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Bacillus cereus, a potential pathogen of snakehead fish *Ophiocephalus argus*

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Abstract

Bacillus cereus is an emerging pathogen that has caused high mortalities in aquaculture animals. Yet the pathogenicity of *B. cereus* in snakehead fish *Ophiocephalus argus* is still unclear. In this study, a virulent strain (CA4) was isolated from diseased snakehead fish suffering from a typical symptom of hepatic hemorrhage with blood vessel congestion and macrophage infiltration, and was identified molecularly and phenotypically as *B. cereus*. It was β-hemolytic, showed an LD_{50} value of 2.57×10^6 CFU mL^{-1} for snakehead fish, and developed multiple resistances to cotrimoxazole, doxycycline, florfenicol, neomycin, sulfisoxazole, and tetracycline in aquaculture use. To the best of our knowledge, this is the first report of snakehead fish- pathogenic *B. cereus.* The findings of this study provide new insights into the potential threat of pathogenic *B. cereus* to snakehead fish.

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Introduction

Snakehead Ophiocephalus argus is a commercially important freshwater fish widely farmed in China, India, Korea, Malaysia, the Philippines, and Thailand (Gu et al., 2019). In particular, by implementing modern farming techniques, snakehead fish aquaculture in China has become a rapidly growing industry with a total production of over 548 kilotons in 2021 (Ministry of Agriculture and Rural Affairs of China, 2022). However, under intensive culture conditions, this industry has been badly contaminated with bacterial pathogens (Gu et al., 2019; Wang et al., 2020). The association of *Aeromonas schubertii*, *A. veronii*, *Citrobacter freundii*, and *Nocardia seriolae* with massive mortalities in snakehead fish has been well documented (Wang et al., 2007; Liu et al., 2012; Gu et al., 2019; Wang et al., 2020). Thus, bacterial pathogens in snakehead fish should be concerned.

Bacillus cereus is a Gram-positive pathogen widely distributed among aquatic environments (Bottone, 2010). It is characterized by the ability to produce a collection of virulence factors such as pore-forming toxins, hemolysins, enterotoxins, proteases, and phospholipases (Tuipulotu et al., 2021). It has caused high mortalities in the Chinese soft-shelled turtle *Pelodiscus sinensis*, Huangsha soft-shelled turtle *Truogx sinensis*, half-smooth tongue sole *Cynoglossus semilaevis*, yellow catfish *Pelteobagrus fulvidraco*, tiger frog *Hoplobatrachus rugulosus*, and tilapia *Oreochromis mossambicus* (Cho et al., 2010; Wang and Huang, 2012; Hu et al., 2016; Yang et al., 2017; Wang et al., 2018; Dou et al., 2019; Meng et al., 2019; Li et al., 2020a). So far, characterization of pathogenic *B. cereus* isolated from the soft-shelled turtle, half-smooth tongue sole, tiger frog, yellow catfish, and tilapia has been documented (Cho et al., 2010; Wang and Huang, 2012; Hu et al., 2016; Yang et al., 2017; Wang et al., 2018; Dou et al., 2019; Meng et al., 2019; Li et al., 2020a), but little information is available on *B. cereus* isolated from diseased snakehead fish.

In this study, *B. cereus* CA4 was demonstrated as a pathogen of diseased snakehead fish with a typical symptom of hepatic hemorrhage, and its taxonomic position, virulence, as well as susceptibility to antibiotics were further examined. As far as we know, this study is the first to identify *B. cereus* as a causative pathogen of snakehead fish. The findings of this study provide new insights into the potential threat of pathogenic *B. cereus* to snakehead fish.

Materials and Methods

Fish and Reagents

Twenty diseased snakehead fish (200.3±2.3 g in weight) were obtained from an infected snakehead fish farming pond with a mortality of over 40% in Huaiyuan, Anhui, China, in June 2022, and immediately placed into ice-cold sterile bags and sent to laboratory according to Hossain et al. (2013). The water quality indicators measured during the disease outbreak were 32 °C, pH 7.9, 6.1 mg L^{-1} dissolved oxygen, 0.3 mg L^{-1} nitrite, and 0.9 mg L^{-1} total ammonia. Healthy snakehead fish (190.1±7.8 g) were acquired from unaffected snakehead fish farming ponds in Hangzhou, Zhejiang, China. They maintained good health with no contamination with *B. cereus*, *A. hydrophila,* and *A. veronii* pathogens by sampling a few individuals for careful examinations (Chu and Lu, 2005; Li et al., 2020b).

Pathogen Assay

The potential pathogens were examined according to Wang et al. (2020). First, the organs (liver, kidney, intestine, muscle, and gill) were dissected from diseased snakehead fish in the laboratory (Gu et al., 2019), compressed manually between two glass slides to prepare thin sections of the organs, and then subjected to a careful observation for potential parasites using light microscopy (Yang, 2018). Second, to determine if viruses caused this disease, two replicate aquaria of healthy snakehead fish (10 fish per aquarium) were artificially infected by intramuscular injection with 0.2 mL of the freshly-prepared bacteria-free organ filtrates (Gong et al., 2010). Another two replicate aquaria of control fish (10 fish per aquarium) were treated intramuscularly with the same volume (0.2 mL)

of sterile normal saline. All the test fish were stocked in aquaria containing 100 L aerated tap water at 32 °C without water being changed and observed for fifteen days to record fish mortalities and any pathological signs (Huang et al., 2013). Third, to determine if this disease was due to bacterial infections, liver samples were streaked onto the thiosulfate citrate bile salt sucrose (TCBS) agar plate (Sinopharm Chemical Reagent Co., Ltd) for bacterial isolation according to Gu et al. (2019), and incubated at 32 °C for 24 h. The uniform isolates were subjected to purification by repeated streaking onto the nutrient agar (NA) plates. The suspensions of pure isolates were further prepared by washing the inoculated NA plates after 24 h incubation at 32 °C. The colony forming units (CFU) in the suspensions were further estimated by calculating CFU on NA plates from a series of 10-fold dilutions in sterile normal saline. Afterward, two replicate aquaria of healthy snakehead fish (10 fish per aquarium) were artificially infected by intramuscular injection (Iqbal et al., 1999) with 0.2 mL of every pure isolate with 3.0×10^5 CFU mL^{-1}. Another two replicate aquaria of control fish (10 per aquarium) were treated with the same volume (0.2 mL) of sterile normal saline intramuscularly. All the test fish were stocked in aquaria containing 100 L aerated tap water at 32 °C without water being changed and observed carefully for seven days to record fish mortalities and any pathological signs (Dou et al., 2019). The challenge isolate was re-isolated from freshly dead fish to confirm the cause of death. Histopathological changes in the liver of infected and healthy fish were also examined, according to Phrompanya et al. (2021).

