



# Isolation and Characterization of Virulent *Citrobacter freundii* Associated with White Muscle Disease in Farmed Red Swamp Crayfish, *Procambarus clarkia*

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## ABSTRACT

In June 2021, a sudden outbreak of white muscle disease coincidence with considerable mortality was occurred in *Procambarus clarkii* which were cultured in Jiangxia fish farm, Wuhan, Hubei province, China. A dominant bacterial colony, named FSN1, was isolated by incubating on Luria-Bertani agar. Morphological, physiological and biochemical characterization as well as 16S rRNA sequencing identified FSN1 isolate as *Citrobacter freundii*. Drug sensitivity test showed the strain has multiple drug resistance and was only sensitive to a few antibiotics (doxycycline, tetracycline, minocycline and ciprofloxacin). PCR amplification of specific genes responsible for antibiotic resistance (extended-spectrum  $\beta$ -lactamases, quinolone resistance determinants and tetracycline-resistance) confirmed antibiotics sensitivity of this bacterium. Artificial infection showed that the strain could cause similar symptoms to those of naturally infected crayfish, and the lethal dose 50% was  $1.52 \times 10^6$  CFU/g crayfish. Serious histopathological changes, such as cell degeneration and necrosis, hemorrhage and inflammatory cell infiltration, were found in diseased crayfish. In conclusion, this study confirmed the pathogen of whitish muscle disease in farmed *P. clarkii*, and preliminarily investigated the biological characteristic, pathogenicity and drug sensitivity of this bacterium. The results of this study will provide scientific guidance for the effective prevention and control of muscle turbidity of *P. clarkii*.

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## Authors' Contribution

HX and XW conducted the experiments, wrote and revised the manuscript. TT and CZ collected the samples. DY and JL analyzed the results.

## Key words

*Procambarus clarkii*, *Citrobacter freundii*, Whitish muscle disease, Antibiotic resistance, Histopathology

## INTRODUCTION

Red swamp crayfish (*Procambarus clarkii*), originally from northeastern Mexico and the southcentral United States, is extensively distributed worldwide (Hobbs, 1989). In 1929, *P. clarkii* was firstly introduced into Nanjing, China from Japan for aquaculture, which then spread to many cities as a special aquatic animal (Yan *et al.*, 2001). Nowadays, *P. clarkii* has been an important commercial freshwater crustacean species in China due to its strong fecundity and high economic value (Cremades *et al.*, 2001). According to the China Fisheries Statistic Yearbook in 2022, the gross production of crayfish has exceeded


2.63 million tons in 2021, which has increased about 10.02% compared to those produced in 2020 (Bureau, 2022). Nevertheless, the appearance of contagious diseases has substantially accumulated with the increased stocking density of intensive practices, which constitutes the major bottlenecks of sustainable development in *P. clarkii* farming.

Bacteria disease has caused significant economic losses in the culture of *P. clarkii*. The major microorganism identified from diseased crayfish including *Aeromonas veronii* (Yuan *et al.*, 2021), *Aeromonas hydrophila* (Jiravanichpaisal *et al.*, 2009) and *Citrobacter freundii* (Liu *et al.*, 2020). Meanwhile, *C. freundii* is a facultative anaerobic Gram-negative bacterium that belongs to the Enterobacteriaceae family (Liu *et al.*, 2020). It is widely distributed in aquatic environment and is an opportunistic pathogen in veterinary and medical sciences (Nawaz *et al.*, 2008). The bacterium was firstly regarded as an emerging fish pathogen from sunfish (*Mola mola*) in Japan (Sato *et al.*, 1982). Recently, there have been reports of deaths of aquatic animals infected with *C. freundii*, including significant infections in silver catfish (*Rhamdia quelen*) (Junior *et al.*, 2018), rohu (*Labeo rohita*) (Behera *et al.*,

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2022), freshwater prawn (*Macrobrachium rosenbergii*) (Zhao *et al.*, 2022) and *P. clarkii* (Liu *et al.*, 2020).

In this study, we firstly report a case of a natural bacterial infection with *C. freundii* as a cause of white muscle disease in farmed crayfish. In addition to the morphological and biochemical characteristics, the molecular determination by PCR was carried out to ascertain the distribution of antimicrobial resistant genes of *C. freundii*. Then, the antibiotic susceptibility of the bacterium and the histopathological changes caused by *C. freundii* in the infected crayfish were investigated.

