Isolation, identification and antimicrobial susceptibility of pathogenic *Aeromonas media* isolated from diseased Koi carp (*Cyprinus carpio koi*)

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Abstract

Aeromonas infections are the most common bacterial disease of cultured fish in China. In this study, a gram-negative bacillus was isolated from the liver of diseased koi carp (*Cyprinus carpio koi*), and named strain KC-2. The results of morphological and biochemical tests, as well as phylogenetic analysis derived from *16S rRNA* and *gyrB* sequences indicated that the isolated strain KC-2 was highly identical to the known *Aeromonas media* ATCC 33907. Experimental infection assays were conducted, and pathogenicity was demonstrated in crucian carp (*Carassius auratus*) and zebrafish (*Danio rerio*). Antimicrobial susceptibility testing showed that the strain KC-2 was sensitive to cefalotin, cefixime, cefotaxime, gentamicin, netilmicin, azithromycin and chloramphenicol. This is the first report on the isolation and identification of *A. media* from diseased, cultured koi fish. The results of the study will provide a scientific reference for prevention of bacterial disease of koi carp and identification of *A. media* in fish.

Keywords: Koi carp, *Aeromonas media*, Isolation, Identification, Antimicrobial susceptibility testing

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Introduction

Koi (*C. carpio koi*) are well-known common carp (*C. carpio*) that belong to the Cyprinidae family. The koi are ornamental varieties of domesticated common carp that have high ornamental and economic values (Blasiola and Earle-Bridges, 1995). In recent years, bacterial pathogens from ornamental carp have increasingly occurred and caused large economic losses in China (Li *et al*., 2010; Jiang, 2012; Han *et al.*, 2013; Shen *et al*., 2014). Of these, the species of *Aeromonas* are most commonly involved in the aetiology of a variety of fish and human diseases (Austin and Adams, 1996; Rahman *et al*., 2002; Janda and Abbott, 2010; Liu and Li, 2012). To date, there are few reports of *A. media* associated with aquatic animals (Gu *et al.*, 2001; Wang *et al*., 2007; Huang *et al*., 2013). Gu *et al*. (2001) reported that the pathogenic *A. media* was isolated from the zebra mussel (*Dreissena polymorpha*). Wang *et al*. (2007) showed that *A. media* was the etiologic agent of the skin ulceration disease of sea cucumber (*Apostichopus japonicus*). Recently, a bacterial strain YZ-2 was isolated from naturally infected Chinese sucker (*Myxocyprinus asiaticus*), it was then identified as the *A. media* by morphology, chemical characters and 16S rDNA sequence analysis (Huang *et al*., 2013). However, there are no reports on isolation and characterization of *A. media* from the koi carp. In October 2012, an epizootic disease occurred in koi carp farm in eastern China, which was found to be caused by *A. media* infection. The suspected fish were presented with clinical signs of anal dilatation with hyperemia, ulcers and petechial hemorrhages on the abdomen, skin and base of fins, and scales fall off. To our knowledge, this is the first report on the isolation and identification of *A. media* from diseased, cultured koi fish, *C. carpio koi*. This study will provide a scientific reference for prevention of bacterial disease of *koi* carp and identification of *A. media* in fish.

Materials and methods

Fish

Moribund koi carp were collected from a fish farm in Xuzhou City, China. The typical disease signs were external haemorrhages, inflammation and ulcers. Healthy crucian carp of approximately 50 g in weight (n=12) with no history of disease were used in experimental infections, and wild zebrafish with an average weight of 0.5 g (n=20) were bought from a local fish supplier. Prior to infection, based on species, fish were separately kept for 15 days in aquaria with aeration. They were fed with commercial dry feeds of 1.0 to 2.0 mm sizes (Tongwei for crucian carp and

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Fengnian for zebrafish, respectively) distributed by hand once daily. Water was replaced daily and maintained at 26°C.

