

Original Research Articles

Haemolytic *Bacillus subtilis*, a potential pathogen of hemorrhage disease in farmed yellow catfish *Pelteobagrus fulvidraco*Chunlei Gai^{1,2a}, Xurui Zheng^{3b}, La Xu^{1,2}, Jing Diao^{1,2}, Haibin Ye^{1,2}, Haipeng Cao^{3,4c}, Xiaoqing Yu^{1,2d}¹ Marine Science Research Institute of Shandong Province (National Oceanographic Center), Qingdao Shandong 266104, China, ² Shandong Key Laboratory of Disease Control in Mariculture, Qingdao Shandong 266104, China, ³ National Pathogen Collection Center for Aquatic Animals, Shanghai Ocean University, Shanghai 201306, China, ⁴ Key Laboratory of Freshwater Fishery Germplasm Resources, Ministry of Agriculture and Rural Affairs of China, Shanghai 201306, ChinaKeywords: *Bacillus subtilis*, characterization, pathogen, *Pelteobagrus fulvidraco*<https://doi.org/10.46989/001c.84493>**Israeli Journal of Aquaculture - Bamidgah**

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The hemorrhagic disease has become a major bottleneck in yellow catfish *Pelteobagrus fulvidraco* aquaculture. Yet scarce information is available on *Bacillus subtilis* as a pathogen of hemorrhagic disease in cultured yellow catfish. In this study, a virulent isolate, temporarily named HS5, was isolated from hemorrhagic disease-infected cultured yellow catfish, and was identified phenotypically and molecularly as *B. subtilis*. In addition, isolate HS5 showed an LD50 value of 5.20×10^6 CFU/ml in yellow catfish, exhibited beta-haemolytic activity, harbored the virulent entFM gene, and displayed high susceptibility to enrofloxacin and florfenicol in aquaculture use. To the best of our knowledge, this is the first report of *B. subtilis* as a pathogen of hemorrhagic disease in farmed yellow catfish.

INTRODUCTION

Yellow catfish *Pelteobagrus fulvidraco* is a favorite freshwater aquaculture fish due to its high meat quality, and has been widely farmed in China, Japan, South Korea and Southeast Asia (Li, 2000). Especially in China, the culture of yellow catfish has increased rapidly in recent years with a total production of over 587,800 tons in 2021.¹ However, the hemorrhagic disease has become a major bottleneck in yellow catfish aquaculture due to its high mortality.² Thus, the hemorrhagic disease in yellow catfish should be emphasized to ascertain the sustainable development of this industry. Recently, hemorrhagic disease in cultured yellow catfish has been caused by several bacterial pathogens such as *Aeromonas veronii*, *Aeromonas jandaei*, *Bacillus cereus*, *Edwardsiella ictaluri*, *Shewanella putrefaciens*, *Staphylococcus sciuri*, and *Streptococcus iniae*,²⁻⁸ indicating the diversity of bacterial pathogens from hemorrhagic disease-infected yellow catfish. *Bacillus subtilis* is a facultative anaerobic Gram-positive bacterium that includes virulent members with the ability to produce haemolysins⁹ and induce high mortality in turbot *Scophthalmus maximus* and tongue sole *Cynoglossus semilaevis*.¹⁰ Yet scarce informa-

tion is available on *Bacillus subtilis* as a causal agent of hemorrhagic disease in cultured yellow catfish.

In this study, a haemolytic isolate HS5 of *B. subtilis* was demonstrated as a pathogen of hemorrhagic disease in farmed yellow catfish, and its taxonomy, virulence, and antimicrobial susceptibility were further tested. As far as we know, this is the first study to identify haemolytic *B. subtilis* as a causative agent of hemorrhage disease in cultured yellow catfish. The findings of this study provide insights into the potential threat of haemolytic *B. subtilis* to cultured yellow catfish.

