Pakistan J. Zool., pp 1-10, 2023. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20221004141003

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

Hongsen Xu*, Xiaoni Wang, Tie Tian, Changyu Zhao, Denghang Yu and Jun Liu

Hubei Key Laboratory of Animal Nutrition and Feed Science, School of Animal Science and Nutritional Engineering, Wuhan Polytechnic University, No. 68, Xuefunan Road, Wuhan 430023, China

Article Information
Received 04 October 2022
Revised 22 October 2022
Accepted 09 November 2022
Available online 19 May 2023
(early access)

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**

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 β-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 × 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*.

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*.,

* Corresponding author: Hsxu1989@163.com
0030-9923/2023/0001-0001 $ 9.00/0

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 ºC 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 1.8×10^9 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	GGTTACCTTGTTACGACTT	55	1465	Liu *et al*., 2020
bla_{CTX}	CGCTTTGCGATGTGCAG	ACCGCGATATCGTTGGT	54	550	Dallenne *et al*., 2010
bla_{TEM}	CATTTCCGTGTCGCCCTTATTC	CGTTCATCCATAGTTGCCTGAC	52	800	Dallenne *et al*., 2010
bla_{SHV}	AGCCGCTTGAGCAAATTAAAC	ATCCCGCAGATAAATCACCAC	52	713	Dallenne *et al*., 2010
bla_{PER}	GCTCCGATAATGAAAGCGT	TTCGGCTTGACTCGGCTGA	52	520	Dallenne *et al*., 2010
bla_{ACC}	AACAGCCTCAGCAGCCGGTTA	TTCGCCGCAATCATCCCTAGC	64	346	Pérez-Pérez and Hanson, 2002
bla_{DHA}	AACTTTCACAGGTGTGCTGGGT	CCGTACGCATACTGGCTTTGC	64	405	Pérez-Pérez and Hanson, 2002
bla_{EBC}	TCGGTAAAGCCGATGTTGCGG	CTTCCACTGCGGCTGCCAGTT	64	302	Pérez-Pérez and Hanson, 2002
bla_{FOX}	AACATGGGGTATCAGGGAGATG	CAAAGCGCGTAACCGGATTGG	64	190	Pérez-Pérez and Hanson, 2002
qnrA	CAGCAAGAGGATTTCTCACG	AATCCGGCAGCACTATTACTC	54	630	Ciesielczuk *et al*., 2013
qnrC	GGGTTGTACATTTATTGAATCG	CACCTACCCATTTATTTTCA	54	307	Kim *et al*., 2009
qepA	GCAGGTCCAGCAGCGGGTAG	CTTCCTGCCCGAGTATCGTG	54	218	Kim *et al*., 2009
tetA (A)	GCTACATCCTGCTTGCCTTC	CATAGATCGCCGTGAAGAGG	55	210	Poirel *et al*., 2005
tetA (B)	TTGGTTAGGGGCAAGTTTTG	GTAATGGGCCAATAACACCG	55	659	Poirel *et al*., 2005
tetA (C)	CTTGAGAGCCTTCAACCCAG	ATGGTCGTCATCTACCTGCC	55	418	Poirel *et al*., 2005
tetA (G)	GCTCGGTGGTATCTCTGCTC	AGCAACAGAATCGGGAACAC	55	468	Poirel *et al*., 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 3.0×10^8 CFU/mL. Then 200 µL 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 1.0×10^8 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 β-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 ± 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 1.0×10^4, 1.0×10^5, 1.0×10^6, 1.0×10^7 and 1.0×10^8 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 µL/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 µm 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.

