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Bacteriology

Molecular characteristics, pathogenicity and medication regimen of *Aeromonas hydrophila* isolated from common carp (*Cyprinus carpio* L.)

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ABSTRACT. *Aeromonas hydrophila* causes disease in fish known as Motile *Aeromonas* Septicemia (MAS), also named as bacterial hemorrhagic septicemia. In this study, a pathogenic *A. hydrophila* strain was isolated from common carp *Cyprinus carpio* L., which were suffering from severe hemorrhagic septicemia. According to the phylogenetic analysis derived from 16S rDNA sequence, the isolate formed a single branch in the *A. hydrophila* group, named AhHN1. Artificial infection results indicated that AhHN1 showed strong pathogenicity in *C. carpio* and the LD_{50} was 1.38 $\times 10^6$ CFU/fish, the clinical symptoms and pathological features of infected fish were similar to those observed in natural infections. The antimicrobial susceptibility testing revealed that AhHN1 resistance to more than 13 kinds of antimicrobial agents. However, the AhHN1 strain exhibited an extremely sensitivity to enrofloxacin, the *in vitro* activities of enrofloxacin were subsequently investigated and drug selection window (MSW) was 0.0016–0.0125 µg/ml. Pharmacokinetics data showed that plasma concentration of enrofloxacin was 0.0016, 0.0148 and 0.0282 µg/ml at 24 hr after orally administered with 2.5, 5 and 10 mg/kg enrofloxacin. Moreover, dosing once a day of 2.5, 5 and 10 mg/kg enrofloxacin, which the relative protection ratio (RPS) was amounted to 33.3, 66.7, and 83.3%, respectively. Therefore, 5 mg/kg enrofloxacin was considered to be the rational regimen for controlling AhHN1 infection in *C. carpio* in the countries where the use of enrofloxacin is permitted in aquaculture. The aim of this study was to establish a scientific medication regimen for the prevention and therapy of the mutidrug-resistant *A. hydrophila* infection.

KEY WORDS: *Aeromonas hydrophila,* common carp (*Cyprinus carpio* L.), enrofloxacin, medication regimen

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Common carp (*Cyprinus carpio* L.) belongs to Cyprinidae, is undoubtedly one of the most cultured fish all over the world and accounts for about 10% (over 3 million metric tons) of global annual freshwater aquaculture production [1]. However, frequent outbreaks of infectious diseases pose a serious threat to the further development of the rapidly expanding carp production industry, which not only caused profound economic losses to fishery production, also affected the quality and safety of aquatic products, as well as human health [10].

Aeromonas hydrophila is a Gram negative bacterium that widely distributed in aquatic environments [7] and an opportunistic pathogen for fish, reptiles, amphibians and humans, which is capable of causing severe hemorrhagic septicemia and skin ulceration in aquatic animals and diarrhea in mammals [16, 19]. It was known that *A. hydrophila* caused motile *Aeromonas* septicemia (MAS), also named as bacterial hemorrhagic septicemia. It is defined as worldwide distributed septicemic disease that affecting numerous species of freshwater and marine fishes, such as carp (*C. carpio & Carassius auratus*) [5, 8, 24], grass carp (*Ctenopharynodon idellus*) [13], channel catfish (*Ictalurus punctatus*) [19] and tilapia (*Oreochromis niloticus*) [17], leading to massive mortalities of wild and farmed fish.

Antimicrobial agents considered to be the most important factors influencing the emergence of resistance in bacterial pathogens. However, the inappropriate overuse of antimicrobial agents in the aquaculture industry has led to the development of bacteria resistance, thereby reducing drug efficacy and affect treatment outcomes for infectious diseases [2, 23]. Recently, *A. hydrophila* display a notable trend of resistance to commonly used antimicrobial agents, multidrug resistant *A. hydrophila* were isolated from different parts of the world [11, 21]. Moreover, the accumulation of antimicrobial agents in animals and water ponds can

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be potentially risky to human health and water environment [12]. Therefore, there should be a continuous and concerted effort to control and stimulate the proper use of antibiotics in aquaculture.