MATERIALS AND METHODS**FISH SAMPLES**

Sixteen hemorrhagic disease-infected yellow catfish (mean weight 3.41 ± 0.51 g) were sampled from infected indoor circular tanks of a fish farm in Fengxian, Shanghai China during November 2022, and were kept in sterile ice-cold bags and immediately transported to the laboratory. Healthy yellow catfish (mean weight 2.11 ± 0.33 g) were acquired from unaffected yellow catfish farming ponds in Huzhou, Zhejiang, China, and were confirmed in good health by

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sampling a few individuals for careful health examinations according to General Administration of Quality Supervision, Inspection and Quarantine of China.¹¹

PATHOGEN ISOLATION

Each sampled diseased fish was externally disinfected with 75% alcohol and dissected according to Yang et al.⁷ Samples from livers of diseased fish were streaked onto nutrient agar (NA) (Sinopharm Chemical Reagent Co., Ltd., China) with a flamed loop. After incubation for 24h at 28°C, isolated bacteria were subcultured on NA plates to check the purity of the isolates. Bacterial suspensions at 5.0×10^6 colony forming units (CFU)/ml were prepared by suspending bacterial colonies in 50 ml of sterile normal saline,¹² which were calculated by counting CFU on NA plates from a series of ten-fold dilutions in sterile normal saline.⁷ Afterwards, bacterial challenge test was conducted in glass aquaria (80 cm × 54 cm × 50 cm) supplemented with 100 L aerated tap water at 25 °C according to Yang et al.⁷ Briefly, healthy yellow catfish were randomly assigned to one control and five treatment groups (two replicate aquaria per group, ten fish per aquarium). Treatment groups of fish were injected intramuscularly with 0.1 ml of bacterial suspensions at 5.0×10^6 CFU/ml, and the control group of fish were injected intramuscularly with 0.1 ml of sterile normal saline. All the test fish were stocked without water change and feeding, and were observed daily for seven days to determine pathological symptoms and mortality.¹³ The challenge strain was re-isolated from freshly dead fish to confirm the cause of death. Meanwhile, careful examinations of parasites and pathogenic viruses were also carried out as described by Wang et al.¹⁴

PATHOGEN IDENTIFICATION

The genomic DNA was extracted from the pathogenic isolate using a DNA extraction kit (Tiangen Biotech (Beijing) Co., Ltd., China). The near complete 16S rRNA gene was amplified from the genomic DNA by PCR according to Zu et al.¹⁵ using primers 27F (5'-AGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'), and the amplified PCR product was further sequenced by Qingdao MD-Bio Biotech Co. Ltd., China. Following the 16S rRNA gene sequence assembly using Editseq and Seqman in DNASTar software, the alignment of the 16S rRNA gene sequence was performed using the Basic Local Alignment Search Tool (BLAST) program at the National Centre for Biotechnology Information (NCBI), and the phylogenetic tree was further constructed by the neighbour-joining method using the pathogenic isolate's 16S rRNA gene sequence and its homologous sequences. In addition, the pathogenic isolate was identified phenotypically using API 50CH test strip (BioMerieux, France) as recommended by Zhou et al.¹⁶ following the instruction of the manufacturer. The test strip was incubated at 37°C and observed after 24h against the API identification index. The phenotypic traits of *B. subtilis* previously reported by He et al.¹⁷ were used as controls.

LD₅₀ ASSAY

Prior to the virulence assay, the suspensions of the pathogenic isolate at 6.0×10^5 , 6.0×10^6 , 6.0×10^7 , and 6.0×10^8 CFU/ml were prepared as described above. The virulence assay was performed in glass aquaria (80 cm × 54 cm × 50 cm) supplemented with 100 L aerated tap water at 25 °C according to Yang et al.,⁷ and consisted of one control and four treatment groups (three replicate aquaria per group, ten fish per aquarium). Treatment groups of fish were injected intramuscularly with 0.1 ml of bacterial suspensions at 6.0×10^5 , 6.0×10^6 , 6.0×10^7 , and 6.0×10^8 CFU/ml, and the control group of fish were injected intramuscularly with 0.1 ml of sterile normal saline. All the test fish were stocked without water change and feeding, and were observed daily for seven days to determine survival rates.¹⁸ Dead fish were immediately sampled to confirm whether the mortality was resulted specifically from the challenge isolate. The mean lethal dose (LD₅₀) value is calculated according to the graphical probit method as recommended by Ogbuagu & Iwuchukwu.¹⁹