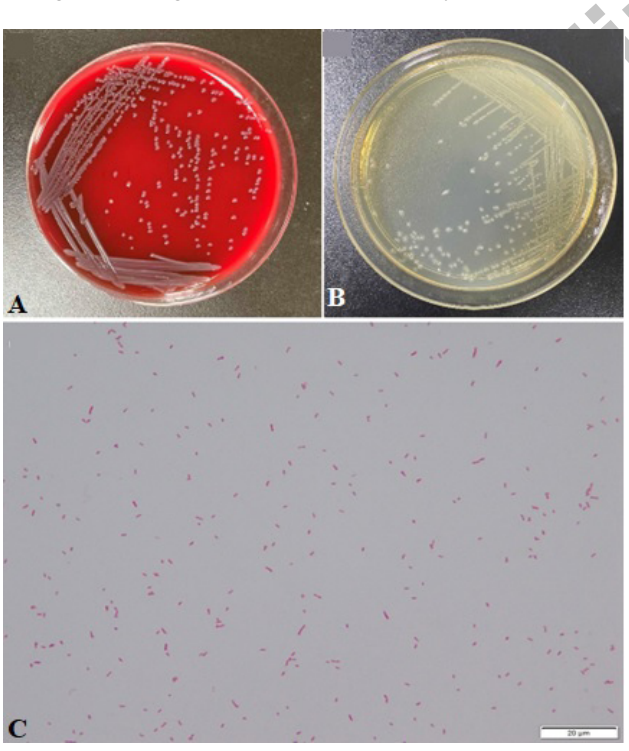

Fig. 2. Morphologies of *C. freundii* FSN1. A: Colony morphologies of FSN1 on LBA plate; B: Colony morphologies of FSN1 on 5% sheep blood agar; C: Gram staining of bacterium FSN1 (100 ×). Scale bars = 20 μm.

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).

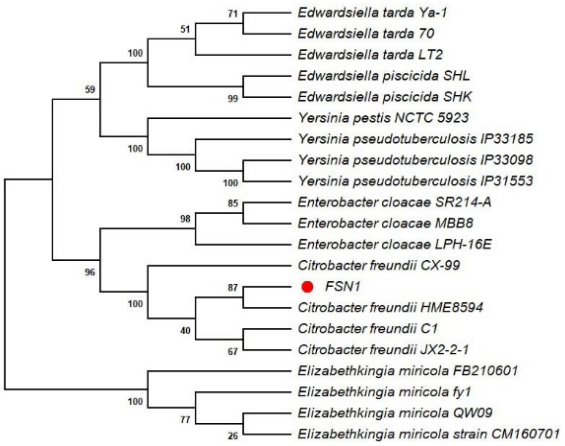

Fig. 3. Phylogenetic tree of isolated FSN1 as determined by 16S rRNA gene sequence.

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

Items	*C. freundii* FSN1	*C. freundii**
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)	+	+
β-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_2S 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).

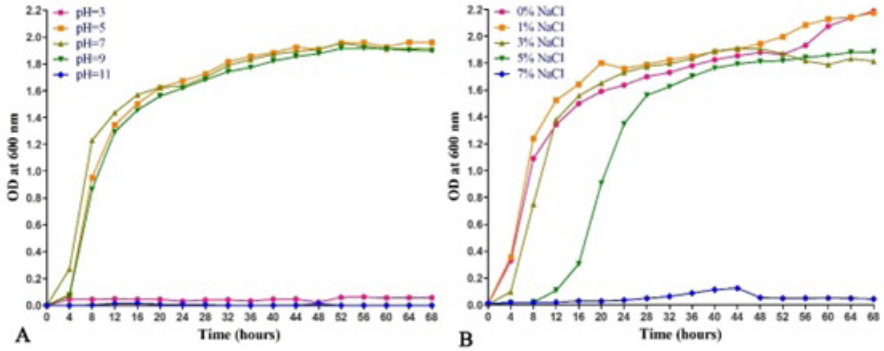

Fig. 4. Growth (OD_{600}) of the strain FSN1 at different pH (A) and NaCl concentration (B).

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

Antibiotics	Concen-tration	R/ mm	I/mm	S/ mm	Diameter of inhibited zone/ mm
					C. freundii FSN1
Aminoglycosides					
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)
Tetracyclines					
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)
Cephalosporins					
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)
Penicillin					
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)
Quinolones					
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)
Sulfonamides					
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).

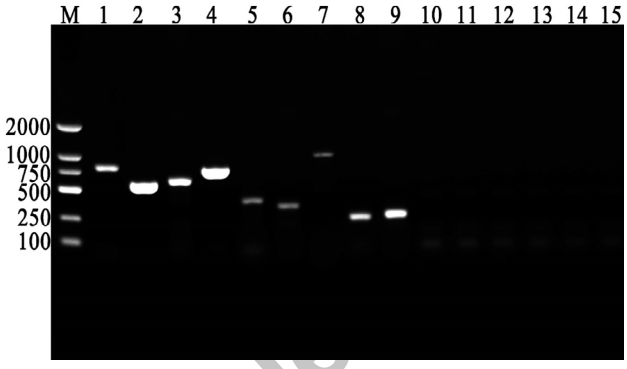

Fig. 5. Agarose gel electrophoresis of fifteen drug-resistant genes for *Citrobacter freundii*. M: DL2000 maker, 1: bla_{TEM}, 2: bla_{PER}, 3: bla_{CTX}, 4: bla_{SHV}, 5: bla_{DHA}, 6: bla_{ACC}, 7: bla_{FOX}, 8: qepA, 9: bla_{EBC}, 10: qnrA, 11: qnrC, 12: TetA, 13: TetB, 14 TetC, 15: TetG.

