Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
31 (5): 669-677, 2025
DOI: 10.9775/kvfd.2025.34626

RESEARCH ARTICLE

First Report of Gastroenteritis Caused by *Citrobacter braakii* in a Yellow-margined Box Turtle (*Cuora flavomarginata*)

Min CHEN [1] Yifan SONG [1] Chongfeng LIU [1] Ningning CHEN [1] Junzeng XUE [1, (*)]

[1] College of Oceanography and Ecological Science, Shanghai Ocean University, Shanghai, 201306, CHINA

(*) **Corresponding author:**
Junzeng Xue
Phone: +86 021 61900428
E-mail: jzxue@shou.edu.cn

How to cite this article?

Chen M, Song Y, Liu C, Chen N, Xue J: First report of gastroenteritis caused by *Citrobacter braakii* in a yellow-margined box turtle (*Cuora flavomarginata*). *Kafkas Univ Vet Fak Derg,* 31 (5): 669-677, 2025. DOI: 10.9775/kvfd.2025.34626

Article ID: KVFD-2025-34626
Received: 14.06.2025
Accepted: 27.09.2025
Published Online: 06.10.2025

Abstract

To determine the pathogen responsible for gastroenteritis mortality in a yellow-margined box turtle *(Cuora flavomarginata)*. A dominant single colony, A1, was isolated from the liver lesions of the diseased *C. flavomarginata* on the farm. Based on 16S rDNA sequencing and comparative analysis in NCBI, the isolate showed 99.58% similarity to *Citrobacter braakii*. Antimicrobial susceptibility testing revealed that strain A1 was resistant to multiple antibiotics. The pathogenicity of the strain was confirmed through experimental infection in red-eared sliders *(Trachemys scripta elegans),* which resulted in clinical signs consistent with gastroenteritis. Histopathological examination of infected tissues revealed inflammatory cell infiltration in the gastric submucosa and lamina propria, as well as degenerative changes in hepatocytes, including cytoplasmic loosening, mild edema, and cholestasis. Additionally, structural alterations were observed in the intestinal mucosa, accompanied by inflammatory infiltration. Our study demonstrates that *C. braakii* is one of the pathogenic agents responsible for gastroenteritis in turtles. Preliminary discussions were conducted from the perspectives of pathogenicity, antimicrobial susceptibility, and histopathology, thereby providing a theoretical foundation for the prevention of turtle diseases caused by this microorganism.

Keywords: Antimicrobial resistance, *Citrobacter braakii, Cuora flavomarginata,* Experimental infection, Histopathology, Turtle

INTRODUCTION

The yellow-margined box turtle *(C. flavomarginata)* belongs to the family *Emydidae* and the genus *Cuora* [1-3]. It is highly valued for its edible, medicinal, and ornamental qualities, and is easily domesticated. These attributes have driven intensive wild harvesting, resulting in severe declines in population. Consequently, it is now afforded legal protection at both national and international levels [4]. Intensive artificial breeding has been developed to meet market demand. However, high-density farming practices often lead to frequent disease outbreaks, including pneumonia, shell rot, peptic ulcer, and cystic disease. In 2021, Du et al.[5] reported fatal duodenal perforation in a juvenile yellow-margined box turtle caused by *Chelonobacter oris,* and in 2016, *Aeromonas hydrophila* was documented to cause keratitis in this species [6]. Among these various diseases, bacterial gastroenteritis is a common and clinically significant problem in *C. flavomarginata*. To date, gastrointestinal diseases related to turtles have been described in Chinese grass turtles *(Mauremys reevesii)* [7], yellow pond turtles *(Mauremys mutica)* [8], leatherback turtle *(Dermochelys coriacea)* [9], and red-footed turtles *(Chelonoidis carbonarius)* [10], which are caused by *Escherichia coli, Salmonella* spp., *Aeromonas punctata, Aeromonas sobria, Vibrio metschnikovii, Photobacterium damselae* subsp. *piscicida,* and *Clostridium perfringens*. However, scarce information is available on *C. braakii* as a bacterial pathogen in turtles.

C. braakii is considered to be an important human and aquaculture pathogen. It has been documented to cause disease in a wide range of aquatic species, including the red claw crayfish *(Cherax quadricarinatus)* [11], black carp *(Mylopharyngodon piceus)* [12], spiny frog *(Quasipaa spinosa)* [13], rainbow trout *(Oncorhynchus mykiss)* [14], catfish *(Silurus asotus)* [15], *Melanotaenia praecox* [16], and other aquatic animals. As a typical opportunistic pathogen, it can also cause inflammation and bacteremia in human tissue [17,18]. Infections with *C. braakii* are frequently characterized by necrotizing inflammation and immunosuppression in host species, especially poikilothermic aquatic ones [19]. The bacterium demonstrates considerable tissue invasiveness and environmental adaptability in such hosts [20]. Given that

C. flavomarginata is a poikilothermic aquatic reptile whose immune function is highly susceptible to environmental stress, its immune responses may share similarities with those reported in fish and amphibians. We therefore hypothesized that *C. braakii* poses a comparable pathogenic threat to this turtle species.

This study demonstrates the association of *C. braakii* with severe gastroenteritis in *C. flavomarginata.* We systematically characterized its phenotypic and biochemical characteristics, pathogenicity, antibiotic resistance profile, and histopathological changes induced by infection. These findings offer a scientific basis for the clinical diagnosis and control of bacterial gastroenteritis in this species. They also hold practical significance for protecting the health of captive populations of this endangered turtle.

Material and Methods

Ethical Statement

All animal experimental procedures in this study strictly adhered to international ethical guidelines for animal experimentation and relevant Chinese regulations. The experimental protocol was reviewed and approved by the Academic Committee of the College of Oceanography and Ecological Science at Shanghai Ocean University and received formal approval from the Shanghai Ocean University Animal Ethics Committee (Approval No. SHOU-DW-2024-300).

