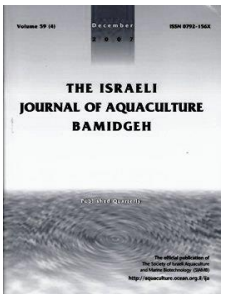

Stenotrophomonas maltophilia: an Emerging Pathogen of Ascites Disease in Farmed Yellow Catfish *Pelteobagrus fulvidraco*

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Abstract

Ascites disease causes significant economic losses in yellow catfish *Pelteobagrus fulvidraco*. Yet only scare information is reported on *Stenotrophomonas maltophilia* as a pathogen for yellow catfish. In this study, a virulent strain, temporarily named WY1, was isolated from diseased yellow catfish fry suffering from ascites disease, identified phenotypically and molecularly as *S. maltophilia*. A phylogenetic tree was constructed to examine isolate WY1 and compare it to other known isolates. In addition, isolate WY1 is apparently susceptible to aminoglycosides and tetracycline drugs 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 *S. maltophilia* as a pathogen of ascites disease in farmed yellow catfish.

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Introduction

Yellow catfish *Pelteobagrus fulvidraco* is a commercially important freshwater fish widely cultivated in China, Japan, Korea, and Southeast Asian countries (Li, 2000). In China, the yellow catfish industry has grown rapidly and is very profitable. In 2016, the total output reached over 417,000 tons (Ministry of Agriculture of China, 2017). However, under intensive culture, this industry has been seriously affected by bacterial diseases (Zhang et al., 2009; Yang et al., 2017). Hence, more attention should be paid to bacteriosis in general, to enable further sustainable development of this industry.

Ascites is one of the most important infectious bacterial diseases in a wide range of fish including yellow catfish (Zhang et al., 2014). This disease resulted in significant economic losses in Ayu *Plecoglossus altivelis* (Nishimori et al., 2000), channel catfish *Ictalurus punctatus* (Wei et al., 2014), Japanese flounder *Paralichthys olivaceus* (Zhu et al., 2006), mandarin fish *Siniperca chuatsi* (Cao et al., 2013) and turbot *Scophthalmus maximus* (Li et al., 2006). Studies have shown that ascites disease in fish can be the result of several bacterial pathogens such as *Aeromonas hydrophila* (Liang et al., 2012), *Aeromonas sobria* (Wei et al., 2014), *Pseudomonas plecoglossicida* (Nishimori et al., 2000), *Vibrio cholerae* (Cao et al., 2013), *Vibrio mimicus* (Zhang et al., 2014). Until now, however, few reports have been published on *Stenotrophomonas maltophilia* as a causal agent of this disease.

Between April and June of 2012 to 2016, ascites, characterized by abdominal distension (Zhang et al., 2009), occurred frequently in yellow catfish cultured in regions of Chongqing, Guangdong, Guangxi, Jiangxi, and Liaoning provinces, China, particularly during the fry culture stage. This disease is highly infectious and lethal, causing 20% to 90% mortality. In the present study, we isolated and identified a *S. maltophilia* pathogen as a causative agent for this disease, and determined its taxonomy and antibiotic susceptibility. To our knowledge, this is the first report of *S. maltophilia* as a pathogen for farmed yellow catfish.

Materials and methods

Fish samples. Sixteen ascites-infected yellow catfish fry averaging 0.3±0.1 g, were sampled from infected ponds of a fish farm in Foshan, Guangdong China in April 2016. The farm has six acres of ponds with yellow catfish stocked at an initial density of 250,000 fry per acre. The water quality during the disease outbreak was pH 8.50, 0.20 mg/L total ammonia, 0.15 mg/L nitrite and 5.12 mg/L dissolved oxygen. Diseased samples were placed in sterile bags, kept in ice and transported to the laboratory.

