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Full Length Research Paper

Grass carp (*Ctenopharyngodon idellus*) infected with multiple strains of *Aeromonas hydrophila*

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***Ctenopharyngodon idellus* is an important commercial fish species in China and many other countries to meet human food habits. However, there is no definitive data to indicate the multi-infection of pathogenic *Aeromonas hydrophila* strains in cultured *C. idellus*. In this study, four strong virulent strains with five virulence genes were simultaneously isolated from the cultured *C. idellus* suffering from septicemia, and identified as different *A. hydrophila* isolates using the ATB 32GN system, phylogenetic analysis, enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR). They were susceptible to chloramphenicol, tobramycin, kanamycin, norfloxacin, spectinomycin, furantoin, azithromycin, and resistant to carbenicillin, penbritin, clindamycin. In addition, to further control the multi-infection of *A. hydrophila*, norfloxacin, the well-known fishery drug widespread used in aquaculture, was employed to treat this disease. Its minimum inhibitory concentrations (MICs) were 2~4 mg/L and good protection effects were also exhibited on the naturally infected fish at a final concentration of 1 and 2 g/Kg in the feed (cal. 20 and 40 mg/Kg of fish). As an important pathogen of a zoonotic and foodborne disease, the multi-infection of *A. hydrophila* strains may be an emerging threat in grass carp farming and food safety, and more importance should be attached to their multi-infection in fish farming.**

Key words: *Ctenopharyngodon idellus*, *Aeromonas hydrophila*, multi-infection, control.

INTRODUCTION

The grass carp (*Ctenopharyngodon idellus*) is widely distributed and cultivated in Asia, Europe, North America (Beveridge, 1984). Especially in China, the grass carp has become an important commercial fish species with the production of above 3 million tons since 1999, accounting for 95.7% of its global production (Liu et al., 2009). However, the grass carp farming has been seriously affected due to diseases, especially aeromonasis, which has led to USD 0.3 billion of economic losses (Jang et al., 2010). Thus, aeromonasis, especially multiple infection of *Aeromonas* pathogens, should be brought more attention for the sustainable development of grass carp farming industry all over the world. *Aeromonas hydrophila* is a common opportunistic fish pathogen, which has been reported as a causative agent of outbreaks such as haemorrhagic septicemia, ulcer syndrome, motile *Aeromonas* septicemia (MAS), and enteritis (Shao et al., 2004; Zhang et al., 2006). These diseases caused by *A. hydrophila* are usually associated with its production of toxins such as cytotoxins, proteases, S-layers, aerolysins and haemolysins (Cahill, 1990). The single pathogenic *A. hydrophila* strain has been isolated from *Clarias gariepinus*, *Oreochromis niloticus*, *Pacifastacus leniusculus*, *Acipenser baeri*, respectively (Angka et al., 1995; Yambot, 1998; Jiravanichpaisal et al., 2009; Cao et al., 2010). However, there is no definitive data to indicate the multi-infection of *A. hydrophila* in cultured grass

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carps.

In this paper, four different strong virulent *A. hydrophila* strains were simultaneously isolated from the diseased grass carps suffering from septicemia in Xiantao, Hubei China, their phenotypic characterizations, taxonomic positions, genotypic analysis, virulence genes, antimicrobial susceptibility were examined, and the treatment of the multi-infection of *A. hydrophila* strains was also further conducted. As far as we know, this is the first report of farmed grass carp infected with multiple strains of *A. hydrophila*.

MATERIALS AND METHODS

Grass carp samples

Forty diseased grass carps (800±10 g in weight) were sampled and transported to the laboratory as described by Ruso (1987) from Dongsen Bioscience Co., Ltd. in Xiantao, Hubei China during June 2011, where 200,000 grass carps were farmed.

Isolation of bacteria

Each sampled moribund grass carp, 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, kidneys were cut to isolate and purify bacteria according to Song et al. (2011).

