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First report of *Rahnella aquatilis* infection in crucian carp *Carassius auratus* in China

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ABSTRACT: *Rahnella aquatilis* infection is rare in aquaculture. Here, a Gram-negative rod-shaped bacterium was isolated from diseased crucian carp *Carassius auratus* in Xuzhou City, Jiangsu Province, eastern China. The isolate was tentatively named strain KCL-5, and subsequently identified as *R. aquatilis* by biochemical properties and molecular techniques. The results showed that the isolate KCL-5 was most closely related to the type strain ATCC33071 (= DSM4594) of *R. aquatilis*, which shared 99.67, 96.26 and 99.58% nucleotide sequence identities for *16S rDNA*, *gyrB* and toxin *yhaV* genes, respectively. Experimental challenges were conducted which demonstrated pathogenicity of the isolate in crucian carp. Antimicrobial susceptibility testing showed that the isolated strain was susceptible to piperacillin, gentamicin, kanamycin, nalidixic acid, norfloxacin, ofloxacin, azithromycin and erythromycin. To our knowledge, this is the first report on *R. aquatilis* infection in crucian carp, and the first evidence of pathogenicity in fish.

KEY WORDS: *Carassius auratus* · *Rahnella aquatilis* · 16S rDNA · gyrB · YhaV · Antimicrobial susceptibility

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INTRODUCTION

Rahnella, which was firstly recognized in 1976 (Gavini et al. 1976, Izard et al. 1979), is a very typical representative of the *Enterobacteriaceae*. *R. aquatilis* can usually be isolated from water (Gavini et al. 1976, Brenner et al. 1998), soil (el-Hendawy et al. 2003, Guo et al. 2012) and clinical infections (Farmer et al. 1985, Tash 2005, Gaitán & Bronze 2010). As an opportunistic pathogen, *R. aquatilis* has primarily been isolated from blood, faeces and sputum in humans (Goubau et al. 1988, Maraki et al. 1994, Menasalvas et al. 1996, Carinder et al. 2001) and has been associated with surgical wound infections, diarrhoea and endocarditis (Funke & Rosner 1995, Matsukura et al. 1996, Reina & Lopez 1996). In addition, the protein toxin YhaV of *R. aquatilis* can cause reversible bacteriostasis and has endonuclease activity *in vitro* (Schmidt et al. 2007). Recently, *R. aquatilis* was found in the frog *Rana temporaria chensinensis* in China (Xue et al. 2013). However, *R. aquatilis* has never before been reported to cause disease in fish.

Crucian carp *Carassius auratus* is one of the most important farmed freshwater species in China (Chen et al. 2007, Xu et al. 2013). In this study, a bacterial strain was isolated from diseased fish and identified as *R. aquatilis* by standard conventional methods including microscopic observations, physiological and biochemical tests, and *16S rDNA* and *gyrB* gene sequencing. Additionally, the *yhaV* gene, encoding a toxin with endonuclease activity, was amplified by polymerase chain reaction (PCR) using specific primers (Lindberg et al. 1998). Antimicrobial susceptibility testing was carried out to determine the degree of sensitivity or resistance to different antibiotics of the pathogen isolated from crucian carp. This report

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represents the first published case of *R. aquatilis* isolated from crucian carp, and the first report of *R. aquatilis* pathogenicity for fish. Consequently, this study will be helpful in describing and understanding *R. aquatilis*-induced disease in fish.

MATERIALS AND METHODS

Fish

Diseased crucian carp (n = 10; each weighing ca. 100 g) were collected from a fish farm in Xuzhou City, Jiangsu Province, in eastern China, from June to August 2014. The typical clinical signs were skin haemorrhages, mainly located on the pectoral fin base, caudal fin and parts of the body. Mortality levels of ~40% were found in the naturally infected fish. The diseased fish gave negative results for virus isolation performed as described by Zhang & Gui (2008). Briefly, a carp leucocyte cell line was used for viral detection tests. The cells were grown in 199 medium (Sigma) supplemented with 5% foetal bovine serum for 7 d at 25°C. Healthy crucian carp with no history of disease, which were collected from the Fisheries Experimental Centre of Henan Normal University, China, were used in experimental infections. Prior to infection, crucian carp were acclimatized for 7 d in aquaria with aeration. A 12:12 h light:dark period was maintained, and fish were fed with commercial feed (Tongwei) once daily. Water was replaced daily and maintained at 26 ± 2°C.