Isolation and Identification of the Pathogen

On November 25, 2024, we obtained a diseased *C. flavomarginata* (230 g in weight) from a commercial farm in Shanghai, exhibiting multiple clinical signs. The individual initially presented with lethargy, significantly reduced food intake, and diminished activity. These symptoms were subsequently accompanied by the excretion of green feces. The abnormal manifestations persisted for more than 48 h and showed no improvement following initial routine surface disinfection and isolation. The condition progressed to include complete anorexia, redness and swelling around the anus, dark brown fecal excretion, limb edema, and sunken orbits. The turtle eventually became severely debilitated and succumbed to the illness. The moribund turtles were immediately transported to the laboratory on ice packs for immediate microbiological analysis. The body surface of dead turtles was disinfected with 75% ethanol (Huankai Microbiology Science and Technology Co., Ltd., China), and necropsy was performed under aseptic conditions. Liver lesions were aseptically streaked with a sterilized loop onto LB agar (Huankai Microbiology Science and Technology Co., Ltd., China) and violet red bile glucose agar (VRBGA) plates (Hangzhou Microbiology Reagent Co., Ltd., China). The inoculated plates were incubated aerobically at 37°C for 24 h. A predominant bacterial colony was repeatedly subcultured on fresh LB agar to obtain a pure isolate, designated strain A1. For phenotypic characterization, strain A1 was cultured on LB agar at 37°C for 24 h, and colony morphology was recorded. Based on relevant literature and the characteristics of *enterobacteria*, Biochemical profiling was performed using Bacterial Biochemical Identification kit (Hangzhou Microbial Reagent Co., China), including tests for glucose fermentation, lysine decarboxylase, ornithine decarboxylase, H_2S production, peptone water, lactose fermentation, dulcitol fermentation, phenylalanine deaminase, urea, citrate, sucrose fermentation [21]. Results were interpreted according to *Bergey's Manual of Systematic Bacteriology*.

The genomic DNA of the pathogenic isolate was extracted using a bacterial DNA kit (Personal Biotechnology Co., Ltd., China), and the 16S rDNA gene region was amplified by PCR using the universal primers 27F (5-AGAGTTTGATCCTGGCTCAG-3)and1492R(5-GGTTACCTTGTTACGACTT-3). The PCR amplification reaction mixture (50 µL) consisted of: 1 µL genomic DNA (20 ng/µL), 5 µL 10xBuffer (containing 2.5 mM Mg^{2+}), 1 µL Taq polymerase (5 U/µL), 1 µL dNTP mix (10 mM), 1.5 µL of each primer (10 µM), and 39.0 µL ddH_2O. The mixture was gently flicked to combine the components, and droplets on the tube wall were collected by brief centrifugation. Amplification was carried out in a thermal cycler under the following conditions: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 90 s; and a final extension at 72°C for 7 min. After amplification, 3 µL of the PCR product was analyzed by 1% agarose gel electrophoresis to confirm the presence of the target amplicon. The PCR products were purified and sequenced by Shenzhen MicroUnion Technology Group Co., Ltd. (China), and the sequencing was performed on an ABI3730-XL genetic analyzer. The obtained sequence was analyzed using the BLAST algorithm on the NCBI website *(https://www.ncbi.nlm.nih.gov,* accessed on December 7, 2024) to identify the species with the greatest sequence similarity to our isolate. Sequences with high similarity scores and from related aquaculture pathogens were selected for further analysis. A phylogenetic tree was constructed using the neighbor-joining method [22] in MEGA5.1 software *(http://www.megasoftware.net/,* accessed on December 7, 2024). The tree was subjected to a confidence test with 1000 bootstrap replications.

Experimental Infection

Trachemys scripta elegans and *C. flavomarginata* both belong to the class *Reptilia,* order *Testudines,* and suborder *Cryptodira* [23,24]. They share numerous ecological and biological traits, including a freshwater habitat, an

omnivorous diet, and similarities in the anatomical structure of the digestive system, physiological functions, and basic immune responses [25-27]. Disease reports also indicate that these species are susceptible to common health issues, such as pneumonia [28] and ophthalmitis [29], which present with similar clinical manifestations upon pathogenic bacterial infection [30]. Furthermore, in adherence to the "3R Principles" (Replacement, Reduction, and Refinement) and due to ethical as well as institutional regulatory constraints -specifically, the lack of approved animal ethics protocol for experimental work involving *C. flavomarginata* at our institution- the present study selected *Trachemys scripta elegans* as the infection model to investigate gastroenteritis caused by *C. braakii*.

After strain A1 was cultured at a constant temperature for 24 h, the bacterial lawn was harvested with 0.85% sterilized saline. The bacterial suspension was adjusted to a concentration of $3x10^8$ CFU/mL. Twenty healthy *Trachemys scripta elegans* (carapace length: 9.0±2.0 cm; body weight: 110±20 g) were purchased from a farm in Jinhua, Zhejiang Province. The turtles were fed ad libitum twice daily for 14 days in an aerated tap water environment maintained at 28°C according to established husbandry protocols. No signs of disease or abnormalities were observed during this period. The turtles were then equally divided into two groups: a control group and an experimental group, with 10 individuals in each group. The experimental group received an intraperitoneal injection of 0.2 mL of the bacterial suspension, whereas the control group received an equal volume of sterile saline. The injected turtles were housed in individual tanks (80 x 47 x 28 cm) and fed normally throughout the experimental period. Survival rates and clinical signs were monitored and recorded daily for 45 days. Mortality of the *Trachemys scripta elegans* was monitored and recorded daily following the initiation of the infection experiment. For the purpose of survival analysis, a death was coded as "1". Censored data -defined as individuals that either survived until the end of the study period or died from causes unrelated to the experimental infection- were coded as "0". All data were compiled and subjected to statistical analysis using GraphPad Prism software (version 10.1.2). Survival rates across groups were compared using the Log-rank (Mantel-Cox) test. A two-tailed *P* value of less than 0.05 was considered statistically significant.

Histopathology

The main organs of the digestive system (liver, intestine, and stomach) were collected from deceased *Trachemys scripta elegans* and fixed in 4% paraformaldehyde solution for histopathological analysis. Following fixation, the tissues were dehydrated through a graded ethanol series, cleared in xylene, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). The prepared sections were then examined under a light microscope for observation, photography, and assessment of pathological changes.

Antimicrobial Susceptibility Assay

The Kirby-Bauer (K-B) disk diffusion method was employed. Four antibiotic disks were placed on each agar plate. The plates were then incubated at 28°C for 18 to 24 h. Following incubation, the diameters of the zones of inhibition were measured using manual calipers. Each experiment was performed in triplicate, and the average diameter was calculated for analysis. The antimicrobial susceptibility results were interpreted according to the standards provided by the Clinical and Laboratory Standards Institute (CLSI) [31].

Results

Autopsy of the diseased Trachemys scripta elegans revealed several pathological changes. These included an enlarged liver and gallbladder with necrotic and congested tissues, a stomach with blood on its outer wall, and intestines that were inflated with blood, had inelastic walls, and showed erosion of the hind-gut *(Fig. 1)*. In the experimental infection, individuals in the experimental group exhibited progressive mortality, culminating in 100% cumulative mortality by day 45. The initial mortality occurred on day 4, with a peak mortality period observed between days 15 and 30. The final death was recorded on day 45. In contrast, all turtles in the control group remained healthy throughout the experimental period, with no mortality observed *(Fig. 2)*. The survival analysis revealed a statistically significant difference between the two groups (Log-rank test, $\chi^2 = 4.907$, df = 1, $^*p^* = 0.0267$). Consistent with the survival curve, the infected group exhibited a significantly lower survival rate compared to the control group, demonstrating that the experimental bacterial infection significantly reduced the survival of *Trachemys scripta elegans*.