Identification of bacteria

Phenotypic identification using ATB 32GN system

The isolates were phenotypically identified using ATB 32GN system as recommended by Altwegg and Zollinger-Iten (1987). Briefly, the isolates were 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 API ID32GN strip (Bio-Merieux, SA) following the manufacturer's instruction. The strip was incubated at 28°C and observed after 48 h for checking against the API identification index and database.

Molecular identification

The genomic DNA extracts of the isolates, as well as their 16S rRNA genes' polymerase chain reaction (PCR) amplification and sequencing were performed according to Cao et al. (2010), then their partial 16S rRNA sequences were assembled using MegAlign, Editseq and Seqman software with a Power Macintosh computer. Searches were 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 sequences of the isolates and their homologous sequences was further constructed using neighbor-joining method.

Strain differentiation assay

The genotyping of the isolates was performed using enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) as recommended by Saxena et al. (2002) and Xiao et al. (2011), a useful tool for typing gram-negative and also gram-positive bacteria (Sampaio et al., 2006), and *A. hydrophila* ATCC7966 was used as the control bacterium.

Bacterial virulence assay

100 healthy grass carps (100±10 g in weight), evaluated by a careful exam of physical appearance and behavior as well as internal organs such as liver and kidney for bacterial pathogens, were obtained from Dongsen Bioscience Co., Ltd. in Xiantao, Hubei China, and were respectively maintained in 40 aquaria (10 grass carps per aquaria) supplied with 100 L de-chlorinated tap water at 25~28°C for 14 days. Prior to the bacterial virulence assay, the isolates' live cells were respectively prepared as recommended by Cao et al. (2010), and their cell densities were determined using the dilution and spread plate technique. 10 healthy grass carps were respectively injected intramuscularly as recommended by Iqbal et al. (1999) with 0.2 ml of the isolate's live cells at a final cell density of 1.0×10^8 cfu/ml. Another 10 healthy grass carps were injected with 0.2 ml of sterile saline as the control. The experimental grass carps were kept at 28°C and observed daily for 7 days. Dead grass carps were immediately removed for pathogen isolation according to Bucke (1989), and the signs and mortalities were recorded. Each experiment was conducted in two parallel. After the virulence assay, another 420 healthy fish were sampled to test the mean lethal dose (LD_{50}) value of the isolates at a final cell density of 10^4~10^8 cfu/ml, the LD_{50} values were calculated using the linear regression method described by Won and Park (2008). Each experiment was conducted in two parallel.

Virulence gene assay

The genomic DNA was extracted from the pure cultures of the isolates using a genomic DNA extraction kit following instructions of the manufacturer (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.). The virulence genes, including the aerolysin (*aerA*) gene, haemolysin (*hlyA*) gene, serine protease (*ahpA*) gene, cytotonic enterotoxin (*alt* and *ast*) genes, 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. hydrophila* strain S1, previously isolated from *Acipenser baeri* with septicemia and phenotypically, molecularly identified (Cao et al., 2010), and *Escherichia coli* DH5α were respectively taken as the positive and negative control bacteria. The specific primers for virulence gene amplification were listed in Table 1. The PCR products were electrophoresed on 1% agarose gel and visualized via ultraviolet trans-illumination.

Antimicrobial susceptibility assay

Susceptibility of the isolates to antimicrobial agents was assayed on NA plates using Kirby-Bauer disk diffusion method as recommended by Jones et al. (2001). Thirteen fishery drug discs were obtained from Hangzhou Tianhe Microorganism Reagent Co., Ltd. The diameters of inhibition zones against the isolates were measured and recorded after the incubation of 24 h. The susceptibility was determined following the susceptibility category criteria of the manufacturer.