In this study, we observed a bacterial disease occurred in cultured common carp (*C. carpio* L.) and the bacterial pathogen was identified by morphological features and 16S rDNA analysis. Based on the antimicrobial susceptibility analysis, the activities of enrofloxacin and the pharmacokinetics parameters of enrofloxacin were investigated, a rational medication regimen to prevent drug resistant bacteria was proposed for controlling AhHN1 infection in *C. carpio*. This study will provide a scientific reference to countries where the use of enrofloxacin is permitted in aquaculture, and provide partial protection against infection with drug-resistant pathogens by drawing a specific dosage guideline of antimicrobial agents.

MATERIALS AND METHODS

Fish

Diseased common carp (*C. carpio* L.) were collected from a local fish farm. The typical disease signs were external haemorrhages, inflammation and ulcers. Healthy *C. carpio* L. were obtained from the breeding farm in Hebi city of Henan province, average weight 20 ± 5 g. Fish were acclimated in 250 *l* aerated water with pH 8.0 ± 0.2, dissolved oxygen 7 ± 0.5 mg/*l*, water hardness 20 ± 1 mg/*l* $CaCO_3$, total ammonia 0.006 ± 0.001 mg/*l*, and nitrite 0.03 ± 0.01 mg/*l*. Temperature was maintained at 25 ± 2°C. Fish were fed twice daily for two weeks before experiments. All procedures and handling of fish were conducted in compliance with the guidelines of the Institutional Laboratory Animal Care and Use Committee, Henan Normal University.

Isolation and identification of bacteria

Gill, intestine, kidney and liver tissues of diseased *C. carpio* were excised and homogenized in 1 m*l* of 0.85% saline. The samples were plated on brain heart infusion (BHI) plates incubated at 28°C for 24 hr. The morphological features of bacterial colonies were observed on plates, dominant colonies were selected for bacteria identification. The purified isolates were amplified in BHI medium and DNA extraction was done with a DNA extraction kit (Sangon, Shanghai, China). Then, the bacteria were subjected to PCR with 16S ribosomal DNA primers (F: 5′-AGAGTTTGATCATGGCTCAG-3′ and R: 5′-CTACGGTTACCTTGTTACGAC-3′). The PCR reaction system contains 1 *µl* of template DNA, 1 *µl* each of 16S rDNA sense and anti-sense primers, 2.5 *µl* of dNTP, 2.5 *µl* 10X PCR Buffer (Mg^{2+} plus), 0.25 *µl* of Ex Taq DNA polymerase and RNase-Free Water in a final volume of 20 *µl*. PCR was carried out for 30 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min with a final extension at 72°C for 10 min. The PCR products were analyzed on 1.5% agarose gel electrophoresis, purified and sequenced in Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). The BLAST search was analysed at the National Center for Biotechnology Information website (NCBI, http://www.ncbi.nih.gov/BLAST/). Phylogenetic tree was constructed using the neighbor-joining method in the software MEGA 5.05.

Pathogenicity assays

Isolated *A. hydrophila* strain (AhHN1) was cultured in BHI medium for 18 hr at 28°C. An appropriate amount of bacterial culture was centrifuged at 12,000 rpm for 5 min, the pellet was washed twice with 0.85% saline and re-suspended in saline, the optical density at 600 nm (OD_{600}) was adjusted to 0.6. LD_{50} dose used for challenge was determined by intraperitoneal injection into *C. carpio* at 25°C. In brief, healthy fish were randomly divided into five groups, each group contained 20 fish. Fish were challenged with 0.1 m*l* *A. hydrophila* suspension (5-fold dilution series of doses ranging from 2×10^6 to 2.5×10^8 CFU/m*l*) intraperitoneally, the control group were injected with an equal volume of saline. *C. carpio* started to show infectious symptoms after 12 hr and had a consistent mortality rate within 48 hr, no further death occurred. The infected fish showed external hemorrhages in fins, which is the typical symptom of *A. hydrophila* infection. Deaths were observed up to 7 days and the LD_{50} was calculated by Reed-Muench method [22].