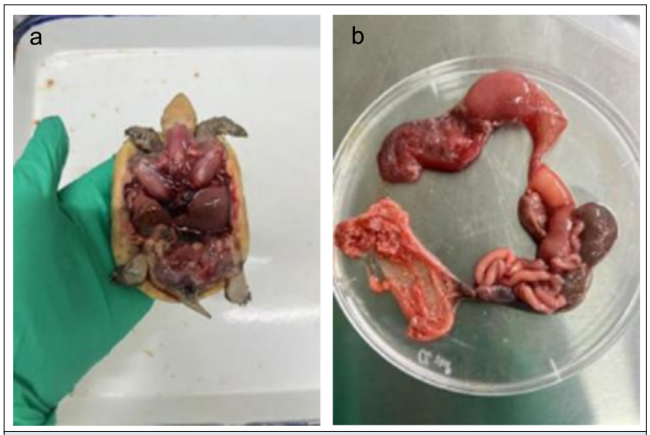

Fig 1. Anatomical diagram of a diseased turtle

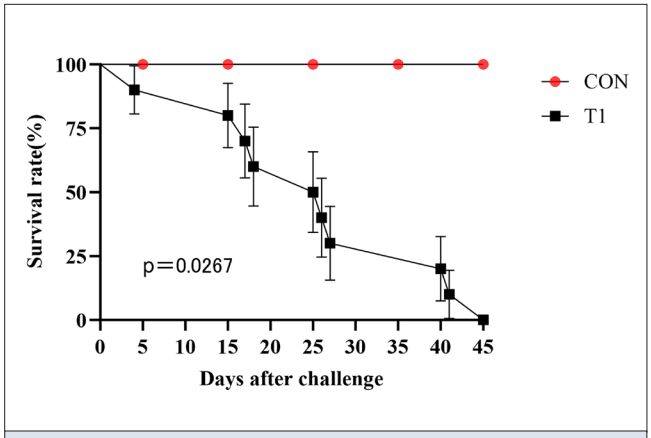

Fig 2. Survivals of experimental *Trachemys scripta elegans* infected intraperitoneally with strain A1 for Forty Five days (CON, 0 CFU/mL; T1, 3×10^8CFU/mL)

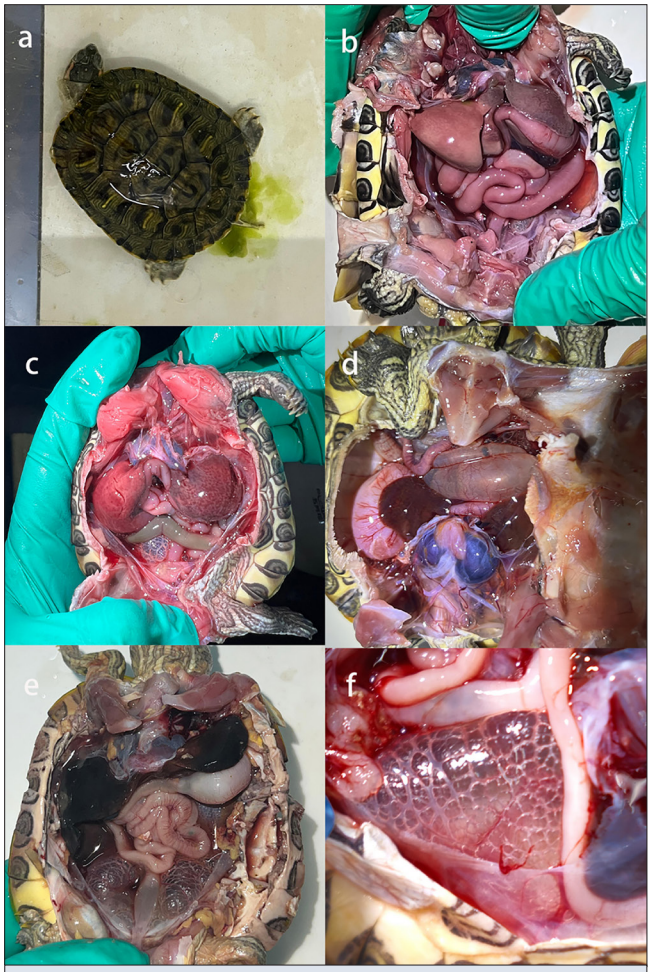

Fig 3. Symptoms and anatomical diagram of the onset of *Trachemys scripta elegans*. **a-** Typical gastroenteritis symptoms of dead turtles, **b, c, d-** dissection of the turtle infected with *C. braakii*, **e, f-** pneumonia symptoms

In the experimental group, infected turtles exhibited a range of clinical signs, including tail floating, anal mucus discharge, diarrhea with greenish stools *(Fig. 3-a)*, and a progressive reduction in food intake leading to complete anorexia. Occasional open-mouth breathing, limb edema and weakness were also observed. Upon dissection, pathological findings included hemorrhagic streaks on the outer stomach wall, intestinal distension with serosal hemorrhage, and swollen and partially eroded livers *(Fig. 3-b,c,d)*. Some affected individuals also showed severe pulmonary emphysema *(Fig. 3-e,f)*. The clinical manifestations and postmortem lesions observed in *Trachemys scripta elegans* were consistent with those previously described in *C. flavomarginata*. The original challenge strain, A1, was successfully reisolated from the infected *Trachemys scripta elegans* and confirmed through molecular and phenotypic analyses. Accordingly, Koch's postulates were fulfilled, confirming the pathogenic potential of *C. braakii* strain A1 in this host species.

Observation and Identification of Strain A1

After 24 h of incubation, strain A1 formed circular, convex, smooth, moist colonies with entire margins, measuring approximately 1-2 mm in diameter. The colonies exhibited a milky white and translucent appearance *(Fig. 4-a)*.