MIC assay

The minimal inhibitory concentrations (MICs) of the susceptible drug against the isolates were determined as described by

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 Reverse: GTGTAGGGATTGAAGAAGCCG	331

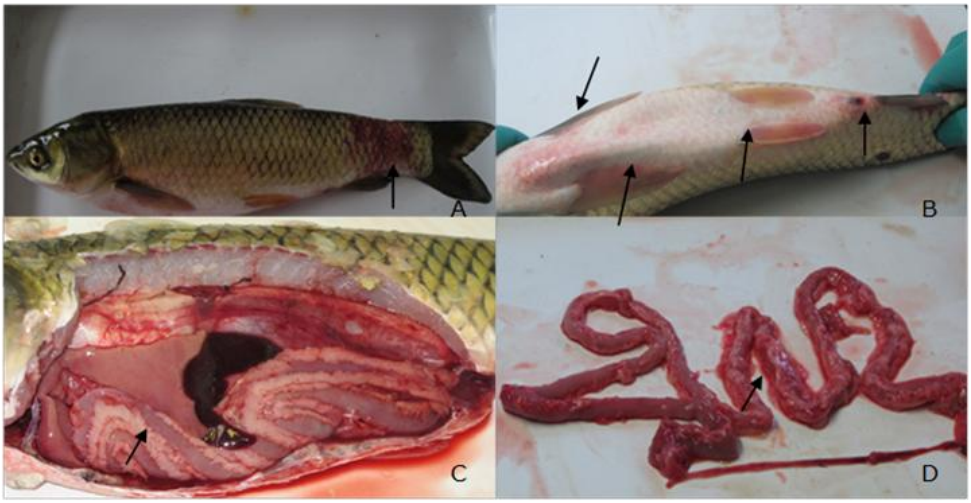

Figure 1. The pathological symptoms of the grass carps suffering from septicemia. A: arrow indicated the hemorrhage of the body; B: arrows indicated the hyperaemia of the pectoral fin, pelvic fin, abdomen, as well as the inflammation of the anus; C: arrow indicated the hemorrhage of the abdominal cavity; D: arrow indicated the hyperaemia of the intestine wall.

Bussmann et al. (2010). The growth of the isolates was observed after the incubation of 48 h, and the MIC was recorded as the lowest concentration that did not permit any visible growth.

Protection test

270 naturally infected grass carps were randomly placed in three 200 L tanks (30 fish per tank, three tank per group) for the three treatments (the control, low dosage and high dosage groups) described below. The tanks used recycled aerating farm water that was kept at 28°C throughout the experiment. Norfloxacin, obtained from Beijing Yujing Biotech. Co. Ltd., was manually incorporated into commercial dry pellets at a final concentration of 1 and 2 g/Kg in the feed (cal. 20 and 40 mg/Kg of fish) for low and high dosages, respectively. Fish were fed approximately 2% of body weight once a day. Fish fed only commercial dry pellets served as a control. Dead fish were immediately removed for pathogen isolation as described by Bucke (1989), and mortalities were recorded each day for 7 days.

RESULTS

Identification and genotyping of the isolates

80~100% of the challenged fish acutely died and showed the same clinical septicemia signs similar to the originally infected fish (Figure 1) after the injection of four isolates' cells (GCLB3, GCkB3, GCSB2, GCSB3), and no acute mortality or visible changes were observed in the control grass carps. The LD_{50} values of the four isolates were

Table 2. LD_{50} values of the four isolates.

Strain	Correlation regression equation between the logarithm of their cell densities (X) and mortalities (Y)	LD_{50} value (cfu/g)
GCLB3	Y=22X-96, R^2=0.9453	4.37×10^4
GCKB3	Y=20X-52, R^2=0.9346	1.26×10^3
GCSB2	Y=23X-90, R^2=0.9653	1.23×10^4
GCSB3	Y=20X-62, R^2=0.9804	3.98×10^3

