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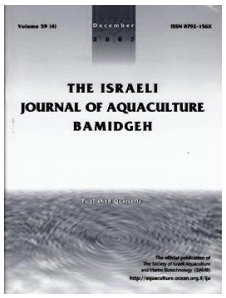

Aeromonas hydrophila: a Causative Agent for Tail Rot Disease in Freshwater Cultured Murray Cod *Maccullochella peelii*

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Abstract

Tail rot disease is the cause of significant economic damage in freshwater farmed Murray cod *Maccullochella peelii*. Only scarce information is available on *Aeromonas hydrophila* as a possible causal agent for this disease. In this study, a virulent strain, temporarily named XY3, was isolated from diseased codfish suffering from tail rot disease, and identified as *A. hydrophila* through phylogenetic analysis and phenotypic characteristics. *A. hydrophila* possesses multiple virulence genes including *aerA*, *ahpA*, *alt*, *ast* and *hlyA* genes. In addition, it appears that isolate XY3 has developed multiple resistances to cephalosporin, chloromycetin, glycopeptides, macrolides, nitrofuran, and penicillin drugs, as well as to aminoglycosides, sulfonamides, and tetracyclines antibiotics for veterinary uses in aquaculture as revealed when screened against a range of common antibiotics. To the best of our knowledge, this is the first report of tail rot disease caused by *A. hydrophila* in freshwater farmed codfish.

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Introduction

Murray cod *Maccullochella peelii,* is one of the world's largest and best-known freshwater fish species. It has high economic value, and has been listed as an endangered species due to the decline in its distribution and abundance (http://www.fishbase.org/summary/10311). With the rapid development of breeding techniques, codfish farming has become a promising industry with great potential for rapid growth (Harford et al., 2006). Production increased to over 150 tons per year in New South Wales and Victoria (Ingram et al., 2005). However, bacterial diseases have become a major cause of mass mortality in codfish (Luo et al., 2015). In order to establish a sustainable codfish farming industry, more attention to bacteriosis is needed.

Tail rot disease is known to cause a significant economic damage in the codfish farming industry (Luo et al., 2015). Several bacterial pathogens such as *Vibrio harveyi*, *Aeromonas sobria,* and *Citrobacter freundii* have been reported to cause tail rot disease in farmed fish (Mei et al., 2010; Haldar et al., 2010; Li & Cai, 2011; Cao et al., 2016). However, scarce information is available on *Aeromonas hydrophila* as a possible causal agent for tail rot disease in freshwater farmed codfish.

In this study, an *A. hydrophila* pathogen was isolated from freshwater cultured codfish suffering from tail rot disease in Shanghai China in June 2015 and our aim was to characterize the phenotype, taxonomic position, potential virulence genes, and antibiotic susceptibility of this strain. To our knowledge, this is the first report of an *A. hydrophila* pathogen as a causative agent for tail rot disease in freshwater farmed *M. peelii*.

Materials and Methods

Murray cod samples. Eighteen diseased freshwater cultured codfish averaging 21.1±2.0 g suffering from tail rot disease were sampled from a codfish farm in Shanghai China during June 2015. The farm had six 200-square meter ponds with codfish stocked at an initial rearing density of 50 juveniles per square meter. Water quality during the disease outbreak was pH 6.82, 0.26 mg/L total ammonia, 0.11 mg/L nitrite and 6.36 mg/L dissolved oxygen. This was the first outbreak of the disease on the farm and it could not be controlled although chlorine dioxide and providone-iodine were applied. Diseased samples were placed in sterile bags, kept in ice and transported to the laboratory.

Isolation of bacteria. Each sampled diseased codfish was externally disinfected with 75% alcohol and dissected. Before conducting a careful microscopic examination for parasites and fungi in diseased codfish, 0.1 g of rotten tail muscle and a liver sample of each codfish was cut and streaked onto nutrient agar (NA) 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 NA plates. Pure isolates of the dominant colonies were stored at -80^0C supplemented with 15% glycerol. A representative of the dominant isolates, temporarily named XY3, was further characterized in the present study.