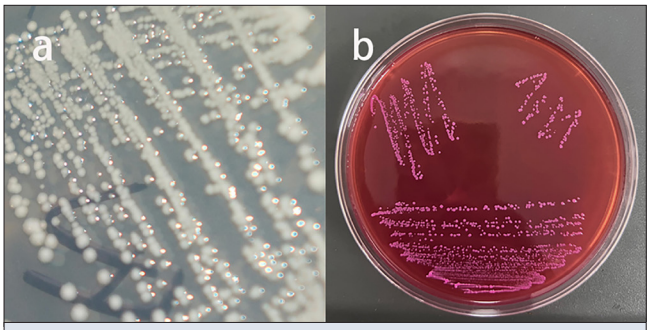

Fig 4. Morphological observation of strain A1

Table 1. *Physiological and biochemical characteristics of strain A1 and the reference strain C. braakii CICC 21598*

Test Item	Strain A1	*C. braakii* CICC 21598
Glucose fermentation	+	+
Lysine decarboxylase	+	+
Ornithine decarboxylase	+	+
H_2S production	+	+
Peptone water	+	+
Lactose fermentation	+	+
Dulcitol fermentation	+	+
Phenylalanine deaminase	-	-
Urea	-	-
Citrate	+	+
Sucrose fermentation	-	-

+ positive; - negative. The reference strain CICC 21598 was obtained from the China Center of Industrial Culture Collection (CICC)

On violet red bile dextrose agar (VRBGA), the colonies developed a distinct pink coloration *(Fig. 4-b)*. According to the results of the biochemical identification test of strain A1, glucose, lysine, ornithine, H_2S production, peptone water, lactose, dulcitol, and citrate were positive, and phenylalanine, urea, and Sucrose were negative *(Table 1)*. These biochemical characteristics are consistent with those of *C. braakii*, supporting the preliminary identification of strain A1 as *C. braakii*. Genomic DNA was extracted from strain A1 using a commercial kit and served as the template for PCR amplification. Amplified products were separated by 1% agarose gel electrophoresis, which yielded a target band of approximately 1, 500 bp *(Fig. 5)*. The 16S rDNA sequence of strain A1 was deposited in GenBank under accession number PX129799.1. BLAST analysis revealed 99.58% similarity with *C. braakii* (accession no. CP113163.1). Phylogenetic analysis further confirmed that strain A1 clusters within the same clade as *C. braakii* reference strains *(Fig. 6)*.

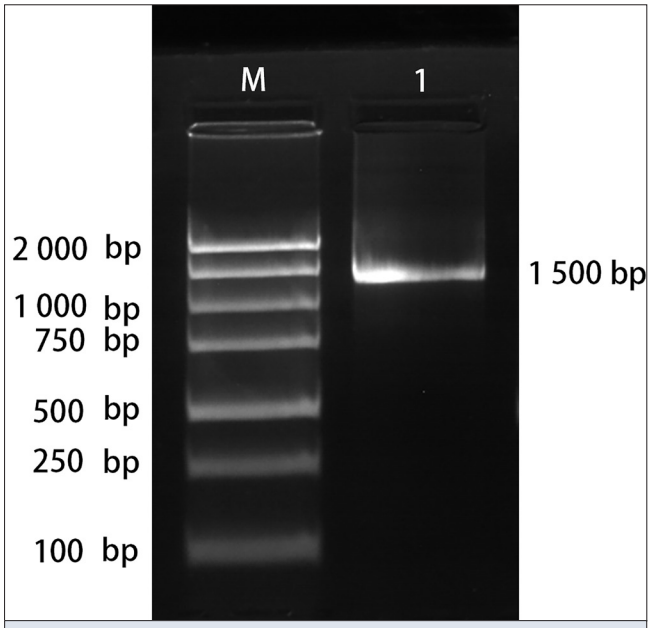

Fig 5. The result of PCR primers specificity test

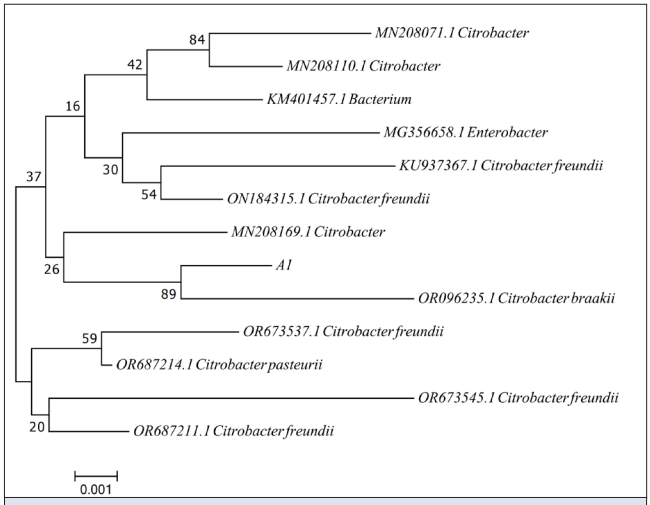

Fig 6. Phylogenetic tree based on the 16S rDNA sequence similarity

Histopathological Analysis

Histopathological examination of the deceased turtles revealed that the hepatocytes were polygonal in shape, with scant eosinophilic cytoplasm and mild hydropic degeneration. Scattered inflammatory cell infiltration and cholestasis were observed within the hepatic parenchyma *(Fig. 7-a, arrow 2)*. Increased brown pigment deposits were noted in focal areas *(Fig. 7-a, arrow 1)*. Marked fibrous tissue proliferation was evident in the portal areas, accompanied by disorganization of the hepatic plate architecture. Hepatic sinusoids were distinct and contained numerous nucleated erythrocytes *(Fig. 7-a, arrow 3)*. In the intestinal tissue, villi were seen projecting into the lumen. The mucosal lamina propria exhibited a significant reduction in glandular structures, with some glands displaying slight architectural irregularity. Prominent fibrous tissue proliferation and extensive inflammatory cell infiltration were observed in the lamina propria *(Fig. 7-c, arrow 2)*. The muscularis mucosae was mildly thickened, and scattered inflammatory cells were present in both the submucosa and the lamina propria *(Fig. 7-c, arrow 1)*. In the stomach, certain glandular structures appeared irregular, with a noticeable decrease in gland number and increased inflammatory cell infiltration *(Fig. 7-e, arrow 2)*. The lamina propria showed substantial fibrous hyperplasia and abundant inflammatory cell infiltration *(Fig. 7-e, arrow 1)*. The

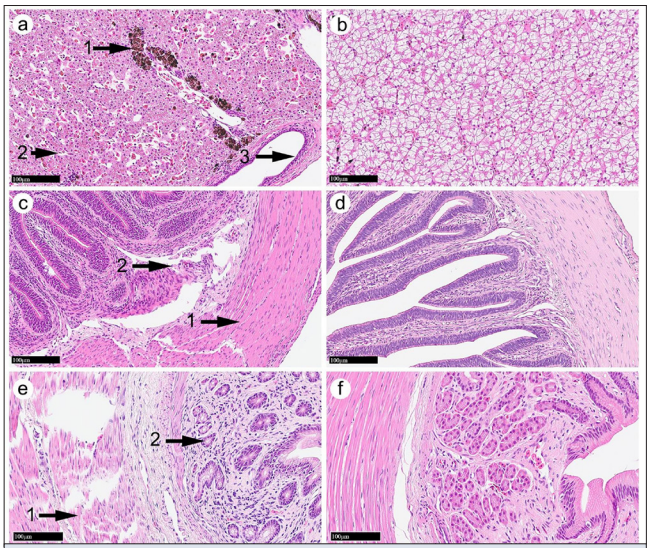

Fig 7. H&E staining of diseased and normal experimental turtles. **a-** Liver of diseased *Trachemys scripta elegans* (20×, H&E), **b-** Liver of healthy *Trachemys scripta elegans* (20×, H&E), **c-** Intestinal tract of diseased *Trachemys scripta elegans* (20×, H&E), **d-** Intestinal tract of healthy *Trachemys scripta elegans* (20×, H&E), **e-** Stomach of diseased *Trachemys scripta elegans* (20×, H&E), **f-** Stomach of healthy *Trachemys scripta elegans* (20×, H&E)