Isolation, characterization and identification of bacteria

In October 2012, an epizootic disease occurred in koi carp (*C. carpio koi*) farm in Xuzhou City, Jiangsu Province, eastern China. All suspected fish were presented with clinical signs of anal dilatation with hyperemia, ulcers and petechial hemorrhages on the abdomen, skin and base of fins, and scales fall off. Bacteria were isolated from the liver of diseased koi carp and the samples were streaked on tryptic soy agar (TSA) plates incubated at 28 °C for 24 h, according to Bergey′s manual (Martin-Carnahan and Joseph, 2005). Presumptive positive colonies for *Aeromonas* were biochemically characterized and identified using commercial microtest systems (Hangzhou Tianhe Microorganism Reagent Co., Ltd, China). Pure cultures of the KC-2 strain were subjected to standard tests, comprising motility, Hugh and Leifson's fermentation test (O/F), gas from glucose, production of catalase and oxidase, hydrogen sulfide (H_2S) production, growth in KCN, methyl red test, Voges-Proskauer test, nitrate reduction, gelatin hydrolysis, urea hydrolysis, lysine decarboxylase, ornithine decarboxylase, arginine dehydrolase, phenylalanine deaminase, ONPG test , indole production, esculin hydrolysis, citrate-Simmons, gluconate, and acids from adonitol, arabitol, dulcitol, mannitol, sorbitol, cellobiose, glucose, lactose, melibiose, raffinose, sucrose, xylose, etc. The test system was incubated at 28°C and the final readings were made after 7 days (Shen *et al*., 2014).

Gram staining of the isolated strain was carried out using the standard Gram reaction and observations made before performing the biochemical tests (Lü *et al.*, 2011). The bacterial strain was cultivated in TSA medium and scanning electron microscopy (SEM) observation was conducted under the HITACHI S-3400N SEM at experimental teaching centre of morphology, Xuzhou Medical College. Bacterial cells were prepared as described by Allen *et al*. (1983).

Antimicrobial susceptibility testing

The susceptibility pattern of bacterial isolate was performed using the standard Kirby-Bauer method (Bauer *et al*., 1966) on Mueller-Hinton agar. The 31 different antimicrobial agents used viz., ampicillin, piperacillin, amoxicillin, cefalexin, cefoperazone, cefalotin, cefixime, cefotaxime, amikacin, kanamycin, neomycin, gentamicin, netilmicin, clarithromycin, erythrocin, azithromycin, norfloxacin, ofloxacin, bacteresulf, trimethoprim,

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sulfamethoxazole/trimethoprim, lincomycin, clindamycin, teicoplanin, vancomycin, chloramphenicol, tetracycline, rifampicin, nystatin, furantoin, metronidazole. Mueller-Hinton agar plates were prepared and commercially available antimicrobial discs were purchased from Hangzhou Tianhe Microorganism Reagent Co., Ltd, China. The plates were incubated at 28°C for 24 h and observed for susceptibility. The zones of inhibition were recorded for all the plates and determination was made as to whether the strains were susceptible, intermediately susceptible or resistant to each antibiotic evaluated using standard criteria. The assay was conducted in triplicate for the strain evaluated.

16S rRNA and gyrB genes analysis

Total genomic DNA of the isolate was extracted from the bacterial cultures in TSB broth by proteinase K digestion in lysis buffer at 55°C for 15 min, following the manufacturer's instructions for the UNIQ-10 column genomic DNA extraction kit (Sangon, China). The nearly full-length 16S rRNA and gyrB genes from extracted DNA were amplified with the bacterial universal primers (Table 1), which were synthesized by Shanghai Sangon Biological Engineering Technology, Ltd. The PCR reaction mixture in a final volume of 25.0 µL consisted of 50 ng genomic DNA, 0.5 µM of each primer, 1.5 mM $MgCl_2$, 200 µM dNTPs, and 0.625 U Taq DNA polymerase (Sangon, China). The amplifications were carried out in a thermal cycler (Gene AMP 9700) with the following parameters: an initial denaturation step at 94°C for 5 min; 35 serial cycles of 94°C for 1 min, primer annealing at 55°C for 30 s and extension at 72°C for 90 s; and a final extension step of 72°C for 10 min (Mirbakhsh *et al*., 2014). The PCR products of about 1500 bp and 1200 bp for *16S rRNA* and *gyrB* genes, respectively, were evaluated by electrophoresis in 1% agarose gel by staining with ethidium bromide.

