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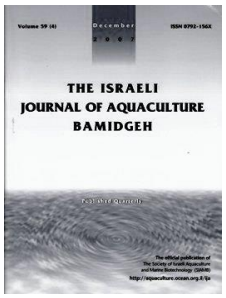

Recovery of *Pseudomonas aeruginosa* from Diseased Grass Carp (*Ctenopharyngodon idella*) in China

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Abstract

An epizootic was discovered in grass carp (*Ctenopharyngodon idellus*) in earthen ponds in Ninghe, Tianjin Province, northern China, from May to June 2013. The cumulative mortality was 30% within 25 days, and the diseased fish presented with hemorrhaging in the abdominal wall, ascites in the abdomen and pale liver. Strains named PA131207 and PA131208 were isolated from the moribund fish, and were identified as *Pseudomonas aeruginosa* according to both biochemical tests and phylogenetic analysis derived from the 16S rRNA gene. The pathogenicity of PA131207 was confirmed by infectivity experiments and histopathological examination. Antimicrobial susceptibility tests showed that PA131207 was resistant to 9 of 15 antimicrobial agents tested.

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Introduction

Pseudomonas comprises a group of rod-shaped, non-spore-forming, Gram-negative bacteria that cause red skin disease in a wide range of freshwater fish (Wang et al., 2009). They are considered to be among the most important fish pathogens (Austin and Austin, 2007). Within the *Pseudomonas* genus, *Pseudomonas fluorescens* (Altinok et al., 2007; Aly et al., 2008; Geng et al., 2006; Shiose et al., 1974; Swain et al., 2003; 2007), *Pseudomonas putida* (Altinok et al., 2006), *Pseudomonas anguilliseptica* (Wakabayashi and Egusa, 1972), *Pseudomonas chlororaphis* (Hatai et al., 1975), and *Pseudomonas plecoglossicida* (Kobayashi et al., 2000) are regarded as opportunistic pathogenic species in aquaculture. The association of the pathogen *Pseudomonas aeruginosa* with human clinical infections has been well documented, but reports of its pathogenicity in fish have rarely been published. In the present study, *P. aeruginosa* was isolated from diseased grass carp, and its pathogenicity in grass carp was confirmed by infectivity experiments.

Materials and methods

Isolation of bacteria. On June 6, 2013, diseased grass carp (15-20 cm long) were sent from a farm in Ninghe, Tianjin Province, China, to our laboratory for diagnosis. The body surfaces and gills of three moribund fish were examined microscopically for the presence of parasites. Then, the fish were surface-disinfected with 70% ethanol and dissected, and a loopful of material from the liver, spleen, and kidney were aseptically streaked on Luria-Bertani (LB) agar plates and incubated at 28 °C for 24 h. Single colonies were selected and re-streaked on the same medium; all colonies on the same plates were identical in morphology and considered to be a pure culture. Strains PA131207 and PA131208 were isolated from the livers of the fish.

Identification of strains PA131207 and PA131208. Strains PA131207 and PA131208 were characterized using standard physiological and biochemical tests including Gram staining, Voges-Proskauer, oxidation / fermentation and hemolysis reaction, indole and hydrogen sulphide (H_2S) production, motility, oxidase, catalase, and methyl red test, utilizing citrate, gelatin, and starch, producing acid from glucose, fructose, maltose and sucrose (Dong and Cai, 2001). Reference strain *P. aeruginosa* ATCC 27853 was used as the control.

Strains PA131207 and PA131208 were cultured separately in 5.0 mL LB broth at 28°C for 24 h with gentle shaking, then their genomic DNA was extracted from a 200-µL bacterial suspension using the Genomic DNA extraction kit (SBS, Shanghai) according to the manufacturer's protocol. 16S rRNA gene fragments were amplified by PCR using the universal bacterial primers (F): 5'-AGAGTTTGATCCT
GGCTCAG-3' and (R): 5'-TACGGCTACCTTGTTACGACTT-3' (Xia *et al*., 2008). The PCR program was as follows: (1) 5 min at 94 °C; (2) 40 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C; and (3) 10 min final extension at 72 °C. The PCR products were purified and inserted into the pMD18T vector. Then, the recombinant plasmids were transformed into *E. coli* strain TOP10 competent cells and sequenced by Bigdye-Terminator in Unigene. The obtained sequences were aligned and compared with previously published sequences in the NCBI GenBank using the BLAST program. The phylogenetic trees were constructed using the neighbor-joining method with MEGA 5 version software (Liu and Li, 2012).