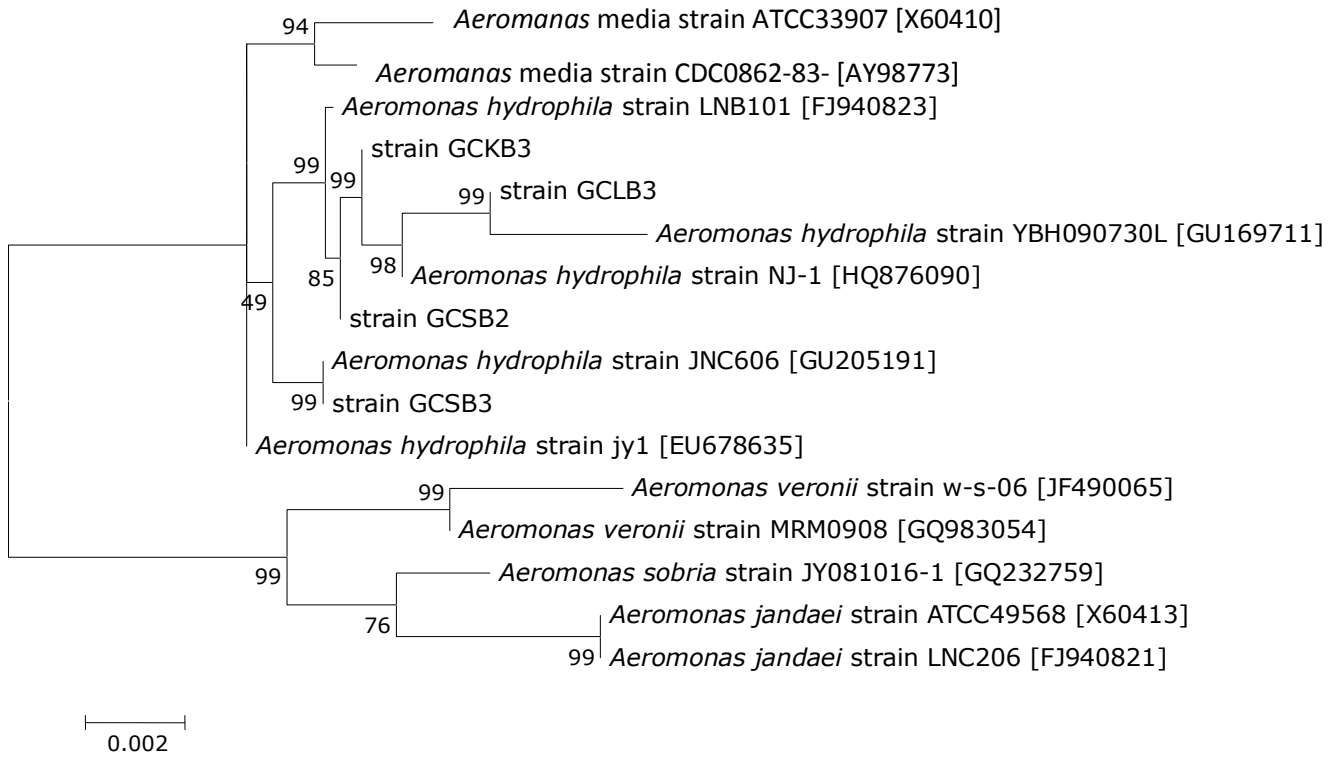

Figure 2. The constructed phylogenetic analysis of the four isolates (GCSB3, GCSB2, GCLB3, GCKB3) using neighbor-joining method.

further respectively tested to be 4.37×10^4, 1.26×10^3, 1.23×10^4 and 3.98×10^3 cfu/g according to the correlation regression equation between the logarithm of their cell densities and mortalities (Table 2), which confirmed the four isolates highly virulent according to Mittal et al. (1980).

The ATB 32GN system identified the four isolates as *A. hydrophila* strains (data not shown), and showed an identity of >99.1% with the type strain ATCC7966 in phenotypic characterization. However, there were some differences among the four strains in the characterization of Salicin, Caprate, L-Alanine and L-Proline, which revealed the characteristic diversity of the four isolates of *A. hydrophila*. In addition, the partial 16S rRNA sequences (ca. 1.4 kb) of the four isolates (strain GCLB3, strain GCkB3, strain GCSB2 and strain GCSB3) were submitted to GenBank database with the accession no. JN400039, JN400040, JN400041, JN400044. Similarities between the 16S rRNA sequences of the four strains and those of *A. hydrophila* strains in the GenBank database were 99~100%, which proved the initial identification. The constructed phylogenetic tree using neighbor-joining method further demonstrated the four isolates as *A. hydrophila* strains (Figure 2). However, the four different banding profiles were obtained from the four isolates (Figure 3), which indicated that the four isolates generated different genotypes.