The PCR products were purified and cloned into pMD18-T (TaKaRa) to transform *E. coli* (DH5a) competent cells. The positive clones were sequenced by Sangon (China). The BLAST search was done at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nih.gov/BLAST/). Alignment was performed using CLUSTAL W method in MEGA 4.1 software. Phylogenetic trees were constructed using the neighbour-joining algorithm of MEGA4.1 software, with 1000 Bootstrap replicates.

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Table 1: The PCR primers for *16S rRNA* and *gyrB* genes.

Gene	Sequences(5'>3')	Product sizes (bp)
16S rRNA	Forward: AGTTTGATCATGGCTCAG Reverse: GTTACCTTGTTACGACTT	1509
gyrB	Forward: ACAACTCCTACAAGGTCTCCG Reverse: TCAGCAGCAGGGTACGGATGT	1215

Pathogenicity test

To test the pathogenic potential of isolate, the strain KC-2 was used for experimental infection of healthy crucian carp and zebrafish acclimatized to 26°C for 15 days prior to challenge. Assays of crucian carp and zebrafish were conducted in 100 L aquaria with 50 L water, and 10 L aquaria with 5 L water with aeration, respectively. Fish were divided into four groups, ie., six fish each for crucian carp and ten for zebrafish, and experimental infection was as previously described (Lü, *et al*., 2011). The infectivity trials in zebrafish were performed by immersion-challenged for 4 h with the KC-2 strain at the doses of approximately 2.0×10^8 CFU/mL, while the crucian carp pathogenicity assay was inoculated intraperitoneally with 0.2 mL of bacterial doses (2.0×10^8 CFU/mL) of the isolate. Clinical signs and mortalities were recorded everyday for 7 days post-infection. Morbid fish were subjected to laboratory examination and bacterial re-isolation. Control fish received PBS alone (Lü *et al*., 2011).

Results

Morphologic and biochemical characteristics

A bacillus was isolated from the liver of diseased koi carp, and tentatively named strain KC-2. Colonies on TSA are circular, convex, buff, moist, smooth, entire, and 1-2 mm in diameter after incubation for 24 h at 28°C. It was gram-negative, straight, nonsporeforming rod-shaped cells with rounded ends (Fig. 1). The biochemical characteristics of isolate are summarized in Table 2. The isolate was positive for oxidase, catalase, arginine dihydrolase, DNase, nitrate reduction and hydrolysis of gelatin, and negative for production of H_2S, Urea, ornithine decarboxylase. It showed a fermentative metabolism and produced acid and gas from glucose, acid from mannitol, sucrose, maltose, fructose, raffinose, trehalose, and mannose.

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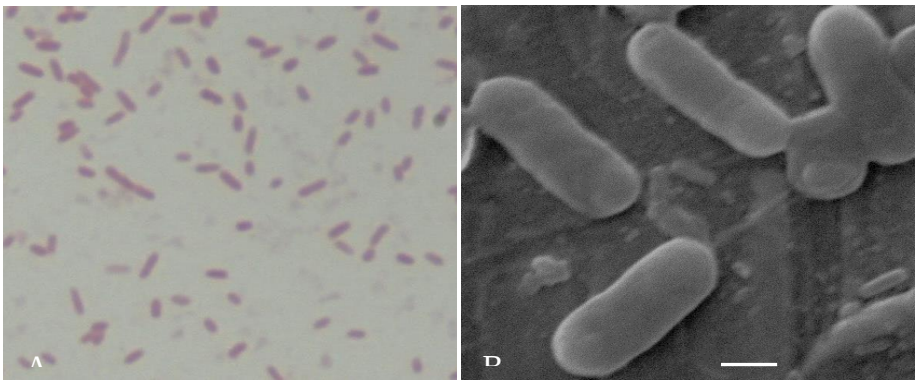

Figure 1: Gram staining (A) and SEM **cells (B) of strain KC-2 (Scale bar = 500 nm).**

Table 2: Biochemical characteristics of *Aeromonas media* isolated from koi carp.