Bacterial isolation. Each sampled diseased fish was externally disinfected with 75% alcohol and dissected. Before conducting a careful detection of parasites and viruses using traditional methods as described by Huang et al. (2010) and Ma et al. (2015), samples from livers and ascetic fluid of diseased fish were streaked onto nutrient agar (NA) (Sinopharm Chemical Reagent Co., Ltd.) with a flamed loop. After incubation for two days at 28^0C, bacteria isolated from fish were subcultured on the same media plate to check the purity of the isolate. Pure isolates of the predominant uniform colonies were stored at -80^0C supplemented with 15% glycerol. A representative isolate, temporarily named WY1, was characterized further in the present study.

Bacterial identification

Molecular identification. The extraction of genomic DNA from isolate WY1, as well as PCR amplification and sequencing of its 16S rRNA gene, were performed as described by Geng et al. (2010). Near complete 16S rRNA gene sequence was assembled using Editseq and Seqman in DNAstar 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 near-complete 16S rRNA gene sequence of the isolate and its homologous sequences was constructed using the neighbor-joining method.

Phenotypic identification. Isolate WY1 was identified phenotypically using API 32E test strips as recommended by Cheng et al. (2013). The test strip was incubated at 37^0C and observed after 24h against the API identification index. The type strain ATCC51331 of *S. maltophilia* was used as the control.

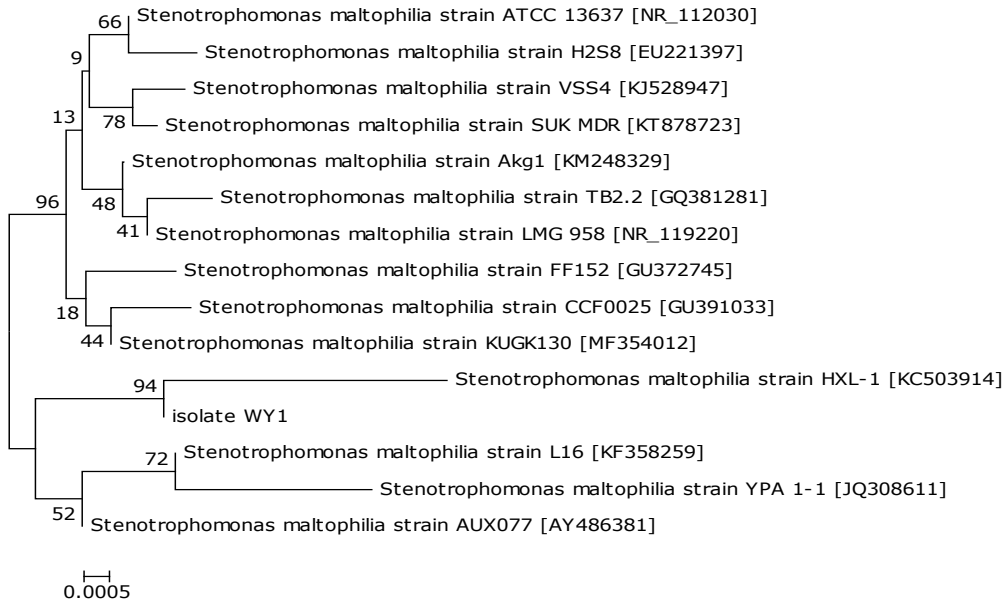
Bacterial virulence assay. Bacterial virulence was examined by experimentally infecting healthy cultured yellow catfish. One hundred healthy yellow catfish averaging 36.1±2.1 g were obtained from Baishazhou fishery Co., Ltd. in Wuhan, China. Their health status was evaluated according to the guidelines recommended by Zheng et al. (2012). The experimental fish were acclimated in ten replicate aquaria (each stocked with ten fish) supplied with 50 L of aerated filtered farming water at 25^0C for 14 days. Prior to the bacterial virulence assay isolate WY1 was inoculated onto NA plate, incubated at 28^0C for 24h, and 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. Two replicates of ten healthy fish in four treatment groups were challenged by intraperitoneal injection with 0.2 mL of isolate WY1 at cell densities of 2.0 $\times10^4$ CFU/mL, 2.0 $\times10^5$ CFU/mL, 2.0 $\times10^6$ CFU/mL and 2.0 $\times10^7$ CFU/mL. Another two replicates of ten healthy fish exposed to the same experimental conditions and injected intraperitoneally with 0.2 mL of normal saline that served as control. The experimental fish were kept at 25^0C and observed daily for seven days without feeding and water change. Any dead fish were immediately removed and sampled to confirm if the mortality was caused specifically by the challenge isolate. The mean lethal dose (LD_{50}) value is calculated according to the graphical probit method as recommended by Ogbuagu & Iwuchukwu (2014).

