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Short communication

Isolation and characterization of *Citrobacter* spp. from the intestine of grass carp *Ctenopharyngodon idellus*

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ABSTRACT

Citrobacter gastroenteritis became an increasingly significant health problem in intensive aquaculture in China. In this study, three bacterial strains were isolated from the intestinal tract of farmed grass carp (*Ctenopharyngodon idellus*), and were tentatively numbered C9414, C0520 and C0934, and then they were identified as *Citrobacter freundii*, *C. gillenii* and *C. werkmanii* by the biochemical properties and molecular techniques. The strain C9414, namely *C. freundii* showed the pathogenicity to mice and zebrafish, respectively. Furthermore, the fingerprint patterns with distinct bands of three isolates were generated by single-strand conformation polymorphism (SSCP) analysis. To our knowledge, this is the first report on the bacteria *Citrobacter* spp. isolated from the intestine of fish in China.

1. Introduction

Grass carp, *Ctenopharyngodon idella*, is one of the main cultured fish species in China. The production of farmed grass carp had exceeded 3.96 million tons in year 2006 in China, which is by far the major producer and holds around 95% of the global total (Chen et al., 2007). However, *Citrobacter* gastroenteritis became an increasingly significant health problem in intensive aquaculture in China (Chen et al., 2006; Lin et al., 2008). The first description of pathology and isolation of *Citrobacter* species from the diseased fish was in 1982 by Sato et al.. The Citrobacters, particularly *C. freundii*, were subsequently isolated from Atlantic salmon, carp and rainbow trout (Baya et al., 1990; Austin et al., 1992; Karunasagar et al., 1992; Toranzo et al., 1994; Jeremic et al., 2003).

To date, studies revealed that gram-negative bacteria like *Aeromonas*, *Acinetobacter*, *Bacteroides*, *Citrobacter*, *Flavobacterium* and *Pseudomonas* etc. were the most common in the intestine of farmed fish (Hovda et al., 2007; Kim et al., 2007; Navarrete et al., 2010). Recently, the *Citrobacter* spp. was isolated from the intestine of farm-raised catfish (Nawaz et al., 2008). However, very little has been reported on the isolation and identification of intestinal *Citrobacter* spp. strains from fish. The objective of this study was to clarify the taxonomy of *Citrobacter* bacteria isolated from the intestine of farmed grass carp and describe their potential pathogenicity. Here, three *Citrobacter* spp. were characterized systematically by morphological observations, biochemical tests, infectivity trials, 16S rDNA sequences and PCR-SSCP analysis.

2. Materials and methods

2.1. Isolation, characterization and identification of bacteria

Bacteria were isolated from the intestinal samples of fifteen seemingly healthy grass carp, *C. idella* (weight ca. 500 g per fish) collected from a freshwater fish farm in Xuzhou, China. The samples were streaked on Luria–Bertani (LB) agar or MacConkey agar plates incubated at 28 °C for 24 h. The Gram staining test was performed using the Hucker method and observations made before performing the biochemical tests. Colonies typical for Citrobacters were biochemically characterized and identified using commercial microtest systems (Hangzhou Tianhe Microorganism Reagent Co., Ltd, China). The test system was incubated at 28 °C and the final readings were made after 7 days.

2.2. DNA extraction, PCR amplification and SSCP analysis

Total genomic DNA of all isolates was extracted by using the UNIQ-10 column genomic DNA extraction kit (Sangon, China) according to the instructions of the manufacturer. The nearly full-length 16S rDNA, V3 region and *fimC* genes were amplified with the bacterial universal primers based on *E. coli* sequences, respectively. The PCR primers were synthesized by Sangon, China, and the three PCR products were amplified with the primers sequences (5′ to 3′) as follows: 16S rRNA-F:AGAGTTTGATCATGGCTCAG, 16S rRNA-R:TACGGTTACCTTGTTACGACTT (Tm = 57.0 °C); V3-F:CCTACGGGAGGCAGCAG, V3-R:ATTACCGCG

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GCTGCTGG (Tm = 51.0 °C); fimC-F:GGGTAGAAAATGCCGATGGTG, fimC-R:CGTCATTTTGGGGGTAAGTGC (Tm = 59.0 °C). 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 MgCl2, 200 μM dNTPs, and 0.625 U Taq DNA polymerase (Sangon, China). The PCR products were evaluated by electrophoresis in 1% agarose gel by staining with ethidium bromide. Amplification of the V3 region of the 16S rDNA for SSCP analysis was performed according to the method of Nair et al. (2002).