Virulence genes of the isolates

The specific PCR amplification of virulence genes was

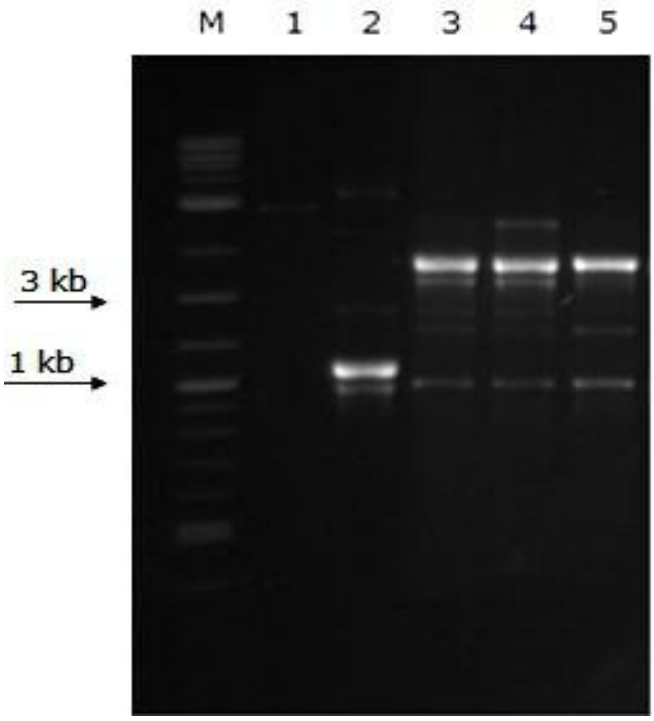

Figure 3. The enterobacterial repetitive intergenic consensus PCR fingerprint of the isolates. M: DNA marker, 1: isolate GCSB3, 2: isolate GCSB2, 3: isolate GCLB3, 4: isolate GCKB3, 5: the control strain ATCC7966.

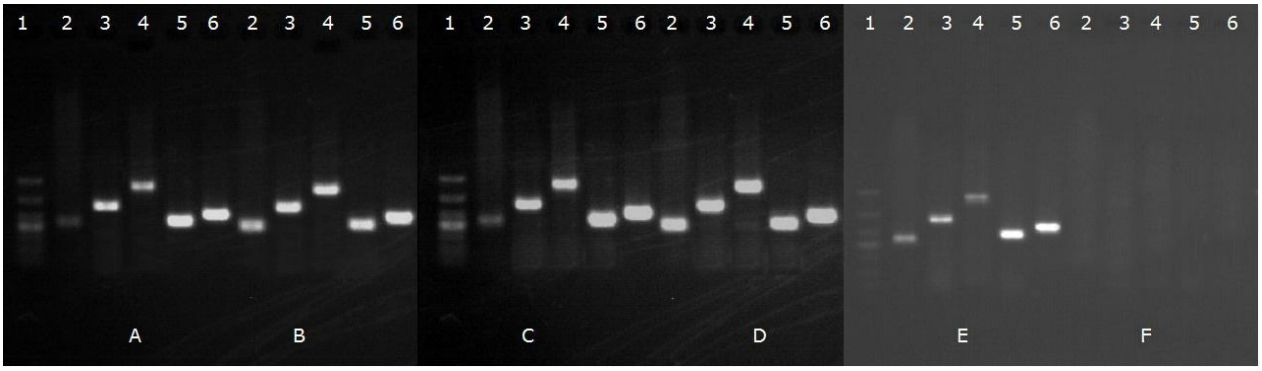

Figure 4. The PCR amplification of the four isolates' virulence genes. A: isolate GCLB3, B: GCkB3, C: GCSB2, D: GCSB3, E: the positive control strain S1; F: the negative control strain DH5α. Lane 1: DNA marker; lane 2: *aerA* gene, lane 3: *hlyA* gene, lane 4: *ahpA* gene, lane 5: *Ast* gene, lane 6: *Alt* gene.