Antibiotic sensitivity assay. The antibiotic sensitivity of isolate WY1 was assayed on NA plates using the Kirby-Bauer disk diffusion method as described by Joseph et al. (2011). Nineteen antibiotic discs were acquired from Hangzhou Tianhe Microorganism Reagent Co., Ltd. The inhibition zones were measured after a 24h incubation period at 28^0C. The antibiotic susceptibility was determined according to the manufacturer's guidelines.

Results

Bacterial identification. A dominant isolate WY1 was isolated from the diseased yellow catfish fry and identified by molecular and phenotypic methods as *S. maltophilia.* Its near complete 16S rRNA gene sequence (1400 nucleotides) was submitted to GenBank database with the accession no. MF458998. A similarity of 99% was observed in the 16S rRNA gene sequence between the WY1 isolate and other *S. maltophilia* isolates from the GenBank database. The phylogenetic tree confirms that the isolate WY1 is identified with *S. maltophilia* strain (Figure 1).

Figure 1. A 16S rRNA gene tree of 14 known bacteria and the WY1 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.



This is again confirmed by the phenotypic features as *S. maltophilia* (Table 1) with 96.88% identity compared to the reference strain. No parasites and viruses were detected in the diseased yellow catfish from which the isolate WY1 was obtained.

Table 1. Phenotypic characteristics of isolate WY1.

Tests	*Reaction*	
	WY1	*ATCC51331*
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 WY1 was virulent to yellow catfish with a LD_{50} value of 1.12×10^5 CFU/mL (Table 2). The infected fish exhibited similar clinical symptoms to those seen in the originally diseased fish (Figure 2). When fish were challenged with a concentration of 2.0×10^7 CFU/mL, acute mortality was observed. The isolate WY1 could be re-isolated from experimentally dead fish. No clinical signs or mortality were noted in the control fish.

Table 2. Cumulative mortality of experimental yellow catfish infected by the isolate WY1.

Group	*Concentration (CFU/mL)*	*Fish no.*	*Dead fish no. on day after challenge*							*Average cumulative mortality (%)*	*LD_{50} value (CFU/mL)*
			1	*2*	*3*	*4*	*5*	*6*	*7*		
Control	0	10	0	0	0	0	0	0	0	0	1.12×10^5
		10	0	0	0	0	0	0	0		
Treatment 1	2.0×10^4	10	0	0	1	1	0	0	0	25	
		10	0	1	1	1	0	0	0		
Treatment 2	2.0×10^5	10	0	1	2	1	1	0	0	55	
		10	1	1	1	2	1	0	0		
Treatment 3	2.0×10^6	10	3	3	1	1	1	0	0	90	
		10	2	3	1	2	0	1	0		
Treatment 4	2.0×10^7	10	4	4	1	1	0	0	0	100	
		10	5	4	1	0	0	0	0		

Figure 2. Pathological symptoms of the diseased yellow catfish fry. A: arrow shows abdominal distension. B: arrow shows dead fry floating to the water surface.

Antibiotic susceptibility. The antibiotic sensitivity of isolate WY1 is shown in Table 3. The data indicate that the isolate WY1 is sensitive to ceftazidine, cefotaxime, doxycycline, gentamycin, kanamycin, neomycin, streptomycin, tetracycline, and resistant to the macrolides, penicillin, quinolones, sulfonamides antibiotics. This suggests that the isolate WY1 has not developed resistance to aminoglycosides and tetracycline antimicrobials.