## MATERIALS AND METHODS

### Bacterial isolation and phenotypic characterization

The bacterial isolation was performed from three clinically moribund crayfish cultured in Jiangxia fish farm, Wuhan, Hubei province, China as described previously (Xu *et al.*, 2022). Briefly, swabs were taken aseptically using a sterile inoculating loop from the white muscle of diseased crayfish, streaked onto Luria-Bertani agar (LBA, Land Bridge Ltd) plates and incubated at 28 °C for 24 h. The bacteria were re-inoculated on LBA plates thrice to obtain the pure culture. Moreover, the isolate was streaked on LBA containing 5% defibrinated sheep blood and incubated at 28 °C for 24 h to observe colony morphology and haemolysis. The morphology of isolated bacteria was certified using a Gram Stain Kit (Solarbio,

Beijing, China). Images were acquired with an imaging microscope (Olympus BX60, Japan) by using 100 × oil immersion objective lens.

The biochemical characteristics of *C. freundii* was detected using standardized API® 20E system (BioMerieux S.A., France) according to the manufacture's guidelines. Briefly, pure cultures of single colonies obtained on LB were suspended in 2 mL of sterile saline and then gently mixed for 10 min to disperse the bacteria. The turbidity of bacterial suspension was adjusted to  $1.8 \times 10^9$  CFU/mL, and 65 µL of each suspension was added to each cupule on the strip. Plates were incubated at 28 °C for 48 h and the biochemical characteristics of *C. freundii* were recorded thereafter.

### Molecular identification of pathogenic bacterium

Molecular identification was based on 16S rRNA gene sequence analysis. Total genomic DNA of isolated bacterium was prepared using bacteria DNA extraction kits (Tiangen Biotech, Beijing Co., Ltd., China) following the manufacturer's instructions. The extracted total genomic DNA was stored at -20 °C for further used as templates for PCR amplification. PCR assay was performed in a final volume of 25 µL using a Biometra T Professional Thermocycler (TaKaRa, Japan) as previous report (Sun *et al.*, 2018). The utilized primers for 16S rRNA gene was shown on Table I. The amplified products were checked on 1% agarose gel

**Table I. Oligonucleotide primer sequences used for PCR amplification of drug-resistant genes of *Citrobacter freundii*.**

Genes	F: Primer sequence (5'-3')	R: Primer sequence (5'-3')	Tm	Product size/bp	References
16s rRNA	AGAGTTTGATCCTGGCTCAG	GGTACCTTGTTACGACTT	55	1465	Liu <i>et al.</i> , 2020
<i>bla</i> <sub>CTX</sub>	CGCTTTGCGATGTGCAG	ACCGGATATCGTTGGT	54	550	Dalenne <i>et al.</i> , 2010
<i>bla</i> <sub>TEM</sub>	CATTTCCGTGTCGCCCTTATTC	CGTTCATCCATAGTTGCCTGAC	52	800	Dalenne <i>et al.</i> , 2010
<i>bla</i> <sub>SHV</sub>	AGCCGCTTGAGCAAATTAAC	ATCCCGCAGATAAATCACCAC	52	713	Dalenne <i>et al.</i> , 2010
<i>bla</i> <sub>PER</sub>	GCTCCGATAATGAAAGCGT	TTCGGCTTGACTCGGCTGA	52	520	Dalenne <i>et al.</i> , 2010
<i>bla</i> <sub>ACC</sub>	AACAGCCTCAGCAGCCGGTTA	TTCGCCGAATCATCCCTAGC	64	346	Pérez-Pérez and Hanson, 2002
<i>bla</i> <sub>DHA</sub>	AACTTTCACAGGTGTGCTGGGT	CCGTACGCATACTGGCTTTGC	64	405	Pérez-Pérez and Hanson, 2002
<i>bla</i> <sub>EBC</sub>	TCGGTAAAGCCGATGTTGCGG	CTTCCACTGCGGCTGCCAGTT	64	302	Pérez-Pérez and Hanson, 2002
<i>bla</i> <sub>FOX</sub>	AACATGGGGTATCAGGGAGATG	CAAAGCGCGTAACCGGATTGG	64	190	Pérez-Pérez and Hanson, 2002
<i>qnrA</i>	CAGCAAGAGGATTTCTCACG	AATCCGGCAGCACTATTACTC	54	630	Ciesielczuk <i>et al.</i> , 2013
<i>qnrC</i>	GGGTTGTACATTTATTGAATCG	CACCTACCCATTTATTTCA	54	307	Kim <i>et al.</i> , 2009
<i>qepA</i>	GCAGGTCCAGCAGCGGGTAG	CTTCTGCCCGAGTATCGTG	54	218	Kim <i>et al.</i> , 2009
<i>tetA</i> (A)	GCTACATCCTGCTTGCCCTTC	CATAGATCGCCGTGAAGAGG	55	210	Poirel <i>et al.</i> , 2005
<i>tetA</i> (B)	TTGGTTAGGGCAAGTTTTG	GTAATGGGCAATAACACCG	55	659	Poirel <i>et al.</i> , 2005
<i>tetA</i> (C)	CTTGAGAGCCTTCAACCCAG	ATGGTCGTCATCTACCTGCC	55	418	Poirel <i>et al.</i> , 2005
<i>tetA</i> (G)	GCTCGGTGGTATCTCTGCTC	AGCAACAGAATCGGGAACAC	55	468	Poirel <i>et al.</i> , 2005

electrophoresis, purified with gel extraction kit (TIANGEN, Beijing, China), and then inserted into the pMD18-T vector. The recombinant vectors were sequenced at TsingKe Biotech (Wuhan, China) and thereafter blasted using the National Center for Biotechnology Information (NCBI) BLASTn (<http://www.ncbi.nlm.nih.gov>). Phylogenetic tree was constructed using the neighbor-joining method in the MEGA X software package following multiple alignments (using clustal W) of the 16S rRNA sequence.