VIRULENCE FACTOR ASSAY

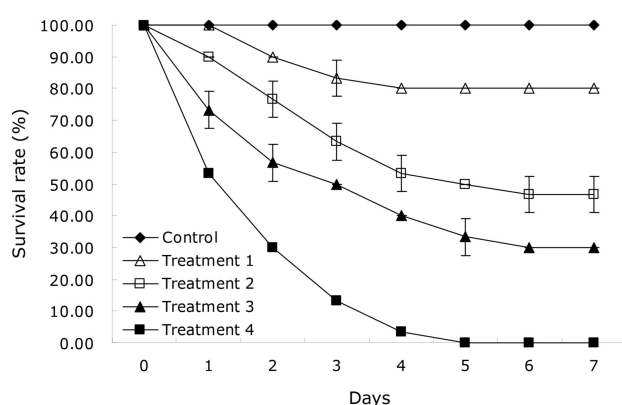
The haemolysis production of the pathogenic isolate was tested with rabbit blood agar (RBA) plates (Guangdong Huankai Microbial Science and Technology Co. Ltd., China), and was observed for the presence of zones around the colony after 24h of growth on RBA plates at 28°C.¹⁴ In addition, the virulence genes of the pathogenic isolate were also detected by PCR according to Yu et al.²⁰ and Owusu-Kwarteng et al.²¹ Briefly, the genomic DNA was extracted from the pathogenic isolate using a DNA extraction kit (Tiangen Biotech (Beijing) Co., Ltd., China). The virulent genes encoding enterotoxins (*entFM*, *nheA*, *nheB*, and *nheC*) were amplified from the genomic DNA by PCR according to Yu et al.²⁰ and Owusu-Kwarteng et al.²¹ using specific primers listed in [Table 1](#). The PCR products were electrophoresed on 1% (w/v) agarose gel and visualized under ultraviolet illumination. The virulent *B. cereus* strain CA4, previously isolated from diseased snakehead fish *Ophiocephalus argus*,¹⁴ was used as the control.

ANTIMICROBIAL SUSCEPTIBILITY ASSAY

The antimicrobial susceptibility of the pathogenic isolate was examined in triplicate using the Kirby-Bauer disk diffusion method as recommended by Yao et al.²² Briefly, the suspension of the pathogenic isolate at 1.5×10^8 CFU/ml was prepared as described above. Afterwards, the pathogenic isolate at 1.5×10^8 CFU/ml was spread on NA plates, and discs of fourteen antimicrobials (amoxicillin, bacitracin, cefotaxime, cefradine, ceftizoxime, doxycycline, enrofloxacin, gentamicin, florfenicol, kanamycin, netilmicin, norfloxacin, roxithromycin, and sulfamethoxazole) (Hangzhou Binhe Microorganism Reagent Co., Ltd., China) were respectively placed on the inoculated plates. Then, the inoculated plates with antimicrobial discs were incubated at 28°C, and the diameters of inhibition zones were measured after 24h of in-

Table 1. Specific PCR primers targeting the virulence genes.

