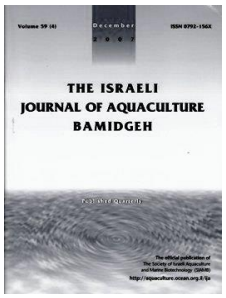

Acinetobacter johnsonii: an Emerging Pathogen for Cultured Blunt Snout Bream *Megalobrama amblycephala*

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Abstract

Bacteriosis has caused significant economic losses in farmed blunt snout bream *Megalobrama amblycephala*. Only scarce information is available on *Acinetobacter johnsonii* as a possible causal agent in *M. amblycephala*. In this study, a virulent strain temporarily named BY3, was isolated from diseased bream, identified phenotypically and molecularly as *A. johnsonii*. Phylogenetic trees of 16S rRNA and *gyr*B gene sequences were constructed to examine the taxonomic position of isolate BY3 and compare it to other known isolates. When screened against a range of common veterinary antibiotics, isolate BY3 has not shown resistance to florfenicol, neomycin, and sulfisoxazole antibiotics in aquaculture. To the best of our knowledge, this is the first report of *A. johnsonii* infection in farmed blunt snout bream.

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Introduction

Cyprinids are highly important food fish and are widely farmed over a large area of Eurasia. As an emerging industry in China blunt snout bream *Megalobrama amblycephala* is an important cyprinids fish species for farming. It grows rapidly and is high in economic value (Chen, 2015) with production of over 0.79 million tons in 2015 (Ministry of Agriculture of China, 2016). However, this emerging industry has been seriously affected by bacterial diseases. These should be given more consideration in order to determine the future development and sustainability of this industry. Studies have revealed that *Acinetobacter* species, particularly *Acinetobacter lwoffii, Acinetobacter baumannii*, *Acinetobacter junii*, cause infections in *Clarias fuocus* (Li et al., 2001), *Ictalurus punctatus* (Xia et al., 2008), *Carassius auratus gibelio*♀×*Cyprinus carpio*♂ (Lu et al., 2010) and *Acipenser schrenckii* (Mao et al., 2013). However, there are few reports on *Acinetobacter johnsonii* infection in *M. amblycephala*.

In April 2016, a severe outbreak of the disease occurred in cultured *M. amblycephala* in outdoor ponds of Xiantao, Hubei province, China. The disease is highly infectious and lethal, causing over 80% mortality. Diseased fish suffer mainly from symptoms of skin and fin ray hemorrhages. In the present paper, we isolated and identified an *Acinetobacter johnsonii* pathogen as a causative agent for this disease, and determined its taxonomy and antibiotic susceptibility. To our knowledge, this is the first report of *A. johnsonii* as an emerging pathogen for *M. amblycephala*.

Materials and methods

Fish samples. Fifteen diseased *M. amblycephala* averaging 506±15 g were sampled from infected ponds of a fish farm in Xiantao, Hubei China during April 2016. The farm had 56 acres of ponds with juvenile blunt snout bream stocked at an initial rearing density of 1,200 juveniles per acre. The water quality during the disease outbreak was pH 7.2, 0.12 mg/L total ammonia, 0.03 mg/L nitrite, and 5.26 mg/L dissolved oxygen. Diseased samples were placed in sterile bags, kept in ice and transported to the laboratory.

Isolation of the pathogen. Each sampled diseased blunt snout bream was externally disinfected with 75% alcohol and dissected. Before conducting a careful examination of parasites and viruses using traditional methods as described by Yang & Yang (2013) and Zeng et al. (2013), samples from spleens, kidneys, and livers of diseased blunt snout bream were streaked onto nutrient brain heart infusion agar (BHIA) plates (Sinopharm Chemical Reagent Co., Ltd.). After incubation for 24h at 28^0C, the dominant uniform isolates were purified by streaking and re-streaking onto BHIA plates. Pure isolates of the predominant uniform colonies were stored at -80^0C supplemented with 15% glycerol. A representative of the dominant isolates, temporarily named BY3, was characterized further in the present study.

Identification of the pathogen

Molecular identification. The extraction of genomic DNA from isolate BY3, as well as PCR amplification and sequencing of the 16S rRNA and *gyr*B genes were performed according to Bing et al. (2010). The near complete 16S rRNA and *gyr*B gene sequences were assembled using Editseq and Seqman in DNAstar software. Searches were performed in the National Centre for Biotechnology Information (NCBI) database for sequence homology using the Basic Local Alignment Search Tool (BLAST) program. Phylogenetic trees of the near complete 16S rRNA and *gyr*B gene sequences from the isolate and the homologous sequences were constructed using the neighbor-joining method.

