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Plesiomonas shigelloides, a potential pathogen of enteritis in *Ictalurus punctatus*

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Abstract

Enteritis has resulted in large economic losses in channel catfish aquaculture. Yet only scarce information is available on *Plesiomonas shigelloides* as a causal agent for this disease. In this study, a virulent strain, temporarily named BD1, was isolated from diseased channel catfish suffering from enteritis, and was identified as *P. shigelloides* through molecular and phenotypic methods. A phylogenetic tree was also constructed to determine its taxonomic position. In addition, the BD1 isolate has developed multiple resistances to aminoglycosides, amphenicols, quinolones, tetracyclines and sulfonamides 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 *P. shigelloides* as a potential pathogen of enteritis in channel catfish.

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Introduction

Channel catfish *Ictalurus punctatus* is widely cultivated in many countries such as America, Canada, China and Mexico (Liu et al., 2006; Zhang et al., 2019). Especially in China, with the rapid development of farming techniques, the channel catfish has become one of the most important commercial freshwater fish species and has brought a great profit in recent years (Zhang et al., 2019). Its production has increased to over 230,000 tons in 2018 (Ministry of Agriculture and Rural Affairs of China, 2019). However, under intensive culture, this industry has been seriously affected by bacterial diseases (Mohammed et al., 2018). Thus, more attention should be given to bacteriosis to make further development of this industry.

Enteritis, characterized by intestinal hyperemia and inflammation, is one of the most important infectious bacterial diseases in channel catfish aquaculture (Wu et al., 2000) and usually results in a high mortality of over 90% (Xie et al., 2019). So far, several bacterial pathogens such as *Aeromonas punctata*, *Aeromonas veronii*, *Enterobacter aerogenes* and *Stenotrophomonas maltophilia* have been reported to cause this disease (Wu et al., 2000; Geng et al., 2006; Huang et al., 2010; Cao et al., 2017). *Plesiomonas shigelloides* is a Gram-negative, rod-shaped, motile bacterium which is widely distributed in freshwater (Khardori et al., 1988; Ekundayo et al., 2020), and its occurrence in clinical infections is usually found in warm waters especially in farming waters with temperatures of 25 to370C and pH values of 5 to 8 (Liu et al., 2015). However, little information is available on *Plesiomonas shigelloides* as a causal agent for enteritis in channel catfish.

The aim of this study is to characterize the phenotype, taxonomic position and antibiotic susceptibility of *P. shigelloides* pathogen isolated from enteritis-infected channel catfish. As far as we know, this is the first report of enteritis caused by *P. shigelloides* in channel catfish.

Materials and Methods

Channel catfish samples

Twenty diseased channel catfish averaging 753.0±5.7 g suffering from enteritis were sampled from a channel catfish farm with a disease morbidity of 80% and water quality parameters of pH 7.80, 0.20 mg L^{-1} total ammonia, 0.01 mg L^{-1} nitrite and 5.61 mg L^{-1} dissolved oxygen in Yancheng, Jiangsu China during May 2019. This farm had 15 acres of ponds with channel catfish stocked at an initial rearing density of 9100 juveniles per acre and fed pellet diets at a rate of 3% of body weight with 3 times per day. Diseased samples were placed in sterile bags, kept in ice and transported to the laboratory according to Zeng et al. (2009).

Confirmation of the pathogen

Each sampled diseased channel catfish was externally disinfected with 75% alcohol and dissected according to Zhu et al. (2001). To verify the potential pathogens, a squash of organs (intestine, liver, kidney, muscle, gill) were made and carefully examined for parasites under the microscope as described by Feng et al. (2019). Meanwhile, the virological examination was also conducted as described by Huang et al. (2013). Briefly, the homogenate of organs (intestine, liver, kidney, muscle, gill) was made and filtered through 0.22-μm-pore-size membrane filter to remove bacteria. Two aquaria of 10 healthy fish were injected muscularly with 0.2 mL of each bacteria-free organ filtrate. Another two aquaria of 10 healthy fish, which were exposed to the same experimental conditions and injected muscularly with 0.2 mL of normal saline, served as the control. Experimental fish were kept at 30^0C without water change. The mortality and any visible changes of the experimental fish were recorded every day for 15 days. In addition, besides the liver, kidney samples of each diseased fish, a section of 0.1 g of affected intestine sample was aseptically cut and rinsed three times with sterile nine-salt solution (Olsson et al., 1992), then was separately homogenized in sterile nine-salt solution and followed by serial tenfold dilution directly streaked onto nutrient agar (NA) plates (Sinopharm Chemical Reagent Co., Ltd.) as recommended by Xu et al. (2008) and Cao et al. (2017). After incubation for 24 h at 30^0C, the dominant uniform isolates were purified by streaking and re-streaking onto NA plates. Only the dominant isolates with dense virtually pure culture growth on NA plates