Pathogenicity testing. In order to test the pathogenic potential of the isolates, strain PA131207 was selected for experimental infection of healthy grass carp (body length: 10 ± 2.2 cm). Sixty grass carp were acclimatized to 25°C for two weeks prior to the challenge. They were then divided equally into two groups and placed in 150 L aquaria containing 75 L dechlorinated municipal water under constant aeration. Isolate PA131207 was cultured in LB at 28°C overnight, and a suspension of PA131207 was prepared in Phosphate Buffered Saline (PBS) solution to achieve a concentration of 1.0×10^8 cfu/mL. Grass carp in the challenge group were IP injected with 0.2 mL bacterial suspension, while those in the control group were injected with the same amount of PBS. The two groups were kept under observation for 30 days and the mortality rates were recorded. Moribund fish were retrieved and subjected to laboratory examination and bacterial re-

isolation. The animal experiments were approved by the Animal Welfare Committee of the Tianjin Fishery Research Institute.

Histopathology. For histological investigations, organs including the spleen, liver, and kidney were collected from the grass carp in the infected and control groups, and the organs were fixed in 75% alcohol and processed for histological examination. 5-μm-thick sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope.

Antimicrobial susceptibility testing. The susceptibility pattern of isolate PA131207 were tested by using the standard method described by Bauer et al. (Bauer *et al*., 1966) on Mueller-Hinton agar (MHA) plates using commercial antibiotic discs (Tianhe, China) including ampicillin (10 μg), streptomycin (10 μg), chloromycetin (30 μg), kanamycin (30 μg), norfloxacin (10 μg), tetracycline (30 μg), doxycycline (30 μg), enoxacin (10 μg), ciprofloxacin (5 μg), gentamycin (10 μg), erythromycin (15 μg), tobramycin (10 μg), florfenicol (30 μg) vancomycin (30 μg), roxithromycin (15 μg). Based on the zone diameters of inhibition, the results were interpreted as sensitive, medium sensitive, or resistant, according to the manufacturer's instructions.

Results

The diseased fish displayed hemorrhages on ventral surfaces, ascites in their abdomens, and pale livers. Dense growth of one colony type was discovered on the LB plates streaked with sections of liver of diseased fish. Only a few colonies were detected from other organs. Strains PA131207 and PA131208 were isolated from the livers of diseased fish and they were Gram-negative, short rod-shaped, non-spore-forming bacteria. After incubation on LB agar plates at 28 °C for 48 h, their colonies were yellow-green, round, raised, and glossy, with a diameter of 1.5–2.0 mm. The biochemical characteristics are summarized in Table 1 and compared with those of the known *P. aeruginosa* ATCC 27853 strain. They exhibited β-hemolysis activity and were oxidase and catalase positive and motile. Also, the strains were capable of utilizing citrate and gelatin, but not starch, and producing acid from glucose and fructose, but not maltose or sucrose. Furthermore, they showed negative results in Voges Proskauer reaction, Indole, and methyl red tests and could not produce H_2S from glucose.