#### *Growing characteristics*

The pH value and NaCl concentration were adjusted based on the LB medium. Briefly, the pH value of LB was adjusted to 3, 5, 7, 9 and 11 with HCl (1 mol/L) or NaOH (1 mol/L), respectively, prior to sterilize by autoclaving. Similarly, the NaCl concentration of LB was adjusted to 0, 1, 3, 5 and 7%, respectively, before sterilization. The FSN1 isolate was incubated in LB until the bacterial suspension reached the  $3.0 \times 10^8$  CFU/mL. Then 200  $\mu$ L of bacterial suspension was added to prepared LB and cultured at 180 rpm in 100-mL conical flask. Growth was observed for 68 h by measuring the OD value at 600 nm every 4 h by spectrophotometer.

#### *Antimicrobial susceptibility assay*

The antibiotic sensitivity or resistivity of isolated *C. freundii* was determined following the standard Kirby-Bauer disk diffusion method (Bauer, 1966). Briefly, the bacterial isolates were cultured in LB broth and the concentration of the bacterial solution was adjusted to  $1.0 \times 10^8$  CFU/mL. The suspension was streaked on LBA plates, and the commercial drug impregnated disks (Hangzhou Tianhe Microorganism Reagent Co., Ltd., China) were applied on the streaked cultures. After 18 h of incubation at 28 °C, the diameter of the zone around the disc was measured and antibiotics were interpreted as susceptible (S), intermediate (I) or drug resistant (R) according to the criteria set by the manufacturer.

#### *Identification of antimicrobial resistant genes*

The *C. freundii* were subjected to PCR amplification to identify extended-spectrum  $\beta$ -lactamases ( $bla_{TEM}$ ,  $bla_{SHV}$ ,  $bla_{CTX}$ ,  $bla_{PER}$ ,  $bla_{DHA}$ ,  $bla_{ACC}$ ,  $bla_{FOX}$  and  $bla_{EBC}$ ), plasmid-mediated quinolone resistance determinants (qnrA, qnrC and qepA) and tetracycline-resistance genes (tetA, tetB, tetC and tetG) using specific primers shown at Table I. The PCR amplicons were separated by electrophoresis on 1% (w/v) agarose gels stained with 4S Green and photographed under Gel Doc EZ Imager (Bio-Rad, Germany).

#### *Experimental infection*

Two hundred healthy *P. clarkii* with an average body

weight of  $15.8 \pm 0.6$  g were purchased from a local crayfish farm (Hubei, China). Prior to the experimental infection, the crayfish were randomly divided into six groups with each containing 30 crayfish, temporarily cultured in tanks (60 L) with temperature 28 °C for two weeks. The concentration of FSN1 isolate was adjusted with PBS to prepare  $1.0 \times 10^4$ ,  $1.0 \times 10^5$ ,  $1.0 \times 10^6$ ,  $1.0 \times 10^7$  and  $1.0 \times 10^8$  CFU/mL bacterial suspensions. Crayfish in five experimental groups were injected at third abdominal segment with the aforementioned bacterial concentrations at a dose of 100  $\mu$ L/crayfish, respectively. The control group was injected with the same dose of PBS. The number of dead crayfish were recorded daily for a period of 14 days and then the mean lethal dose 50% ( $LD_{50}$ ) value was calculated according to a modified arithmetical method of Reed and Muench (Reed and Muench, 1938). Investigations were performed strictly following the guidelines allowed by the Committee of the Ethics on Animal Care and Experiments at Wuhan Polytechnic University (No. WPU202011002).

#### *Histopathological assessment*

Histopathology assays were conducted in infected crayfish tissues including the muscle, gill, intestine and hepatopancreas with typical clinical manifestations. These tissues were sampled from the diseased crayfish, trimmed to the appropriate size, and fixed in 10% neutral buffered formalin for 48 h. Afterwards, the fixed samples were routinely dehydrated in series increased ethanol solutions, equilibrated the alcohol in the tissues with xylene, followed by embedding in paraffin blocks. Serial 5  $\mu$ m thicknesses sections of paraffin blocks were prepared using a microtome (Leica RM2245, Germany), mounted onto gelatinized slides and then stained with hematoxylin and eosin (H and E) for histopathological examination. The slides were observed and photographed under an imaging microscope (Olympus BX60, Japan) equipped with an image acquisition software (Cell Sens Standard, Olympus, Tokyo, Japan).