Pathogen Identification

The pathogenic isolate was identified using 16S rRNA gene sequencing analysis and biochemical tests (Cho et al., 2010). Briefly, the total DNA was extracted from the pathogenic isolate by the TIANamp DNA Kit (Tiangen Biotech. Co., Ltd., Beijing, China) following the manufacturer's instructions. Then, the amplification of the 16S rRNA gene was conducted as described by Liu and Fu (2021) using the universal primer pair 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'), and the amplified product was subjected to sequencing through the ABI 3730 XL DNA Sequencer (Applied Biosystems, Waltham, MA, USA). Finally, the 16S rRNA gene sequence of the pathogenic isolate was subjected to the Basic Local Alignment Search Tool (BLAST) to search the closest related sequences in GenBank, and the construction of a phylogenetic tree was carried out using the neighbor-joining (NJ) method. Furthermore, the phenotypic identification of the pathogenic isolate was conducted by API 50CHB strips (Biomerieux, France) following the manufacturer's guidance (Oguntoyinbo and Oni, 2004). The phenotypic features of the *B. cereus* reference strain (Liu et al., 2017) were used as controls.

Hemolytic Activity Assay

The hemolytic activity of the pathogenic isolate was tested according to Sun et al. (2016) and Wang et al. (2018). Briefly, the pathogenic isolate was inoculated onto rabbit blood agar (RBA) plates (Guangdong Huankai Microbial Science and Technology Co., Ltd., Guangzhou, China), and then observed after 24 h of incubation at 32 °C for the presence of zones around its colonies. The presence of complete transparent zones surrounding the colonies was interpreted as β-hemolysis, the presence of incomplete transparent zones surrounding the colonies was interpreted as α-hemolysis, and the absence of zones surrounding the colonies was interpreted as γ-hemolysis.

LD_{50} Assay

The mean lethal dose (LD_{50}) of the pathogenic isolate in snakehead fish was examined according to Wang and Huang (2012). Three replicate aquaria of healthy snakehead fish (10 fish per aquarium) were artificially infected by intramuscular injection with 0.2 mL of the pathogenic isolate with 3.0×10^5 to 3.0×10^8 CFU mL^{-1}. Another three replicate aquaria of control fish (10 fish per aquarium) were treated intramuscularly with the same volume (0.2 mL) of sterile normal saline. All the test fish were stocked in aquaria containing 100 L

aerated tap water at 32 °C without water being changed, and observed carefully for seven days to record fish mortalities and any pathological signs. The challenge isolate was re-isolated from freshly dead fish to confirm the cause of death. The medium lethal dose (LD_{50}) value was estimated using the Bliss method (Finney, 1985) to evaluate the virulence of the pathogenic isolate.

Susceptibility to Antibiotics Assay

The antibiotic susceptibility of the pathogenic isolate was tested in triplicate by the Kirby-Bauer disk diffusion method (Joseph et al., 2011). Briefly, the pathogenic isolate was spread onto NA plates. The amikacin, ampicillin, cotrimoxazole, doxycycline, enrofloxacin, florfenicol, gentamycin, kanamycin, levofloxacin, neomycin, norfloxacin, ofloxacin, penicillin, polymyxin B, streptomycin, sulfisoxazole, tetracycline, and vancomycin discs (Hangzhou Binhe Microorganism Reagent Co., Ltd., Hangzhou, China) were then immediately placed on the inoculated plates. Afterward, inoculated plates with antibiotic discs were incubated at 32 °C for 24 h to measure the diameters of inhibition zones surrounding the antibiotic discs. The susceptibility of the pathogenic isolate to the eighteen antimicrobials was evaluated following the manufacturer's instruction. The antibiotic susceptibilities of *B. cereus* previously reported by Hu et al. (2016), Yang et al. (2017), Tian et al. (2018), and Li et al. (2020a) were used as references.

Results

Confirmation and Virulence of Pathogen

No parasites were detected in the naturally diseased snakehead fish, and no mortality or visible disease signs were observed in the control or test fish challenged with the bacteria-free organ filtrate (data not shown), revealing that the disease did not result from parasites or viruses. In addition, a totally of 4 bacterial strains, temporarily named as CA1, CA2, CA3, and CA4, were isolated from the liver of naturally-diseased snakehead fish, and no disease signs or mortalities were noted in control or challenged fish with isolates CA1, CA2, and CA3. Only isolate CA4 was found to be pathogenic to snakehead fish, which was β-hemolytic (**Figure 1**) and showed an LD_{50} value of 2.57×10^6 CFU mL^{-1} (**Figure 2**). The challenged fish with isolate CA4 displayed a hepatic hemorrhage sign similar to the naturally-infected fish (**Figure 3**). The same strain (CA4) confirmed by phenotypic and molecular identification was re-isolated from the experimental diseased fish. Furthermore, blood vessel congestion and macrophage infiltration were observed in the liver of artificially and naturally infected snakehead fish (**Figure 4**), similar to those observed in *B. cereus*–infected *Pelodiscus sinensis* (Cheng et al., 2021). Thus, isolate CA4 was identified as snakehead fish's causative pathogen associated with hepatic hemorrhage.