Table 2. *Susceptibility of strain A1 to antimicrobials*

Antimicrobials	Content (μg/disk)	Zone Diameter Breakpoints (mm)			Inhibition Zone Diameter (mm)	Susceptibility
		S	I	R		
Ampicillin (AMP)	10	≥17	14~16	≤13	11	R
Amikacin (AMK)	30	≥17	15~16	≤14	18	S
Piperacillin (PIP)	100	≥21	18~20	≤17	28	S
Cephalexin (CN)	30	≥18	15~17	≤14	11	R
Cefazolin (CZ)	30	≥18	15~17	≤14	6	R
Cefuroxime (CXM)	30	≥18	15~17	≤14	23	S
Cefoperazone (CPZ)	75	≥21	16~20	≤15	30	S
Ceftriaxone (CTR)	30	≥27	25~26	≤25	30	S
Ceftazidime (CAZ)	30	≥18	15~17	≤14	28	S
PenicillinG (PEN)	1	≥21	18~20	≤17	6	R
Gentamicin (GEN)	10	≥15	13~14	≤12	22	S
Kanamycin (KAN)	30	≥18	14~17	≤13	9	R
Streptomycin(S)	10	≥15	12~14	≤11	11	R
Tetracycline (TET)	30	≥19	15~18	≤14	15	I
Minocycline (MI)	30	≥19	15~18	≤14	18	I
Doxycycline (DO)	30	≥16	13~15	≤12	12	R
Polymyxin B (PB)	300	≥12	8~11	≤7	11	I
Vancomycin (VAN)	30	≥13	9~12	≤8	6	R
Lincosamide (LCM)	2	≥31	24~30	≤23	6	R
Erythromycin (E)	15	≥23	14~22	≤13	6	R

S: *susceptible;* ***I:*** *intermediately susceptible;* ***R:*** *resistant*

muscularis mucosae was slightly thickened, and scattered inflammatory cells were identified within the submucosa and lamina propria.

Antimicrobial Susceptibility of Pathogenic Strain

The antimicrobial susceptibility profile of strain A1 is summarized in *Table 2*. The strain was susceptible to amikacin, gentamicin, piperacillin, cefuroxime, cefuroxime, cefoperazone, ceftazidime, and ceftriaxone. Conversely, it was resistant to ampicillin, cephalexin, cefazolin, penicillin G, kanamycin, streptomycin, doxycycline, vancomycin, lincosamide, and erythromycin. Intermediate susceptibility was observed for tetracycline, minocycline, and polymyxin B. These results indicate that strain A1 exhibits a multidrug-resistant phenotype, with resistance observed to narrow-spectrum beta-lactams (penicillin G and first-generation cephalosporins) and specific aminoglycosides (kanamycin and streptomycin).

Discussion

This study is the first to identify and confirm *C. braakii* as an emerging pathogen associated with lethal gastroenteritis in *C. flavomarginata*. Notably, we observed and documented several previously unreported clinical signs -including limb edema, respiratory distress- which provide critical insights into its unique pathogenic presentation in turtles. The findings reveal specific gross lesions, antimicrobial resistance patterns, and characteristic histopathological changes induced by this bacterium. Together with physiological, biochemical, and pathogenicity analyses, this research not only expands the known spectrum of reptilian bacterial pathogens but also establishes a crucial reference for understanding the pathogenic mechanisms of *C. braakii* in *chelonians*. These results offer valuable theoretical and practical guidance for developing targeted disease control strategies for this species.

Our findings indicate that *C. braakii* is a primary causative agent of severe gastroenteritis in turtles. Turtle gastroenteritis, a common reptilian disease, is characterized by well-documented clinical signs and pathological features. In the initial stages, infected turtles typically exhibit lethargy, reduced mobility, and anorexia. Their feces are soft and poorly formed, ranging in color from green to yellow-brown or dark brown. In severe cases, watery diarrhea with a foul odor is observed. Advanced disease is marked by sunken eyes

and abnormal feces, which may be egg-white-like, black, or liver-colored. Terminal stages often involve complete anorexia, leading to death from progressive debilitation. Gross pathological examination revealed a pronounced inflammatory response in the digestive tract. The gastrointestinal mucosa exhibits erosions and ulcerations, often accompanied by excessive mucus production, severe hemorrhage, or petechiae. The lumen may contain a white, clear, or milky gelatinous exudate. The liver frequently exhibits pale yellow lesions, consistent with secondary hepatic involvement [32-34]. However, limb edema and severe pulmonary emphysema appear to be a novel feature of *C. braakii* infection, distinguishing it from infections caused by common pathogens such as *Salmonella* spp., *Escherichia coli*, or *Aeromonas hydrophila* [35]. These findings are consistent with a study in night herons *(Nycticorax nycticorax)* [36], which reported that *C. braakii* infection can cause depression, anorexia, dyskinesia, and diarrhea. Furthermore, this pathogen has been isolated from the liver and intestine of diseased *Procambarus clarkii* and *Andrias davidianus* exhibiting diarrhea and dehydration [37,38]. The high virulence of *C. braakii* strains from rhesus monkeys *(Macaca mulatta)* was demonstrated in animal models. Collectively, these reports from diverse species corroborate our findings and underscore the significant pathogenic capacity of *C. braakii*. This cross-species pathogenicity underscores its role as an emerging multi-host opportunistic pathogen. The mechanism underlying its gastroenteritic effects likely involves the induction of significant inflammation and damage to the gastrointestinal mucosa [39,40].