Isolation and culture of bacterial pathogen from diseased crucian carp

Samples of internal organs (liver, kidney, spleen) were collected from the diseased fish and streaked onto separate Luria-Bertani (LB) agar plates for each tissue under a sterile environment and incubated at 28°C for 18 to 24 h as previously described by Sreedharan et al. (2011). All bacterial colonies obtained in the original plates of affected fish had identical morphology. Three colonies selected from the liver, kidney and spleen were obtained, respectively. Single colonies were picked and restreaked 3 times to ensure purity. The Gram staining test was conducted by the Hucker method, and the morphological characteristics of the 3 colonies were observed on a microscope before performing physiological and biochemical tests (Doetsch 1981).

Physiological and biochemical tests

The biochemical identification tests were performed using a micro-bacteria biochemical test system (Tianhe) including motility, dextrin, gelatinase, lactose, oxidase, glucose, ornithine decarboxylase, lysine decarboxylase, arginine dihydrolase, methyl red, Voges-Proskauer, glucose (gas), phenylalanine deaminase, nitrate reductase, H_2S production, glucosamine, citrate, gluconate, malonate, urease, esculin and indole. The micro-bacteria biochemical test tube was incubated in an incubator at 28°C for 48 h. The results of the test were interpreted following Holt et al. (1994).

16S rDNA, *gyrB* and *yhaV* gene sequence analysis

Total genomic DNA of the 3 colonies was extracted using the UNIQ-10 column genomic DNA extraction kit (Sangon) according to the instructions of the manufacturer. The *16S rDNA* and *gyrB* fragments were amplified by a PCR kit (BioTeke) using the purified genomic DNA as template. The universal primers 27F (5′-AGA GTT TGA TCA TGG CTC AG-3′) and 1492R (5′-TAC GGT TAC CTT GTT ACG ACT T-3′) (T_m = 55°C) were used to amplify *16S rDNA* (Lane 1991). A pair of specific primers was used in the *gyrB* PCR reactions, gyrB-F2 (5′-CTC ACT TAG CCG GTT TCC GT-3′) and gyrB-R2 (5′-CAG CAA CAG GGT ACG GAT GT-3′) (T_m = 55°C), which was designed by Primer5.0 software based on the known *Rahnella aquatilis* genome sequence (GenBank accession no. CP003244) (Martinez et al. 2012), and their specificity was tested by NCBI Primer-Blast software. The endonuclease activity gene *yhaV* was amplified using primers YhaV-F3 (5′-AAC AAA CGG TAC TGC TGG-3′) and YhaV-R3 (5′-ACC CTT CTC GCT CAT CCT-3′) (T_m = 50°C) (Lindberg et al. 1998). For the 3 genes, PCR amplification was performed as follows: preheating at 94°C for 2 min; followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C or 50°C for 30 s and elongation at 72°C for 1 min; and a final extension step at 72°C for 10 min. The PCR products were visualised by electrophoresis in a 1.0% agarose gel by staining with ethidium bromide.

The PCR products were purified using a QIAquick PCR purification kit (Qiagen) and cloned into pMD18-T (TaKaRa) to transform *Escherichia coli* (DH5a) competent cells. Three positive clones from the original 3 colonies were sequenced in both directions by Shanghai Sangon, China. A homology search with the re-

sulting sequence data was performed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and ClustalX software was used for multiple sequence alignment analysis. Phylogenetic trees were constructed using the neighbour-joining method of MEGA5.0 software (Tamura et al. 2011), and support for grouping of strains was determined with 1000 bootstrap replicates.