shown in Figure 4. The specific virulence gene (*aerA*, *hlyA*, *ahpA*, *alt* and *ast*) fragments were obtained with strain GCLB3, strain GCkB3, strain GCSB2 and strain GCSB3 using a pair of *aerA*-specific primers, *hlyA*-specific primers, *ahpA*-specific primers, *alt*-specific primers and *ast*-specific primers, respectively, which was in accordance with that with the control *A. hydrophila* strain S1. The result demonstrated that the virulence genes (*aerA*, *hlyA*, *ahpA*, *alt* and *ast*) were all present in the four isolates.

Antimicrobial susceptibility

The susceptibility of the four isolates to antimicrobial agents was shown in Table 3. The result indicated that the four isolates were all susceptible to chloramphenicol, tobramycin, kanamycin, norfloxacin, spectinomycin,

Table 3. Susceptibility of the four isolates to antimicrobial agents.

Antimicrobial agent	Content (μg/disc)	Inhibiton zone diameter (mm)			
		GCSB2	GCSB3	GCKB3	GCLB3
Carbenicillin	100	0^{R}	7.0^{R}	6.5^{R}	9.0^{R}
Medemycin	30	13.5^{I}	12.5^{R}	16.0^{S}	13.0^{I}
Penbritin	100	8.0^{R}	7.5^{R}	10.5^{R}	9.0^{R}
Chloramphenicol	30	24.0^{S}	23.0^{S}	30.0^{S}	25.0^{S}
Tobramycin	10	17.5^{S}	17.0^{S}	16.5^{S}	16.0^{S}
Kanamycin	30	18.5^{S}	18.0^{S}	20.5^{S}	18.0^{S}
Furazolidone	300	16.0^{I}	16.0^{I}	18.0^{S}	17.5^{S}
Norfloxacin	10	31.0^{S}	34.0^{S}	36.0^{S}	25.0^{S}
Spectinomycin	100	31.0^{S}	28.0^{S}	33.0^{S}	23.0^{S}
Furantoin	300	17.5^{S}	18.0^{S}	20.0^{S}	21.0^{S}
Vancomycin	30	10.0^{R}	9.5^{R}	9.0^{R}	17.0^{S}
Clindamycin	15	0^{R}	9.5^{R}	8.0^{R}	9.0^{R}
Azithromycin	15	28.0^{S}	35.0^{S}	30.0^{S}	33.0^{S}

^{S}Susceptible; ^{I}Intermediate; ^{R}Resistant.

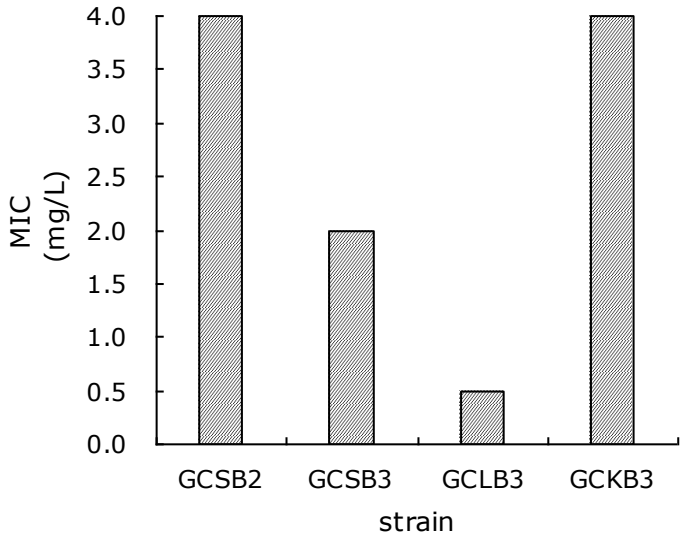

Figure 5. The minimal inhibitory concentrations of norfloxacin against the four isolates (GCSB3, GCSB2, GCLB3, GCKB3).

furantoin, azithromycin, and resistant to carbenicillin, penbritin, clindamycin. Only 25% of these isolates were susceptible to vancomycin, and 50% were intermediate susceptible to furazolidone and medemycin. Due to the effectiveness and well-known widespread use of norfloxacin in aquaculture, norfloxacin was chosen for further study.