Virulence genes	Primer (5' to 3')	Amplicon (bp)
<i>entFM</i>	TTAGTATGCTTTTGTGTAACC	610
	ATGAAAAAGTAATTTCAGG	
<i>nheA</i>	TACGCTAAGGAGGGGCA	480
	GTTTTTATTGCTTCATCGGCT	
<i>nheB</i>	CTATCAGCACTTATGGCAG	754
	ACTCCTAGCGGTGTTCC	
<i>nheC</i>	CGGTAGTGATTGCTGGG	564
	CAGCATTCTGACTTGCCAA	

**Figure 1. Survival rates of experimental yellow catfish infected by isolate HS5.**

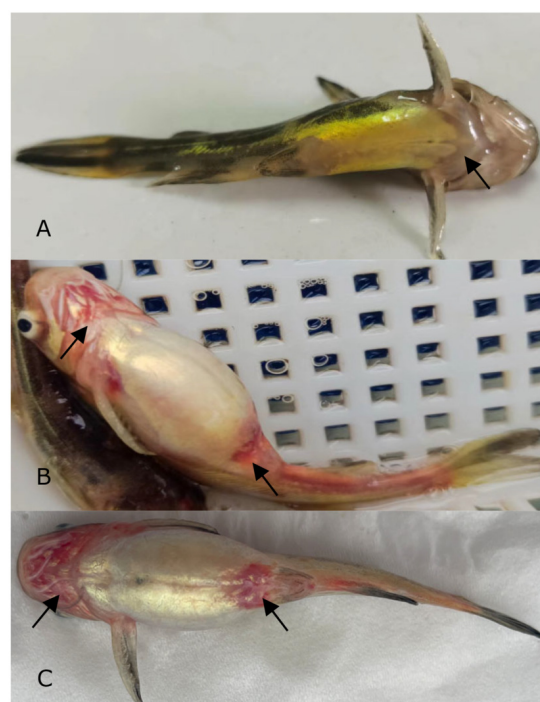
Control, 0 CFU/ml; Treatment 1, 6.0×10^5 CFU/ml; Treatment 2, 6.0×10^6 CFU/ml ; Treatment 3, 6.0×10^7 CFU/ml; Treatment 4, 6.0×10^8 CFU/ml. Data are presented as mean $\pm$ standard deviation.

cubation. The antimicrobial susceptibility is determined according to the manufacturer's instruction.

RESULTS

PATHOGEN ISOLATION

A total of five bacterial isolates, temporarily named HS1, HS2, HS3, HS4, and HS5, were isolated from the liver of diseased yellow catfish, and only isolate HS5 was found to be pathogenic to yellow catfish, showing an LD_{50} of 5.20×10^6 CFU/ml in yellow catfish (Figure 1). The experimental fish challenged with isolate HS5 displayed the typical hemorrhage sign similar to the naturally-infected fish (Figure 2). The same strain (HS5) that was demonstrated through phenotypic and molecular identification was re-isolated from the experimental diseased fish. In addition, no visible pathological signs or mortality were observed in the control or treatment fish challenged with isolates HS1, HS2, HS3, and HS4, and no parasites or viruses were detected in the naturally diseased fish from which isolate HS5 was obtained. These data indicated that this disease was caused by isolate HS5.

**Figure 2. Gross signs of diseased yellow catfish.**

A. Healthy fish. Arrow shows normal skin. B. Naturally diseased fish. Arrows show skin hemorrhage. C. Artificially infected fish. Arrows show skin hemorrhage.

PATHOGEN IDENTIFICATION

The near complete 16S rRNA gene sequence (1400 nucleotides) of isolate HS5 was submitted to GenBank database with the accession no. QQ926614. A similarity of 99% was noted in the 16S rRNA gene sequence between isolate HS5 and other *B. subtilis* strains from the GenBank database. The phylogenetic tree further confirmed that isolate HS5 was a *B. subtilis* strain (Figure 3). Furthermore, the phenotypical features also identified isolate HS5 as *B. subtilis*, which showed a 100% identity to the reference strain of *B. subtilis*. It was positive for amido, amygdalin, arbutin, D-cellobiose, D-fructose, D-glucose, D-maltose, D-mannitol, D-mannose, D-melibiose, D-raffinose, D-ribose, D-saccharose, D-sorbitol, D-trehalose, D-xlyose, esculin, glycerin, glycogen, inositol, inulin, L-arabinose, L-rhamnose, Methyl- α D-glucopyranosid, N-acetylglucosamin, and salicin, and negative for D-adonitol, D-arabinose, D-ara-

