 µg/disc), ceftriaxone sodium (30 µg/disc), ofloxacin (5 µg/disc), piperacillin (100 µg/disc), clindamycin (2 µg/disc), rifampicin (5 µg/disc), imipenem (10 µg/disc), erythromycin (15 µg/disc) and chloramphenicol (30 µg/disc) were used for investigation. Two strains of bacteria, D4 and HU201301, were used in antibiotic susceptibility test. A bacterial suspension (100 µL) was spread onto nutrient agar plates and then chemotherapeutic agent discs were placed on. The plates were incubated at 28 ^{o}C for 24 h. The diameter of each inhibition zone (including the diameter of the disc) was measured and recorded in mm. The responses were illustrated, which is resistant (R), intermediate (I) or sensitive (S) according to manufacturer's recommendation.

F. Molecular analysis

Bacteria were incubated on the agar medium at 28 ^{o}C for 24 hours. The bacterial suspension was prepared by being diluted in sterile double-distilled water and centrifuged at 12,000 rpm in 5 min at 4 °C. Genomic DNA was extracted using ammonium acetate precipitation technique as described previously (Bruford et al. 1998) with slight modification. The quality of genomic DNA was evaluated using electrophoresis in 1% agarose gels. The quantity of genomic DNA samples was measured by using a Nanodrop 2000 (Thermo Scientific, USA). DNA was stored at -20^{o}C until use. Polymerase Chain Reaction (PCR) was performed on all strains. Two universal primers, forward primer 27F: 5'-agagtttgatcctggctcag-3' and reverse primer 1492R: 5'-tacggctaccttgttacgactt-3' (Sangon Biotech (Shanghai) Co., Ltd.), were used to amplify 16S genomic DNA gene. The PCR amplifications were performed in a final volume of 10 µL containing 5 U Taq DNA polymerase, 1.0 µL 10× buffer (with Mg^{2+}) for Taq DNA polymerase, 0.25 µL dNTP, 0.25 µL each primer and 50 ng genomic DNA. A negative control (without template DNA) was also carried out. The PCR amplification program was set with initial denaturation at 94 °C for 5 min, 30 cycles at 94 °C for 30 s, 58 °C for 45 s, 72 °C for 1.5 min, and followed by final extension step at 72 °C for 10 min. The PCR products were analysed by using electrophoresis in 1% agarose gels. The PCR products were sequenced in Sangon Biotech (Shanghai) Co. Ltd. by the Sanger's sequencing method using ABI 3730. The identity of 16S genomic DNA gene sequences was obtained based on BLAST search in GeneBank database (Altschul et al. 1990).

III. Results

Clinical signs

The first-observed mortality time was 8 h after infection. The external clinical signs were observed with impaired swimming, exophthalmia and anorexia, swollen eyes, and haemorrhage at the site of injection and ventral surface of moribund and dead fish. Internal clinical signs observation showed haemorrhage in the abdominal cavity, red odiferous fluid, swollen and colour-deep spleen, pale liver and sometimes swollen kidney recognized. The clinical signs of fish challenged to *A. hydrophila* were similar to those of the naturally infected fish recorded (Fig. 1).

The cumulative mortality

The cumulative mortality of BSB challenged with *A. hydrophila* at 7 days post-inoculation was showed in Fig. 2. The different mortalities were found in different bacterial suspension densities. The mortality was recorded as 10, 10, 70 and 100% after bacterial intraperitoneal injection with 1.7×10^4, 1.7×10^5, 1.7×10^6 and 1.7×10^7 cfu/fish, respectively. Non-died fish was recorded in negative control group during experiment carried out. The median lethal dose (LD_{50}) was 5×10^5 cfu/fish in intraperitoneal injection of *A. hydrophila* to BSB.

Re-isolation and re-identification

A total of 15 bacterial strains were re-isolated from spleen, liver and kidney of moribund artificially injected fish with clinical signs. No evident external lesions or died fish in control group were recorded and no bacteria were isolated from either the control experimental fish.

A similarity was found in biochemical characteristics between experimental original strain (D4) and re-isolated strains (HU201301, HU201302 and HU201303) shown in Table 1. For the morphological characteristics, these bacterial strains grew on nutrient agar at 28 ^{o}C for 24 hours with mucoid yellow colour colonies. All can growth on AHM medium at 28 ^{o}C. Microscopic observation exhibited a phonotypical homogenization of short rod-shaped. All were gram-negative, motile, oxidase-positive and catalase-negative. Biochemical testing resulted in positive

reaction of all tested bacterial strains for production of indole and H_2S, utilization of citrate, hydrolysis of starch and esculin, fermentation of glucose, lactose, arabinose and mannitol and Methyl Red/Voges-Proskauer. Nevertheless, negative reaction was shown for fermentation of sucrose and salicine in all tested strains.