## RESULTS

#### *Clinical signs and gross pathology*

The natural diseased crayfish showed the clinical symptoms of anorexia, lethargy and inactivity, and exhibited a sudden onset of high mortalities. Figure 1 displayed the clinical picture of the diseased crayfish with opaque or whitish muscles, particularly noticeable in the abdomen.

#### *Morphological and biochemical characteristics*

The FSN1 isolate formed white, smooth and convex colonies both on LB and blood agars (Fig. 2A and B).



Fig. 1. Pathological features of diseased crayfish. A: Diseased crayfish lateral caudal muscle; B: Diseased crayfish inner tail muscle.

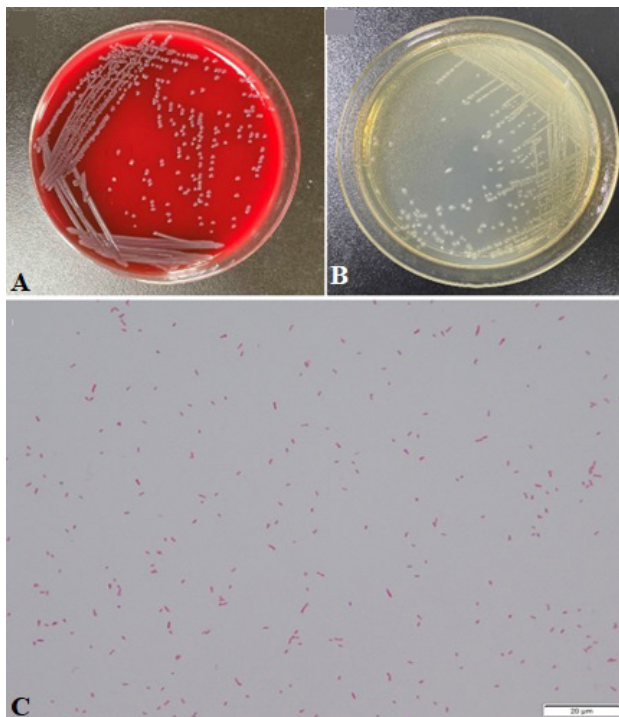


Fig. 2. Morphologies of *C. freundii* FSN1. A: Colony morphologies of FSN1 on LBA plate; B: Colony morphologies of FSN1 on 5% sheep blood agar; C: Gram staining of bacterium FSN1 (100 ×). Scale bars = 20 µm.

They were not pigmented and did not induce hemolysis on blood agar (Fig. 2B). The isolate was a typically Gram-negative rod-shaped bacterium (Fig. 2C). The biochemical characterization of *C. freundii* showed that the bacterium was positive for citrate, sucrose, β-galactosidase, sorbitol, rhamnose, mannitol, glucose, arginine, lysine, ornithine and gelatin reaction, but negative for arabinose, inositol, tryptophan and acetoin, and production from indole and urease (Table II).

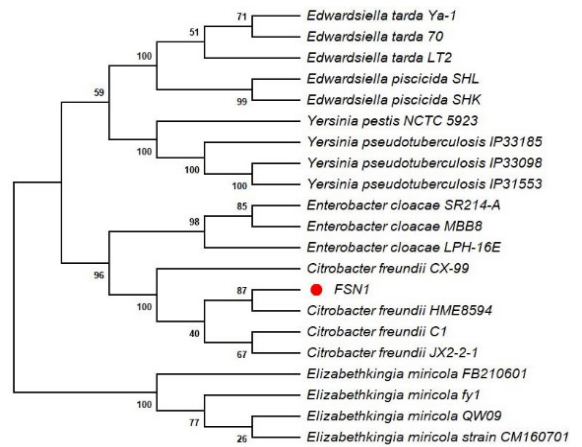


Fig. 3. Phylogenetic tree of isolated FSN1 as determined by 16S rRNA gene sequence.



**Table II. API 20E result for isolates and reference strains of *Citrobacter freundii*.**

Items	<i>C. freundii</i> FSN1	<i>C. freundii</i> *
Gram stain	Negative	Negative
Cell morphology	Rod	Rod
Motility	+	+
Catalase	+	+
Oxidase	-	-
Hemolysis of sheep RBCs	-	+
Indole production (IND)	-	V
Urease production (URE)	-	-
Citrate utilization (CIT)	+	V
Sucrose fermentation (SAC)	+	+
$\beta$ -galactosidase (ONPG)	+	+
Sorbitol fermentation (SOR)	+	+
Rhamnose fermentation (RHA)	+	V
Melibiose fermentation (MEL)	-	+
Amygdalin fermentation (AMY)	-	-
Arabinose fermentation (ARA)	-	V
Inositol fermentation (INO)	-	V
Mannitol fermentation (MAN)	+	+
Glucose fermentation (GLU)	+	+
H <sub>2</sub> S production	+	V
Arginine dihydrolase (ADH)	+	+
Lysine decarboxylase (LDC)	+	-
Ornithine decarboxylase (ODC)	+	-
Tryptophan deaminase (TDA)	-	-
Gelatin hydrolysis (GEL)	+	+
Acetoin production (VP)	-	-

+, positive; —, negative; \* Reference strain data compiled from Bergey's manual.