Antimicrobial susceptibility testing revealed that *C. braakii* exhibited high resistance to lincosamide and macrolide antibiotics, consistent with findings in red crayfish [41]. In contrast, its susceptibility to β-lactams, tetracyclines, and aminoglycosides differed from profiles reported elsewhere. Notably, predominant resistance was observed against narrow-spectrum β-lactams, a finding corroborated by Li et al.[37]. These variations in antimicrobial susceptibility may be attributed to differences in host species, environmental conditions, and selective pressures from the unregulated or excessive use of broad-spectrum antibiotics. Such practices promote the emergence and dissemination of resistant strains. For example, the multidrug resistance gene cfr was detected in *C. braakii* isolates from chicken farm environments in Jiangxi, China [42]. Additionally, the carbapenemase gene blaKPC-2 and the plasmid-mediated colistin resistance gene mcr-1 have been identified in this species [43,44]. The presence of these resistance genes poses serious challenges for clinical management and complicates the control of *C. braakii* infections. Furthermore, *C. braakii* infection often triggers a multi-tissue inflammatory response, leading to clinical manifestations including anorexia, digestive dysfunction, and diarrhea. In severe cases, the infection can be fatal [45]. Characteristic histopathological features include gastric mucosal erosion or ulceration, submucosal hemorrhage with inflammatory cell infiltration, and mild to moderate glandular hyperplasia with structural disorganization, congestion, edema, and inflammation. Additional features involve swelling of the intestinal epithelial mucosa with extensive submucosal inflammatory cell infiltration, as well as hepatic erythrocyte accumulation and pigment deposition [46]. The pathological observations in this study were consistent with these previously described changes. However, we identified additional specific alterations, including polygonal hepatocytes with cytoplasmic vacuolization and cholestasis, fibrotic tissue proliferation in portal areas, disorganized hepatic cord structures, abnormal protrusion of intestinal villi into the lumen, and fibrosis in the gastric lamina propria. These discrepancies may arise from variations in host immune status, bacterial virulence, environmental stressors, and genetic background [47]. The stomach, intestine, and liver were identified as the primary organs affected by *C. braakii*. This targeting of vital digestive organs likely explains the high mortality rates associated with clinical symptoms such as anorexia and diarrhea [48].

These findings have direct clinical and managerial implications for aquaculture. In cases where farmed turtles exhibit symptoms including anorexia, lethargy, and digestive disturbances, infection with *C. braakii* should be included as a differential diagnosis. This suspicion is reinforced by necropsy findings such as dull yellow hepatic lesions with white flocculent material, gastrointestinal hemorrhage, necrosis, and the presence of gelatinous exudate. Confirmatory diagnosis should rely on bacteriological isolation followed by 16S rDNA sequencing. Given that the isolated strain exhibited multi-drug resistance to commonly used β-lactams (e.g., ampicillin, penicillin G), aminoglycosides (e.g., kanamycin), and macrolides (e.g., erythromycin), empirical use of these antibiotics should be avoided to prevent treatment failure and the spread of resistance. Instead, alternative strategies such as phage therapy (e.g., phage vB-CbrM-HP1, which has proven effective in reducing *C. braakii* load in fish [49]) could be considered due to its high specificity and favorable safety profile [50]. From a management perspective, stringent biosecurity measures are essential. These include optimizing water quality, ensuring feed hygiene, maintaining controlled stocking densities, and implementing regular disinfection of ponds and equipment. Furthermore, quarantine protocols for newly introduced animals are critical to prevent pathogen introduction. Finally, as *C. braakii* is a zoonotic pathogen capable of infecting humans, these

measures are also vital for protecting occupational health and preventing cross-contamination.

However, this study has several limitations. Firstly, the bacterial isolate was derived from a single dominant strain (A1) obtained from a limited sample size (n=1) at one farm, which may not represent the full genetic diversity of *C. braakii* pathogens. Secondly, although justified by ethical considerations and the physiological similarities of the digestive system, the use of a surrogate model *(Trachemys scripta elegans)* for the challenge experiment may not perfectly recapitulate the natural infection process in the primary host, *C. flavomarginata*. Finally, while our work focused on pathogen identification, pathological characterization, and antimicrobial susceptibility testing, the underlying molecular mechanisms -including key virulence factors (e.g., adhesins, toxins) and resistance genes (e.g., AmpC β-lactamases, aminoglycoside-modifying enzymes)- remain uncharacterized. Future studies should expand the sample size to include geographically diverse strains, prioritize challenge experiments in the original host species where feasible, and employ genomic and molecular techniques to comprehensively elucidate the pathogenic and resistant mechanisms of *C. braakii*.

Declarations

Availability of Data and Materials: The data given in this study may be obtained from the corresponding author (Junzeng XUE) on reasonable request.

Acknowledgements: We sincerely thank all those who contributed to this research and the manuscript submission process. We are also grateful to the reviewers for their valuable comments, which significantly improved the quality of this paper.

Funding Support: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical Approval: All animal experimental procedures in this study strictly adhered to international ethical guidelines for animal experimentation and relevant Chinese regulations. The experimental protocol was reviewed and approved by the Academic Committee of the College of Oceanography and Ecological Science at Shanghai Ocean University and received formal approval from the Shanghai Ocean University Animal Ethics Committee (Approval No. SHOU-DW-2024-300).

Competing Interests: The authors declare that there is no conflict of interest.

Declaration of Generative Artificial Intelligence (AI): The authors declare that the article, tables and figures were not written/created by AI and AI-assisted Technologies.

Author Contributions: MC: Conceptualization, Study Design, Data Curation, Methodology, Formal Analysis, Investigation, Writing - Original Draft. YS: Supervision, Writing - Review and Editing. CL: Supervision, Visualization. NC: Investigation, Supervision. JX: Project Administration, Funding Acquisition, Final Approval.

References

1. Liao PF, Li L, Liu CT, Ding YJ, Huang QY, Hu YW, Ge CT, Xu JH, Zhang XD: Isolation, identification, biological characteristics, and genomic study of a *Providencia rettgeri* strain. *Acta Hydrobiol Sin,* 48 (12): 2109-2121, 2024. DOI: 10.7541/2024.2024.0085

2. Wang H, Duan GQ, Hua XH, Hu YT, Liu AM: Development and characterization of 40 SNP markers of endangered species *Cuora flavomarginata* by whole-genome resequencing. *Conserv Genet Resour,* 16 (4): 273-277, 2024. DOI: 10.1007/s12686-024-01366-2

3. Cheng AP, Huang CC, Tsai CF, Chan FC, Zheng YT, Wang CC, Chen HC, Hung KH: Optimization and application of a forensic microsatellite panel for two endangered freshwater turtle species *(Cuora flavomarginata* and *Mauremys mutica)* in Taiwan. *Glob Ecol Conserv,* 58:e03502, 2025. DOI: 10.1016/j.gecco.2025.e03502

4. Lin YF, Wu SH, Lin TE, Mao JJ, Chen TH: Population status and distribution of the Endangered yellow-margined box turtle *(Cuora flavomarginata)* in Taiwan. *Orys,* 44 (4): 581-587, 2010. DOI: 10.1017/S0030605310000797

5. Du MZ, Zhao Y, Jia YG: Exploration of the pathogen of infection in the death case of the yellow-margined box turtle. *Chinese J Anim Sci,* 57 (7): 88-91, 2021.