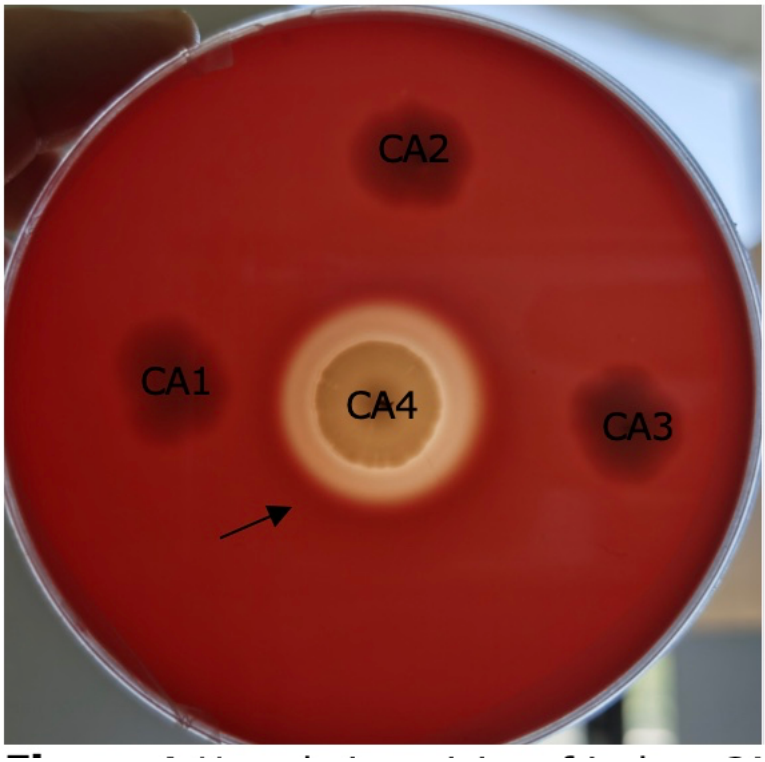

Figure 1 Hemolytic activity of isolate CA4 and the other three isolates (CA1, CA2 and CA3). The arrow shows the clear β-hemolytic zone on the RBA plate.

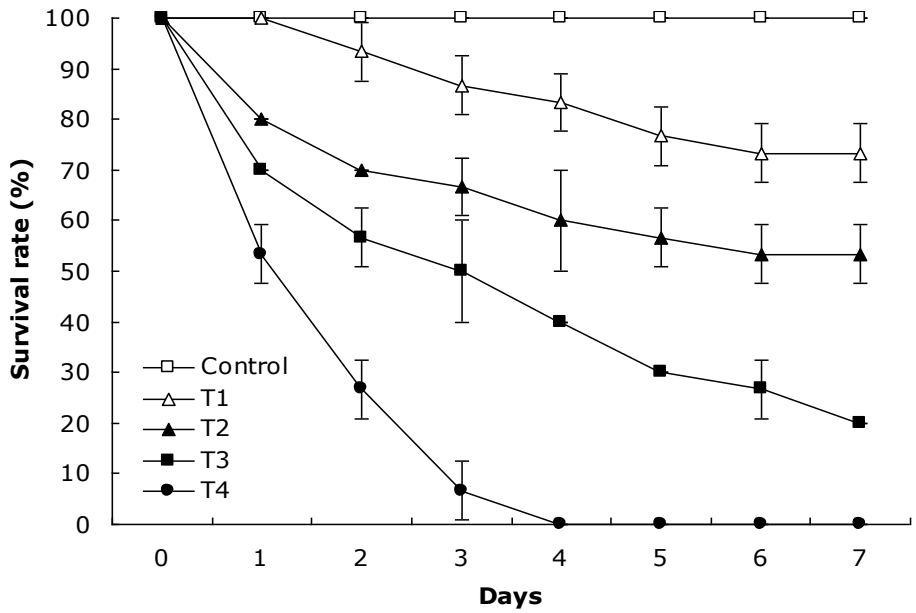

Figure 2 Survival rates of experimental snakehead fish infected by isolate CA4. Control, 0 CFU mL^{-1}; T1, 3.0×10^5 CFU mL^{-1}; T2, 3.0×10^6 CFU mL^{-1} ; T3, 3.0×10^7 CFU mL^{-1} ; T4, 3.0×10^8 CFU mL^{-1}. Data are presented as mean ± standard deviation.

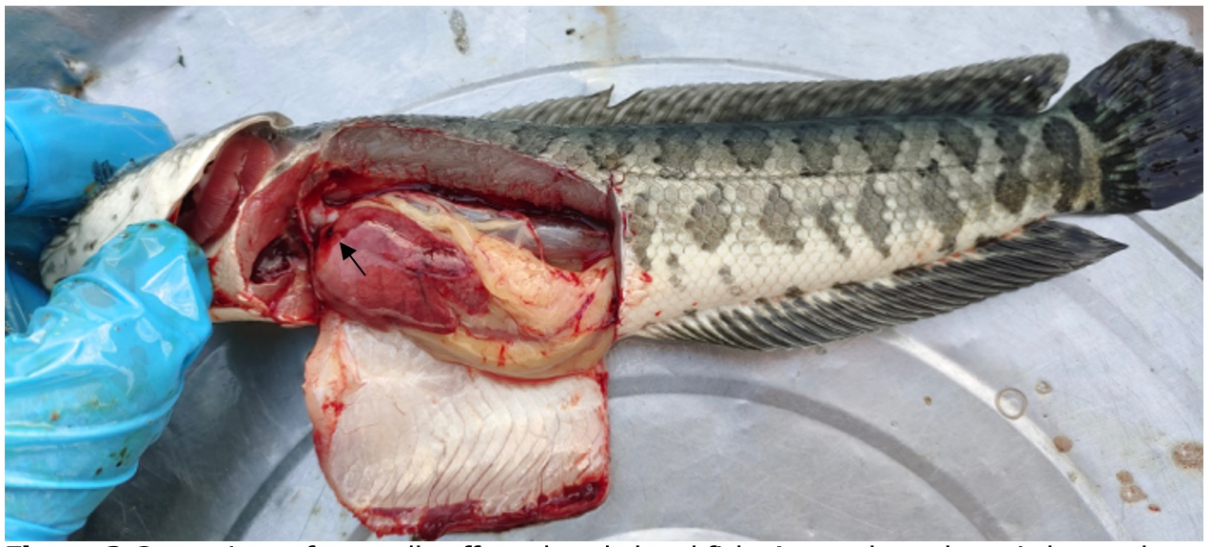

Figure 3 Gross signs of naturally affected snakehead fish. Arrow shows hepatic hemorrhage.