Phenotypic identification. Isolate BY3 was identified phenotypically by API 32E analysis according to our previous study (Cao et al., 2016) where the isolate BY3 was grown on nutrient agar (NA) plates (Sinopharm Chemical Reagent Co., Ltd.) at 28^0C for 24h, and the bacterial suspension was then used to inoculate the API 32E test strips (Biomerieux, France) following the manufacturer's instruction. The test strip was incubated at 37^0C and observed after 18h for checking against the API identification index. The type strain ATCC17909 of *Acinetobacter johnsonii* was used as the control.

Bacterial virulence assay. Bacterial virulence was examined by experimentally infecting healthy blunt snout bream. Fifty healthy fish averaging 100±10 g were obtained

from Baishazhou fishery Co., Ltd. in Wuhan China. Their health status was assessed according to the guidelines in our previous study (Cao et al., 2013). The experimental fish were maintained in five replicate aquaria (ten fish per aquarium) supplied with 50 L aerated filtered farming water at 20^0C for 14 days to acclimate. Prior to the bacterial virulence assay, isolate BY3 was inoculated onto NA plate, incubated at 28^0C for 24h, and then washed with normal saline into a sterile tube. Its cell density was determined by counting colony forming units after a ten-fold serial dilution in sterile distilled water. Ten healthy fish in each treatment group were challenged by intramuscular injection with 0.2 mL of isolate BY3 at a concentration of 2.0×10^4 CFU/mL to 2.0×10^7 CFU/mL. Another ten healthy fish exposed to the same experimental conditions were injected intramuscularly 0.2 mL of normal saline. The experimental fish were kept at 20^0C and observed daily for seven days without feeding and water change. Any dead fish were immediately removed and sampled to re-isolate and confirm if mortality was caused specifically by the challenge isolate. The mean lethal dose (LD_{50}) value was calculated using the probit analysis as described by Li et al. (2012).

Antibiotic sensitivity assay. The antibiotic sensitivity of isolate BY3 was assayed on NA plates using the Kirby-Bauer disk diffusion method as recommended by Jones et al. (2001). Seventeen antibiotic discs were acquired from Hangzhou Binhe Microorganism Reagent Co., Ltd. The zones of inhibition were measured after a 24h incubation period at 28^0C. The antibiotic susceptibility was determined according to the manufacturer's guidelines.

Results

Pathogen identification. A dominant isolate BY3 was isolated from the diseased farmed *M. amblycephala* and identified by molecular and phenotypic methods as *A. johnsonii.* Its near complete 16S rRNA and *gyr*B gene sequences were submitted to GenBank database with the accession numbers KY118919 and KY118918. Similarities of 99% and 97% are observed in the 16S rRNA and *gyr*B gene sequences between the BY3 isolate and other *A. johnsonii* isolates from the GenBank database. The phylogenetic trees indicated that the isolate BY3 is an *A. johnsonii* strain (Figures 1 and 2). This was again confirmed by the phenotypic features as *A. johnsonii* (Table 1) with 100% identity compared to the type strain. No parasites and viruses were detected in diseased blunt snout bream from which isolate BY3 was obtained.

Figure 1. A 16S rRNA gene tree of 14 known bacteria and the BY3 isolate constructed using the neighbor-joining method. The bootstrap values (%) are shown besides the clades, accession numbers are indicated beside the names of strains, and scale bars represent distance values.