MIC and MBC determination

For antimicrobial susceptibility testing, 14 antibacterial agents including enrofloxacin, Balofloxacin, florfenicol, gentamicin, kanamycin, streptomycin, amoxicillin, ampicillin, penicillin G, ceftriaxone, neomycin sulfate, sulfamethazine, sulfamethoxazole and sulfadiazine were chose. The minimum inhibitory concentration (MIC) for AhHN1 were determined using the broth microdilution protocol following VET04-A2 guideline of Clinical and Laboratory Standards Institute (CLSI) [15]. In brief, two-fold dilutions of antimicrobial agents were added to 96-well plates with adjusted bacterial concentration (about 1×10^5 CFU per well), negative control contained only inoculated BHI medium at 28°C for 24 hr. The MIC is the lowest concentration of antimicrobial agents that visually inhibits the growth of microorganisms. *A. hydrophila* ATCC 7966 was reference strain in parallel with AhHN1 for quality control (QC) purposes. The MICs were determined by the broth microdilution method wtih *A. hydrophila* ATCC 7966 and AhHN1 on three different days, each tray contained three biological replicates. Thus, each strain produced 9 MIC results for each antimicrobial agent, the mean MIC was calculated.

For minimum bactericidal concentration (MBC) determination, 0.1 m*l* of culture solution from all wells in the MIC broth dilution assay were plated on BHI agar plates, were incubated for 18 hr at 28°C. The colonies on the agar was counted and the lowest concentration of reagents that could kill 99.9% of the bacteria was determined as the MBC. Three biological replicates for each antimicrobial agent were performed.

MPC and MSW determination

The determination of the mutation preventive concentration (MPC) was described previously [4]. Briefly, a single colony of AhHN1 was inoculated on BHI for overnight, 0.1 m*l* cultures contained about 3 × 10^9 CFU were plated on BHI agar plates containing 1–10 folds MIC enrofloxacin, and each drug concentration had three plates, incubated at 28°C overnight. To estimate the MPC of enrofloxacin, logarithms of bacterial numbers were plotted against enrofloxacin. The MPC was taken as the minimum drug that completely inhibited growth. According to the hypothesis of the mutant selection window (MSW) [26], the concentration range from the MIC to the MPC, within which it is proposed that resistant mutants are enriched or selected.

Plasma sample collection

To detect plasma concentrations of enrofloxacin, the fish were divided into three groups, enrofloxacin was administered orally at 2.5, 5, 10 mg/kg body weight, as described previously [27]. Following dosing, blood samples were collected periodically via a needle, and heparin-treated syringe at 0, 0.5, 1, 3, 6, 9, 12, 24, 48 and 72 hr after administration of enrofloxacin, 3 fish were sampled at each time point. Blood samples were centrifuged at 3,500 rpm for 15 min at 4°C and the plasma was then collected and stored at −20°C until analyzed.

Ultraperformance liquid chromatography-mass spectrometry (UPLC-MS) for detection of enrofloxacin

Concentrations of enrofloxacin in plasma was measured on an Acquity UPLC system (Waters, Milford, MA, U.S.A.), and the separations were achieved using an Acquity UPLC BEH C18 column (1.7 *µ*m particle size, 50 mm ×2.1 mm). Separations were performed using binary gradient mobile phases, consisting of acetonitrile (eluent A) and 0.1% formic acid in water (eluent B) at a flow rate of 0.3 m*l*/min. The separation was performed at 40°C, applying the following gradient program: 0–1 min, 5% A; 1–4 min, linear increase to 90% A; 4–6 min, 5% A. The samples were kept in an autosampler at 16°C. The mass spectrometry analyses were carried out using a Quattro Micro triple quadrupole mass spectrometer equipped with an electrospray ionization source (ESI) (Waters, Milford, MA, U.S.A.). The mass spectrometer was settled in the positive ion mode (ES+), with a capillary voltage of 0.5 kV, cone voltages of 30 V, collision energies of 19 eV, a source temperature of 120°C and a desolvation temperature of 500°C. The nitrogen gas flow was 800 *l*/hr and 50 *l*/hr for desolvation and cone, respectively. Data acquisition was performed using MassLynx V4.1 software with the Quanlynx program.