Identification of the pathogen:

Molecular identification. Extraction of genomic DNA from isolate XY3, as well as PCR amplification and sequencing of 16S rRNA gene were performed according to our previous study (Cao et al., 2010). The near complete 16S rRNA gene sequence was assembled using MegAlign, Editseq, and Seqman software. A search was performed in the National Centre for Biotechnology Information (NCBI) database for sequence homology using the Basic Local Alignment Search Tool (BLAST) program. A phylogenetic tree from the near complete 16S rRNA gene sequence of the isolate and its homologous sequences was constructed using the neighbor-joining method.

Phenotypic identification. Isolate XY3 was identified phenotypically by API 20E system recommended by Topic Popovic et al. (2007). Isolate XY3 was grown on NA plates (Sinopharm Chemical Reagent Co., Ltd.) at 28^0C for 24h, and the bacterial suspension was then used to inoculate the API 20E test strips (Biomerieux, France) following the manufacturer's instruction. The plate was incubated at 37^0C and observed after 18h for checking against the API identification index and database. Information related to *A. hydrophila* in *General manual of systematic and determinative bacteriology* (Dong & Cai, 2001) serves as a reference.

Bacterial virulence assay. Bacterial virulence was examined by experimentally infecting healthy freshwater cultured codfish. One hundred healthy codfish averaging 26.3±1.8 g were obtained from Qinhuang fishery Co., Ltd. in Shanghai China. Their health status was assessed according to the guidelines in our previous study (Cao et al., 2013). The codfish were maintained in ten replicate aquaria (ten codfish per aquarium) supplied with 100 L aerated filtered farming water at 25^0C for 14 days to acclimate. Prior to the bacterial virulence assay, isolate XY3 was inoculated onto NA plate, incubated at 28^0C for 24h, then washed with normal saline into a sterile tube. Cell density was determined by counting colony forming units after a ten-fold serial dilution in sterile distilled water. Two groups of ten healthy codfish were challenged by intramuscular injection in the tail of 0.1 mL of isolate XY3 at a concentration of 5.0×10^5 CFU/mL to 5.0×10^8 CFU/mL. Another two groups of ten healthy codfish exposed to the same experimental conditions were injected intramuscularly with 0.1 mL of normal saline and served as control. The experimental codfish were kept at 25^0C and observed daily for seven days without feeding and water change. Any dead codfish 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 linear regression method as recommended by Won & Park (2008).

Virulence gene assay. The PCR amplification of the virulence genes in isolate XY3 was performed according to our previous study (Zheng et al., 2012). The specific primers for PCR amplification of *A. hydrophila* virulence genes are listed in Table 1, including the aerolysin (*aerA*) gene, serine protease (*ahpA*) gene, cytotonic enterotoxin (*alt* and *ast*) genes, and hemolysin (*hlyA*) gene. *Escherichia coli* DH5α was used as a control. The PCR product was determined by electrophoresis on 1% agarose gel and visualized via ultraviolet trans-illumination.

Table 1. Target-specific primers designed by Zhu et al. (2006) for PCR amplification of virulence genes in *A. hydrophila*.

Virulence gene	Primer (5′→3′)	Sequence length (bp)
aerA	Forward: CCTATGGCCTGAGCGAGAAG Reverse: CCAGTTCCAGTCCCACCACT	431
ahpA	Forward: ATTGGATCCCTGCCTATCGCTTCAGTTCA Reverse: GCTAAGCTTGCATCCGTGCCGTATTCC	1011
alt	Forward: TGACCCAGTCCTGGCACGGC Reverse: GGTGATCGATCACCACCAGC	442
ast	Forward: TCTCCATGCTTCCCTTCCACT Reverse: GTGTAGGGATTGAAGAAGCCG	331
hlyA	Forward: GGCCGGTGGCCCGAAGATACGGG Reverse: GGCGGCGCCGGACGAGACGGGG	592

Antibiotic sensitivity assay. The antibiotic sensitivity of isolate XY3 was assayed on NA plates using the Kirby-Bauer disk diffusion method as recommended by Jones et al. (2001). Twenty-four fishery 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

Identification of the pathogenic isolate. A dominant isolate XY3 was isolated from the diseased freshwater farmed codfish and identified by molecular and phenotypic methods as *A. hydrophila.* Its near complete 16S rRNA gene sequence (1300 nucleotides) was submitted to GenBank database with the accession no. KU249217. 99% of similarity is observed in the 16S rRNA gene sequence between the XY3 isolate and other *A. hydrophila* isolates from the GenBank database. The phylogenetic tree confirms that the isolate XY3 is an *A. hydrophila* strain (Figure 1). This was again confirmed by the

phenotypic features as *A. hydrophila* (Table 2) with 95.2% identity compared to the reference strain. No parasites and fungi were detected in the diseased codfish from which isolate XY3 was obtained.