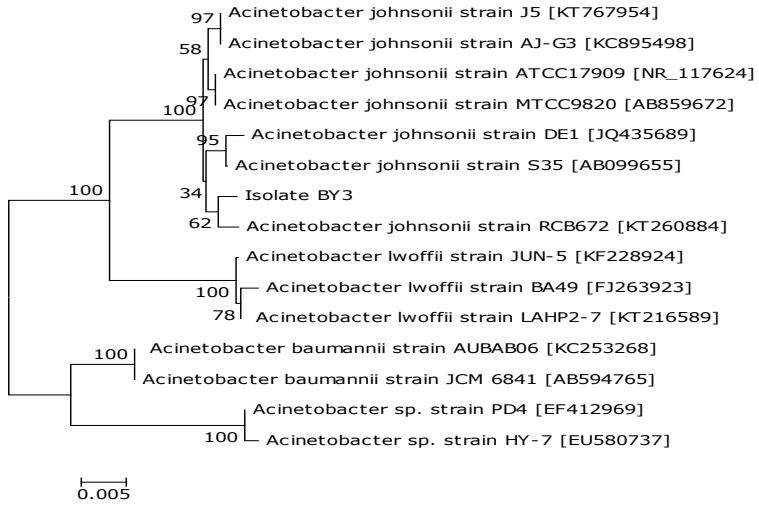

Figure 2. A *gyr*B gene tree of 15 known bacteria and the BY3 isolate constructed using the neighbor-joining method. The bootstrap values (%) are shown besides the clades, accession numbers are indicated beside the names of strains, and scale bars represent distance values.

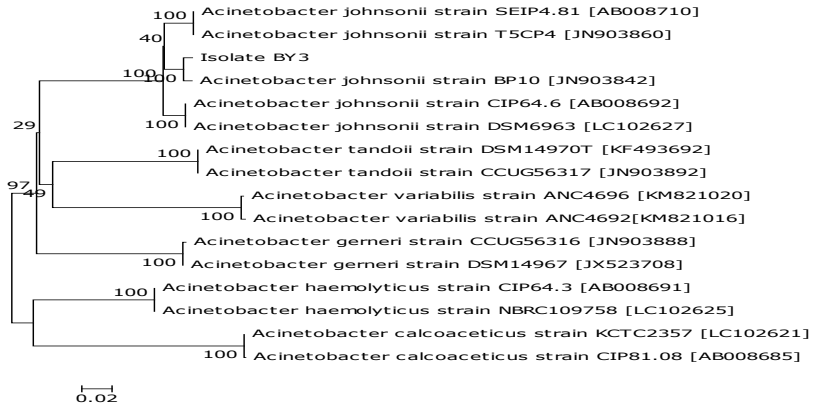

Table 1. Phenotypic characteristics of isolate BY3 in comparison with the type train ATCC17909 of *A. johnsonii*.

Item	*Reaction*	
	BY3	*ATCC17909*
Arginine dihydrolase	R^-	R^-
Lysine decarboxylase	R^-	R^-
Lipase	R^-	R^-
L-aspartate aminase	R^+	R^+
N-acetyl-β-glucosaminidase	R^-	R^-
α-galactosidase	R^-	R^-
α-glucosaccharase	R^-	R^-
α-maltosidase	R^-	R^-
β-galactosidase	R^-	R^-
β-glucosaccharase	R^-	R^-
β-glucuronidase	R^-	R^-
Urease	R^-	R^-
Orinithine decarboxylase	R^-	R^-
Indole production	R^-	R^-
Malonate utilization	R^-	R^-
Acid production from		
Adonitol	R^-	R^-
Galacturonic acid	R^-	R^-
Inositol	R^-	R^-
L-arabinose	R^-	R^-
L-arabitol	R^-	R^-
L-rhamnose	R^-	R^-
D-arabitol	R^-	R^-
D-cellobiose	R^-	R^-
D-glucose	R^-	R^-
D-maltose	R^-	R^-
D-mannitol	R^-	R^-
D-sorbitol	R^-	R^-
D-sucrose	R^-	R^-
D-trehalose	R^-	R^-
5-ketone-potassium gluconate	R^-	R^-
Palatinose	R^-	R^-
Sodium pyruvate	R^-	R^-

R^+: positive reaction; R^-: negative reaction.

Isolate BY3 exhibits pathogenicity in an experimental challenge. During the seven-day bacterial virulence experiment, 30%-100% of the experimental fish challenged with isolate BY3 died at a concentration of 2.0 $\times 10^5$ CFU/mL to 2.0 $\times 10^7$ CFU/mL (Table 2) with a LD_{50} value of 6.32×10^5 CFU/mL and showed disease signs of skin and fin ray hemorrhages, similar to those seen in the originally diseased fish (Figure 3). When challenged with isolate BY3 at a concentration of 2.0 $\times 10^7$ CFU/mL, the fish died quickly:

six fish died on day 1, three on day 2, and one on day 3. Re-isolates from the experimentally diseased fish are identified phenotypically and molecularly as the BY3 isolate. No clinical signs or mortality were noted in the control fish.

Table 2. Experimental infections of *M. amblycephala* with isolate BY3.