Experimental challenge

All procedures involving the handling and treatment of fish used during this study were approved by the Henan Normal University Institutional Animal Care and Use Committee (HNU-IACUC) prior to initiation. Ninety apparently healthy crucian carp were used for challenge experiments. Experimental fish were confirmed to be culture negative for bacterial infection by culturing liver and kidney samples from representative groups of fish on LB plates. Prior to experiments, 5 fish were maintained in each 30 l tank (filled with 20 l of water) in a water recirculation system with a hydrocyclone prefilter (Hailea). Exponential-phase bacteria grown in shaking flasks at 28°C in LB broth were pelleted and resuspended in sterile phosphate-buffered saline (PBS) to achieve a final concentration of 1.0×10^{10} CFU ml^{-1}. The bacterial concentration was determined using CFU ml^{-1} by plating 10 µl of 10-fold serial dilutions onto LB agar plates. Fish were infected by intraperitoneal (i.p.) injection with 0.2 ml of the bacterial suspensions. The i.p. 50% lethal dose (LD_{50}) was calculated on the total cumulative mortality (%) by the method of Reed & Muench (1938). For LD_{50} determination, 15 fish per dilution were injected with a suspension of strain KCL-5 at 10^6, 10^7, 10^8, 10^9 and 10^{10} CFU ml^{-1}. Meanwhile, 1 control group was injected with 0.2 ml of sterile 0.65% physiological saline. Triplicate tanks (5 fish per tank) were used for each group within the same experiment. During the trial, fish were offered feed once daily at a rate of 3% of their body weight (Tongwei). The experimental challenge was allowed to run for 14 d, and data on time of death, morbidity and numbers of fish dying were recorded.

Antimicrobial susceptibility test

The antimicrobial sensitivity of strain KCL-5 was tested with 30 different antibiotics (Tianhe) including kanamycin, sulphamethoxazole, gentamicin, sulphamethoxazole/trimethoprim, amoxicillin, netilmicin, chloramphenicol, nystatin, ampicillin, nitrofurantoin, lincomycin, clindamycin, tetracycline, norfloxacin, trimethoprim, metronidazole, rifampin, piperacillin, teicoplanin, nalidixic acid, cefixime, azithromycin, cephalothin, cephalexin, cefamandole, cefotaxime, cefoperazone, erythromycin, ofloxacin and vancomycin. The diameter of the inhibition zone was measured to determine the antimicrobial susceptibility or resistance, and the criteria were as follows: inhibition zone diameter (Φ) ≥15 mm indicates high sensitivity (S), 15 mm > Φ ≥10 mm shows intermediate sensitivity (I), Φ < 10 mm indicates resistance (R). The sensitivity pattern of the bacterial isolate KCL-5 was determined by the standard Kirby-Bauer method (Bauer et al. 1966).

RESULTS

Isolation and morphological and biochemical characteristics of the KCL-5 strain

A Gram-negative rod-shaped bacterium was isolated from diseased crucian carp in China and tentatively named strain KCL-5. All strain colonies were circular, smooth, white, translucent and convex with an entire edge, 1.0–2.0 mm in diameter after 24 h incubation, and Gram-negative and short rod-shaped cells (0.5 µm × 2.0–3.0 µm) belonging to the *Enterobacteriaceae* were observed by microscopic examination (Fig. 1). The strain was positive for oxidase, lysine decarboxylase, ornithine decarboxylase, nitrate reduction, citrate utilization and hydrolysis of esculoside; and produced acid and gas from glucose, arabinose, galactose, fructose, mannose, melibiose, malonate, maltose, xylose, gluconate, raffinose, carbamide, lactose, rhamnose, cellobiose, sucrose, mannitol, dulcite and sorbitol. It was negative for arabitol, adonitol, phenylalanine deaminase, arginine hydrolase, indole, methyl red, Voges-Proskauer, hydrogen sulphide and gelatin liquefaction. Biochemical characteristics and differentiation of strain KCL-5 are summarized in Tables 1 & 2, and showed that this strain belongs to the genus *Rahnella* based on Bergey's manual of determinative bacteriology (Holt et al. 1994).

16S rDNA, *gyrB* and *yhaV* gene sequence analysis

The *16S rDNA*, *gyrB* and *yhaV* genes of strain KCL-5 were amplified by PCR for sequencing. The *16S rDNA* fragments from all colonies gave the same