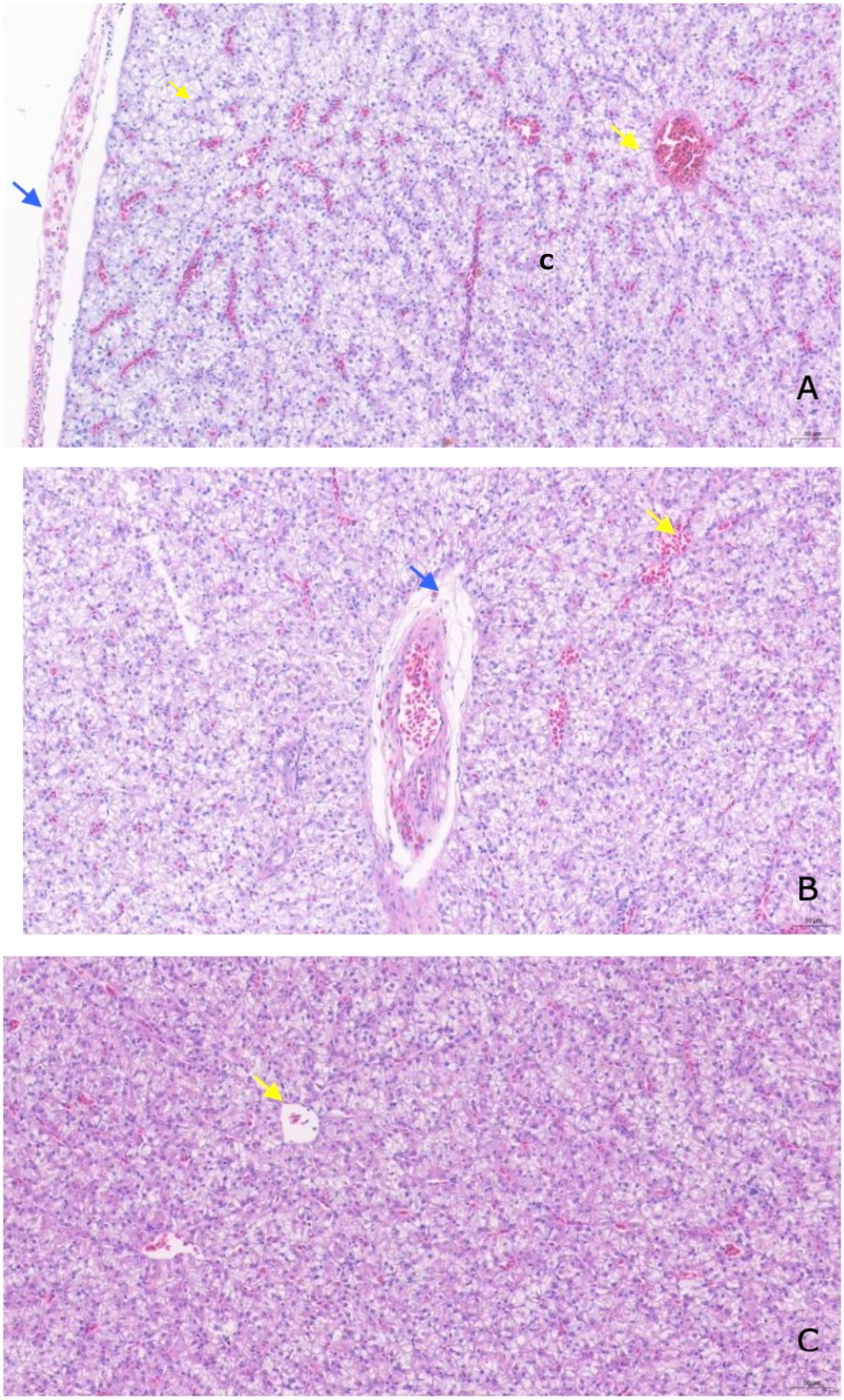

Figure 4 Histopathological changes in the liver of diseased snakehead fish. A. Blood vessel congestion (yellow arrow) and macrophage infiltration (blue arrow) in the artificially infected liver (20×); B. Blood vessel congestion (yellow arrow) and macrophage infiltration (blue arrow) in the naturally infected liver (20×); C. Normal blood vessel (yellow arrow) in the healthy liver (20×).

Identification of Pathogen

The 16S rRNA gene sequence was submitted to GenBank under the accession number ON231759. A similarity of 99% to 100% was observed between isolate CA4 and other *B. cereus* strains in the GenBank database and further demonstrated as a *B. cereus* strain through the phylogenetic tree (**Figure 5**). In addition, the phenotypic characterization also identified isolate CA4 as *B. cereus*, identical to the reference strain of *B. cereus* **(Table 1**). It was positive for amidon, arbutin, catalyse, D-cellobiose, D-fructose, D-glucose, D-lactose, D-maltose, D-melibiose, D-ribose, D-saccharose, D-turanose, D-trehalose, esculin, glycerin, glycogen, kaliumgluconat, N-acetylglucosamin, and salicin, and negative for D-adonitol, amygdalin, D-arabinose, D-arabitol, D-fucose, D-galactose, D-lyxose, D-mannitol, D-mannose, D-melezitose, D-raffinose, D-sorbitol, D-tagatose, D-xlyose, dulcitol, erythritol, gentiobiose, inositol, inulin, kalium-2-ketogluconat, kalium-5-ketogluconat, L-arabinose, L-arabitol, L-fucose, L-rhamnose, L-sorbose, L-xylose, methyl-αD-mannopyranoside, methyl-αD-glucopyrannoside, methyl-βD-xylopyranoside, and xylitol. Thus, isolate CA4 was identified molecularly and phenotypically as *B. cereus.*