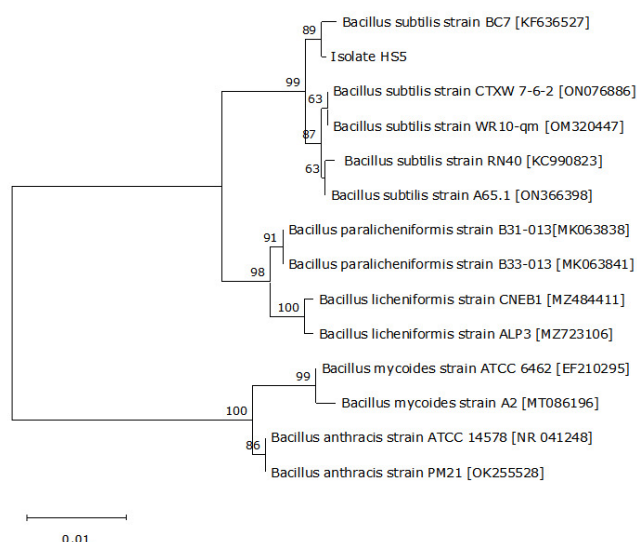


Figure 3. A 16S rRNA gene tree of thirteen known bacteria and isolate HS5 constructed using the neighbour-joining method.

The bootstrap values (%) are shown beside the clades, accession numbers are indicated beside the names of strains, and scale bars represent distance values.

bitol, D-fucose, D-galactose, D-lactose, D-lyxose, D-melezitose, D-tagatose, D-turanose, dulcitol, erythritol, gentiobiose, kaliumgluconat, kalium-2-ketogluconat, kalium-5-ketogluconat, L-arabitol, L-fucose, L-sorbose, L-xylose, methyl- α D-mannopyranosid, methyl- β D-xylopyranosid, and xylitol (Table 2). Thus, isolate HS5 was identified molecularly and phenotypically as *B. subtilis*.

VIRULENCE FACTORS OF PATHOGEN

Isolate HS5 formed clear zones of haemolysis on the RBA plate (Figure 4), indicating that isolate HS5 was beta-haemolytic. Furthermore, isolate HS5 was found to harbor the *entFM* gene (Figure 5).

ANTIMICROBIAL SUSCEPTIBILITY OF PATHOGEN

The result (Table 3) showed that isolate HS5 was highly susceptible to enrofloxacin, gentamicin, florfenicol, kanamycin, netilmicin, and norfloxacin, intermediately susceptible to doxycycline, and resistant against amoxicillin, bacitracin, cefotaxime, cefradine, ceftizoxime, roxithromycin, and sulfamethoxazole. These data indicated that isolate HS5 had not developed multiple resistances to quinolones, phenicols, and tetracycline antimicrobials in aquaculture use.

DISCUSSION

Recently, *Bacillus* species have been documented to cause diseases in aquaculture animals. Isolate LYS1 of *B. mycoides* has been demonstrated to induce ulcerative disease in largemouth bass *Micropterus salmoides*.²³ Isolate CA4 of *B. cereus* has been confirmed to cause hepatic hemorrhage of snakehead fish.¹⁴ However, there is limited information on

B. subtilis as a causative pathogen of yellow catfish. In the present study, we characterized the phenotype, taxonomic position, virulence, and antimicrobial susceptibility of *B. subtilis* HS5. To our knowledge, this is the first report of haemolytic *B. subtilis* as a causative agent of hemorrhagic disease in yellow catfish.