Character	KC-2	*A. media*	Character	KC-2	*A. media*
Motility	-	v	Glucose (acid)	+	-
Catalase	+	+	Lactose	-	+
Oxidase	+	+	Galactose	±	-
O/F	F	F	Fructose	+	-
Indole	-	v	Sucrose	+	+
Urea	-	-	Raffinose	+	-
ONPG	+	+	Melibiose	-	-
Citrate	-	v	Cellobiose	-	v
Gluconate	+	-	Sorbose	-	nd
Ammonium dextrose	+	nd	Xylose	-	-
Malonate	-	-	Arabinose	-	+
KCN	-	v	Maltose	+	+
Voges–Proskauer	-	-	Rhamnose	-	-
Methyl red	-	v	Trehalose	+	+
Nitrate reduction	+	+	Mannose	+	+
H_2S	-	-	Mannitol	+	+
Lysine decarboxylase	-	-	Inositol	-	-
Ornithine decarboxylase	-	-	Sorbitol	-	-
Arginine dehydrolase	+	v	Arabitol	-	-
Phenylalanine deaminase	-	v	Dulcitol	-	-
DNase	+	v	Xylitol	-	nd
Gelatin	+	+	Erythritol	-	-
Esculin hydrolysis	-	v	Adonitol	-	-
Salicin	-	v	Starch	+	+
Amygdalin	-	-	Dextrin	+	nd
Glucose (gas)	+	-	5%NaCl	-	-

Note: "＋" is positive, "－" is negative, "±" is weak positive,"v" is variable (negative or positive), "nd" is not determined**.**

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Sequencing and phylogenetic tree analysis

Pathogenicity

The pathogenicity of *A. media* isolate KC-2 was confirmed in crucian carp and zebrafish by artificial infection. The mortality rates were 100% in which zebrafish were immersion challenged and crucian carp were intraperitoneally inoculated with the strain KC-2, respectively. In crucian carp, the clinical signs, lesions and microscopic signs produced by experimental inoculation were similar to those observed in natural infections, including petechial haemorrhages on the skin, at the base of the fins, an accumulation of ascites in the abdomen. In zebrafish, haemorrhages on the abdomen and base of fins, hyperaemia in the gills and anal dilatation with hyperemia were observed. The same bacterial strains were reisolated and identified from the moribund diseased fish. No control fish developed clinical signs or died, and no bacteria were reisolated.

Antimicrobial susceptibility

The susceptibility pattern of the isolate from 31 antibacterial agents was carried out (Table 3). The strain KC-2 was sensitive to cefalotin, cefixime, cefotaxime, gentamicin, netilmicin, azithromycin and chloramphenicol; while resistant to ampicillin, piperacillin, amoxicillin, cefalexin, cefoperazone, amikacin, kanamycin, neomycin, clarithromycin, erythrocin, norfloxacin, ofloxacin, bacteresulf, trimethoprim, sulfamethoxazole/trimethoprim, lincomycin, clindamycin, teicoplanin, vancomycin, tetracycline, rifampicin, nystatin, furantoin and metronidazole.

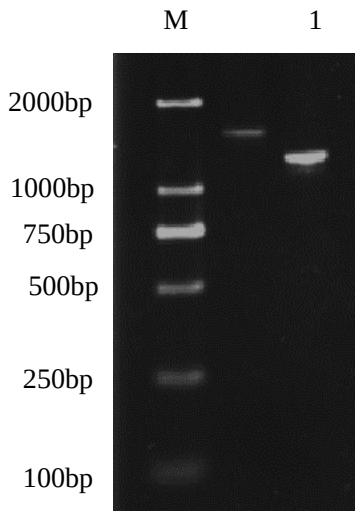

Figure 2: The electrophoresis result of PCR amplification of *16S rRNA* (Lane 1) and *gyrB* gene (Lane 2) form the strain KC-2. M is DL2000 DNA Marker.