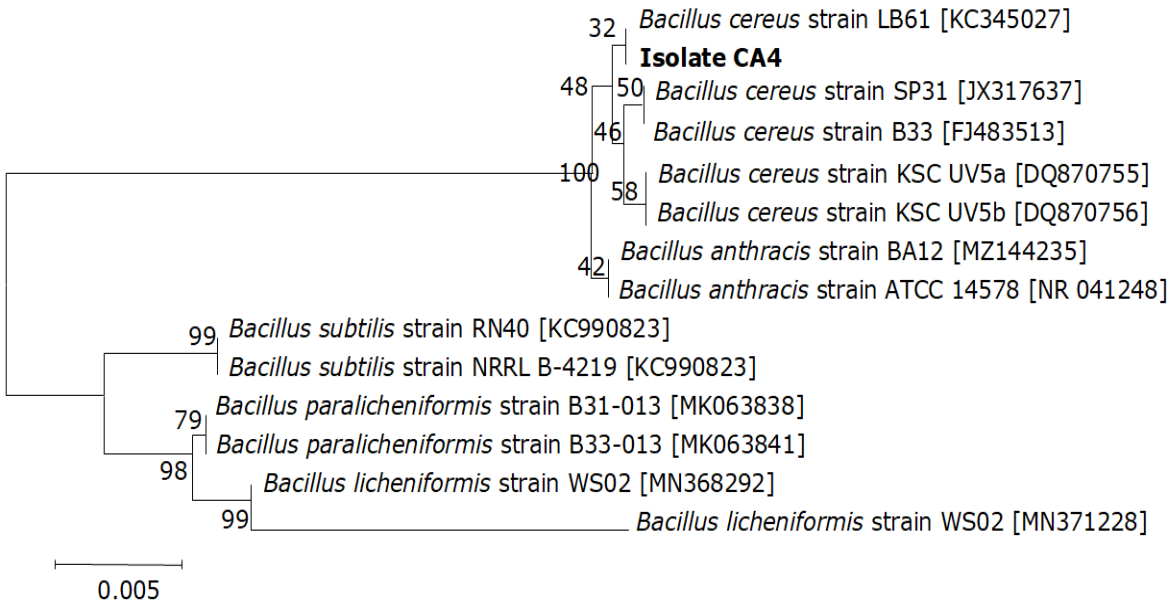

Figure 5 A 16S rRNA gene tree of 13 known bacteria and the CA4 isolate constructed using the neighbor-joining method. The bootstrap values (%) are shown beside the clades, accession numbers are indicated beside the names of strains, and scale bars represent distance values.

Table 1 Phenotypic characterization of isolate CA4.

Tests	Reaction	
	Isolate CA4	*B. cereus*[a]
Amidon	R^+	R^+
Amygdalin	R^-	R^-
Arbutin	R^+	R^+
Catalyse	R^+	R^+
D-adonitol	R^-	R^-
D-arabinose	R^-	R^-
D-arabitol	R^-	R^-
D-cellobiose	R^+	R^+
D-fructose	R^+	R^+
D-fucose	R^-	R^-
D-galactose	R^-	R^-
D-glucose	R^+	R^+
D-lactose	R^+	R^+
D-lyxose	R^-	R^-
D-maltose	R^+	R^+
D-mannitol	R^-	R^-
D-mannose	R^-	R^-
D-melezitose	R^-	R^-
D-melibiose	R^+	R^+
D-raffinose	R^-	R^-
D-ribose	R^+	R^+
D-saccharose	R^+	R^+
D-sorbitol	R^-	R^-
D-tagatose	R^-	R^-
D-turanose	R^+	R^+
D-trehalose	R^+	R^+
D-xlyose	R^-	R^-
Dulcitol	R^-	R^-
Erythritol	R^-	R^-
Esculin	R^+	R^+
Gentiobiose	R^-	R^-
Glycerin	R^+	R^+
Glycogen	R^+	R^+
Inositol	R^-	R^-
Inulin	R^-	R^-
Kaliumgluconat	R^+	R^+
Kalium-2-ketogluconat	R^-	R^-
Kalium-5-ketogluconat	R^-	R^-
L-arabinose	R^-	R^-
L-arabitol	R^-	R^-
L-fucose	R^-	R^-
L-rhamnose	R^-	R^-
L-sorbose	R^-	R^-
L-xylose	R^-	R^-
Methyl-αD-mannopyranosid	R^-	R^-
Methyl-αD-glucopyrannosid	R^-	R^-
Methyl-βD-xylo-pyranosid	R^-	R^-
N-acetylglucosamin	R^+	R^+
Salicin	R^+	R^+
Xylitol	R^-	R^-

R^+: positive reaction; R^-: negative reaction; a: data previously reported by Liu et al. (2017)

Antibiotic Susceptibility of Pathogen

Isolate CA4 was sensitive to enrofloxacin, levofloxacin, norfloxacin, and ofloxacin and resistant to amikacin, ampicillin, cotrimoxazole, doxycycline, florfenicol, gentamycin, kanamycin, neomycin, penicillin, polymyxin B, streptomycin, sulfisoxazole, tetracycline, and vancomycin (**Table 2**). These findings indicated that isolate CA4 was multiply resistant to aminoglycosides, chloramphenicols, sulfonamides, and tetracyclines drugs in aquaculture use.

Table 2 Susceptibility of isolate CA4 to antibiotics.

Antibiotics	Content (μg disc^{-1})	Inhibition zone diameter (mm)	Susceptibility	
			Isolate CA4	*B. cereus*[a]
Amikacin	30	0±0	R	R
Ampicillin	10	0±0	R	R
Cotrimoxazole*	1.25/23.75	0±0	R	R
Doxycycline*	30	0±0	R	R
Enrofloxacin*	5	20.1±0.2	S	S
Florfenicol*	75	0±0	R	R
Gentamycin	10	0±0	R	R
Kanamycin	30	0±0	R	R
Levofloxacin	5	23.5±0.5	S	S
Neomycin*	30	0±0	R	R
Norfloxacin	10	20.7±0.8	S	S
Ofloxacin	5	23.1±0.6	S	S
Penicillin	10	0±0	R	R
Polymyxin B	30	0±0	R	R
Streptomycin	10	0±0	R	R
Sulfisoxazole*	300	0±0	R	R
Tetracycline*	30	0±0	R	R
Vancomycin	30	0±0	R	R

Data are presented as mean ± standard deviation.
a: the antibiotic susceptibility for *B. cereus* was reported by Hu et al. (2016), Yang et al. (2017), Tian et al. (2018), and Li et al. (2020a).
S: sensitive; R: resistant; *: antibiotics in aquaculture use.