The LD₅₀ value is a frequently-used indicator for bacterial virulence assessment.²⁴ *B. subtilis* strains G7 and NCIB 3610^T showed the LD₅₀ values of 3.2×10^5 CFU/g and 4.55×10^5 CFU/g in turbot *Scophthalmus maximus*, and resulted in hemorrhage signs.¹⁰ In our study, isolate HS5 exhibited the LD₅₀ value of 5.20×10^6 CFU/ml, and also caused hemorrhage symptom which was in agreement with the previous findings.¹⁰ As stated by Triyaningsih et al.,²⁵ a bacterial strain can be regarded as virulent when its LD₅₀ value below $10^{7.0}$ CFU/ml. Thus, isolate HS5 was considered as a virulent strain that could probably cause severe threats to yellow catfish health.

Haemolysin is a primary virulence factor of pathogenic *Bacillus* species,²⁶ and the haemolysin-producing *Bacillus* strains have been found to cause bacterial diseases in aquaculture animals.^{14,27} Several haemolytic strains of *B. subtilis* have been found to be pathogenic to *S. maximus* and *C. semilaevis*.¹⁰ In the present study, isolate HS5 of *B. subtilis* was demonstrated to produce beta-haemolysins. This is probably attributed to the involvement of *spoVG* gene in *B. subtilis*.⁹ In addition, the virulent *entFM* gene was also discovered in isolate HS5, which was involved in bacterial adhesion to epithelial cells, vacuolization of macrophages, and virulence.²⁸ The presence of these virulence factors in isolate HS5 probably confer its pathogenicity for yellow catfish.

The antimicrobial resistance in fish-pathogenic *Bacillus* species has been increasing because of the rising use of antimicrobials to treat fish diseases.²⁹ The largemouth bass-pathogenic *B. mycoides* LYS1 has been found to develop multiple resistances against cephalosporins, penicillins, and sulfonamides antimicrobials.²³ The snakehead fish-pathogenic *B. cereus* CA4 has been discovered to be multiply resistant to penicillins, polypeptides, and sulfonamides antimicrobials.¹⁴ In this study, multiple resistances were also found in *B. subtilis* HS5 against cephalosporins, penicillins, polypeptides, and sulfonamides. This is consistent with the findings observed in pathogenic *B. mycoides* LYS1 and *B. cereus* CA4.^{14,23} Thus, more attention should be given to the control of fish-pathogenic *Bacillus* strains. Enrofloxacin and florfenicol are widely used veterinary antibiotics for bacterial disease treatment in aquaculture.³⁰ Dietary supplementation with enrofloxacin at 10 mg/kg body weight for five days has been found to effectively control ulcerative disease in the common carp *Cyprinus carpio* (Liu and Wang, 2004). Oral administration of florfenicol at 5 to 15 mg/kg body weight for ten days has been discovered to significantly control *Aeromonas hydrophila* infection in gibel carp *Carassius auratus gibelio*.¹⁸ In the present study, *B. subtilis* HS5 was highly susceptible to enrofloxacin and florfenicol. This serves as a reminder that enrofloxacin and florfenicol can be used to treat *B. subtilis* infection in yellow catfish. Certainly, some prevention measures should also be

Table 2. Phenotypic characterization of isolate HS5.

Tests	Reaction	
	Isolate HS5	<i>B. subtilis</i> ^a
Amidon	R ⁺	R ⁺
Amygdalin	R ⁺	R ⁺
Arbutin	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- β D-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 He et al.¹⁷