Challenge test

To determine the pathogenicity of the isolated FSN1, the challenge assay was carried out in healthy crayfish. The crayfish injected with 1.0×10^5 to 1.0×10^8 CFU per mL bacteria rapidly died from the 3rd to 8th day post-injection, whereas no death was observed in those crayfish injected with PBS and 1.0×10^4 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_{50} value of isolated *C. freundii* was calculated as 1.52×10^6 CFU/g crayfish weight.

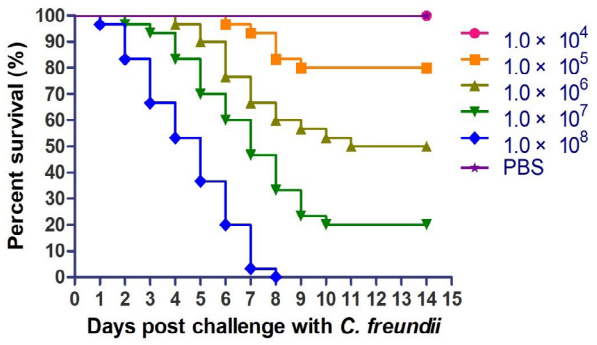

Fig. 6. The survival rates of crayfish challenged by various dose of FSN1 during 14 days post infection.

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, scare 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. clarkia* 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 β-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 the16S 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*.

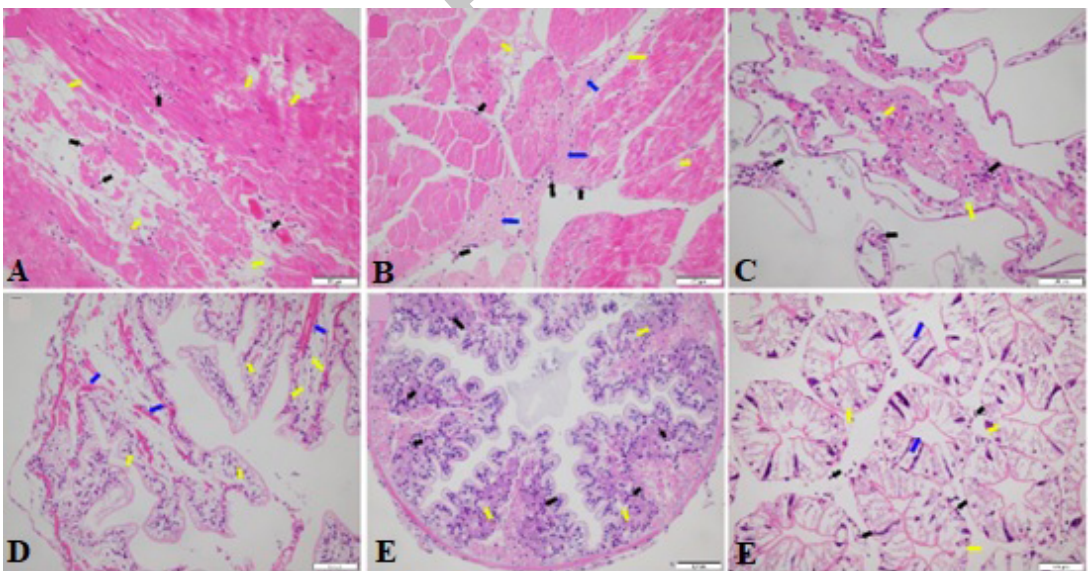

Fig. 7. Histopathological observation of various organs in diseased *P. clarkia*. A-B: The diseased crayfish muscle tissue showed inflammatory cell infiltration (black arrow), cell necrosis (yellow arrow) and fibrinoid degeneration (blue arrow). C: A large number of inflammatory cells infiltrate into 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 enrofloxaci, 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 β-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 β-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.

Funding

This study was supported by the Natural Science Foundation of Hubei Province (grant no. 2021CFB265), National Natural Science Foundation of China (grant no. 31902389) and Key Laboratory of Special Aquatic Formula Feed (grant no. TMKJZ1706 and TMKJZ1912).

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.