Discussion

Hepatic hemorrhage is a common symptom of bacterial diseases in fish. To date, several pathogens have been reported to cause the symptom of hepatic hemorrhage in fish, including *A. hydrophila* (He et al., 2010), *Edwardsiella ictaluri* (Du et al., 2013), *Yersinia ruckeri* (Li et al., 2014), and *Streptococcus agalactiae* (Xie et al., 2019). Nevertheless, scarce information is available on *B. cereus* that causes hepatic hemorrhage in snakehead fish. Previous studies have indicated that the fish liver is the primary target organ in bacterial infections (Oh et al., 2019; Malick et al., 2020). Thus, in this study, we isolated bacteria from the liver of diseased fish and further demonstrated *B. cereus* CA4 as the causative agent of snakehead fish with hepatic hemorrhage according to Koch's postulates (Fredericks and Relman, 1996). To our knowledge, this is the first report of *B. cereus* pathogenic to snakehead fish.

The production of hemolytic toxins plays a vital role in the pathogenicity of bacterial pathogens (Radu et al., 2003). For example, *B. cereus,* which can produce β-hemolysin, is pathogenic to fish (Wang et al., 2018). Thus, β-hemolysis can be used as an indicator for the virulence assessment of *B. cereus*. In the present study, isolate CA4 was found to possess hemolytic activity identical to that seen in the half-smooth tongue sole–pathogenic *B. cereus* (Wang et al., 2018). This could contribute to the lysis of erythrocytes to subsequently cause the disease sign of hemorrhage (Allan and Stevenson, 1981; Zhu et

al., 2006). Besides, isolate CA4 was pathogenic for snakehead fish with an LD_{50} value of 2.57×10^6 CFU $mL^{-1,}$ significantly lower than that in yellow catfish-pathogenic *B. cereus* (Dou et al., 2019). This suggests that the pathogenic *B. cereus* can probably threaten the health of snakehead fish. In addition, the snakehead fish can grow well in ponds with water quality parameters of pH7.20-8.20, dissolved oxygen of 3.28-7.30 mg L^{-1}, nitrite of 0.015-0.039 mg L^{-1}, and total ammonia of 0.01-1.45 mg L^{-1} (Rahman et al., 2012). Thus, our study's infected pond had water quality parameters within acceptable limits. Surely, other primary factors, such as poor feed quality, could probably contribute to this disease (Di et al., 2019; Wen et al., 2019).

Multiple antibiotic resistances in pathogenic *B. cereus* have emerged as an issue of global concern because of the dissemination of antibiotic resistance plasmids (Choudhury et al., 2012). This study also found multiple resistances in *B. cereus* CA4 against chloramphenicols, penicillins, sulfonamides, and tetracyclines. Similar findings are also observed with other pathogenic isolates of *B. cereus* in fish (Yang et al., 2017; Wang et al., 2018; Dou et al., 2019). Thus, more attention should be given to the control of fish-pathogenic *B. cereus*. Enrofloxacin is a widely used veterinary antibiotic for bacterial disease treatment in aquaculture (Rico et al., 2013). For example, the oral administration of enrofloxacin effectively controls mortality from *S. iniae* of hybrid striped bass *Morone saxatilis* (Stoffregen et al., 1996). In the present study, *B. cereus* CA4 was highly susceptible to enrofloxacin. This serves as a reminder that enrofloxacin can treat *B. cereus* infection in snakehead fish.

In conclusion, the findings of this study for first time, identified *B. cereus* CA4 as a causative pathogen of diseased snakehead fish and provided new insights into the potential threat of pathogenic *B. cereus* to snakehead fish.

Acknowledgments

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References

Allan B.J., and R.M.W. Stevenson, 1981. Extracellular virulence factors of *Aeromonas hydrophila* in fish infections. *Can. J. Microbiol.*, 27, 1114-1122. https://doi.org/10.1139/m81-174

Bottone E.J., 2010. *Bacillus cereus*, a volatile human pathogen. *Clin. Microbiol. Rev.*, 23(2), 382-398. https://doi.org/10.1128/CMR.00073-09

Cheng L., Rao S., Poudyal S., Wang P. and S. Chen, 2021. Genotype and virulence gene analyses of *Bacillus cereus* group clinical isolates from the Chinese softshell turtle (*Pelodiscus sinensis*) in Taiwan. *J. Fish Dis.,* 44(10), 1515-1529. https://doi.org/10.1111/jfd.13473

Cho H., Liu L., Liu K., Zhu Y. and X. Yang, 2010. Phenotypic characterization and phylogenetic analysis of a virulent *Bacillus cereus* strain from the tiger frog, *Hoplobatrachus rugulosus* Wiegmann. *Afr. J. Microbiol. Res.,* 4(24), 2780-2786. https://doi.org/10.1007/978-3-642-17913-6_14

Choudhury R., Panda S. and D.V. Singh, 2012. Emergence and dissemination of antibiotic resistance: a global problem. *Indian J. Med. Microbiol.*, 30(4), 384-390. https://doi.org/10.4103/0255-0857.103756

Chu W. and C. Lu, 2005. Multiplex PCR assay for the detection of pathogenic *Aeromonas hydrophila*. *J. Fish Dis.*, 28, 437-441. https://doi.org/10.1111/j.1365-2761.2005.00628.x

Di Y., Li B., Dong P., Li J., Yang X., Fang Z., Gao Y., and Z. Wu, 2019. Investigation and analysis of microbial contamination in biological feed additives. *Feed Ind.*, 40(18), 20-24. https://doi.org/10.13302/j.cnki.fi.2019.18.005

Dou P., Wang L., Fang Q. and J. Li, 2019. Isolation, identification and drug resistance analysis of *Bacillus cereus* isolated from fish. *China Anim. Husb. Vet. Med.*, 46(9), 2745-2752. https://doi.org/10.16431/j.cnki.1671-7236.2019.09.030

Du Z., Geng Y., Wang K., Liao Y., Zhou Z. and X. Huang, 2013**.** Dynamic pathology and pathogen distribution study on yellow catfish *Pelteobagrus fulvidraco* infected with *Edwardsiella ictaluri*. *Ocean. Limn. Sin.*, 44(6), 1519-1523.