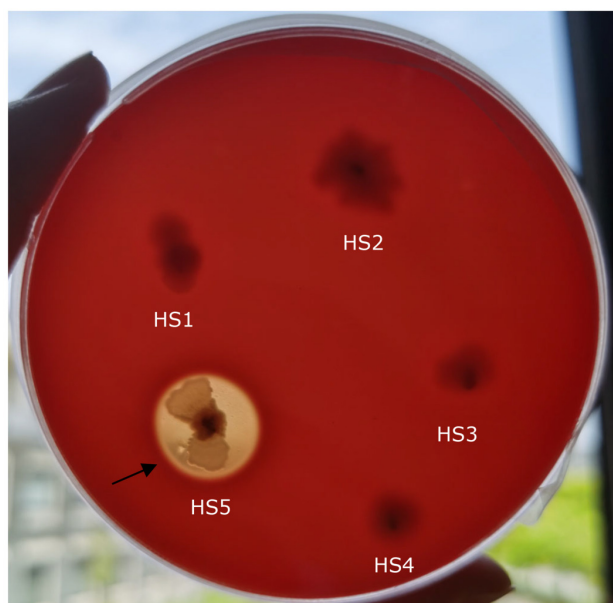


Figure 4. Haemolytic activity of isolate HS5 and the other four isolates (HS1, HS2, HS3 and HS4).

Arrow shows the clear beta-haemolytic zone on the RBA plate.

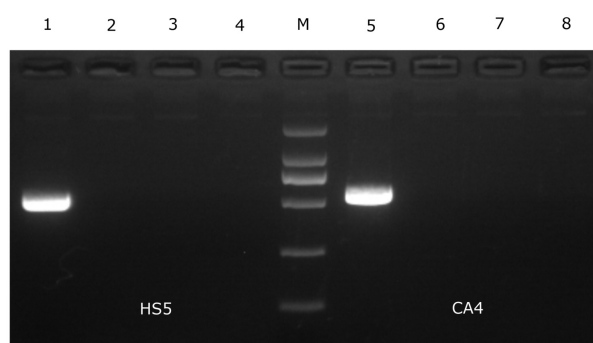


Figure 5. The PCR amplification of virulence genes in the HS5 isolate.

Lane M: DNA marker; lanes 1 and 5: *entFM* gene, lanes 2 and 6: *nheA* gene, lanes 3 and 7: *nheB* gene, lanes 4 and 8: *nheC* gene.

taken to reduce the chance of the outbreak of this disease, such as moderate stocking density, less feed waste and routine monitoring of pathogen population.

In conclusion, the findings of this study for the first time identified haemolytic *B. subtilis* HS5 as a causative pathogen in farmed yellow catfish, and provided new in-

sights into the potential threat of haemolytic *B. subtilis* to yellow catfish.

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AUTHORS' CONTRIBUTION ACCORDING TO CREDIT

Methodology: Chunlei Gai (Equal), Xurui Zheng (Equal). Formal Analysis: Chunlei Gai (Equal), Xurui Zheng (Equal), La Xu (Supporting), Jing Diao (Supporting). Investigation: Chunlei Gai (Equal), Xurui Zheng (Equal), La Xu (Supporting), Jing Diao (Supporting). Funding acquisition: Chunlei Gai (Lead). Resources: Xurui Zheng (Lead). Writing – review & editing: Haibin Ye (Equal), Xiaoqing Yu (Equal). Supervision: Haibin Ye (Lead). Conceptualization: Haipeng Cao (Equal), Xiaoqing Yu (Equal). Writing – original draft: Haipeng Cao (Lead).

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Table 3. Susceptibility of isolate HS5 to antimicrobials.

Antimicrobials	Content (µg/disc)	Inhibition zone diameter (mm)
Amoxicillin	20	0±0 ^R
Bacitracin	0.04	0±0 ^R
Cefotaxime	30	0.00±0.00 ^R
Cefradine	30	0.00±0.00 ^R
Ceftizoxime	30	8.23±0.21 ^R
Doxycycline*	30	14.30±0.79 ^I
Enrofloxacin*	10	16.43±0.40 ^S
Florfenicol*	30	20.43±0.51 ^S
Gentamycin	10	18.00±0.70 ^S
Kanamycin	30	17.77±0.68 ^S
Netilmicin	30	25.97±0.45 ^S
Norfloxacin	10	16.07±0.40 ^S
Roxithromycin	15	8.37±0.45 ^R
Sulfamethoxazole*	300	0.00±0.00 ^R

Data are presented as mean ± standard deviation.

S: susceptible; I: intermediately susceptible; R: resistant; *: antimicrobials in aquaculture use.



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