Group	*Concentration (CFU/mL)*	*Fish no.*	*Dead fish no. on day after challenge*							*Cumulative mortality (%)*
			1	*2*	*3*	*4*	*5*	*6*	*7*	
Control	0	10	0	0	0	0	0	0	0	0
Treated 1	2.0×10^4	10	0	0	0	0	0	0	0	0
Treated 2	2.0×10^5	10	1	2	0	0	0	0	0	30
Treated 3	2.0×10^6	10	3	2	2	1	0	0	0	80
Treated 4	2.0×10^7	10	6	3	1	0	0	0	0	100

Figure 3. The pathological symptoms of diseased *M. amblycephala*. Arrows show skin and fin ray hemorrhages.

Antibiotic sensitivity. The antibiotic susceptibility of isolate BY3 is shown in Table 3. The data indicate that isolate BY3 is resistant to clindamycin and oxacillin, and intermediately sensitive or sensitive to the other fifteen tested antibiotics. This suggests that isolate BY3 has not developed resistance to florfenicol, neomycin, and sulfisoxazole antibiotics in aquaculture.

Table 3. Susceptibility of isolate BY3 to antibiotics.

Antibiotics	*Content (μg/disc)*	*Inhibition zone diameter (mm)*
Chloramphenicol	300	22.94 ± 0.36^S
Cefotaxime	30	24.86 ± 0.21^S
Cefradine	30	23.05 ± 0.04^S
Ciprofloxacin	5	29.61 ± 0.63^S
Clindamycin	2	0 ± 0^R
Doxycycline	30	29.63 ± 0.27^S
Erythrocin	15	2.03 ± 0.10^I
Florfenicol*	75	28.01 ± 0.13^S
Furazolidone	300	13.95 ± 0.11^R
Levofloxacin	5	31.87 ± 0.07^S
Norfloxacin	10	24.79 ± 0.17^S
Neomycin*	30	23.95 ± 0.13^S
Oxacillin	1	0 ± 0^R
Penicillin	10	20.02 ± 0.04^I
Rifampicin	5	21.98 ± 0.13^S
Streptomycin	10	21.32 ± 0.63^S
Sulfisoxazole*	300	29.83 ± 0.19^S

Data are presented as the mean ± standard deviation; [S]Sensitive; [I]Intermediately sensitive; [R]Resistant. *Veterinary antibiotics used in aquaculture.

Discussion

The connection between *A. johnsonii* with cyprinids fish diseases in aquaculture has been little documented. Only isolates of *A. johnsonii* from diseased rainbow trout have been characterized to some extent (Kozinska et al., 2014). Little is known about *A. johnsonii* isolates from diseased farmed blunt snout bream. In this study, we characterized the phenotype, taxonomic position and antibiotic susceptibility of *A. johnsonii* BY3. To our knowledge, this is the first report of an *A. johnsonii* pathogen as a causative agent for farmed blunt snout bream.

Cultured cyprinids fish are sensitive to *A. johnsonii* infection (Kozinska et al., 2015). In the present study, the BY3 isolate was found to cause mortality in healthy cyprinid fish *M. amblycephala* with a LD_{50} value of 6.32×10^5 CFU/mL. This further demonstrates the potential threat of *A. johnsonii* to cyprinids fish farming. Various virulence factors are involved in the pathogenicity of *Acinetobacter*, like adherence to epithelial cells, production of extracellular enzymes and toxins, as well as the ability to protect against phagocytosis (Braun, 2008). The pathogenicity of isolate BY3 could probably be associated with the production of these virulent factors. Apart from the virulence of the BY3 isolate, there might be other secondary factors that induce this infection in *M. amblycephala* such as use of contaminated feed and high breeding densities that should be of concern.

Intensive aquaculture has resulted in the use of antimicrobials to retard bacterial infections. The use of antibiotics has caused emergence of antibiotic resistant bacteria. The BY3 isolate in our study is sensitive to florfenicol, neomycin, and sulfisoxazole antibiotics that can be used as potent antimicrobial agents in the treatment of *A. johnsonii* infection in cyprinids fish. However, *Acinetobacter* strains are commonly known as antibiotic resistance gene transmitters (Kozinska et al., 2014). Isolate BY3 might have a great impact on antibiotic resistance transfer in cyprinids fish farming.

In conclusion, the present study demonstrates *A. johnsonii* as an emerging bacterial pathogen for farmed *M. amblycephala*. The pathogenicity of the BY3 isolate supports this infection as a potential threat in blunt snout bream farming.

Acknowledgments

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