Figure 1. A 16S rRNA gene tree of 12 known bacteria and the XY3 isolate constructed using the neighbour-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.

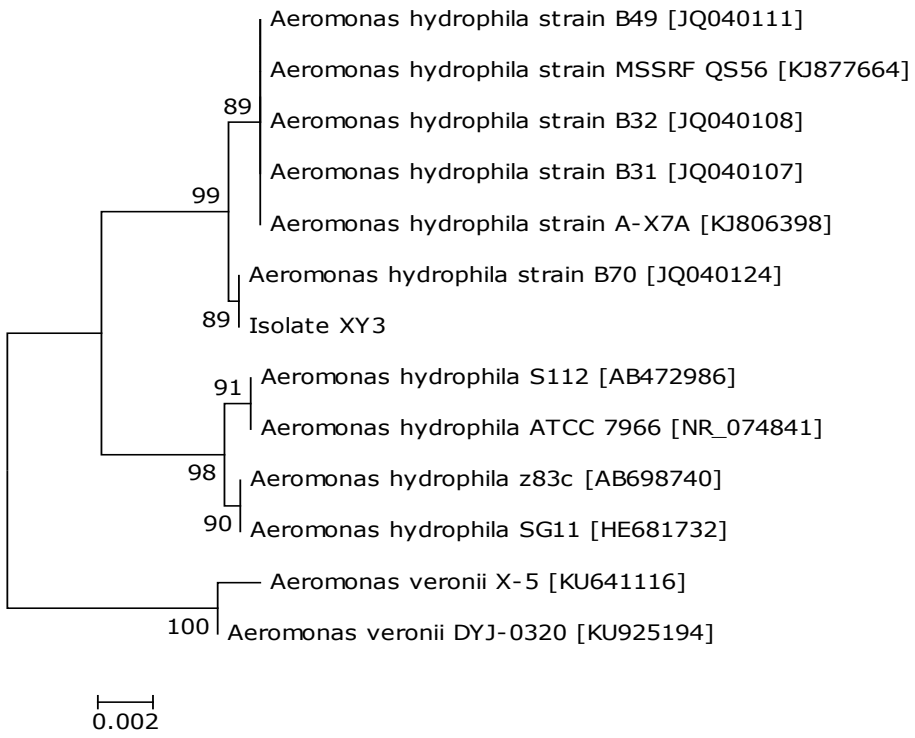


Table 2. Phenotypic characterization of isolate XY3.

Tests	*Reaction*	
	XY3	*A. hydrophila*[a]
Arginine dihydrolase	R^+	R^+
Cytochrome oxidase	R^+	R^+
β-Galactosidase	R^+	R^+
Gelatinase	R^+	R^+
Lysine decarboxylase	R^-	R^+
Ornithine decarboxylase	R^-	R^-
Tryptophan deaminase	R^+	R^+
Urease	R^-	R^-
Citrate utilization	R^-	R^-
Acetoin production	R^+	R^+
Indole production	R^+	R^+
H_2S production	R^+	R^+
Arabinose fermentation	R^+	R^+
Amygdalin fermentation	R^-	R^-
Glucose fermentation	R^+	R^+
Inositol fermentation	R^-	R^-
Mannitol fermentation	R^+	R^+
Melibiose fermentation	R^-	R^-
Rhamnose fermentation	R^-	R^-
Sucrose fermentation	R^-	R^-
Sorbitol fermentation	R^-	R^-

R^+: positive reaction; R^-: negative reaction.
[a]The reference strain data are in accordance with those previously reported (Dong & Cai, 2001).

Isolate XY3 was found to be pathogenic in an experimental challenge. The death of the codfish increased gradually over time after the challenge. 40%-100% of the codfish challenged with isolate XY3 died at a concentration of 5.0×10^5 CFU/mL to 5.0×10^8 CFU/mL (Table 3) with a LD_{50} value of 1.19×10^6 CFU/mL and exhibited tail rot, similar to that seen in the originally diseased codfish (Figure 2). Re-isolated bacteria from experimentally dead codfish were identified phenotypically and molecularly as isolate XY3. No clinical signs or mortality were noted in the control codfish.