MIC determination

The MICs of norfloxacin against the four isolates were shown in Figure 5. The MIC values of norfloxacin varied by the isolates, ranging from 0.5 to 4 mg/L. The lowest MIC value (0.5 mg/L) was recorded in strain GCLB3, and followed by strain GCSB3 (2 mg/L), GCSB2 (4 mg/L) and strain GCKB3 (4 mg/L). According to National Committee for Clinical Laboratory Standards (NCCLS) (2002), MIC of norfloxacin ≤4 mg/L was considered as susceptibility to the isolate. Thus, the four isolates' susceptibility to norfloxacin was consistent with that found through Kirby-Bauer disk diffusion method (Jones et al., 2001).

Protective effect

The effect of norfloxacin on naturally infected grass carps was shown in Figure 6. The cumulative mortality was 75.64% lower in the high dosage group than that in the control group, and the cumulative mortality was also 39.45% lower in the low dosage group than that in the control group. The death of all the test fish observed in the control group was caused by *A. hydrophila*, as determined by bacterial isolation and ATB 32GN system (data not shown). The result exhibited the protective effect of norfloxacin against multi-infection of *A. hydrophila* strains in naturally infected grass carps.

DISCUSSION

Bacterial septicemia is responsible for millions of USD in annual losses to the cultured freshwater fish industry in the US, China and other countries (Shoemaker et al., 2002; Chen et al., 2011). Thus, more attention should be paid to its pathogen and control. So far, several virulent bacteria such as *Edwardsiella ictaluri*, *Edwardsiella tarda*,

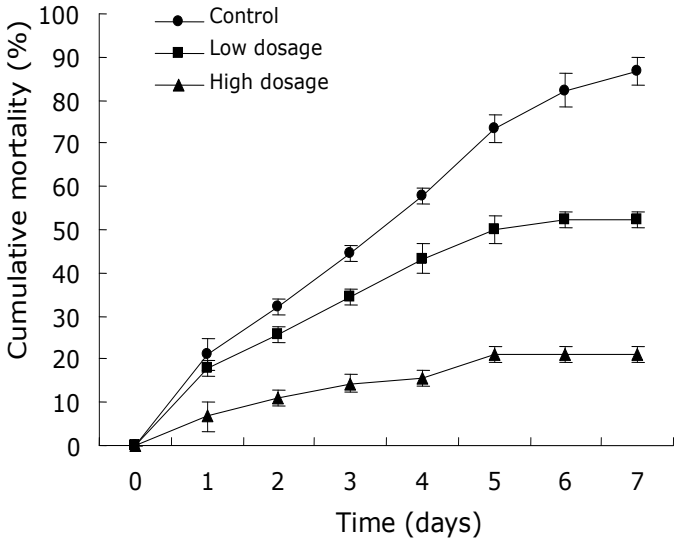

Figure 6. The protective effect of norfloxacin on the naturally infected grass carps, using a final concentration of 20 and 40 mg/Kg of fish as low and high dosages.

Aeromonas hydrophila have been reported to cause septicemia of the farmed *Ictalurus punctatus*, *Oreochromis niloticus, Acipenser baerii* Brandt (Wolters and Johnson, 1995; Pirarat et al., 2007; Cao et al., 2010). However, no relevant information is available about the multi-infection of different virulent *A. hydrophila* strains in the grass carps. In this study, we simultaneously isolated four highly virulent strains of *A. hydrophila* from the cultured grass carps with septicemia, assayed their phenotypic characteristics, taxonomic positions, virulence genes, genotypic analysis and antimicrobial susceptibility, and conducted the treatment of the multi-infection of *A. hydrophila*. As far as we know, this is the first report of farmed grass carp infected with multiple strains of *A. hydrophila*.