6. Musgrave KE, Diehl K, Mans C: *Aeromonas Hydrophila* keratitis in freshwater turtles. *J Exot Pet Med,* 25 (1): 26-29, 2016. DOI: 10.1053/j.jepm.2015.12.003

7. Zhao CG, Zhao NX: Occurrence and prevention of gastrointestinal diseases in *Mauremys reevesii*. *Sci Fish Farm,* 1, 65-66, 2018.

8. He CW, Song XQ, Tang HY: Isolation and identification of pathogens associated with diarrhea in *Mauremys mutica*. *Chinese Vet Sci,* 8, 56-57, 2003. DOI: 10.16656/j.issn.1673-4696.2003.08.017

9. Poppi L, Zaccaroni A, Pasotto D, Dotto G, Marcer F, Scaravelli D, Mazzariol S: Post-mortem investigations on a leatherback turtle *Dermochelys coriacea* stranded along the Northern Adriatic coastline. *Dis Aquat Organ,* 100 (1): 71-76, 2012. DOI: 10.3354/dao02479

10. Weese JS, Staempfli HR: Diarrhea associated with enterotoxigenic *Clostridium perfringens* in a red-footed turtle *(Geochelone carbonaria). J Zoo Wildl Med,* 31 (2): 265-266, 2000. DOI: 10.1638/1042-7260 (2000) 031[0265:DAWECP]2.0.CO;2

11. Zhang SY, Zheng RZ, Ai TS, Yu YZ, Zhang LQ, Zhou WD, Deng P, Ding GZ: Isolation, identification and antimicrobial susceptibility test of *C. braakii* from *Procambarus clarkii. Jiangsu Agric Sci,* 46 (12): 129-131, 2018.

12. Wang JZ, Geng XY, Zhu SX, Dong WL, Jia XY, Shan XF, Gao YF: Isolation, identification and drug susceptibility test of *C. braakii* from *Mylopharyngodon piceus*. *Chinese Vet Sci,* 46 (5): 602-606, 2016.

13. Chen XY, Zheng QZ, Song TT, Zheng SJ, Zheng RQ: Pathogen identification and antimicrobial susceptibility testing of cataracts in spiny *frogs (Quasipaa spinosa). J Zhejiang Agric Sci,* 57 (7): 1141-1143+1151, 2016.

14. Altun S, Duman M, Buyukekiz AG, Ozyigit MO, Karatas S, Turgay E: Isolation of *C. braakii* from rainbow trout *(Oncorhynchus mykiss). Isr J Aquac Bamidgeh,* 65:1, 2013.

15. Nawaz M, Khan AA, Khan S, Sung K, Steele R: Isolation and characterization of tetracycline-resistant *Citrobacter* spp. from catfish. *Food Microbiol,* 25 (1): 85-91, 2008. DOI: 10.1016/j.fm.2007.07.008

16. Jatobá A, Silva BC, Vieira FD, Mouriño JLP, Seiffert WQ: Isolation and characterization of hemolytic bacteria Fish disc and Neon Rainbow. *Semin Cienc Agrar,* 33 (2): 763-768, 2012.DOI: 10.5433/1679-0359.2012v33n2p763

17. Mocerino R, Jolly J, Awerbuch E: *C. braakii* bacteremia of unclear source: A literature review and discussion. *Am J Respir Crit Care Med,* 197:A5298, 2018. DOI: 10.1164/ajrccm-conference.2018.197.1

18. Pasquali F, Crippa C, Lucchi A, Francati S, Dindo ML, Manfreda G: *C. braakii* isolated from salami and soft cheese: An emerging food safety hazard. *Foods,* 14 (11):1887, 2025. DOI: 10.3390/foods14111887

19. Vega-Manriquez DX, Davila-Arrellano RP, Eslava-Campos CA, Salazar Jimenez E, Negrete-Philippe AC, Raigoza-Figueras R, Muñoz-Tenería FA: Identification of bacteria present in ulcerative stomatitis lesions

of captive sea turtles *(Chelonia mydas). Vet Res Commun,* 42, 251-254, 2018. DOI: 10.1007/s11259-018-9728-y

20. Nowakiewicz A, Ziółkowska G, Zięba P, Dziedzic BM, Gnat S, Wójcik M, Dziedzic R, Kostruba A: Aerobic bacterial microbiota isolated from the cloaca of the European pond turtle *(Emys orbicularis)* in Poland. *J Wildl Dis,* 51 (1): 255-229, 2015. DOI: 10.7589/2013-07-157

21. Hu SF, Yu GY, Li R, Xia XZ, Xiao XL, Li XF: Real-time TaqMan PCR for rapid detection and quantification of *Coliforms* in chilled meat. *Food Anal,* 9, 813-822, 2016. DOI: 10.1007/s12161-015-0271-y

22. Wang GH, Clark CG, Liu CY, Pucknell C, Munro CK, Kruk T, Caldeira R, Woodward DL, Rodgers FG: Detection and characterization of the hemolysin genes in *Aeromonas hydrophila* and *Aeromonas sobria* by multiplex PCR. *J Clin Microbiol,* 41 (3): 1048-1054, 2003. DOI: 10.1128/jcm.41.3.1048-1054.2003

23. Chen M, Qin Y, Lin YH, Ren SQ, Ye JZ, Zheng SJ: Genetic diversity analysis of *Cuora flavomarginata* based on COI genes in Zhejiang province. *Chinese J Wildl,* 45 (2): 367-377, 2024. DOI: 10.12375/ysdwxb.20240216

24. Guo SL, Shao P: Breeding methods and tips for pet *Trachemys scripta elegans*. *Agric Technol,* 44 (21): 101-105, 2024. DOI: 10.19754/j.nyyjs.20241115021

25. Zhao HX, Huang B: Anatomy of the digestive system and respiratory system of the yellow-margined box turtle. *Sichuan J Zool,* 29 (1): 59-62, 2010.

26. Pedram S, Zehtabvar O,Rostami A, Akbarein H, Zand S, Rezvani Y, Mahdi SM, Mollaei Z: Laparoscopic anatomy of the coelomic cavity organs in female red-eared slider *(Trachemys scripta elegans). Vet Med Int,* 2025:4041679, 2025. DOI: 10.1155/vmi/4041679

27. Miyai N, Kozono T, Kuriki T, Todoroki M, Murakami T, Shinohara K, Yoshida T, Kigata T: Macro-and microscopic anatomy of the digestive tract in the red-eared slider (Emydidae: *Trachemys scripta elegans). PlosOne,* 19 (12):e0315737, 2024. DOI: 10.1371/journal.pone.0315737

28. Wu CY, Ren SY: A brief analysis of the diagnosis, treatment, and prevention of respiratory disease in *Trachemys scripta elegans. Chinese J Anim Husb Vet Med,* 2023:3, 2023.