Figure 2. Pathological symptoms of the freshwater cultured *M. peelii* suffering from tail rot disease. Arrows show the rotten tails.

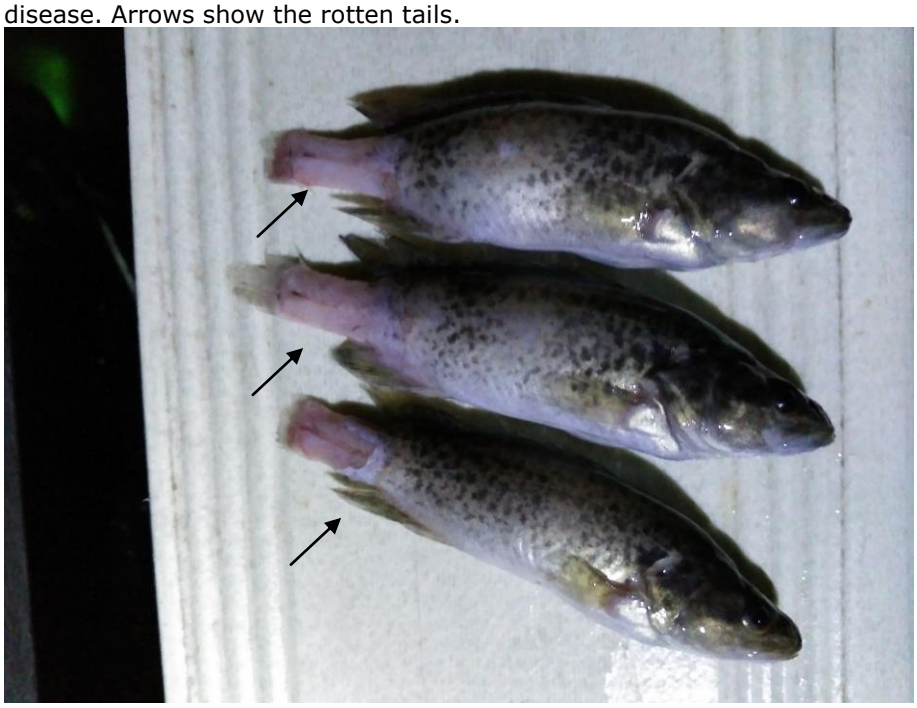
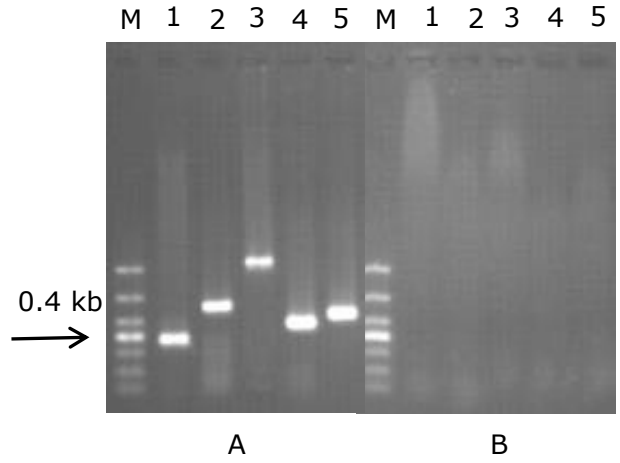


Table 3. Cumulative mortality of experimental codfish infected by isolate XY3.

Group	*Concentration (CFU/mL)*	*No. of codfish*	*Cumulative mortality (%)*	*Average cumulative mortality (%)*
Control	0	10	0	0
		10	0	
Treated 1	5.0×10^5	10	40	40
		10	40	
Treated 2	5.0×10^6	10	60	65
		10	70	
Treated 3	5.0×10^7	10	90	95
		10	100	
Treated 4	5.0×10^8	10	100	100
		10	100	

Virulence genes. The virulence genes of isolate XY3 are shown in Figure 3. The virulent *aerA*, *ahpA*, *alt*, *ast,* and *hlyA* gene fragments were present in isolate XY3 (Figure 3A), which were not found in the control strain (Figure 3B). This indicates that isolate XY3 possesses multiple virulence genes including *aerA*, *ahpA*, *alt*, *ast* and *hlyA* genes.