were obtained according to different colony shape, colony color and colony size as recommended by Xu et al. (2008) and Orozova et al. (2012) and were stored at -80^{0}C supplemented with 15% glycerol according to Zhang et al. (2018). The pure isolates were inoculated onto an NA plate, incubated at 28^{0}C for 24 h, and washed with sterile normal saline into a sterile tube. Their cell densities were determined by counting colony forming units after a ten-fold serial dilution in sterile saline as described by Zhu et al. (2001). Induced infection of the pure isolates was performed according to Cao et al. (2017). Briefly, two aquaria of ten healthy fish were challenged by intraperitoneal injection as recommended by Xu et al. (1988) with 0.2 mL of the isolate at a concentration of 2.6×10^7 CFU mL^{-1}. Another two aquaria of ten healthy fish exposed to the same experimental conditions and injected intraperitoneally 0.2 mL of normal saline remained unchallenged and served as control. The experimental fish were kept at 30^{0}C and observed daily for seven days without feeding and water change. Dead fish were immediately removed to re-isolate and identify the challenge isolate as described above to confirm if the mortality was caused by the challenge isolate.

Identification of the pathogen

Molecular identification

The genomic DNA was extracted from the pathogenic isolate using the TIANamp DNA Kit (Tiangen Biotech. Co., Ltd.). Its 16S rRNA gene was amplified by PCR according to Chen et al*.* (2012) and was sequenced by ABI 3730 XL DNA Sequencer (Applied Biosystems, USA). A homology search was performed in the National Centre for Biotechnology Information (NCBI) database for 16S rRNA gene sequences 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 neighbour-joining method.

Phenotypic identification

The pathogenic isolate was identified phenotypically by API 32GN system recommended by Altwegg et al. (1987), where the isolate BD1 was grown on NA plates at 28^{0}C for 24h, and the bacterial suspension was then used to inoculate the API 32GN test strips (Biomerieux, France) following the manufacturer's instruction. The plate was incubated at 37^{0}C and observed after 18h for checking against the API identification index and database. Information related to *P. shigelloides* previously reported (Wang et al., 2013; Cao et al., 2007) serves as a reference.

Bacterial virulence assay

The experiment was conducted in strict accordance with the Regulations on Experimental Animals Administration of China (Publication No. 676). Bacterial virulence was examined by experimentally infecting healthy channel catfish. One hundred healthy channel catfish averaging 75.2±1.3 g were obtained from a channel catfish farm in Hubei China. The experimental fish were acclimated in ten replicate aquaria (ten fish per aquarium) supplied with 100 L of aerated filtered farming water at 30^{0}C for 14 days. Prior to the bacterial virulence assay the pathogenic isolate was inoculated onto NA plate, incubated at 30^{0}C for 24h, 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 saline as described by Zhu et al. (2001). Two replicates of ten healthy fish were challenged by intraperitoneal injection as recommended by Xu et al. (1988) and Cao et al. (2017) with 0.2 mL of the pathogenic isolate at a concentration of 2.6×10^5 CFU mL^{-1} to 2.6×10^8 CFU mL^{-1}. Another two replicates of ten healthy fish exposed to the same experimental conditions and injected intraperitoneally 0.2 mL of normal saline remained unchallenged and served as control. The experimental fish were kept at 30^{0}C 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 the mortality was caused specifically by the challenge isolate according to Liu et al. (2006). Briefly, dead fish were sampled to re-isolate the challenge isolate which was confirmed phenotypically and molecularly as described above. The mean lethal dose (LD_{50}) value is calculated using the linear regression method as recommended by Spielmann et al. (1999).

Antibiotic susceptibility assay

The antibiotic susceptibility of the pathogenic isolate was assayed on NA plates using the Kirby-Bauer disk diffusion method as recommended by Yang et al. (2014). Twenty-one antibiotic discs were acquired from Hangzhou Binhe Microorganism Reagent Co., Ltd.. The zones of inhibition against the isolate were measured after a 24h incubation period at 28^0C, and its susceptibility to antibiotics was determined according to the manufacturer's guidelines.