Genomic DNA sequence analysis

The identification of *A. hydrophila* based on morphological and biochemical characteristics were carried out. Subsequently, a number of colonies of strains incubated for 24 hours were chosen for PCR amplification. PCR products of 16S genomic DNA gene of D4 and HU201301 were sequenced to validate for *A. hydrophila* identification. A similarity of identity percentage (98%) with the nucleotide sequences of genomic DNA gene of two strains was showed; and a high similarity (99% identity) to other available *A. hydrophila* sequences in the GenBank database by using BLAST network search.

Antimicrobial susceptibility test

The pattern of antibiotic susceptibility of two strains of *A. hydrophila* (D4 and HU201301) is shown in Table 2. This present study found the relevant similarities between the original bacterial strain (D4) used in conducting challenge experiment and the re-isolated strain (HU201301). Both strains showed resistance to amoxicillin, and clindamycin and sensitive to novobiocin, florfenicol, levofloxacin, tetracycline, neomycin, doxycycline, gentamicin, norfloxacin, aztreonan, fortum, kanamycin, ceftriaxone sodium, ofloxacin, piperacillin, rifampicin, and chloramphenicol. Interestingly, the D4 strain showed resistance to imipenem and sensitive to erythromycin, while HU201301 showed intermediate to those of antibiotic agents.

IV. DISCUSSION

In this study, a challenge test with *A. hydrophila* demonstrated similarities in the clinical signs recorded by artificially challenged and naturally infected BSB. The observed symptoms in the present study resembled those reported in other fish (Popovic et al. 2000; Nielsen et al. 2001; He et al. 2006; Duc et al. 2013). Three among fifteen strains re-isolated from tested fish were used to conduct experiment on biochemical tests. Based on biochemical characteristics, it was determined that the four strains (D4, HU201301, HU201302 and HU201303) of bacteria were homogenous, which is comparable to previous reports (Lee et al. 2000; Abbott et al. 2003). All four strains grew on the previously described AH medium (Kaper et al. 1979), used for species identification, confirming that these bacterial strains are *A. hydrophila*.

Molecular method for detecting of *A. hydrophila* was introduced and applied in numerous previous studies (Nielsen et al. 2001; Swaminathan et al. 2004; Yogananth et al. 2009). The genomic DNA was the coding region of ribosomal RNA, which was used for phylogenetic studies as it is highly conserved between different species of bacteria and archaea and as important taxonomic tools (Weisburg et al. 1991; Coenye and Vandamme 2003) such as taxonomy of the genera *Aeromonas* (Lee et al. 2002; Chen et al. 2012; Liu and Li 2012; Sarkar et al. 2012). Two universal primers, 27F and 1492R, were developed in amplification of genomic DNA genes and had allowed discriminating of identification up to the species level and typing of other bacteria (Jiang et al. 2006; Sarkar et al. 2012). It has been indicated previously that levels of similarity between genomic DNA gene sequences higher than 97% suggest that the strains in question belong to the same species (Stackebrandt and Goebel 1994), so a 98% identity of sequences of the D4 and HU201301 strains implies that they belong to the same species. This challenge test experiment did fulfil Koch's postulates (Walker et al. 2006).

The high cumulative mortality (100%) of BSB (challenged with 1.7×10^7 cfu/fish by intraperitoneal injection method) in this study was comparable to the results previously reported (Mostafa et al. 2008). They have challenged *Heteropneustes fossilis* with *A. hydrophila* at a dose of 9.6×10^7 cfu/fish using intraperitoneal and intramuscular injection, which also resulted in 100% mortality of the tested fish within 1-9 days. Similarly, the reported mortality of some other fish species challenged by intraperitoneal injection with lower bacterial density, such as walking catfish *C. batrachus* with 10^6 cfu/fish (Angka 1990) and snakehead *C. striata* with 10^6 cfu/fish (Duc et al. 2013) was 100%. Also, several fish species (*Labeo rohita*, *Catla catla* and *Cirrhinus cirrhosis*) challenged by intramuscular method at a dose of 2×10^6 cfu/fish had 80 to 100% mortality, while *Hypophthalmichthys molitrix* (Valenciennes, 1844) and *C. carpio* at a dose of 2×10^5 cfu/fish had 60-80% mortality within 2-12 days (Sabur 2006) (cited by Sarkar and Rashid 2012). The LD_{50} value in this study was 5×10^5 cfu/fish, similar to the LD_{50} of *A. hydrophila* challenged to snakehead fingerling (Duc et al. 2013), and lower than for some other fish species, such as *Anguilla anguilla* ($10^{6.2}$ to $10^{7.4}$ cfu/fish) (Esteve et al. 2004), *Channa punctatus* (3.42×10^7 cfu/fish) (Yesmin et al., 2004) and *Brycon amazonicus* (6.66×10^{11} cfu/fish) (Oliveira et al. 2012), indicating that isolates used in challenge test were strongly virulent to BSB.