Figure 3. The PCR amplification of virulence genes in the XY3 isolate. A: isolate XY3; B: strain DH5α. Lane M: DL1000 DNA marker; Lane 1: *ast* gene; Lane 2: *hlyA* gene; Lane 3: *ahpA* gene; Lane 4: *aerA* gene; Lane 5: *alt* gene.



Antibiotic sensitivity. The antibiotic susceptibility of isolate XY3 is shown in Table 4. The data indicates that isolate XY3 is sensitive to ciprofloxacin, enrofloxacin, norfloxacin, ofloxacin, but resistant to the other twenty tested antibiotics. This suggests that isolate XY3 developed resistance to aminoglycosides, sulfonamides and tetracyclines antibiotics for aquaculture use, as well as to cephalosporin, chloromycetin, glycopeptides, macrolides, nitrofuran and penicillin veterinary drugs.

Table 4. Susceptibility of isolate XY3 to antibiotics.

Antibiotics	*Content (μg/disc)*	*Inhibition zone diameter (mm)*
Amoxicillin	10	0 ± 0^{R}
Azithromycin	15	0 ± 0^{R}
Carbenicillin	100	0 ± 0^{R}
Cefaran	30	0 ± 0^{R}
Cefobid	75	0 ± 0^{R}
Ceftazidime	30	0 ± 0^{R}
Chloromycetin	30	10 ± 0^{R}
Ciprofloxacin	5	23.5 ± 2.1^{S}
Clindamycin	2	0 ± 0^{R}
Cotrimoxazole*	23.75/1.25	0 ± 0^{R}
Doxycycline*	30	14.8 ± 0.4^{R}
Enrofloxacin*	5	26.5 ± 0.7^{S}
Erythromycin	15	13.2 ± 1.2^{R}
Furantoin	30	0 ± 0^{R}
Gentamicin	10	7.6 ± 0.6^{R}
Lincomycin	2	0 ± 0^{R}
Medemycin	30	0 ± 0^{R}
Neomycin*	30	0 ± 0^{R}
Netilmicin	30	9.7 ± 0.5^{R}
Norfloxacin	10	20.7 ± 0.4^{S}
Ofloxacin	5	23.3 ± 1.1^{S}
Rifampicin	5	0 ± 0^{R}
Sulfamethoxydiazine*	5	0 ± 0^{R}
Vancomycin	30	0 ± 0^{R}

Data are presented as the mean ± standard deviation;
^{S}Sensitive; ^{R}Resistant.*Veterinary antibiotics used in aquaculture.

Discussion

The association of *A. hydrophila* in fish aquaculture has been well documented with massive mortality reported in climbing perch *Anabas testudineus* (Hossain et al., 2011), grass carp *Ctenopharynngodon idellus* (Zheng et al., 2012), silver carp *Hypophthalmichthys molitrix* (Rashid et al., 2013), southern catfish *Silurus meridionalis* Chen (Zhu et al., 2011), and white bream *Parabramis pekinensis* (Ye et al., 2013). However, there is limited information on *A. hydrophila* infection in freshwater cultured codfish. In this study, we reported tail rot disease in infected codfish caused by *A. hydrophila*.

Aeromonas virulence derives from extracellular enzymes, cytotonic enterotoxins, and hemolysins that it produces (Daskalov, 2006). The occurrence of genes encoding these virulence factors may contribute to the strong pathogenesis of this species (Zheng et al., 2012). In the present study, the XY3 isolate was found to have multiple virulence genes that caused mortality in healthy codfish with a LD_{50} value of 1.19×10^6 CFU/mL. This further demonstrates the potential threat of *A. hydrophila* to the freshwater farming of codfish. Apart from the virulence of the XY3 isolate, there may be other secondary factors that induce tail rot disease in *M. peelii* such as use of contaminated feed and inferior farming water quality (Cao et al., 2016) which should be of concern.

Antibiotic resistance in *A. hydrophila* has been reported in aquaculture. The XY3 isolate in our study was also resistant to multiple fishery antibiotics including cotrimoxazole, doxycycline, neomycin, and sulfamethoxydiazine used in fish farming regions, suggesting that the outbreak of this disease may have resulted from abuse of antibiotics.

In conclusion, the present study for the first time reports an *A. hydrophila* isolate as a causal agent for tail rot disease in freshwater cultured *M. peelii*. The pathogenicity and multiple drug resistance of the XY3 isolate support this infection as a potential threat in the codfish farming.

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