Enrofloxacin was obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Stock standard solution of enrofloxacin was prepared by dissolving 10 mg compound in 10 m*l* of acetonitrile to obtain a final concentration of 1 mg/m*l*. Working standard solution was prepared in the range 0.001–10.00 *µ*g/m*l* by diluting the stock standard solutions in 50:50 acetonitrile: water. The precursor and product ions of enrofloxacin were 360.2 and 316.1, the standard curves constructed by plotting the area of enrofloxacin against the working standard concentrations of enrofloxacin (0.001, 0.01, 0.1, 1, 5 and 10 *µ*g/m*l*). The calibration curves were obtained and the sample concentration was calculated by comparing peak area with external calibration curve.

C. carpio infection and enrofloxacin therapy

Healthy *C. carpio* were randomly divided into four groups, 20 fish for each groups, each group of test fish were challenged with 0.1 m*l* 5 × 10^7 CFU/m*l* AhHN1. After 1 hr, three test groups including 2.5, 5 and 10 mg/kg of enrofloxacin were administered orally once a day for three consecutive days, respectively. The control group was administered with an equal volume of saline. After treatment with enrofloxacin, the mortality of the infected fish was observed every 12 hr for 7 days. The protective ability of the drug was calculated using the equation: drug protective rate=[1−(Mortality rate of drug administration group / Mortality rate of the control group)] × 100%.

RESULTS

Morphologic and molecular characteristics

The pathogenic bacteria isolated from the four tissues of the diseased fish, colonies on BHI plates were slightly yellow pigmented, circular, convex, smooth, and about 1 mm in diameter after incubation for 24 hr at 28°C (data not shown).

For molecular characterization, a phylogenetic tree was constructed based on BLAST search between isolated strain and other homologous sequences. From Fig. 1, the 16S rDNA sequence of isolated strain showed above 99% similarity with various species of *A. hydrophila*. Combined with the morphologic and molecular characteristics, the isolated strain was identified as *A. hydrophila,* named AhHN1.

Pathogenicity for C. carpio

The pathogenicity of AhHN1 strain was confirmed in common carp *C. carpio* by artificial infection. After challenged with AhHN1 strain, the infected fish showed external hemorrhages in fins or intestine and death occurred at 12 hr in high dose groups (5 × 10^7 CFU/m*l* and 2.5 × 10^8 CFU/m*l* groups), the symptoms were similar to those observed in natural infections. The 48-hr mortality rate was 10 to 100% in four infection groups, respectively. However, there was no death or no sign of disease in fish infected with saline (Fig. 2). According to the cumulative method, the LD_{50} of AhHN1 was 1.38 × 10^6 CFU/fish after experimental infection to *C. carpio.*

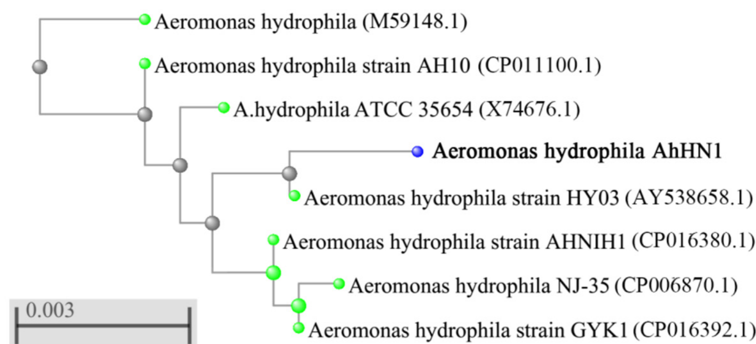

Fig. 1. Phylogenetictree of isolated strain based on 16S rDNA nucleotide sequences. The scale bar represents 0.003 substitutions per site. The tree was generated using neighbour-joining method by the MEGA software and displayed the high similarities between isolated strain and *Aeromonas hydrophila* group.