Chemotherapeutic agents were previously introduced to treat *A. hydrophila* infection in cultured fish (Saitanu and Chularak 1983). In this study, the antibiotic sensitivity patterns of two strains (D4 and HU201301) were evaluated and confirmed to be mostly similar. This test was conducted in order to confirm the resemblance of re-isolated strain (HU201301) and experimental inoculated bacterial strain (D4) in sensitive ability to antimicrobials, and also to determine the suitable therapeutical medicines for controlling *Aeromonas* infection in BSB. Both strains were sensitive to most tested drugs, except for amoxicillin and clindamycin. D4 was also resistant to imipenem, while HU201301 was only intermediately resistant to this drug. In previous studies, *A. hydrophila* was reported to be sensitive to chloramphenicol, erythromycin, kanamycin, neomycin (Boonyaratpalin 1989) and resistant to amoxicillin and clindamycin (Belem-Costa and Cyrino 2006; Adanir and Turutoglu 2007; Jayavignesh et al. 2011). The results from the present study were similar to these, but different from the results reported by Son et al. (1997, p. 480) and Vivekanandhan et al. (2002, p. 166), who found that *A. hydrophila* was resistant to chloramphenicol, erythromycin, kanamycin, tetracycline, rifampicin and novobiocin.

The susceptibility of the experimental bacterial strains (D4 and HU201301) to some beta-lactam antibiotics was also

investigated. In comparison to a previous report (Morita et al. 1994), the results showed the similarity in susceptibility to aztreonam and imipenem, and difference in resistance to piperacillin and ceftriaxone. These differences may be related to the source of bacterial isolates and the source of antibiotic agents used in the experiment. Moreover, it is known that the bacteria can receive and transmit antibiotic resistant genes from and to other gram-negative bacteria (Marchandin et al. 2003). Thus, aeromonad-infection in BSB may still be controlled by using correct drugs. Nevertheless, reduction of the use of chemicals in aquaculture is nowadays considered a good management practice. Certain types of chemicals, especially antibiotics, if used inappropriately, not only cause damages to animals and the environment, but also increase production costs and adverse consequences (Tonguthai 2000). Furthermore, the observation of disease odd usually correlates with anorexia (Duc et al. 2013); hence the utilization of antibiotics at the disease outbreak time may not be effective. Also, the extensive use of antibiotic and other chemotherapeutics to prevent and treat fish diseases may cause drug-resistance phenomenon in cultured fish (Son et al. 1997). Vaccination treatment was recommended for use in disease prevention in cultured fish (Bakopoulos et al. 1995; Sommerset et al. 2005) and herbal concoction was used in the therapy of *A. hydrophila* infection in goldfish (Harikrishnan et al. 2009).

V. CONCLUSION

This study demonstrated *A. hydrophila* as a potential pathogen, which can cause haemorrhagic septicaemia clinical signs in BSB. In the antimicrobial susceptibility test, both tested bacterial strains showed sensitivity to most used drugs.

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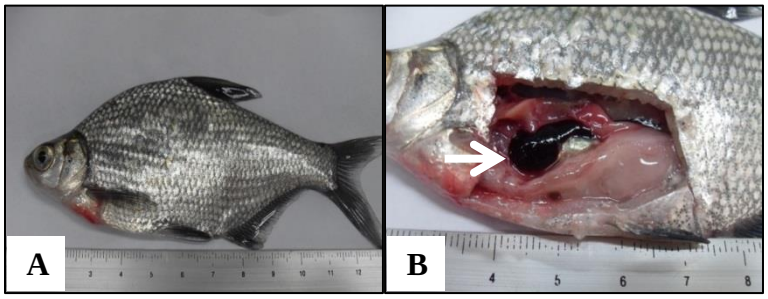

Fig. 1. Clinical signs of BSB (*M. amblycephala*) intraperitoneally challenged to *A. hydrophila* (1.7×10^7 cfu/fish). (A) Haemorrhage at the site of injection and ventral surface; (B) Red odiferous fluid, swollen and colour-deep spleen (arrow)