Results

Identification of the pathogen

No parasites were found in the diseased channel catfish, and all of the experimental fish challenged with the bacteria-free organ filtrate survived with no visible changes (data not shown), indicating that this disease was not caused by parasites or viruses. A total of eight dominant isolates, temporarily numbered from BD1 to BD8, were recovered from diseased fish, and only isolate BD1, which could be isolated from intestine, liver, kidney of all the sampled fish, was confirmed as the pathogen for this disease according to Koch's postulate: (i) The BD1 isolate could be isolated from diseased channel catfish. (ii) The death of the experimental fish was increased gradually over time after the challenge with isolate BD1. 30%-100% of the challenged fish died at an LD_{50} value of 2.42×10^6 CFU mL^{-1} (**Table 1**) and exhibited signs of intestinal hyperemia and inflammation similar to that seen in the originally diseased channel catfish (**Figure 1**), which was consistent with the clinical symptoms of bacterial enteritis in channel catfish reported by Wu (2000). No clinical signs or mortality are noted in the control channel catfish. (iii) The BD1 isolate could be re-isolated from experimentally dead fish, which was determined through phenotypical and molecular identification. These findings demonstrated that isolate BD1 was the causative agent of this disease.

Table 1 Cumulative mortality of experimental channel catfish infected by isolate BD1.

Group	Concentration (CFU mL^{-1})	Fish no.	Dead fish no. on day after challenge							Average cumulative mortality (%)	LD_{50} value (CFU mL^{-1})
			1	2	3	4	5	6	7		
Control	0	10	0	0	0	0	0	0	0	0	2.42×10^6
		10	0	0	0	0	0	0	0		
Treatment 1	2.6×10^5	10	0	2	1	0	0	0	0	30	
		10	1	1	1	0	0	0	0		
Treatment 2	2.6×10^6	10	2	1	1	0	0	0	0	40	
		10	1	1	1	1	0	0	0		
Treatment 3	2.6×10^7	10	2	3	1	1	1	0	0	80	
		10	3	1	2	2	0	0	0		
Treatment 4	2.6×10^8	10	5	2	3	0	0	0	0	100	
		10	4	4	2	0	0	0	0		

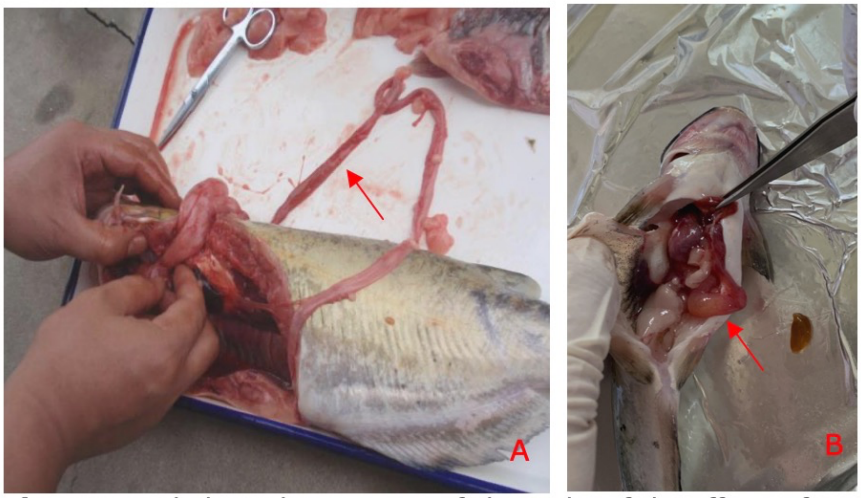

Figure 1 Pathological symptoms of channel catfish suffering from enteritis. (A) Naturally affected fish. (B) Experimental fish challenged with isolate BD1. Arrows show intestinal hyperemia and inflammation.

Identification of the pathogen

Isolate BD1 was identified by molecular and phenotypic methods as *P. shigelloides.* Its near complete 16S rRNA gene sequence (1400 nucleotides) was submitted to GenBank database with the accession no. MT250047. A similarity of 99% to 100% is observed in the 16S rRNA gene sequence between the BD1 isolate and other *P. shigelloides* isolates from the GenBank database. The phylogenetic tree indicates that the BD1 isolate is identified as a *P. shigelloides* strain (**Figure 2**). Besides, isolate BD1 was also confirmed by the phenotypic features as *P. shigelloides* (**Table 2**) with 100% identity compared to the reference strain. No parasites and viruses were detected in the diseased channel catfish from which isolate BD1 was isolated.