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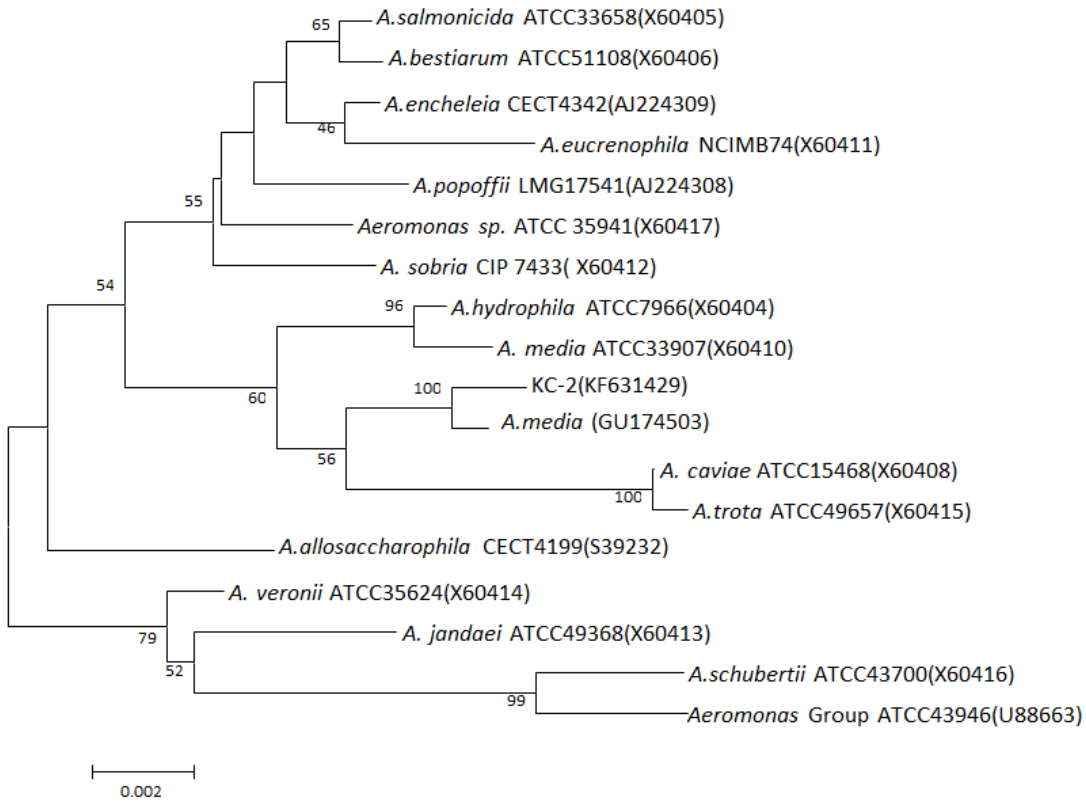

Figure 3: Phylogenetic tree analysis of *Aeromonas* species based on *16S rRNA* nucleotide sequences. Unrooted tree was generated using neighbour-joining method by the MEGA4.1 software. The numbers next to the branches indicate percentage values for 1000 bootstrap replicates. The scale bar represents 0.002 substitutions per site. Bootstrap values above 45% are shown at the nodes.

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Table 3: Antimicrobial susceptibility patterns of *A. media* strain KC-2.

Antimicrobial agents	Dose of antibiotic (µg)	Zone of inhibition (mm)	Susceptibility
Ampicillin	10	-	R
Piperacillin	100	-	R
Amoxicillin	10	-	R
Cefalexin	30	13	R
Cefoperazone	75	11	R
Cefalotin	30	19	**S**
Cefixime	5	31	**S**
Cefotaxime	30	34	**S**
Amikacin	30	13	R
Kanamycin	30	13	R
Neomycin	30	-	R
Gentamicin	10	17	**S**
Netilmicin	30	21	**S**
Clarithromycin	15	-	R
Erythrocin	15	13	R
Azithromycin	15	19	**S**
Norfloxacin	10	10	R
Ofloxacin	5	-	R
Bacteresulf	300	-	R
Trimethoprim	5	12	R
Sulfamethoxazole/trimethoprim	23.75/1.25	12	R
Lincomycin	2	-	R
Clindamycin	2	-	R
Teicoplanin	30	-	R
Vancomycin	30	-	R
Chloramphenicol	30	25	**S**
Tetracycline	30	8	R
Rifampicin	5	11	R
Nystatin	100	-	R
Furantoin	300	13	R
Metronidazole	5	-	R