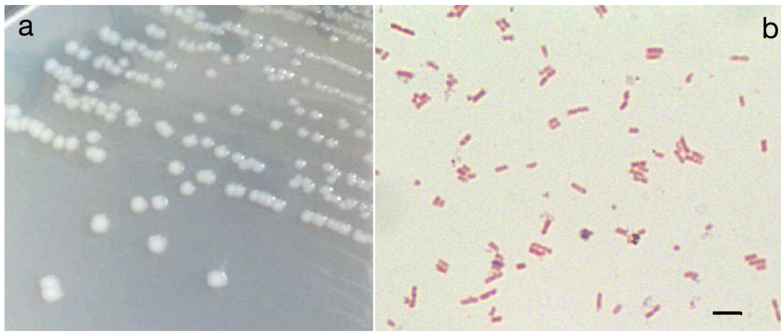

Fig. 1. (a) Bacterial colonies and (b) Gram-negative rod-shaped cells (scale bar = 5 µm) of *Rahnella aquatilis* strain KCL-5 isolated from diseased crucian carp *Carassius auratus*

sequence of 1507 bp, and the *gyrB* fragments had the same sequence of 695 bp. These sequences were submitted to GenBank under accession numbers KR070962 and KR070963, respectively. The results of BLAST analysis showed that the isolate KCL-5 was most closely related to type strain ATCC33071 (= DSM4594) of *R. aquatilis*, with *16S rDNA* (GenBank accession no. AJ233426) and *gyrB* (WP_014333278) sequence identities of 99.67 and 96.26%, respectively. Furthermore, the *yhaV* gene was amplified from KCL-5, giving a gene fragment of 251 bp in length, which shared 99.58% nucleotide sequence and 98.72% amino acid sequence identity with *R. aquatilis* ATCC 33071 (WP_014333375). The phylogenetic analysis based on *16S rDNA* gene sequences confirmed that the isolate KCL-5 was most closely related to *R. aquatilis* reference strains, falling within a group including the *R. aquatilis* type strain ATCC33071 from water and strain bf008 (GenBank accession no. KC480178) from frogs, *R. aquatilis* from soil (GABIT-M100, T7, OV744 and HX2) and *Rahnella* sp. from water (CDC2987-79 and Y9602). A number of isolates (e.g. Y9602, HX2, bf008) formed a subclade within the group with KCL-5, indicating a closer relationship (Fig. 2).

Pathogenicity *in vivo*

The isolate KCL-5 was confirmed as pathogenic to crucian carp by challenge experiments. The results indicated that the *R. aquatilis* strain KCL-5 had a cumulative mortality rate of 73.3% at a dose of 2.0 × 10^9 CFU per fish after 14 d and the clinical signs produced by experimental infections were similar to the signs observed in the cultured diseased carp from which the strain was originally isolated, with LD_{50} values of 1.7 × 10^8 CFU ml^{-1} in crucian carp (Table 3). The moribund fish exhibited sluggish behaviour, skin haemorrhages mainly located on pectoral fin base, caudal fin and parts of the body, typically in internal ascites and petechiae in liver and kidney

Table 1. Biochemical and physiological characteristics of *Rahnella aquatilis* strain KCL-5. +: positive; −: negative

Characteristic	KCL-5	Characteristic	KCL-5
Motility	−	Esculin	+
Gelatinase	−	Indole	−
Oxidase	+	Dextrin	−
Ornithine decarboxylase	+	Lactose	+
		Glucose	+
Lysine decarboxylase	+	Melibiose	+
Arginine dihydrolase	−	Fructose	+
Methyl red	−	Arabinose	+
Voges-Proskauer	−	Sucrose	+
Rhamnose	+	Galactose	+
Glucose (gas)	+	Cellobiose	+
Phenylalanine deaminase	−	Raffinose	+
		Xylose	+
Nitrate reductase	+	Mannose	+
H_2S production	−	Maltose	+
Glucosamine	+	Sorbitol	+
Citrate	+	Adonitol	−
Gluconate	+	Dulcitol	+
Malonate	+	Mannitol	+
Urease	+	Arabitol	−

Table 2. Biochemical differentiation of *Rahnella aquatilis* strain KCL-5 from both the type strain and strain bf008 of *R. aquatilis*. Reference data on the *R. aquatilis* type strain and strain bf008 were compiled from Holt et al. (1994) and Xue et al. (2013), respectively. +: positive; −: negative; v: variable (positive or negative); n: not given