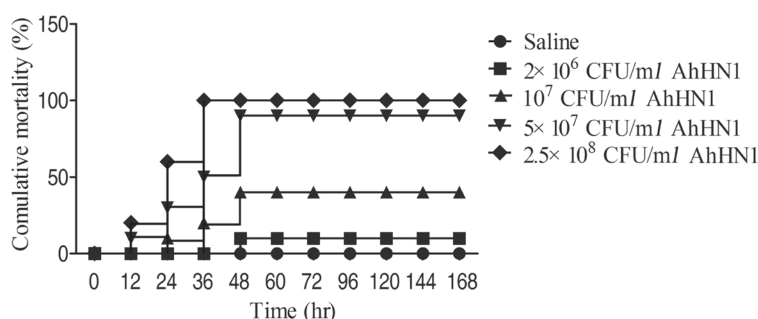

Fig. 2. The cumulative mortality rates of *Cyprinus carpio* after challenged with AhHN1 strain.

Antimicrobial susceptibility

The susceptibility pattern of *A. hydrophila* AhHN1 to 14 antibacterial agents was measured using microdilution method and *A. hydrophila* ATCC 7966 was used in this study as a control strain. The MIC and MBC results showed that AhHN1 was resistant to balofloxacin, florfenicol, gentamicin, kanamycin, streptomycin, ceftriaxone and neomycin sulfate, compared with control strain. However, *A.hdrophila* including AhHN1 and control strain, was highly resistance to amoxicillin, ampicillin, penicillin G, sulfamethazine, sulfamethoxazole and sulfadiazine (MIC ≥64 μg/m*l*), we speculated that *A. hdrophila* can harbour the corresponding antibiotic resistance genes. However, AhHN1 showed extremely sensitive to enrofloxacin in all tested antibacterial agents, the MIC and MBC was 0.0016 μg/m*l* and 0.0031 μg/m*l*, respectively (Table 1). These results indicated that AhHN1 is a typical multidrug resistant bacteria and enrofloxacin maybe an effective antibacterial agent for the prevention and treatment of this infectious disease.

Activities of enrofloxacin on AhHN1 in vitro

The *in vitro* activities of enrofloxacin were subsequently investigated. Based on the MIC and MBC determination, we detected the MPC and MSW of enrofloxacin to AhHN1. MPC values should be considered in drawing dosing strategies since traditional MIC-based dosing level might give rise to treatment failure due to the selection of drug-resistant mutant. The MPC of enrofloxacin against AhHN1 was determined as 8 MIC (0.0125 μg/m*l*). The MSW of enrofloxacin on isolate AhHN1was determined as 0.0016–0.0125 μg/m*l*, which reflected the difference between the measured MIC and MPC values. Existence of the MSW allowed us to predict that the likelihood for resistance selection or prevention based on achievable and therapeutic drug concentration.

Pharmacokinetics of enrofloxacin in common carp

UPLC-MS presents a simple, rapid, and reliable analytical method for detection and quantification of enrofloxacin. The plasma concentration-time curves for enrofloxacin was shown in Fig. 3. The maximum concentration (C_{max}) was 0.5824 and 0.9326 μg/m*l* at 1 hr after orally administered of 2.5 and 5 mg/kg enrofloxacin, respectively. High dose (10 mg/kg) reached the peak level with 1.3620 μg/m*l* in a shorter time (0.5 hr). Twenty-four hr post drug delivery, plasma concentration of enrofloxacin was 0.0016, 0.0148 and 0.0282 μg/m*l* for the three doses, respectively. For 5 and 10 mg/kg group, the plasma concentration of enrofloxacin was above the MPC value till 24 hr, while in 2.5 mg/kg group, the plasma concentration of enrofloxacin fell inside the MSW from 9–24 hr. However, enrofloxacin levels of 2.5 mg/kg group at 48 and 96 hr treatment were below the limit of detection (<0.0015 μg/m*l*).