Antibiotic susceptibility of the pathogen

The antibiotic susceptibility of isolate BD1 is shown in **Table 3**. The data indicate that isolate BD1 is sensitive to furazolidone, but resistant to amoxicillin, cefotaxime, cephradine, chloramphenicol, ciprofloxacin, clindamycin, doxycycline, enrofloxacin, erythromycin, florfenicol, kanamycin, levofloxacin, neomycin, norfloxacin, oxacillin, penicillin, rifampicin, streptomycin, sulfisoxazole and tobramycin. This suggests that isolate BD1 has developed multiple resistances to aminoglycosides, amphenicols, quinolones, tetracyclines and sulfonamides drugs used in aquaculture.

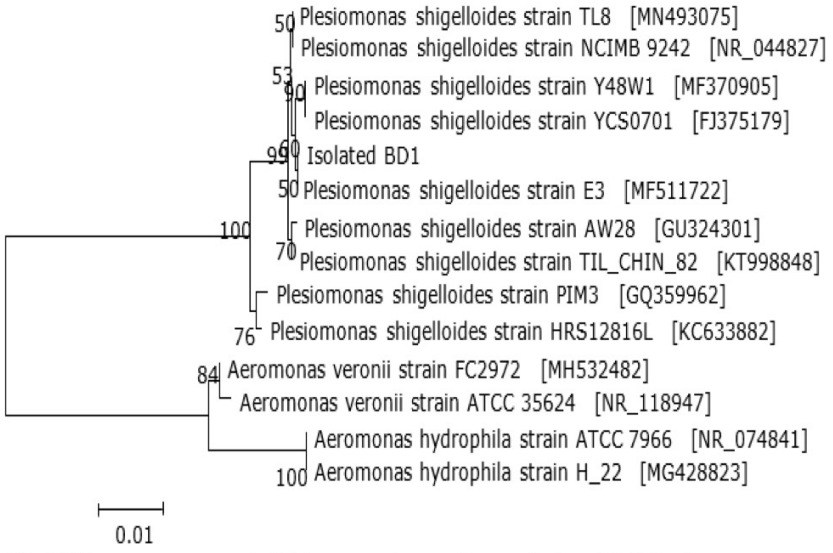

Figure 2 A 16S rRNA gene tree of 13 known bacteria and the BD1 isolate constructed using the neighbor-joining method. The bootstrap values (%) are shown besides the clades, accession numbers are indicated beside the names of strains, and scale bars represent distance values.

Table 2 Phenotypic characterization of isolate BD1.

	Reaction	
	Isolate BD1	***P. shigelloides*[a]**
Rhamnose	R^-	R^-
N-Acetyl-glucosamine	R^+	R^+
Ribose	R^+	R^+
Inositol	R^+	R^+
Saccharose	R^-	R^-
Maltose	R^+	R^+
Itaconate	R^-	R^-
Suberate	R^-	R^-
Malonate	R^-	R^-
Acetate	R^-	R^-
DL-Lactate	R^-	R^-
L-Alanine	R^-	R^-
5-Keto-gluconate	R^-	R^-
Glycogen	R^-	R^-
3-Hydroxy-benzoate	R^-	R^-
L-Serine	R^-	R^-
Mannitol	R^-	R^-
D-Glucose	R^+	R^+
Salicin	R^-	R^-
D-Melibiose	R^-	R^-
L-Arabinose	R^-	R^-
L-Fucose	R^-	R^-

D-Sorbitol	R^-	R^-
Propionate	R^-	R^-
Caprate	R^-	R^-
Valerate	R^-	R^-
Citrate	R^-	R^-
Histidine	R^-	R^-
2-Keto-gluconate	R^-	R^-
3-Hydroxy-butyrate	R^-	R^-
4-Hydroxy-benzoate	R^-	R^-
L-Proline	R^+	R^+

R^+: positive reaction; R^-: negative reaction.
[a]The reference strain's data are in accordance with those previously reported (Wang et al., 2013; Cao et al., 2007).

Table 3 Susceptibility of isolate BD1 to antibiotics.