Characteristics	KCL-5	*R. aquatilis*	bf008
Oxidase	+	−	n
Ornithine decarboxylase	+	−	−
Lysine decarboxylase	+	−	−
Methyl red	−	+	n
Voges-Proskauer	−	+	n
Phenylalanine deaminase	−	+	−
Citrate	+	v	+
Urease	+	−	+
Acid from:			
Glucose	+	+	+
Arabinose	+	+	n
Sorbitol	+	+	+
Arabitol	−	−	−

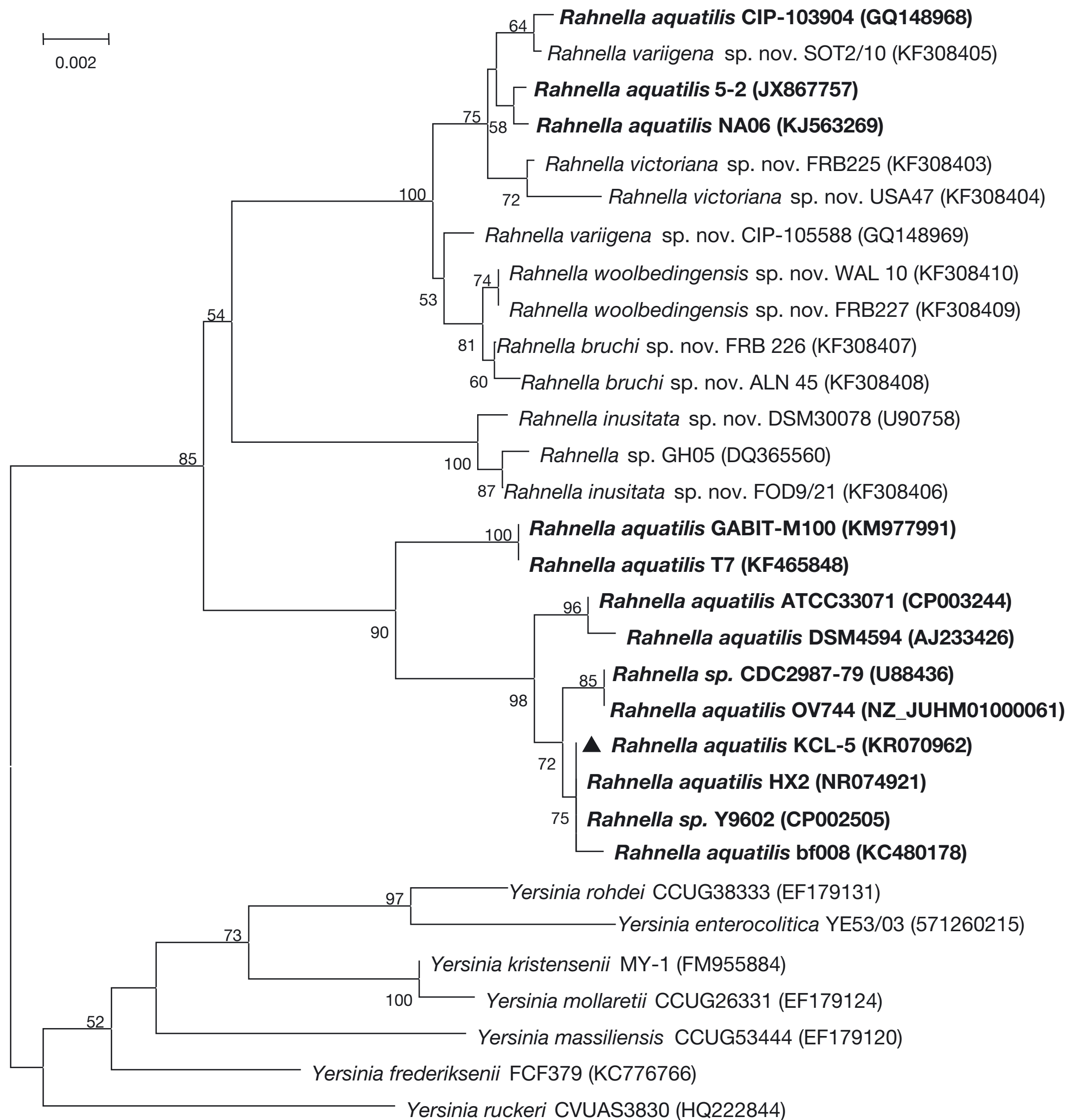

Fig. 2. Neighbour-joining phylogenetic tree of *Rahnella aquatilis* (**bold** text) strain KCL-5 (triangle) based on 16S rDNA sequences. The numbers at the nodes indicate percentage values for 1000 bootstrap replicates; only values above 50% are shown. The scale bar represents 0.002 substitutions site^{-1}

(Fig. 3). *R. aquatilis* was reisolated and identified from the moribund diseased fish. No clinical signs were observed in the control fish.