2.3. Sequence, alignment and phylogenetic tree analysis

The PCR products of *fimC* genes were purified with the QIAquick PCR Purification kits (Qiagen) and directly sequenced using the ABI 377 sequencer from both directions. The nearly full-length 16S rDNA PCR products were cloned into the pMD19-T vector (TaKaRa) and transformed into competent *E. coli* DH5a. Positive clones were sequenced with M13 primers by automatic sequencing. 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.

2.4. Pathogenicity assays in zebrafish and mice

Zebrafish and mice were used for evaluation of pathogenicity of three *Citrobacter* isolates in present study. The infectivity trials in wild zebrafish (ca. 0.25 g per fish) were performed by immersion-challenged for 15 min with the *Citrobacter* strain at the doses of approximately 10^8 cfu/ml, and observed for 7 days post-infection. Sixty fish were maintained at 25 °C with aeration during the course of the experiment. The mouse pathogenicity assay was performed with twelve Kunming mice weighing ca. 22 g (provided by Xuzhou Medical College), which were inoculated intraperitoneally with 0.3 ml of bacterial doses (about 10^8 cfu/ml) of each *Citrobacter* isolate. Morbid fish and mice were subjected to laboratory examination and bacterial re-isolation. Control animals received PBS alone.

3. Results

3.1. Characterization of the Citrobacter isolates

Three bacterial strains were isolated from the intestinal tract of farmed grass carp and were provisionally numbered C9414, C0520 and C0934, respectively. They all were non-sporing, Gram-negative rods that produced colonies in diameter 2–3 mm, low convex, moist, translucent, and gray with a shiny surface and entire edge on LB agar plates, while colonies with white, pink and red phenotypes on MacConkey agar plates, respectively. The comparison of biochemical characteristics of the three isolates in this study with that of the reference strains of genus *Citrobacter* in Bergey's Manual are shown in Table 1.

3.2. Sequencing and phylogenetic tree analysis

The nearly full length sequences of 16S rDNA (approximately 1.5 kb) were amplified from the isolated strains (C9414, C0520,

Table 1
Morphological and biochemical properties of *Citrobacter* spp. isolated grass carp.

Characteristics	Strain C9414	*C. freundii*[a]	Strain C0520	*C. gillenii*[a]	Strain C0934	*C. werkmanii*[a]
Gram reaction	−	−	−	−	−	−
Shape	Rod	Rod	Rod	Rod	Rod	Rod
Motility	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Oxidase	−	−	−	−	−	−
Glucose (Hugh and Leifson)	F	F	F	F	F	F
Citrate (Simmons)	+	v	−	v	+	+
H_2S	+	v	+	v	+	+
KCN growth	+	+	+	+	+	+
Methyl Red	+	+	+	+	+	+
Voges-Proskauer	−	−	−	−	−	−
Nitrate reductase	+	+	+	+	+	+
Gelatinase	−	−	−	−	−	−
Urease	−	v	−	−	+	+
Lysine decarboxylase	−	−	−	−	−	−
Ornithine decarboxylase	−	−	−	−	−	−
Phenylalanine deaminase	−	−	−	−	−	−
ONPG	+	+	+	+	+	+
Indole	−	v	−	v	−	−
Esculin	+	−	+	−	+	−
Gluconate	−	v	−	v	−	v
Tartrate	+	v	−	−	+	+
Glucose (gas)	+	v	+	+	+	+
Acids from						
Adonitol	−	−	−	−	−	−
Sorbitol	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+
Dulcitol	−	v	−	−	−	−
Arabitol	−	−	−	−	−	−
Glucose	+	+	+	+	+	+
Lactose	−	v	+	v	+	v
Sucrose	+	+	−	v	+	−
Cellobiose	+	v	−	v	−	v
Melibiose	−	v	+	+	+	−
Raffinose	−	v	−	−	−	−
Xylose	+	+	+	+	+	+

[a] Reference strain data compiled from Bergey's Manual (Frederiksen, 2005). +, positive; −, negative; v, variable (positive or negative).