#### Phylogenetic analysis based on 16S rRNA gene sequence

The gene sequence for the 16S rRNA of isolate FSN1 was submitted and deposited in the GenBank database under accession no. OP355145. The BLAST alignment of

16S rRNA gene sequence showed that strain FSN1 shared 99.93% sequence similarity with the sequence of *C. freundii* strain HME8594 (GenBank accession no. JX426059.1), and the phylogenetic tree further demonstrated that FSN1 clustered together with known species of *C. freundii* strains (Fig. 3). These results showed that FSN1 have been related to *C. freundii*, which is supported by a high bootstrap value.

#### Growing characteristics

The growing characteristics of FSN1 on pH value and NaCl concentration were investigated. As shown in Figure 4A, the bacterium has a wide pH range and can grow normally at pH 5–9, whose optimal value was around pH 5, whereas the growth was inhibited at pH 3 and 11. As shown in Figure 4B, the growth and concentration of FSN1 reached the maximum level at 1% NaCl. The latent phase of growth was extended markedly at 5% NaCl. At 0% and 3% NaCl, the growth trends were similar, even though the considerable final concentration was higher in 0% NaCl than those cultured in 3% NaCl. Growth was halted at 7% NaCl. The present results revealed that *C. freundii* has a wide range of pH and salinity tolerance.

#### Determination of antimicrobial resistance

The antibiotic resistance patterns of *C. freundii*, valued by the size of the inhibition zones around each disc, showed that the strain FSN1 was resistant (R) to kanamycin, streptomycin, amikacin, gentamycin, neomycin, ceftriaxone, cefpimizole, ceftazidime, ampicillin, piperacillin, carbenicillin, oxacillin, levofloxacin, rifampicin, trimethoprim, and sulfisoxazole, intermediate sensitive (I) to cefepime, enrofloxacin, norfloxacin and florfenicol, and susceptible (S) to four antibiotics including doxycycline, tetracycline, minocycline and ciprofloxacin, indicating that isolate FSN1 had multiple resistances to aminoglycosides, cephalosporins, penicillin and sulfonamides antimicrobials used in aquaculture (Table III).

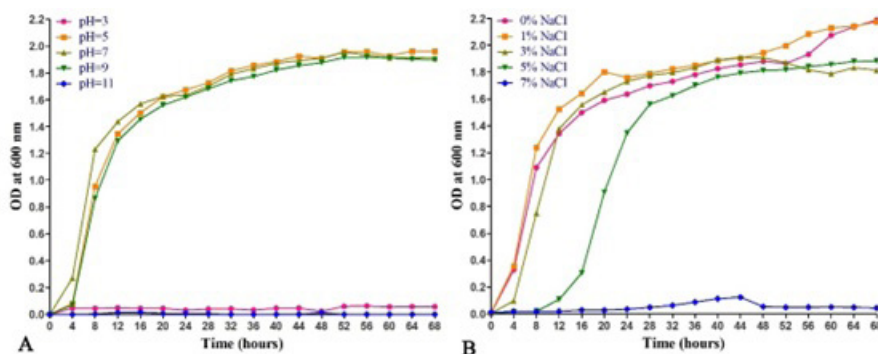


Fig. 4. Growth (OD<sub>600</sub>) of the strain FSN1 at different pH (A) and NaCl concentration (B).