Antimicrobial susceptibility results

The results of antimicrobial susceptibility tests are summarized in Table 4. Of the antibiotics/concentrations tested, the isolate was highly susceptible to chloramphenicol, both macrolides, 3 quinolones, and 2/3 aminoglycosides (exception: netilmicin), but resistant to all 6 cephalosporins, all 3 sulphonamides, 2/3 penicillins (exception: piperacillin), and all other antibiotics in the panel

DISCUSSION

The occurrence of infections by opportunistic pathogens has become an increasingly important health problem for farmed fish in China (Lü et al. 2011, Xu et al. 2013). Recently, *Rahnella aquatilis* strain bf008, isolated from the frog *Rana temporaria*, was identified by phenotypic characterization and *16S rDNA* sequencing (Xue et al. 2013), and there

Table 3. Cumulative mortality of crucian carp *Carassius auratus* (n = 15 fish per concentration, injected i.p. with 0.2 ml bacterial suspension) and lethal dose (LD_{50}) testing of *Rahnella aquatilis* strain KCL-5

Concentration ($CFU\ ml^{-1}$)	Individual mortality after 12 h	24 h	48 h	7 d	14 d	Cumulative mortality (%)
1.0×10^{10}	1	5	9	11	11	73.3
1.0×10^{9}	0	4	5	8	8	53.3
1.0×10^{8}	0	0	3	5	5	33.3
1.0×10^{7}	0	0	0	1	1	6.7
1.0×10^{6}	0	0	0	0	0	0
Control	0	0	0	0	0	0

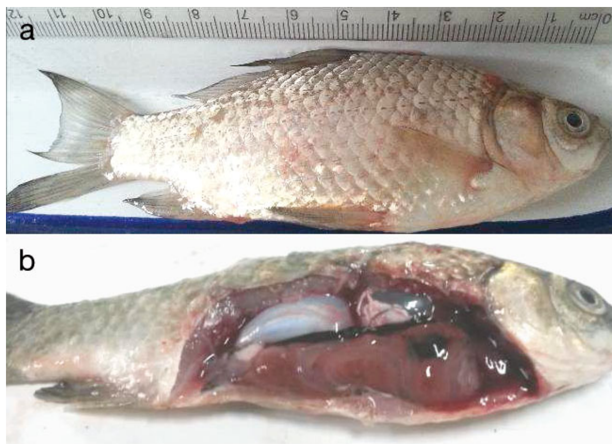

Fig. 3. Clinical signs of experimental infection with *Rahnella aquatilis* strain KCL-5 in crucian carp *Carassius auratus*. The moribund fish exhibited (a) haemorrhages on skin and (b) petechiae in liver and kidney

Table 4. Results of antimicrobial sensitivity test of *Rahnella aquatilis* strain KCL-5. Inhibition zone diameter (Φ) ≥15 mm indicates high sensitivity (S), 15 mm > Φ ≥10 mm shows intermediate sensitivity (I), Φ < 10 mm indicates resistance (R)

Antibiotic	Concentration ($\mu g\ disc^{-1}$)	Φ (mm)	Sensitivity
Penicillins			
Ampicillin	10	0	R
Amoxicillin	10	0	R
Piperacillin	100	16	S
Cephalosporins			
Cephalothin	30	0	R
Cephalexin	30	0	R
Cefamandole	30	0	R
Cefixime	30	0	R
Cefoperazone	75	0	R
Cefotaxime	30	0	R
Aminoglycosides			
Gentamicin	10	16	S
Kanamycin	30	16	S
Netilmicin	30	0	R
Quinolones			
Norfloxacin	10	20	S
Nalidixic acid	30	21	S
Ofloxacin	5	20	S
Sulphonamides			
Sulphamethoxazole	300	0	R
Sulphamethoxazole/ trimethoprim	23.75/1.25	0	R
Trimethoprim	5	0	R
Macrolides			
Azithromycin	15	15	S
Erythromycin	15	16	S
Others			
Chloramphenicol	30	15	S
Nitrofurantoin	300	0	R
Tetracycline	30	0	R
Rifampin	5	7	R
Vancomycin	30	0	R
Teicoplanin	30	0	R
Clindamycin	2	0	R
Lincomycin	2	0	R
Metronidazole	5	0	R
Nystatin	100	0	R