Table 3. Susceptibility of isolate WY1 to antibiotics.

Antibiotics	Content (μg/disc)	Inhibition zone diameter (mm)
Amoxicillin	10	0 ± 0^{R}
Ampicillin	10	0 ± 0^{R}
Ceftazidine	30	24.80 ± 0.42^{S}
Cefotaxime	30	29.50 ± 0.71^{S}
Ciprofloxacin	5	0 ± 0^{R}
Doxycycline*	30	23.65 ± 0.64^{S}
Enrofloxacin*	5	0 ± 0^{R}
Erythromycin	15	0 ± 0^{R}
Furazolidone	30	17.75 ± 1.06^{R}
Gentamycin	10	20.20 ± 0.28^{S}
Kanamycin	30	18.75 ± 0.07^{S}
Neomycin*	30	23.25 ± 0.21^{S}
Norfloxacin	10	0 ± 0^{R}
Ofloxacin	5	0 ± 0^{R}
Penicillin	10	0 ± 0^{R}
Streptomycin	10	15.25 ± 0.35^{S}
Sulfamethoxazole*	300	0 ± 0^{R}
Sulfamethoxydiazine	5	0 ± 0^{R}
Tetracycline	30	21.00 ± 1.41^{S}

Data are presented as the mean ± standard deviation;
^{S}Sensitive; ^{R}Resistant.*Antibiotics for aquaculture use.

Discussion

S. maltophilia in aquaculture has been documented and associated with mortality in African catfish *Clarias gariepinus* (Abraham et al., 2016), channel catfish *Ictalurus punctatus* (Geng et al., 2010), turbot *Scophthalmus maximus* (Wu et al., 2014). However, there is limited information on *S. maltophilia* as a causal agent for cultured yellow catfish. In this study, we characterized the phenotype, taxonomic position, and antibiotic susceptibility of *S. maltophilia* WY1. To our knowledge, this is the first report of a *S. maltophilia* pathogen as a causative agent for ascites in farmed yellow catfish.

S. maltophilia is considered a fish pathogen with the ability to produce enzymatic virulence factors such as protease, lipase, and hemolysin (Geng et al., 2006). Fish diseases caused by *S. maltophilia* are usually associated with the production of these virulent factors (Chen et al., 2008). In the present study, the WY1 isolate of *S. maltophilia* attained an LD_{50} mortality in healthy yellow catfish when challenged with a concentration of 1.12×10^5 CFU/mL. This further demonstrates the potential threat of WY1 to yellow catfish farming. Apart from the virulence of the WY1 isolate, there might be other causes of ascites that should be raised as concerns, such as high density stocking, lack of food disinfection, high ammonia and nitrite levels in farming waters (Cao et al., 2013; Zhang et al., 2015).

Antibiotic resistance of *S. maltophilia* has developed due to massive use of antimicrobials for treatment of fish bacteriosis (Hu et al., 2015). Resistance to sulphamethoxazole is an indicator of multiple drug resistance (Zhao et al., 2017). In our study, the resistance to sulfamethoxazole is observed in the WY1 isolate, which is consistent with the fact of multiple resistance of isolate WY1. This is usually associated with the presence of *sul* and *dfrA* genes in integrons (Zhao et al., 2017). The resistance to penicillins and quinolone antibiotics is also noted in *S. maltophilia* isolated from *I. punctatus* (Geng et al., 2010). The WY1 isolate exhibited sensitivity to doxycycline and neomycin used in the fish farming regions, suggesting that the outbreak of this disease may not have resulted from the abuse of these antibiotics.

In conclusion, the present study for the first time reports a *S. maltophilia* isolate as a pathogen for cultured yellow catfish. The pathogenicity of the WY1 isolate supports this infection as an emerging threat in yellow catfish farming.

Acknowledgments

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