Note: S is Susceptibility, R is Resistance**.**

Discussion

In the past decades, the genus *Aeromonas* has received great attention in fish and human (Austin and Austin, 1990; Austin and Adams, 1996; Janda and Abbott, 2010). *A. media* was first reported by Allen *et al*. (1983), which isolated from fish ponds and other aquatic sources (Gibson, *et al*., 1998). In fact, *A. media* was also commonly occurring species in

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pond and river waters (Figueira *et al*., 2011; Carvalho *et al*., 2012), which may be an important intracellular pathogen in fish (Kenzaka *et al.*, 2014). In October 2012, an epizootic disease occurred in koi carp farm in eastern China, which was found to be caused by *A. media* infection. In this study, the *A. media* strain KC-2 was isolated from diseased koi carp, and pathogenicity was confirmed in crucian carp and zebrafish by artificial infection. In accordance, Hu *et al*. (2012) showed that a pathogenic *A. media* strain NJ-30 was isolated from the gills of healthy crucian carp in Jiangsu Province, China, and the medium lethal concentration (LC_{50}) of 1.0×10^6 CFU/mL to zebrafish was observed by intraperitoneal injection. The pathogenicity of *A. media* isolates was present in fish, and the strain KC-2 studied is in agreement with previous results due to the geographical region of bacterial isolation from the eastern China (Hu *et al*., 2012). Interestingly, *A. media* has been shown to be a potential probiotic for the control of bacterial and fungal pathogens in aquaculture (Gibson *et al*., 1998; Lategan and Gibson, 2003; Lategan *et al*., 2004; Lategan *et al*., 2006). A bacteriocin-like inhibitory substance (BLIS) produced by *A. media* strain A199 inhibited the growth of *Saprolegnia sp. in vitro*, an opportunistic pathogen isolated from eels (*Anguilla australis*) (Lategan and Gibson, 2003). Chasanah *et al*. (2011) reported that *A. media* isolated from shrimp waste secreted chitosanase, and the chitooligosccharide produced by this enzyme was able to inhibit some pathogenic bacteria. Lategan *et al*. (2006) revealed that the indole produced by *A. media* strain A199 contained inhibitory activity against fish pathogens, which thus endowed A199 with its probiotic properties. In bacteria, the indole has been shown to cause toxicity by acting on cell membrane lipids causing derangement (Deslandes *et al*., 2001; Lategan *et al*., 2006). Gibson *et al*. (1998) reported that *A. media* strain A199 could prevent death of the oyster (*Crassostrea gigas*) larvae when challenged with *Vibrio tubiashii*. Lategan *et al*. (2004) concluded that *A. media* strain A199 is a potential agent for the control of winter outbreaks of saprolegniosis in eels (*A. australis*). *A. media* is widely distributed in aquatic environments, and non-pathogenic potential probiotic strains have been found in the diseased, healthy fish and waters (data not shown), but the mechanism of the probiotic effect provided by *A. media* is still unclear.

Today, the genus *Aeromonas* is regarded as an important disease-causing pathogen of fish and other coldblooded species (Janda and Abbott, 2010**)**. *Aeromonas* infections are the most common bacterial disease of cultured fish in China (Li *et al.*, 2010; Liu and Li,