Antibiotics	**Content (μg/disc)**	**Inhibition zone diameter (mm)**
Amoxicillin	20	0 ± 0^R
Cefotaxime	30	0 ± 0^R
Cephradine	30	0 ± 0^R
Chloramphenicol	30	0 ± 0^R
Ciprofloxacin	5	0 ± 0^R
Clindamycin	2	0 ± 0^R
Doxycycline*	30	0 ± 0^R
Enrofloxacin*	5	0 ± 0^R
Erythromycin	15	0 ± 0^R
Florfenicol*	30	0 ± 0^R
Furazolidone	30	21 ± 0^S
Kanamycin	30	0 ± 0^R
Levofloxacin	5	11.5 ± 0.71^R
Neomycin*	30	10.25 ± 0.35^R
Norfloxacin	10	0 ± 0^R
Oxacillin	1	0 ± 0^R
Penicillin	10	0 ± 0^R
Rifampicin	5	0 ± 0^R
Streptomycin	10	10.25 ± 0.35^R
Sulfisoxazole*	300	0 ± 0^R
Tobramycin	10	11.25 ± 0.35^R

Data are presented as the mean ± standard deviation. [S]Sensitive; [I]Intermediately sensitive; [R]Resistant.
*Antibiotics for aquaculture use (Ministry of Agriculture of China, 2013).

Discussion

The association of *P. shigelloides* in aquaculture has been well documented with mortality of *Anguilla japonica* (Sugita et al., 1993), *Callinectes sapidus* (Marshall et al., 1996), *Trionyx sinensis* (Wu et al., 2004), *Takifagu obscurus* (Fang et al., 2005), *Ostrea gigas thunberg* (Gu et al., 2006), *Garra rufa* (Yu et al., 2009), *Oreochromis niloticus* (Nadirah et al., 2012; Martins et al., 2018), *Ctenopharyngodon idellus* (Hu et al., 2014), *Andrias davidianus* (Yang et al., 2014), *Carassius auratus* (Zhang et al., 2015), *Macrobrachium rosenbergii* (Liu et al., 2015), *Mylopharyngodon piceus* (Ye et al., 2016), *Lota lota* (Zhang et al., 2018) and *Pelteobagrus fulvidraco* (Dou et al., 2019). However, there is limited information on *P. shigelloides* isolates as causal agents for enteritis in channel catfish. In the present work, a *P. shigelloides* pathogen was isolated from channel catfish suffering from enteritis, and its phenotype, taxonomic position and antibiotic susceptibility were characterized. To our knowledge, this is the first report of a *P. shigelloides* pathogen as a causative agent for enteritis in channel catfish.

P. shigelloides is widely distributed in aquatic ecosystems and possesses a variety of virulence factors, including adhesive ability, hemolysins, cytotoxins, cholerae-like toxins and other exoenzymes associated with pathogenicity (Salerno et al., 2010; Edwards et al., 2019). Thus, *P. shigelloides* is a well-recognized fish opportunistic pathogen causing both intestinal and extra-intestinal diseases (Salerno et al., 2010; Zhang et al., 2019). Enteritis caused by *P. shigelloides* is probably associated with the production of these virulent factors. The intraperitoneal injection has been demonstrated as an effective challenge way to induce enteritis of fish (Xu et al., 1988; Cao et al., 2017), which is better than oral challenge and immersion exposure (Ye et al., 2000; Liu et al., 2006; Liang et al., 2007). This is probably because that the bacterial pathogen could grow in the intestinal vessel through intraperitoneal injection, release its virulent toxins which impair the permeability of vessel, damage the vessel wall and cause the erythrocytes escape, eventually resulted in the clinical signs of intestinal hyperemia and inflammation (Xu et al., 1988). Thus, the intraperitoneal injection is chosen in the challenge test. In the present study, the BD1 isolate was found to cause mortality in healthy *I. punctatus* with an LD_{50} value of 2.42×10^6 CFU mL^{-1}. This further demonstrates the potential threat of *P. shigelloides* to channel catfish farming. Apart from the virulence of the BD1 isolate, there might be other secondary factors that induce enteritis in channel catfish such as the varieties of degradation, the misuse of feed additives, and over intensification of stocking density (Feng et al., 2019); these should also be raised as concerns.

The development of antimicrobial resistance in *P. shigelloides* is a matter of concern (Ekundayo et al., 2020). For example, *P. shigelloides* isolate from diseased *T. sinensis* exhibited resistance to aminoglycosides, amphenicols and tetracycline antimicrobials (Wu et al., 2004), *P. shigelloides* isolate from diseased *Silurus asotus* showed resistance to amphenicols and tetracycline antibiotics (Abdelhamed et al., 2018). In our study, isolate BD1 also developed multiple resistances to aminoglycosides, amphenicols, quinolones, tetracyclines and sulfonamides drugs used in fish farming regions, suggesting that the outbreak of this disease may have resulted from the abuse of antibiotics.

In conclusion, the present study for the first time reports a *P. shigelloides* isolate as a causal agent for enteritis in *I. punctatus*. The pathogenicity of the BD1 isolate supports this infection as a potential threat in channel catfish farming.

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