Table 1. Antimicrobial susceptibility patterns of AhHN1 and *Aeromonas hydrophila* ATCC 7966

Antimicrobial agents	MIC (µg/m*l*)		MBC (µg/m*l*)	
	Aeromonas hydrophila ATCC 7966	AhHN1	*Aeromonas hydrophila* ATCC 7966	AhHN1
Enrofloxacin	0.25	0.0016	0.5	0.0031
Balofloxacin	1.56	6.25	1.56	6.25
Florfenicol	0.5	8	2	32
Gentamicin	0.5	4	1	8
Kanamycin	0.5	4	1	8
Streptomycin	0.25	64	0.5	128
Amoxicillin	256	512	>512	>512
Ampicillin	64	64	>512	>512
Penicillin G	256	256	>512	>512
Ceftriaxone	0.156	5	0.312	10
Neomycin sulfate	0.125	2	0.5	8
Sulfamethazine	256	256	>512	>512
Sulfamethoxazole	128	128	>512	>512
Sulfadiazine	128	128	>512	>512

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration.

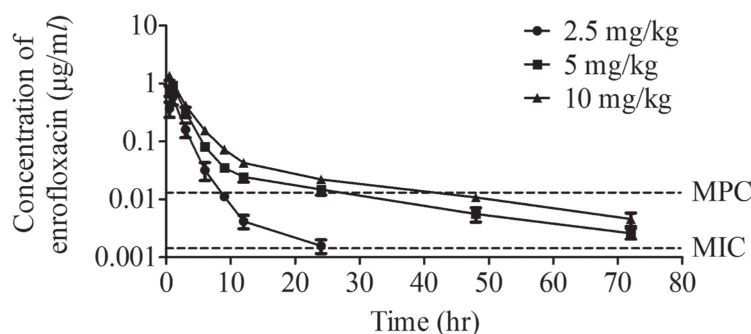

Fig. 3. Plasma enrofloxacin-time curves in plasma of *Cyprinus carpio* at different administration doses.

Medication regimen of enrofloxacin

In order to study the efficacy of enrofloxacin, oral dose of 2.5, 5 and 10 mg/kg enrofloxacin was chose in this study. Healthy *C. carpio* were infected with 5×10^7 CFU/m*l* AhHN1, then treatment with different dose of enrofloxacin, the results showed that the relative protection rates of 2.5, 5 and 10 mg/kg of enrofloxacin were 33.3, 66.7, and 83.3%, respectively (Fig. 4). Compared with control, the death time of fish in the administration group was postponed at least 12 hr. Combined with pharmacokinetics of enrofloxacin, dosing once a day of 5 mg/kg was determined to be the rational regimen for controlling AhHN1 infection and preventing mutant selection in common carp.

DISCUSSION

In the past decades, the genus *Aeromonas* has received great attention in fish and human [7]. The taxonomy of *Aeromonas* has been extended since the development of biochemical and molecular techniques, these species can be accurately identified. In this study, a *A. hydrophila* strain was isolated from the diseased common carp *C. carpio*. In fact, *A. hydrophila* is also commonly occurred in species inpond and river waters [18]. The pathogenicity was confirmed in *C. carpio* by artificial infection and similar symptoms were observed, which verified that the isolated *A. hydrophila* AhHN1 strain showed highly pathogenicity to *C. carpio*.

Reports of growing bacterial resistance to drugs in aquaculture were revealed [22], and which thus are necessary for antimicrobial susceptibility testing to guide clinical medicine. In this study, the isolates of AhHN1 showed serious drug resistant to 3 kinds of penicillins, established MICs for these drug were over 64 µg/m*l*. These discovery attributed to the fact that *Aeromonads* spp. produce different β-lactamases, which confer resistance to a broad spectrum of β-lactam antibiotics [3]. Several studies demonstrated that all investigated *A. hydrophila* strains were resistant to ampicillin [6, 21]. Furthermore, AhHN1 also showed highly resistance to sulfonamides, balofloxacin, florfenicol, gentamicin, kanamycin, streptomycin, ceftriaxoneand neomycin sulfate. These results indicated that the isolated strain is a typical multidrug resistant *A. hydrophila*. However, among all tested antimicrobial agents, AhHN1 only showed sensitivity to enrofloxacin, whether the isolated strain is sensitive to other antibiotics,