**Table III. Antibiotics susceptibility of strain FSN1 against 24 antimicrobial agents.**

Antibiotics	Concentration	R/mm	I/mm	S/mm	Diameter of inhibited zone/mm
<i>C. freundii</i> FSN1					
<b>Aminoglycosides</b>					
Kanamycin	30 µg/disc	≤13	14~17	≥18	0 (R)
Streptomycin	10 µg/disc	≤11	12~14	≥15	0 (R)
Amikacin	30 µg/disc	≤14	15~16	≥17	0 (R)
Gentamycin	10 µg/disc	≤12	13~14	≥15	0 (R)
Neomycin	30 µg/disc	≤12	13~16	≥17	5 (R)
<b>Tetracyclines</b>					
Doxycycline	30 µg/disc	≤12	13~15	≥16	24 (S)
Tetracycline	30 µg/disc	≤14	15~18	≥19	26 (S)
Minocycline	30 µg/disc	≤14	15~18	≥19	30 (S)
<b>Cephalosporins</b>					
Ceftriaxone	30 µg/disc	≤13	13~21	≥21	21 (R)
Cefpimizole	30 µg/disc	≤14	15~17	≥18	9 (R)
Ceftazidime	30 µg/disc	≤17	18~20	≥21	15 (R)
Cefepime	30 µg/disc	≤14	15~17	≥18	16 (I)
<b>Penicillin</b>					
Ampicillin	10 µg/disc	≤13	14~16	≥17	11 (R)
Piperacillin	100µg/disc	≤17	18~20	≥21	15 (R)
Carbenicillin	100 µg/disc	≤18	19~23	≥24	14 (R)
Oxacillin	1 µg/disc	≤10	11~12	≥13	0 (R)
<b>Quinolones</b>					
Enrofloxacin	5 µg/disc	≤16	17~22	≥23	20 (I)
Norfloxacin	10 µg/disc	≤12	13~16	≥17	13 (I)
Ciprofloxacin	5 µg/disc	≤15	16~20	≥21	27 (S)
Levofloxacin	5 µg/disc	≤12	13~16	≥17	12 (R)
Florfenicol	30 µg/disc	≤12	13~17	≥18	17 (I)
Rifampicin	5 µg/disc	≤16	17~19	≥20	0 (R)
<b>Sulfonamides</b>					
Trimethoprim	25 µg/disc	≤23	24~32	≥33	0 (R)
Sulfisoxazole	30 µg/disc	≤10	11~15	≥16	6 (R)

The diameter of the antibacterial zone includes the diameter of the drug sensitive tablet. S, highly sensitive; I, intermediately sensitive; R, low or not sensitive.

*Determination of antimicrobial resistant genes*

PCR assay was conducted to screen the antibiotic resistant genes of isolated *C. freundii*. The PCR profiles of antibiotic resistance genes including  $bla_{TEM}$ ,  $bla_{SHV}$ ,  $bla_{CTX}$ ,

$bla_{PER}$ ,  $bla_{DHA}$ ,  $bla_{ACC}$ ,  $bla_{FOX}$ ,  $bla_{EBC}$  and *qepA* were detected in the *C. freundii* isolate, whereas the *qnrA*, *qnrC*, *tetA*, *tetB*, *tetC* and *tetG* genes were lost in FSN1 (Fig. 5).

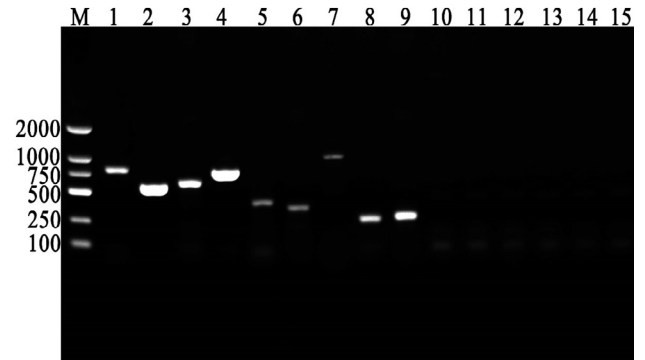


Fig. 5. Agarose gel electrophoresis of fifteen drug-resistant genes for *Citrobacter freundii*. M: DL2000 maker, 1:  $bla_{TEM}$ , 2:  $bla_{PER}$ , 3:  $bla_{CTX}$ , 4:  $bla_{SHV}$ , 5:  $bla_{DHA}$ , 6:  $bla_{ACC}$ , 7:  $bla_{FOX}$ , 8: *qepA*, 9:  $bla_{EBC}$ , 10: *qnrA*, 11: *qnrC*, 12: TetA, 13: TetB, 14 TetC, 15: TetG.

*Challenge test*

To determine the pathogenicity of the isolated FSN1, the challenge assay was carried out in healthy crayfish. The crayfish injected with  $1.0 \times 10^5$  to  $1.0 \times 10^8$  CFU per mL bacteria rapidly died from the 3<sup>rd</sup> to 8<sup>th</sup> day post-injection, whereas no death was observed in those crayfish injected with PBS and  $1.0 \times 10^4$  CFU/mL during the experimental challenge (Fig. 6). Moreover, the crayfish experimentally infected with FSN1 exhibited same symptoms as observed in the diseased crayfish. The LD<sub>50</sub> value of isolated *C. freundii* was calculated as  $1.52 \times 10^6$  CFU/g crayfish weight.