The pathogenesis of *Aeromonas* infections is complex and multi-factorial with the involvement of a number of virulence factors, including extracellular enzymes, cytotonic enterotoxins, haemolysins, etc. (Daskalov, 2006). Previous reports showed that the occurrence of genes encoding haemolysin, aerolysin, serine protease, cytotonic enterotoxins (*hlyA, aerA, ahpA, alt* and *ast*) may contribute to the virulence of *Aeromonas* pathogens, and *Aeromonas* isolates with *hlyA, aerA* and *ahpA* genes were confirmed as strong virulent strains (Wong et al., 1998; Sha et al., 2002; Zhu et al., 2006). In the present study, the four isolates were highly virulent to the healthy grass carps (Table 2) and contained the five virulence genes (Figure 4), which was in accordance with the previous findings (Wong et al., 1998; Sha et al., 2002; Zhu et al., 2006). Apart from pathogenicity of these *A. hydrophila* isolates, there might be other causes for the incidence of bacterial septicemia such as high densities of fish, lack of food disinfection and water degradation (Cao et al., 2010), that should also be raised concerns.

The excellent performance and accuracy of ATB 32GN system allows its good use in routine identification of gram-negative bacteria in the clinical laboratory (Altwegg and Zollinger-Iten, 1987). For example, Lamy et al. (2010) identified 51 (100%) isolates at the species level using ATB 32GN system. In the present study, the four isolates were also correctly identified as distinct *A. hydrophila* strains using the ATB 32GN system for their differentiation in phenotypic characterization possibly due to genetic changes (Rosselló-Mora and Amann, 2001) (data not shown). Additionally, in order to make a better understanding of their taxonomic positions, molecular phylogenetic studies based on the gene sequences was conducted as recommended by Lee et al. (2000). The identification result from phylogenetic analysis (Figure 2) was consistent with that found through the ATB 32GN system (data not shown), and simultaneously revealed the remarkable heterogeneity among the different *A. hydrophila* isolates (Figure 3), which agreed with the previous finding obtained by Rivera (2008).

Multiple antibiotic resistance among *A. hydrophila* strains have been reported from many parts of the world, as a result of wide use of antibiotics to treat their infections and incorporation of subtherapeutic dose of antibiotics into feeds for cultured organisms (Pettibone et al., 1996; Son et al., 1997; Ko et al., 1998). The four isolates also exhibited multiple resistances to human and veterinary drugs, including carbenicillin, penbritin and clindamycin (Table 4). Thus, it was worthwhile to find out the prevalence of antibiotic resistance of *A. hydrophila* pathogens. However, the four isolates were susceptible to the common fishery drugs such as norfloxacin (Table 4, Figure 5), and the mortality of the naturally infected fish was totally controlled when norfloxacin treatment was applied (Figure 6), suggesting that the outbreak of the disease should not be resulted from the abuse of fishery antimicrobial agents. A decrease in dissolved oxygen concentration and an increase in ammonia have been shown to promote stress in fish and trigger *A. hydrophila* infections (Abulhamd, 2010). Thus, when the treatment was stopped, it is essential to fully monitor these environmental variables to avoid their stress in fish and aeromonasis outbreak. At the same time, the proper use of norfloxacin in the grass carps should also be insured because it might contribute to inhibit other necessary gram-negative bacterium groups in the body, and using it for a long time might result in drug resistance and threaten human food security.

In conclusion, the present study for the first time reported a multi-infection of *A. hydrophila* and its control. The pathogenicity and virulence genes presence of the isolates, as well as their antibiotic resistance to several human and veterinary drugs, supported this multi-infection as an emerging threat in grass carp farming and food safety.

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