REFERENCES

Barlow, M., and Hall, B.G., 2002. Origin and evolution of the ampc β-lactamases of *Citrobacter freundii*. *Antimicrob. Agents Chemother*., **46**: 1190-1198. https://doi.org/10.1128/AAC.46.5.1190-1198.2002

Bauer, A., 1966. Antibiotic susceptibility testing by a standardized single disc method. *Am. J. clin. Pathol*., **45**: 149-158. https://doi.org/10.1093/ajcp/45.4_ts.493

Behera, B.K., Paria, P., Das, A., and Das, B.K., 2022. Molecular identification and pathogenicity study of virulent *Citrobacter freundii* associated with mortality of farmed *Labeo rohita* (Hamilton, 1822), in India. *Aquaculture*, **547**: 737437. https://doi.org/10.1016/j.aquaculture.2021.737437

Bureau, M., 2022. *China fishery statistical yearbook*. National aquatic product technology extension station, fisheries, C.S.O. 2022. Agricultural Press of China, Beijing.

Ciesielczuk, H., Hornsey, M., Choi, V., Woodford, N. and Wareham, D., 2013. Development and evaluation of a multiplex pcr for eight plasmid-

mediated quinolone-resistance determinants. *J. med. Microbiol*., **62**: 1823-1827. https://doi.org/10.1099/jmm.0.064428-0

Cremades, O., Ponce, E., Corpas, R., Gutierrez, J., Jover, M., Alvarez-Ossorio, M., Parrado, J., and Bautista, J., 2001. Processing of crawfish (*Procambarus clarkii*) for the preparation of carotenoproteins and chitin. *J. Agric. Fd. Chem*., **49**: 5468-5472. https://doi.org/10.1021/jf0104174

Dallenne, C., Da Costa, A., Decré, D., Favier, C., and Arlet, G., 2010. Development of a set of multiplex pcr assays for the detection of genes encoding important β-lactamases in enterobacteriaceae. *J. Antimicrob. Chemother.*, **65**: 490-495. https://doi.org/10.1093/jac/dkp498

Gallani, S.U., Sebastiao, F.D.A., Valladão, G.M., Boaratti, A.Z., and Pilarski, F., 2016. Pathogenesis of mixed infection by *Spironucleus* sp. and *Citrobacter freundii* in freshwater angelfish *Pterophyllum scalare*. *Microb. Pathog*., **100**: 119-123. https://doi.org/10.1016/j.micpath.2016.09.002

Hobbs, Jr, H.H., 1989. *An illustrated checklist of the american crayfishes (decapoda, astacidae, cambaridae, parastacidae)*. https://doi.org/10.5479/si.00810282.480

Huang, X., Li, M., Wang, J., Ji, L., Geng, Y., Ou, Y., Yang, S., Yin, L., Li, L. and Chen, D., 2021. Effect of bacterial infection on the edibility of aquatic products: The case of crayfish (*Procambarus clarkii*) infected with *Citrobacter freundii*. *Front. Microbiol*., **12**: 722037. https://doi.org/10.3389/fmicb.2021.722037

Jiravanichpaisal, P., Roos, S., Edsman, L., Liu, H. and Söderhäll, K., 2009. A highly virulent pathogen, *Aeromonas hydrophila*, from the freshwater crayfish *Pacifastacus leniusculus*. *J. Invertebr. Pathol*., **101**: 56-66. https://doi.org/10.1016/j.jip.2009.02.002

Junior, G.B., Dos Santos, A.C., de Freitas Souza, C., Baldissera, M.D., dos Santos Moreira, K.L., da Veiga, M.L., de Vargas, A.P.C., da Cunha, M.A. and Baldisserotto, B., 2018. *Citrobacter freundii* infection in silver catfish (*Rhamdia quelen*): Hematological and histological alterations. *Microb. Pathog*., **125**: 276-280. https://doi.org/10.1016/j.micpath.2018.09.038

Kim, H.B., Park, C.H., Kim, C.J., Kim, E.C., Jacoby, G.A., and Hooper, D.C., 2009. Prevalence of plasmid-mediated quinolone resistance determinants over a 9-year period. *Antimicrob Agents Chemother*., **53**: 639-645. https://doi.org/10.1128/AAC.01051-08

Liu, X.D., He, X., An, Z.H., Sun, W., Chen, N., Gao, X.J., Li, X.X., and Zhang, X.J., 2020. *Citrobacter freundii* infection in red swamp crayfish (*Procambarus clarkii*) and host immune-related gene expression profiles. *Aquaculture*, **515**: 734499. https://doi.org/10.1016/j.aquaculture.2019.734499