Finney D. J., 1985. The median lethal dose and its estimation. *Arch. Toxikol.*, 56, 215-218. https://doi.org/10.1007/BF00295156

Fredericks D.N. and D.A. Relman, 1996. Sequence-based identification of microbial pathogens: a reconsideration of Koch's postulates. *Clin. Microbiol. Rev.*, 9, 18-33. https://doi.org/10.1128/CMR.9.1.18-33.1996

Gong Q., Gao S., Shan X., Guo W., Meng Q. and W. Wang, 2010. Isolation and identification of pathogenic *Aeromonas veronii* from *Cyprinus carpio*. *Chin. J. Prev. Vet. Med.*, 32(12), 981-983. https://doi.org/10.3969/j.issn.1008-0589.2010.12.18

Gu Y., Wang H., Guo C., Chen J. and H. Cao, 2019. *Citrobacter freundii*: a causative agent for ulcer disease in snakehead fish *Ophiocephalus argus* (Cantor). *Isr. J. Aquacult. – Bamid.*, 71, 1-8. https://doi.org/10.46989/001c.20982

He Z., Ren H., Yang D., Yang G., Bian Y. and S. Wang, 2010. The histopathological study of hemorrhagic septicemia by *Aeromonas hydrophila* isolated from rice field eel (*Monopterus albus*). *Freshw. Fish.*, 40(4), 56-61. https://doi.org/10.1631/jzus.A1000244

Hossain M.F., Rashid M.M. and M.A. Saved, 2013. Experimental infection of indigenous climbing perch *Anabas testudineus* with *Aeromonas hydrophila* bacteria. *Prog. Agr.*, 22, 105-114. https://doi.org/10.3329/pa.v22i1-2.16472

Hu Z., Song W., Zhang T., Fu L., Li H., Yao F. and P. Yang, 2016. Identification and drug sensitivity test of pathogen *Bacillus cereus* from yellow catfish (*Pelteobagrus fulvidraco*) with red-head disease. *Chin. J. Fish.*, 29(4), 33-37.

Huang J., Huang Y., Hu D., Luo H., Shi J., Peng M., Xuan J., Tan L., Teng Z. and K. Zeng, 2013. Characterization of white plastron disease pathogens and detection of six known virulence genes in *Truogx sinensis*. *Acta Hydrob. Sin.*, 5, 844-854. https://doi.org/10.7541/2013.108

Iqbal M.M., Tajima K. and Y. Ezura, 1999. Pathogenicity of motile *Aeromonas* species isolated from fishes with epizootic ulcerative syndrome (EUS) in Southeast Asian countries. *Bull. Fac. Fish. Hokkaido Univ.*, 50(2), 93-100.

Joseph N. M., Sistla S., Dutta T. K., Badhe A. S., Rasitha D. and S.C. Parija, 2011. Reliability of Kirby-Bauer disk diffusion method for detecting meropenemresistance among non-fermenting gram-negative bacilli. *Indian J. Pathol. Microbiol.*, 54, 556–560.

Li S., Wang D., Feng J. and T. Lu, 2014. Isolation, identification and pathogenicity of a pathogenic bacterium *Yersinia ruckeri* associated with hemorrhage of cultured Amur sturgeon (*Acipenser schrenckii*). *Ocean. Limn. Sin.*, 45(3), 561-567. https://doi.org/10.11693/hyhz20130121001

Li S., Wu L., Xu P., Sheng X., He X., Xiao S., Qiu Z., Liang J., Han S. and J. Huang, 2020a. Isolation, identification and antibiotic sensitivity test of pathogenic *Bacillus cereus* from Huangsha turtle. *Southwest China J. Agr. Sci.*, 33(3), 673-680. https://doi.org/10.16213/j.cnki.scjas.2020.3.032

Li T., Raza S. H. A., Yang B., Sun Y., Wang G., Sun W., Qian A., Wang C., Kang Y. and X. Shan, 2020b. *Aeromonas veronii* infection in commercial freshwater fish: a potential threat to public health. *Int. J. Food Microbiol.*, 10, 608. https://doi.org/10.1016/S0168-1605(96)01163-4

Liu J. and A. Li, 2012. First case of *Aeromonas schubertii* infection in the freshwater cultured snakehead fish, *Ophiocephalus argus* (Cantor), in China. *J. Fish Dis.*, 35(5), 335-342. https://doi.org/10.1111/j.1365-2761.2012.01350.x

Liu X. and D. Fu, 2021. Isolation and identification of a lipase producing strain of *Bacillus cereus*. *China Trop. Agric.*, 6, 68-73. https://doi.org/10.3969/j.issn.1673-0658.2021.06.014

Liu Y., Du J., Lai Q., Zeng R., Ye D., Xu J. and Z. Shao, 2017. Proposal of nine novel species of the *Bacillus cereus* group. *Int. J. Syst. Evol. Microbiol.*, 67(8), 2499-2508. https://doi.org/10.1099/ijsem.0.001821

Malick R.C., Bera A.K., Chowdhury H., Bhattacharya M., Abdulla T., Swain H.S., Baitha R., Kumar V. and B.K. Das, 2020. Identification and pathogenicity study of emerging fish pathogens *Acinetobacter junii* and *Acinetobacter pittii* recovered from a disease outbreak in *Labeo catla* (Hamilton, 1822) and *Hypophthalmichthys molitrix* (Valenciennes, 1844) of freshwater wetland in West Bengal, India. *Aquac. Res.* 51(2), 1-11. https://doi.org/10.1111/are.14584

Meng Q., Yin F., Fu C., Chen F., Liu C., Yuan N., Wang L., Zhang H. and D. Qian, 2019. Isolation, identification and pathogenicity analysis of *Bacillus cereus* from Chinese soft-shelled turtles, *Pelodiscus sinensis*. *Acta Hydrob. Sin.*, 43(3), 570-578. https://doi.org/10.7541/2019.069

Ministry of Agriculture and Rural Affairs of China, 2022. China Fishery Statistics Yearbook. China Agriculture Press. *Beijing*.