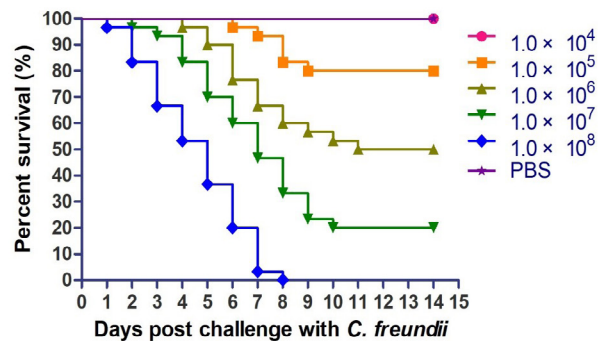


Fig. 6. The survival rates of crayfish challenged by various dose of FSN1 during 14 days post infection.

*Histopathology in crayfish infected by C. freundii*

Histological analysis showed pathological changes in

the muscle, gill, hepatopancrea and intestine from infected crayfish. Specifically, the muscle tissue of diseased crayfish showed cell necrosis and fibrinous degeneration with inflammatory cell infiltration (Fig. 7A and B). The gill of crayfish infected by *C. freundii* demonstrated leukocytes infiltration and vacuolar degeneration (Fig. 7C). A large number of inflammatory cells infiltrated the intestinal tract, accompanying with serious vacuolar and hyaline degeneration (Fig. 7D and E). The hepatopancreatic tubule lumen is dilated and disappeared star structure, the original structure of cells was disappeared and vacuolated, and more inflammatory cells infiltrated in the interstitium (Fig. 7F).

## DISCUSSION

Diseases are major limiting factors in the healthy development of aquaculture industry throughout the world and various diseases are frequently occurred in the breeding process of *P. clarkii* with the high culture density. White muscle disease is a frequent problem for cultured *Litopenaeus vannamei* (Zhou *et al.*, 2012) and *Macrobrachium rosenbergii* (Wang *et al.*, 2008) caused by virus or bacteria. However, scarce information is available on white muscle disease occurred in *P. clarkii*. Previous research revealed that crayfish infected with *C. freundii*

showed high mortality even though the diseased crayfish showed no clinical symptom of whitish muscle (Liu *et al.*, 2020). In the present study, *C. freundii* was isolated from diseased *P. clarkii* and firstly confirmed its association in contributing to the pathogenesis of white muscle and crayfish mortality.

The biochemical characteristics of the FSN1 isolates were basically coincidence with those of the *C. freundii* HME8594, even though the reactions of  $\beta$ -galactosidase, melibiose, arabinose, lysine and ornithine were different. In the current study, based on the results of the phenotypic characterization and biochemical identification, FSN1 was preliminary identified as *C. freundii*. It is worthwhile identifying phenotypic features of bacteria, however, this becomes implausible when only classic phenotypic characteristics being considered (Zhang *et al.*, 2011). Therefore, the molecular identification method based on the sequence analysis of 16S rRNA gene was proposed. In this research, blast alignments exhibited that 16S rRNA gene sequence of the FSN1 isolate was most closely to *C. freundii*. Additionally, the phylogenetic trees construct based on the 16S rRNA gene sequence displayed that the FSN1 isolate belonged to the *C. freundii* strains. Based on morphology, phenotypic characteristics and phylogenetic properties, the FSN1 isolated from the diseased crayfish were confirmed as *C. freundii*.