has been a report of *R. aquatilis* (strain NA06, GenBank accession no. KJ563269) isolated from fish in the Red Sea, albeit with no indication of disease associated with this isolation (Alikunhi et al. 2016). In the present study, the strain KCL-5 was isolated from cultured crucian carp showing clinical signs of disease, and *16S rDNA* and *gyrB* gene sequences were used for the molecular characterization of the isolate KCL-5, which was most closely related to the type strain of *R. aquatilis*, isolated from water. The isolate KCL-5 was further identified as *R. aquatilis* by biochemical and additional molecular analysis. BLAST analysis based on the *16S rDNA* sequence showed that the *R. aquatilis* strain KCL-5 from crucian carp had sequence identity greater than 99% to both *R. aquatilis* type strain ATCC33071 from water (GenBank accession no. AJ233426) and strain bf008 from frogs in China (GenBank accession no. KC480178).

However, several biochemical characteristics of isolate KCL-5 differed from those presented for *R. aquatilis* (i.e. type strain and bf008) in the literature (Holt et al. 1994, Xue et al. 2013). Biochemical differences were detected, especially in oxidase, urease, ornithine decarboxylase and lysine decarboxylase, and are likely associated with the origins of the strains. The analysis showed that some of the strains, including KCL-5 and the *Rahnella* strains Y9602 and bf008, formed a second clade within the group of *R. aquatilis*. Brady et al. (2014) suggested that *Rahnella* sp. Y9602 might be sufficiently differ-

ent genetically to be considered a different species to the type strain *R. aquatilis*. The *R. aquatilis* strain NA06 (GenBank accession no. KJ563269), the only other strain isolated from fish, falls within a different grouping to that of the reference *R. aquatilis* type strain and KCL-5, and groups more closely with other *Rahnella* spp. The virulence factor heat-labile toxin gene (*lt*) of *R. aquatilis* was detected in fish (Lindberg et al. 1998). In our study, the toxin *yhaV* gene was detected in the *R. aquatilis* strain KCL-5 from crucian carp.

In previous studies, antibiograms revealed that *R. aquatilis* from humans is susceptible to aminoglycosides, quinolones, sulphonamides and third-generation cephalosporins (Goubau et al. 1988, Carinder et al. 2001). A human infection caused by *R. aquatilis* was treated successfully with ciprofloxacin (Gaitán & Bronze 2010), and *R. aquatilis* from frogs was found to be susceptible to cefoperazone and cefoxitin (Xue et al. 2013). In our study, results showed that isolate KCL-5 was susceptible to aminoglycosides and quinolones, but was resistant to cephalosporins and sulphonamides. The results for antibiotic resistance of KCL-5 do not agree with those for *R. aquatilis* from humans and frogs (Goubau et al. 1988, Carinder et al. 2001, Xue et al. 2013), which provides information for clinical treatment and infection prevention caused by *R. aquatilis* in fish.

In previous studies, *R. aquatilis* was found in liver of frogs (Xue et al. 2013), muscle of fish (Alikunhi et al. 2016) and whole fish without skin (Lindberg et al. 1998). Our results showed that the *R. aquatilis* strain KCL-5 was found in all examined internal organs (liver, kidney and spleen) of crucian carp, but its tissue tropism remains uncertain in aquatic organisms.

In conclusion, this is the first report of *R. aquatilis* from naturally diseased fish. Moreover, experimental challenges were conducted and demonstrated the pathogenicity of the *R. aquatilis* isolate for crucian carp. *16S rDNA*, *gyrB* and *yhaV* gene sequence analysis of isolate KCL-5 provides scientific reference data for fish disease diagnostics. The data presented are also helpful in describing the clinical signs and understanding *R. aquatilis*-induced disease in fish.

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