Oguntoyinbo F. A. and O. M. Oni, 2004. Incidence and Characterization of *Bacillus cereus* Isolated from Traditional fermented meals in Nigeria. *J. Food Protect.*, 67(12), 2805-2808. https://doi.org/10.4315/0362-028x-67.12.2805

Oh W.T., Kim J.H., Jun J.W., Giri S.S., Yun S., Kim H.J., Kim S.G., Kim S.W., Han S.J., Kwon J. and S.W. Kim, 2019. Genetic characterization and pathological analysis of a novel bacterial pathogen, *Pseudomonas tructae*, in rainbow trout (*Oncorhynchus mykiss*). *Microorganisms*, 7(10), 432. https://doi.org/ 10.3390/microorganisms7100432

Phrompanya P., Panase P., Saenphet S. and K. Saenphet, 2021. Histopathology and oxidative stress responses of Nile tilapia *Oreochromis niloticus* exposed to temperature shocks. *Fish. Sci.*, 87, 491–502. https://doi.org/10.1007/s12562-021-01511-y

Radu, S., Ahmad, N., Ling, F.H. and A. Reezal, 2003. Prevalence and resistance to antibiotics for *Aeromonas* species from retail fish in Malaysia. *Int. J. Food Microbiol.*, 81(3), 261-266. https://doi.org/10.1016/S0168-1605(02)00228-3

Rahman M.A., Arshad A. and S.M.N. Amin, 2012. Growth and production performance of threatened snakehead fish, *Channa striatus* (Bloch), at different stocking densities in earthen ponds. *Aquac. Res.*, 43, 297-302. https://doi.org/10.1111/j.1365-2109.2011.02830x

Rico A., Phu T.M., Satapornvanit K., Min J., Shahabuddin A.M., Henriksson P.J.G., Murray F., Little D.C., Dalsgaard A. and P.J.V. Brink, 2013. Use of veterinary medicines, feed additives and probiotics in four major internationally traded aquaculture species farmed in Asia. *Aquaculture*, 412-413, 231-243.

Stoffregen D.A., Backman S.C., Perham R.E., Bowser P.R. and J.G. Babish, 1996. Initial disease report of *Streptococcus iniae* infection in hybrid striped (sunshine) bass and successful therapeutic intervention with the fluoroquinolone antibacterial enrofloxacin. *J. world Aquacult. Soc.*, 27(4), 420-434. https://doi.org/10.1111/j.1749-7345.1996.tb00626.x

Sun J., Zhang X., Gao X., Jiang Q., Wen Y. and L. Lin, 2016. Characterization of virulence properties of *Aeromonas veronii* isolated from diseased gibel carp (*Carassius gibelio*). *Int. J. Mol. Sci.*, 17, 496. https://doi.org/10.3390/ijms17040496

Tian J., Zhang R., Long H., Liu L., He S., Du X., Jiang M., Zhao Y. and J. Tang, 2018. Analysis of virulence gene detection and antimicrobial susceptibility of *Bacillus cereus* isolated from fresh food. *Sci. Technol. Food Ind.*, 39(6), 135-139. https://doi.org/j.issn1002-0306.2018.06.025

Tuipulotu D.E., Mathur A., Ngo C. and S.M. Man, 2021. *Bacillus cereus*: epidemiology, virulence factors, and host-pathogen interactions. *Trends Microbiol.*, 29(5), 458-471. https://doi.org/10.1016/j.tim.2020.09.003

Wang G., Xu Y., Jin S., Zhu J. and S. Yuan, 2007. Nocardiosis in snakehead, *Ophiocephalus argus* Cantor. *Aquaculture*, 271(1-4), 54-60. https://doi.org/10.1016/j.aquaculture.2007.06.019

Wang H., Gu Y., Luo G. and H. Cao, 2020**.** *Aeromonas veronii*, a potential pathogen of enteritis in snakehaed fish *Ophiocephalus argus*. *Isr. J. Aquacult.–Bamid.*, 72, 1-11. https://doi.org/10.46989/001c.21691

Wang Y., Gao S., Shan J., Luo Z. and J. Liu, 2018. Virulence and drug susceptibility of a pathogenic bacterium from half-smooth tongue-sole. *Fish. Sci.*, 37(1), 59-65. https://doi.org/10.16378/j.cnki.1003-1111.2018.01.009.

Wang Z. and H. Huang, 2012. Isolation, identification and histopathologic observation of a pathogen from hemorrhage tilapia. *J. Huaqiao Univ. (Nat. Sci.)*, 33(6), 660-666.

Wen H., Li M., Li J., Gao Y. and Z. Wu, 2019. Isolation, identification and testing of the cereulide gene in feed and feed additives. *Feed Res.*, 4, 40-43. https://doi.org/10.13557/j.cnki.issn1002-2813.2019.04.011.

Xie Y., Feng J., Liu C., Deng Y., Wang J. and Y. Su, 2019. Comparative pathological study of tilapia naturally infected with *Streptococcus agalactiae* and virulence gene profiling of isolated strains. *South China Fish. Sci.*, 15(2), 47-57. https://doi.org/10.12131/20180185.

Yang X., 2018. Fish parasitology. Science Press. *Beijing*.

Yang Y., Yu L., Liu Y., Yang Q., Su Z., Song Y. and X. Ai, 2017. Isolation, identification and antibiotic sensitivity of *Bacillus cereus* from tilapia. *Freshw. Fish.*, 47(4), 51-56. https://doi.org/10.3969/j.issn.1000-6907.2017.04.009.

Zhu K., Chi Z., Li J., Zhang F., Li M., Yasoda H.N. and L. Wu, 2006. The surface display of haemolysin from *Vibrio harveyi* on yeast cells and their potential applications as live vaccine in marine fish. *Vaccine*, 24(35-36): 6046-6052. https://doi.org/10.1016/j.vaccine.2006.05.043