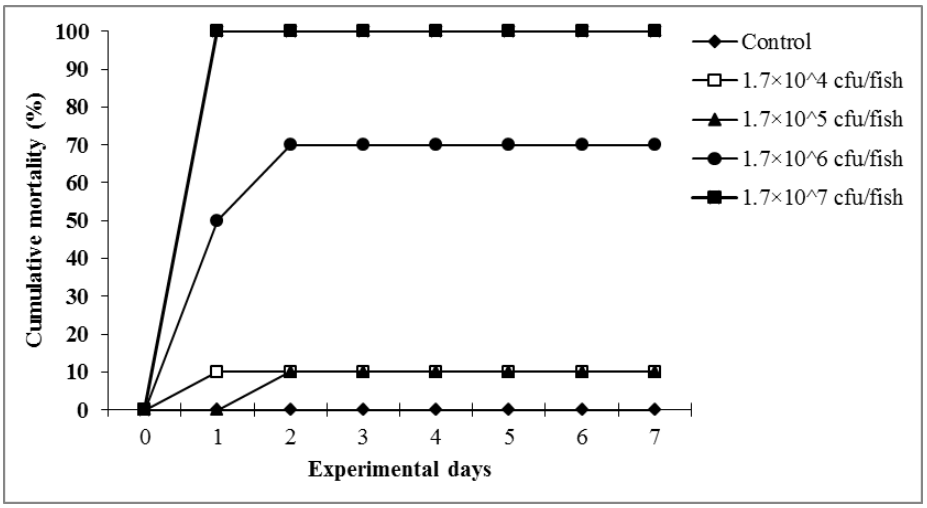

Fig. 2. Cumulative mortality of BSB (*M. amblycephala*) challenged with *A. hydrophila*

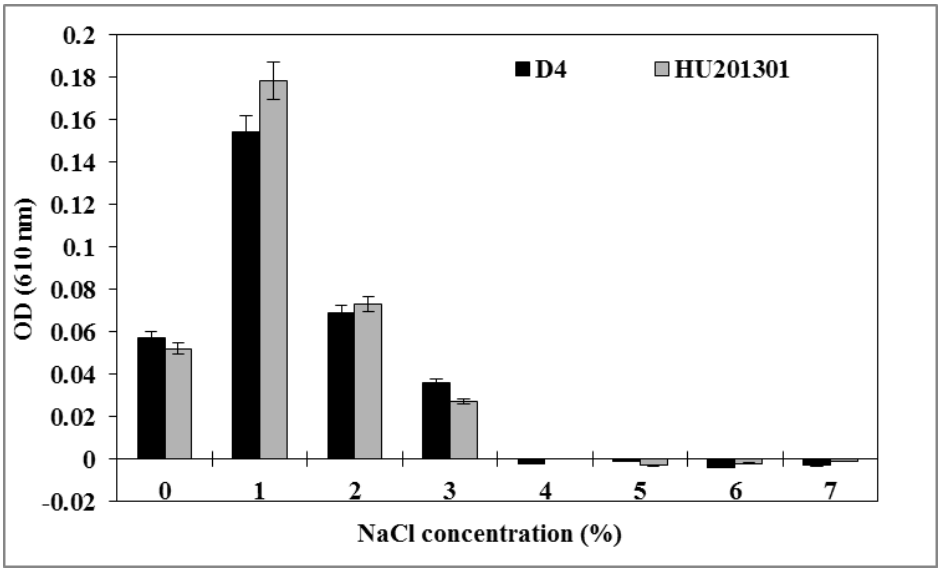

Fig. 3. Effect of NaCl concentrations (%) on growth of D4 and HU201301

Table 1. Biochemical profile of D4 and re-isolated strains of *A. hydrophila*

Biochemical test	D4	HU201301	HU201302	HU201303
Gram stain	-	-	-	-
Motility	+	+	+	+
Oxidase	+	+	+	+
Catalase	+	+	+	+

Glucose	+	+	+	+
Sucrose	-	-	-	-
Lactose	+	+	+	+
Arabinose	+	+	+	+
Mannitol	+	+	+	+
Salicine	-	-	-	-
MR	+	+	+	+
VP	+	+	+	+
Citrate utilization	+	+	+	+
Esculine hydrolysis	+	+	+	+
Starch hydrolysis	+	+	+	+
Indole production	+	+	+	+
H_2S production	+	+	+	+
AHM	+	+	+	+

(+) = positive reaction; (-) = negative reaction

Table 2. Antimicrobial susceptibility testing of experimental original strain (D4) and re-isolated strain of *A. hydrophila* HU201301

Antibiotic agents (µg/disc)	D4	HU201301
Amoxicillin (10)	R	R
Florfenicol (75)	S	S
Levofloxacin (5)	S	S
Tetracycline (30)	S	S
Neomycin (30)	S	S
Novobiocin (30)	S	S
Doxycycline (30)	S	S
Gentamicin (10)	S	S
Norfloxacin (10)	S	S
Aztreonam (30)	S	S
Fortum (30)	S	S
Kanamycin (30)	S	S
Ceftriaxone sodium (30)	S	S
Ofloxacin (5)	S	S
Piperacillin (100)	S	S
Clindamycin (2)	R	R
Rifampicin (5)	S	S
Imipenem (10)	R	I
Erythromycin (15)	S	I
Chloramphenicol (30)	S	S

R = resistance; I = Intermediate; S = sensitivity