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2012; Jiang 2012; Huang *et al*., 2013). As an opportunistic pathogen, *A. media* has been isolated from water environment, infected fish and human diarrheal stools (Singh, *et al*., 2000). Recently, the pathogenic *A. media* were sequentially isolated from the diseased fish (Gu *et al*., 2001; Wang *et al*., 2007; Huang *et al*., 2013), e.g., the abdomen hemorrhages and anal dilatation with hyperemi showed in the infected Chinese sucker (Huang *et al*., 2013), an *A. media* population observed in the moribund zebra mussels tissue (Gu *et al.*, 2001), and the skin ulceration disease in sea cucumber (Wang et al., 2007). Singh *et al*. (2000) reported that enterotoxigenic *A. media* from the skin ulcers of catfish produced a heat-labile and pH-stable enterotoxin. It was recently reported that the high-melanin-yielding *A. media* was associated with the virulence and pathogenicity (Chai *et al*., 2012; Wang *et al*., 2015). In this study, the strain KC-2 isolated from the liver of diseased koi carp was firstly identified as the pathogenic *A. media* by biochemical characteristics, *16S rRNA* and *gyrB* sequences analysis and pathogenicity tests. Wang *et al*. (2007) showed that *A. media* strain Y-1 was the causative agent of the skin ulceration disease in *A. japonicus*, with a mortality rate of 100% at the doses of 8.0×10^8 CFU/mL by artificial infection. The zebra mussels (*D. polymorpha*) were inoculated intraperitoneally with 10 μL doses of the *A. media* (about 10^7 CFU/mL), the mortality rate was 100% at 5 days post-infection (Gu *et al*., 2001). Therefore, experimental infection assays were conducted with the KC-2 strain 2.0×10^8 CFU/mL, and pathogenicity was demonstrated in crucian carp (*C. auratus*) and zebrafish (*D. rerio*), a cumulative mortality of 100% was observed at 2 days post-infection. The *A. media* strains were recovered from the infected fish that developed clinical signs of disease similar to those observed in natural infections. These results revealed a pathogenic *A. media* KC-2 strain to koi, crucian carp and zebrafish, which can cause great economic losses in aquaculture and ornamental fish breeding.

There are reports of growing bacterial resistance to drugs in aquaculture (Li *et al*., 2010; Jiang, 2012; Han *et al*., 2013; Shen *et al.*, 2014), and which thus are necessary for antimicrobial susceptibility testing to guide clinical medicine (Gu *et al*., 2001; Wang *et al*., 2007; Huang *et al*., 2013). Alcaide *et al*. (2010) determined the antibiotic resistance patterns of *Aeromonas* from freshwater and eels origin, and showed that *A. media* strains were resistant to quinolones. In this study, antimicrobial susceptibility testing for *A. media* isolate from diseased carp was performed using Kirby-Bauer disc diffusion method, and results showed that

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A. media strain KC-2 was also resistant to quinolones (norfloxacin and ofloxacin). It is suggested that the quinolone resistance is related to mutation of *gyrA* gene in *A. media* (Alcaide *et al.*, 2010; Figueira *et al*., 2011). Additionally, our data are in accordance with the other studies that found resistance to ampicillin and sensitivity to gentamycin and chloramphenicol in strains of *A. media* isolated from South Africa aquaculture systems and Russia Eltsovka-1 river (Jacobs *et al*., 2007; Carvalho *et al*., 2012; Lobova *et al*., 2015). Antimicrobial susceptibility tests showed that *A. media* strain Y-1 from sea cucumber (*A. japonicus*) was only susceptible to florfenicol (Wang *et al*., 2007), and strain YZ-2 from Chinese sucker (*M. asiaticus*) was highly sensitive to gentamicin, cephalothin and kanamycin, etc (Huang *et al*., 2013). Picao *et al*. (2008) reported that *A. media* strain A72 isolated from the activated sludge was sensitive to cefalotin, cefotaxime, and imipenem, etc. In our study, *A. media* strain KC-2 from the ornamental carp was susceptible to cefalotin, cefotaxime, gentamicin, azithromycin and chloramphenicol, which will contribute to the treatment of *A. media* infection in fish. Interestingly, there are differences in the antibiotic susceptibility patterns among the *A. media* isolates (Wang *et al*., 2007; Picao *et al.*, 2008; Huang *et al*., 2013), which may be associated with the bacterial sources, and clinical medicine practices in treatment of fish diseases.

In conclusion, the strain KC-2 was isolated from the liver of diseased koi carp, it was then identified as *A. media* on the basis of morphological and biochemical characteristics and phylogenetic analysis derived from 16S rRNA and gyrB housekeeping gene sequences. To our knowledge, this is the first description of the isolation or identification of *A. media* from ornamental fish, which will provide a scientific reference to prevention of bacterial disease of ornamental carp and identification of *A. media* in fish.

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