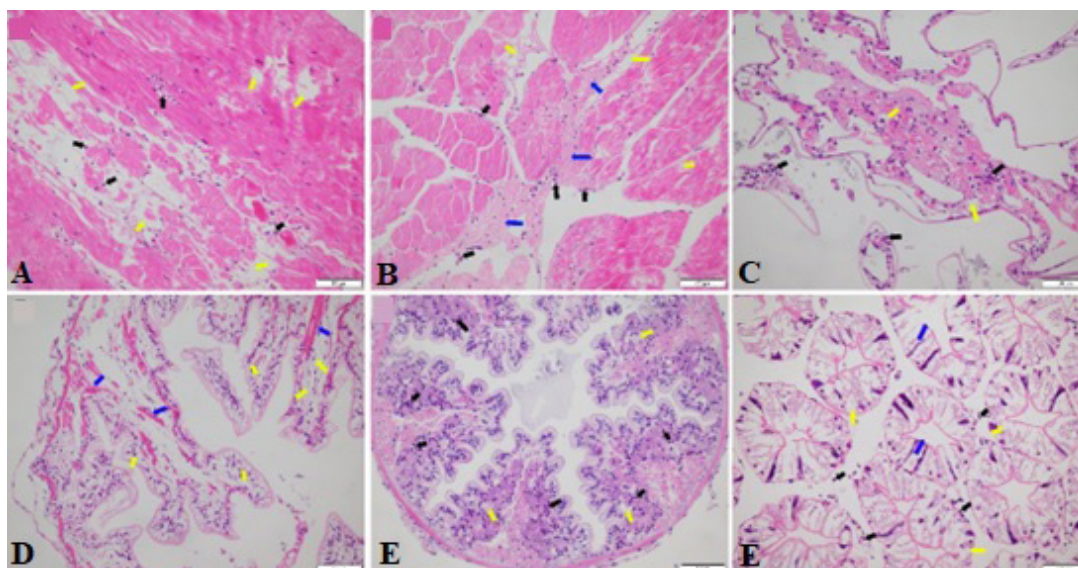


Fig. 7. Histopathological observation of various organs in diseased *P. clarkii*. A-B: The diseased crayfish muscle tissue showed inflammatory cell infiltration (black arrow), cell necrosis (yellow arrow) and fibrinoid degeneration (blue arrow). C: A large number of inflammatory cells infiltrate into the gill fila of the diseased crayfish (black arrow), vacuolar degeneration (yellow arrow). D-E: A large number of inflammatory cells infiltrate the intestines of the diseased crayfish (black arrow), showing vacuolar (yellow arrow) and hyaline degeneration (blue arrow). F: The lumen of the hepatic tubule dilates, the star-shaped structure disappears (the blue arrow), hepatocyte structure collapse, vacuolization (the yellow arrow), interstitial inflammatory cell infiltration (the black arrow).

Appearance of antibiotic resistance is one of the most serious problems in the case of pathogenic bacteria like *C. freundii*. For example, a *C. freundii* isolated from diseased *Pterophyllum scalare* has been found to develop resistance against enrofloxacin, cefalotin, ampicillin, florfenicol and oxytetracycline (Gallani *et al.*, 2016), and a *C. freundii* isolate from diseased *Labeo rohita* has been documented to be resistant to dicloxacillin, rifampicin and trimethoprim (Behera *et al.*, 2022). In the present study, the isolated bacterium was resistant to various  $\beta$ -lactam antibiotics, such as aminoglycosides, penicillin, and cephalosporins (except cefepime). However, the bacterium showed sensitive to tetracyclines. To illustrate the antibiotic resistance mechanism, we conducted genetic screening. The  $bla_{TEM}$ ,  $bla_{SHV}$ ,  $bla_{CTX}$ ,  $bla_{PER}$ ,  $bla_{DHA}$ ,  $bla_{ACC}$ ,  $bla_{FOX}$  and  $bla_{EBC}$  genes, which are well known for conferring resistance to carbapenems, were examined first (Barlow and Hall, 2002). The results showed positive reaction of  $\beta$ -lactamase genes ( $bla_{TEM}$ ,  $bla_{SHV}$ ,  $bla_{CTX}$ ,  $bla_{PER}$ ,  $bla_{DHA}$ ,  $bla_{ACC}$ ,  $bla_{FOX}$  and  $bla_{EBC}$ ) in *C. freundii*. Correspondingly, the bacterium exhibited negative reaction to *TetA*, *TetB*, *TetC* and *TetG* genes, which further explained the sensitive reaction of FSN1 to tetracycline. Even though FSN1 strain exhibited antimicrobial resistance to multiple antibiotics, the pathogens could be regulated by the application of antibiotics, such as doxycycline, tetracycline, minocycline and ciprofloxacin. It is worthy to note that the diseased crayfish successfully recovered as a result of the doxycycline treatment and no further mortality was observed after administration for 7 consecutive days.

Histological changes of immune-related organs including hepatopancreas, gill and intestine can be regarded as a potential indicator for the general health status of crayfish. In the present study, *C. freundii* infected crayfish exhibited leukocytes infiltration and vacuolar degeneration in the sampled tissues. Previous research performed by Liu *et al.* (2020) also showed similar symptom in *C. freundii* infected crayfish (Liu *et al.*, 2020). Interestingly, the typical symptom of crayfish infected with *C. freundii* was white muscle and the histopathology of muscle showed cell necrosis, fibrinoid degeneration and inflammatory cell infiltration. Similarly, previous research exhibited that *C. freundii* infection destroyed the arrangement of crayfish muscle fibers, with fractured muscle fibers, abnormal density, nucleus fixation, and red staining of the sarcoplasm, suggesting that *C. freundii* infection could affect the structural integrity of muscle (Huang *et al.*, 2021). The reason was possibly due to the fact that invading bacteria can use crayfish muscle as a rich source of nutrients to support their own survival and replication.

## CONCLUSIONS

In conclusion, the present study firstly reports a *C. freundii* isolate as a causal agent for white muscle disease in *P. clarkia*. Furthermore, the pathology and pathogenesis study of this bacterium would help in development of therapies and managing the disease caused by this pathogen in the aquaculture systems.

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### IRB approval

For this study, approval was obtained from Institutional Review Board of Wuhan Polytechnic University, Wuhan, China.

### Ethical statement

These investigations were approved by the Ethics Committee for Animal Care and Experiments at Wuhan Polytechnic University, Wuhan, China.

### Statement of conflict of interest

The authors have declared no conflict of interest.

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