Nawaz, M., Khan, A.A., Khan, S., Sung, K., and Steele, R., 2008. Isolation and characterization of tetracycline-resistant *Citrobacter* spp. from catfish. *Fd. Microbiol*., **25**: 85-91. https://doi.org/10.1016/j.fm.2007.07.008

Pérez-Pérez, F.J. and Hanson, N.D., 2002. Detection of plasmid-mediated ampc β-lactamase genes in clinical isolates by using multiplex PCR. *J. clin. Microbiol*., **40**: 2153-2162. https://doi.org/10.1128/JCM.40.6.2153-2162.2002

Poirel, L., Héritier, C. and Nordmann, P., 2005. Genetic and biochemical characterization of the chromosome-encoded class b β-lactamases from *Shewanella livingstonensis* (slb-1) and *Shewanella frigidimarina* (sfb-1). *J. Antimicrob. Chemother.*, **55**: 680-685. https://doi.org/10.1093/jac/dki065

Reed, L.J. and Muench, H., 1938. A simple method of estimating fifty per cent endpoints. *Am. J. Epidemiol*., **27**: 493-497. https://doi.org/10.1093/oxfordjournals.aje.a118408

Sato, N., Yamane, N. and Kawamura, T., 1982. Systemic *Citrobacter freundii* infection among sunfish *Mola mola* in matsushima aquarium. *Bull. Jpn. Soc. Sci. Fish*., **48**: 1551-1557. https://doi.org/10.2331/suisan.48.1551

Sun, H.Y., Cao, X.H., Jiang, Y.F., Ni, L.Y., Mo, Z.Q., Qin, Q.W., Li, Y.W., and Dan, X.M., 2018. Outbreak of a novel disease associated with *Citrobacter freundii* infection in freshwater cultured stingray, *Potamotrygon motoro*. *Aquaculture*, **492**: 35-39. https://doi.org/10.1016/j.aquaculture.2018.03.058

Wang, P.C., Lin, Y.D., Liaw, L.L., Chern, R.S., and Chen, S.C., 2008. *Lactococcus lactis* subspecies lactis also causes white muscle disease in farmed giant freshwater prawns *Macrobrachium rosenbergii*. *Dis. Aquat. Organ*., 79(1): 9-17. https://doi.org/10.3354/dao01868

Xu, H., Xu, R., Wang, X., Liang, Q., Zhang, L., Liu, J., Wei, J., Lu, Y. and Yu, D., 2022. Co-infections of *Aeromonas veronii* and *nocardia seriolae* in largemouth bass (*Micropterus salmoides*). *Microb. Pathogen*., pp. 105815. https://doi.org/10.1016/j.micpath.2022.105815

Yan, X., Zhenyu, L., Gregg, W.P. and Dianmo, L., 2001. Invasive species in China. An overview. *Biodivers. Conserv*., **10**: 1317-1341. https://doi.

org/10.1023/A:1016695609745

Yuan, G., Zhu, L., Jiang, X., Zhang, J., Pei, C., Zhao, X., Li, L., and Kong, X., 2021. Diagnosis of co-infection with white spot syndrome virus and *Aeromonas veronii* in red swamp crayfish *Procambarus clarkii*. *Aquaculture*, **532**: 736010. https://doi.org/10.1016/j.aquaculture.2020.736010

Zhang, X., Qin, G., Bing, X., Yan, B., and Bi, K., 2011. Phenotypic and molecular characterization of *Photobacterium damselae*, a pathogen of the cultured tongue sole *Cynoglossus semilaevis* in china. *N. Z. J. Mar. Freshw. Res*., **45**: 1-13. https://doi.org/10.1080/00288330.2010.531745

Zhao, C., Wen, H., Huang, S., Weng, S. and He, J., 2022. A novel disease (water bubble disease) of the giant freshwater prawn *Macrobrachium rosenbergii* caused by *Citrobacter freundii*: Antibiotic treatment and effects on the antioxidant enzyme activity and immune responses. *Antioxidants*, **11**: 1491. https://doi.org/10.3390/antiox11081491

Zhou, J.F., Fang, W.H., Yang, X.L., Zhou, S., Hu, L.L., Li, X.C., Qi, X.Y., Su, H., and Xie, L.Y., 2012. A nonluminescent and highly virulent *Vibrio harveyi* strain is associated with bacterial white tail disease of *Litopenaeus vannamei* shrimp. *PLoS One*, **7**: https://doi.org/10.1371/journal.pone.0029961