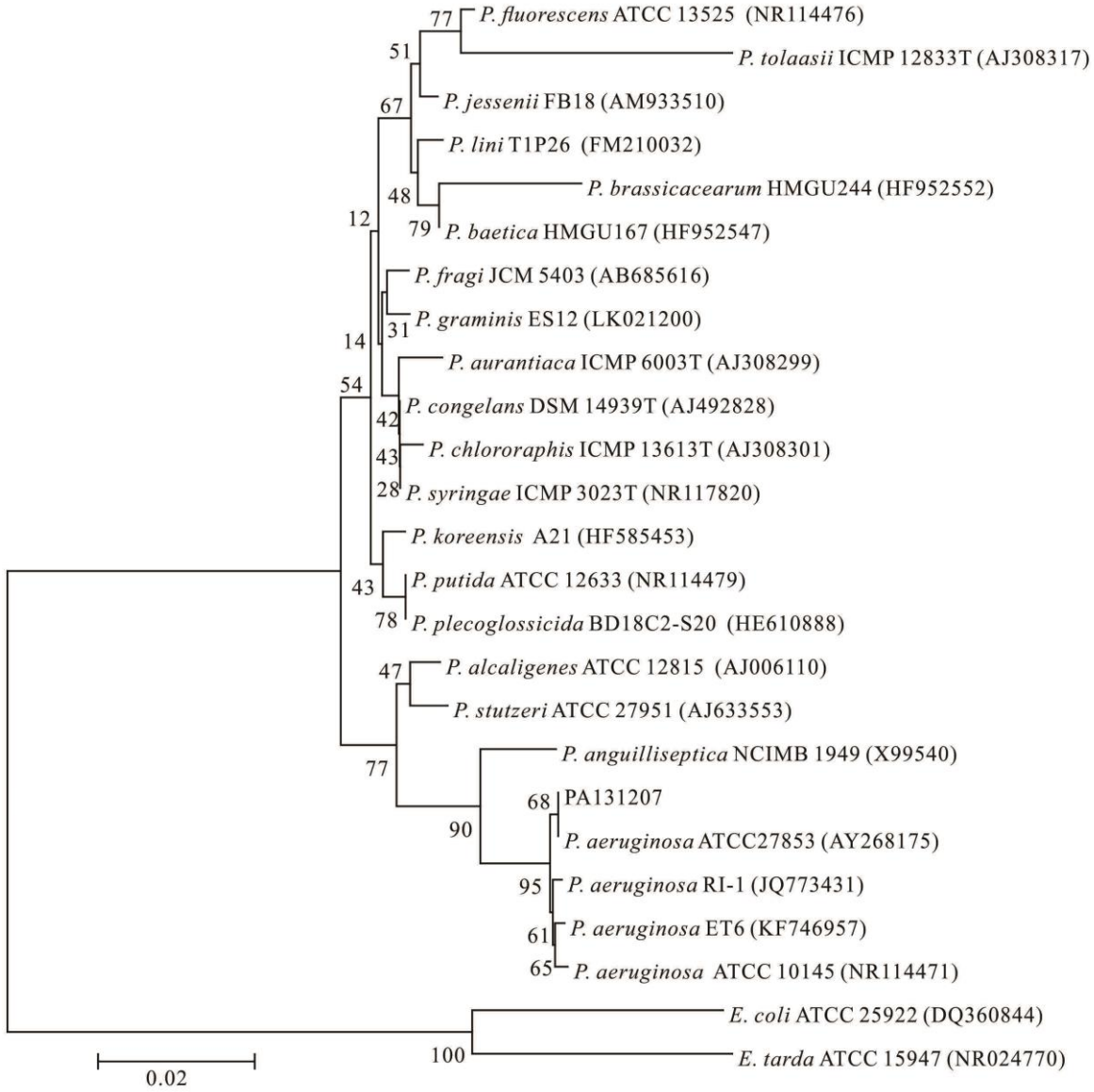
Table 1. Comparison of phenotypic characteristics of isolates PA131207 and PA131208 with *Pseudomonas aeruginosa* ATCC 27853

Biochemical test	*PA131207*	*PA131208*	*ATCC 27853*
Oxidase test	+	+	+
Catlase	+	+	+
Indol production	-	-	-
H_2S production	-	-	-
Methyl red test	-	-	-
Gelatin	+	+	+
Starch	-	-	-
Glucose	+	+	+
Fructose	+	+	+
Maltose	-	-	-
Sucrose	-	-	-
Voges-Proskauer	-	-	-
Hemolysis	β	β	β
Oxidation/fermentation	O	O	O
Motility	+	+	+
Citrate utilization	+	+	+

The 16S rRNA genes were amplified and sequenced for strains PA131207 and PA131208, and they had identical gene sequences. The sequence showed 99% similarity with *P. aeruginosa* ATCC 27853, *P. aeruginosa* ET6, *P. aeruginosa* RI-1, and *P. aeruginosa* ATCC10145 respectively. The 16S rRNA gene sequence of isolate PA131207 (GenBank accession no. KM272634) was selected for construction of a phylogenetic tree by the neighbor-joining method. It formed a single cluster with the 16S rRNA gene

sequences of *P. aeruginosa* ATCC 27853, then clustered with *P. aeruginosa* ET6 , *P. aeruginosa* RI-1, and *P. aeruginosa* ATCC10145 (Fig. 1).

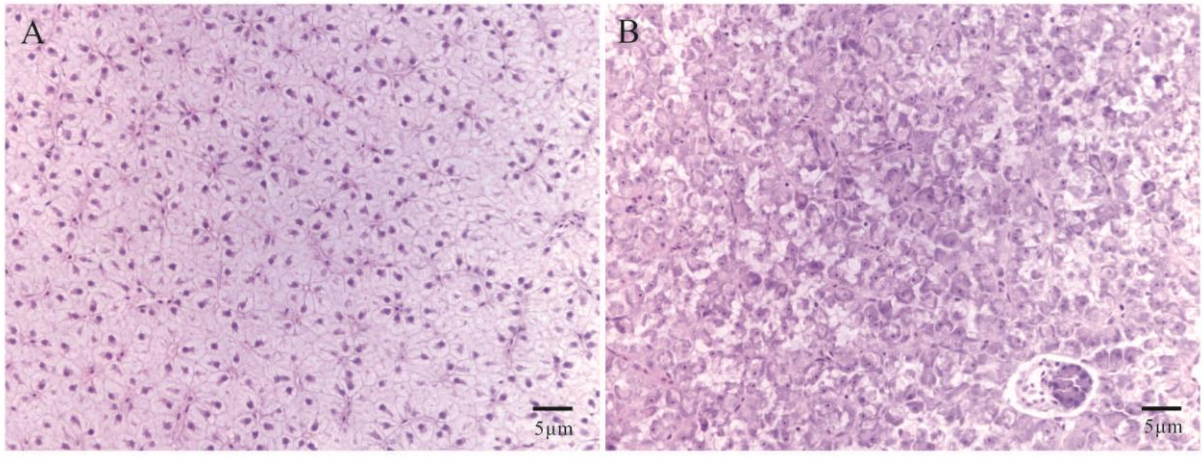
Fig. 1. The phylogenetic tree, based on 16S rRNA gene sequences available in GenBank, was generated. Accession numbers of all strains are given in brackets.