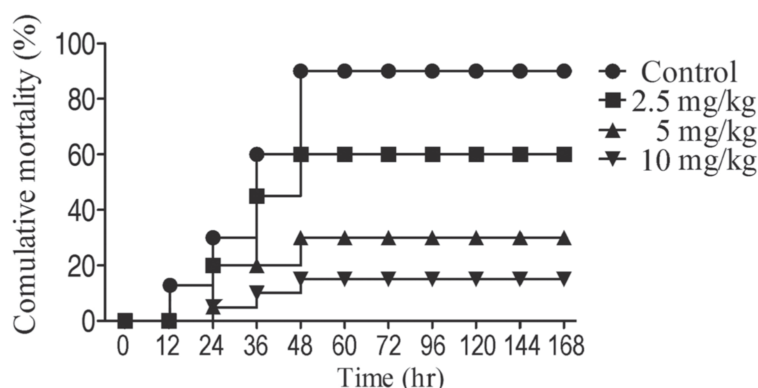

Fig. 4. The cumulative mortality rates of *Cyprinus carpio* after treatment with different dose of enrofloxacin.

such as erythromycin and oxytetracycline, requires further verification. Furthermore, enrofloxacin can be used for treatment and prevention of bacterial disease caused by AhHN1. Enrofloxacin, as the first of the fluoroquinolones approved for use in animals, was approved in the late 1980s. Martinez *et al.* reported that the newer quinolones such as enrofloxacin are among the most important antimicrobial agents for treatment of severe and invasive infections in human and animals [14]. However, enrofloxacin are strictly banned in some countries such as Japan, Canada, Scotland, Vietnam, etc and allowed for use in Chinese and Thailand aquaculture. Thus, the use of fluoroquinolones in aquaculture should, therefore, always be carefully considered and controlled by the food and drug national authority. The aim of this study was to establish a scientific medication regimen to reduce the use of enrofloxacin and prevent drug resistant bacteria in the countries where the use of enrofloxacin is permitted in aquaculture environment.

In general, the plasma concentration was the key factor for drug effectivity, AUC_{24}/MIC >100 and C_{max}/MIC >8 predicted a clinical outcome of enrofloxacin [9]. Based on the UPLC-MS results, C_{max}/MIC=364, 583 and 850 at 2.5, 5 and 10 mg/kg of enrofloxacin group, respectively. These data were similar with PK parameters of enrofloxacin in grass carp *C. idellus* [25]. Furthermore, to determine the enrofloxacin therapy efficacy *in vivo*, three dosage of enrofloxacin treatment significantly improved the survival rate of carp, especially 5 and 10 mg/kg groups. However, the recommended dose of enrofloxacin for Atlantic salmon, rainbow trout, sea bass and sea bream is 10 mg/kg [20]. According to the MSW hypothesis, resistant mutants are selected or enriched at antibiotic concentrations above the MIC but below the MPC. This assumption can be used to estimate the optimal dose, because many traditional dosing regimens may constitute misuse of antimicrobial agents. To determine a rational therapeutic guideline, treatments should maximize the time during which enrofloxacin concentrations above the MPC. In our study, plasma concentration of enrofloxacin above MPC was observed at 9, 24 and 24 hr for the three doses, respectively. At the dose of 2.5 mg/kg of enrofloxacin, the plasma concentration of enrofloxacin fell inside the MSW from 9–24 hr, that resistant mutants may enriched selectively. Thus, a dosage of 5 mg/kg was a safe dose, might achieve effective therapeutic result for controlling bacterial disease in *C. carpio*, and also reduce the occurrence probability of enrofloxacin-resistant.

In conclusion, the drug parameters (MIC, MBC, MPC and MSW) of *A. hydrophila* AhHN1, isolated from common carp *C. carpio* were characterized. Our approach for combining antimicrobial susceptibility with drug parameters (including MPC and MSW), provided a more scientific and effective strategy to face the challenge of drug-resistant bacteria by drawing a specific dosage guideline of antimicrobial agents.

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