Pathogenicity experiments showed that isolate PA131207 was pathogenic to grass carp, and the cumulative mortality was 33% in the treatment group. The fish began to die on the fifth day post-challenge. The clinical signs (hemorrhages on the abdomen and ascites in the abdomen) observed in the experimentally infected fish were similar to those observed in natural infections. *P. aeruginosa* were re-isolated from the livers of the morbid fish. No mortalities or clinical signs were observed in the control group.

The histology of the control fish livers revealed a normal, typical parenchymatous appearance. Their livers were composed of hepatocytes with a central irregular, densely stained nucleolus (Fig. 2A), while hepatocytes in the livers of experimentally infected fish were damaged and many irregular vacuolations were seen (Fig. 2B). No obvious histopathological differences were observed in the spleens and kidneys between the experimental group and the control group.

Fig. 2. Histological sections of livers from healthy grass carp showed a normal, typical parenchymatous appearance (A) whereas diseased fish showed many irregular vacuolations (B).



Isolate PA131207 was tested for susceptibility to 15 antimicrobial agents. The results showed that it was sensitive to enoxacin, ciprofloxacin, tobramycin, and doxycycline; moderately sensitive to streptomycin and chloromycetin; and resistant to ampicillin, kanamycin, norfloxacin, tetracycline, gentamycin, erythromycin, florfenicol, vancomycin, and roxithromycin.

Discussion

Grass carp belong to one of the so-called four major carp groups in China (Wang et al., 2010). Because of their well-adapted, rapid growth and high market demand, grass carp have been widely cultured in all provinces in China, with an annual output of 2,296,750 tons (Bureau of Fisheries, 2012). However, accompanying the increase in production have been increased risks of disease outbreaks, probably because of the high density of cultured grass carp in intensive systems. According to recent reports, the bacterial pathogens associated with cultured grass carp include *Pseudomonas fluoroscens* (Geng et al., 2006), *Flavobacterium columnare* (Wang et al., 2010), *Aeromonas hydrophila* (Song et al., 2014), *Vibrio cholerae* (Li et al., 2011), and *Vibrio mimicus* (Zhang et al., 2014). In the present study, the bacterium isolated from diseased grass carp was identified as *P. aeruginosa* according to morphological and biochemical characteristics and phylogenetic analysis based on 16S rRNA gene sequences. In the challenge experiment, it was demonstrated that *P. aeruginosa* was pathogenic to grass carp though the mortalities were not 100% and it may reveal the pathogen was not as virulent as *P. fluoroscens* for grass carp. However, the disease outbreak caused a 30% loss and in intensive cultural conditions any possible virulent pathogen could not be neglected.

According to histopathological characteristics, damaged hepatocytes were detected in the livers of experimentally infected fish, while similar symptoms were not observed in the control group. This supports the hypothesis that *P. aeruginosa* is pathogenic to grass carp and indicate that the liver is one of the major target organs of the pathogen in grass carp. *P. aeruginosa* is considered a potential probiotic because it effectively improves innate immunity and affords protection against *A. hydrophila* infection in rohu (*Labeo rohita*) (Giri et al., 2012). However, safety should be taken into account because it is pathogenic in grass carp. Similarly, *P. aeruginosa* was also found to be pathogenic in tilapia (*Oreochromis mossambicus*) (John Thomas et al., 2014).

The farm epizootic lasted more than one month and the economic losses were more than 30%. Based on the results of antimicrobial susceptibility testing, grass carp were treated by oral administration of doxycycline and mortality ceased within 2 weeks. This implies that antimicrobial susceptibility testing is useful for selecting the correct

therapeutic medicines. It is worth noting that isolate PA131207 displayed multi-drug resistance to most commonly used antibiotics. *P. aeruginosa* that are resistant to a wide range of antibiotics, including cefazolin, cefotetan, ceftazidime, tobramycin, ciprofloxacin, and ofloxacin, have been reported previously (Bae et al., 2014; Qi et al., 2014). In order to avoid using antibiotics, other preventive management techniques should be established to prevent *P. aeruginosa* infection in aquaculture.

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