29. Ye MH, Hu XC, Lv AJ, Sun JF, Liu XX, Song YJ: Isolation and identification of pathogens causing eye disease in *Trachemys scripta elegans* and histopathological study. *J Dalian Ocean Univ,* 2018:5, 2018.

30. Bai J, Zhou MH, Hu XC, LV AJ, Ye MH, Sun JF: Isolation and identification of *Citrobacter freundii* and susceptibility characteristics of *Citrobacter freundii*. *J Dalian Ocean Univ,* 2029:6, 2019.

31. CLSI (Clinical and Laboratory Standards Institute): Performance standards for antimicrobial susceptibility testing (34th ed.), Informational Supplement M100-ED34. Clinical and Laboratory Standards Institute, Wayne, *PA*, 2024.

32. Chen QM: Studies on pathological conditions in hatchling and juvenile yellow pond turtles *(Mauremys mutica)* under aquaculture conditions. *Agric Technol,* 34 (10): 188, 2014.

33. Liu YN, Zhang YS, Pan LD, Xu PP, Zheng JR: The sea turtle conservation and research progress of the disease. *J Aquac,* 36 (1): 35-40, 2015.

34. Martinson SA, Greenwood SJ, Wadowska D, Martin K: Histopathological, ultrastructural and molecular phylogenetic analysis of a novel microsporidium in a loggerhead sea turtle *(Caretta caretta). Dis Aquat Org,* 129 (1): 31-39, 2018. DOI: 10.3354/dao03234

35. Pasmans F, De Herdt P, Dewulf J, Haesebrouck F: Pathogenesis of infections with *Salmonella enterica subsp. enterica serovar* Muenchen in the turtle *Trachemys scripta scripta. Vet Microbiol,* 87 (4): 315-325, 2002. DOI: 10.1016/S0378-1135(02)00081-0

36. Li Y, Hu QX, Li C, Qing B, Duan WB, Niu KS, Lv N, Zhang YJ, Guo RH, Wang XL: Isolation, identification and drug resistance analysis of *C. braakii* from night heron. *Anim Husb Vet Med,* 55 (7): 53-57, 2023.

37. Zhu RL, Yang CQ, Jiang SD, Shen JJ, Zhang XH, Bao CH, Tang SS: Isolation, identification and antibiotic sensitivity analysis of *Citrobter braakii* from *Procambarus clarkii*. *J Anhui Agric Univ,* 45 (4): 617-620, 2018.

38. Zhu YM, Zhu HY, Wang YP, Feng XD, Zhao ZL, Liu J, Luo ZL, Liu X, Yang XW, Zhao GW: Pathogenic analysis and histopathological observation of multiple bacterial infections in cultured Chinese giant salamander *(Andrias davidianus). Chinese J Vet Sci,* 43 (6): 1304-1309, 2023. DOI: 10.16303/j.cnki.1005-4545.2023.06.28

39. Wei Y: The effect and mechanism of gastric derived *C. braakii* induced inflammatory response in gastric epithelial cells. *MSc Thesis*, Qingdao University, 2024.

40. Yu MC, Xie FY, Xu CZ, Yu T, Wang YX, Liang SZ, Dong QJ, Wang LL: Characterization of cytotoxic *C. braakii* isolated from human stomach. *Febs Open Bio,* 14 (3): 487-497, 2024. DOI: 10.1002/2211-5463.13770

41. Huang YM, Li CL, Qin SJ, Lu W, Zhang BZ, Sun JY, Zhang WQ, Tong T, Zhang Q: Isolation, identification and drug resistance analysis of *C. braakii* from *Cherax quadricarinatus*. *Chinese J Vet Med,* 60 (1): 59-65, 2024.

42. Feng YM, Ding PY, Shen JX, Xu YK, Zheng MX, Pan YS,Yuan L, Hu GZ, He DD: Detection of cfr in *C. braakii* and the characterization of two cfr-carrying plasmids from a chicken farm. *J Antimicrob Chemother,* 79 (8): 2071-2074, 2024. DOI: 10.1093/jac/dkae152

43. Dong DD, Mi ZQ, Li DJ, Gao MM, Jia N, Li ML, Tong YG, Zhang XL, Zhu YQ: Novel IncR/IncP6 hybrid plasmid pCRE3-KPC recovered from a clinical KPC-2-producing *C. braakii* isolate. *mSphere,* 5 (2):e00891-19, 2025. DOI: 10.1128/mSphere.00891-19

44. Liu JX, Yang YX, Li YX, Liu D, Tuo HM, Wang HN, Call DR, Davis M, Zhang A: Isolation of an IncP-1 plasmid harbouring mcr-1 from a chicken isolate of *C. braakii* in China. *Int J Antimicrob Agents,* 51 (6): 936-940, 2018. DOI: 10.1016/j.ijantimicag.2017.12.030.

45. Pan LF, Yang YH, Peng YN, Li DJ, Khan TA, Chen P, Yan L, Hu SB, Ding XZ, Sun YJ, Xia LQ, Yi GF: The novel pathogenic *Citrobacter freundii* (CFC202) isolated from diseased crucian carp *(Carassius auratus)* and its ghost vaccine as a new prophylactic strategy against infection. *Aquaculture,* 533:736190, 2021. DOI: 10.1016/j.aquaculture.2020.736190

46. Sharma PC, McCandless M, Sontakke SP, Varshney N, Brodell RT, Kyle PB, Daley W: Navigating viral gastroenteritis: Epidemiological trends, pathogen analysis, and histopathological findings. *Cureus,* 16 (5):e61197, 2024. DOI: 10.7759/cureus.61197

47. Abreu REF, Magalhaes TC, Souza RC, Oliveira STL, Ibelli AMG, Demarqui FN, Gouveia JJS, Costa MM, Gouveia GV: Environmental factors on virulence of *Aeromonas hydrophila*. *Aquacult Int,* 26 (2): 495-507, 2018. DOI: 10.1007/s10499-017-0230-2

48. Luo FL, Niu YC, Hu XC, Lv AJ, Ye MH, Sun JF: Clinical signs and histochemical observation of *Citrobacter freundii* disease in turtle *(Trachemys scripta elegans). J Tianjin Agric Univ,* 27 (2): 44-47, 2020. DOI: 10.19640/j.cnki.jtau.2020.02.010

49. Huang CZ, Feng C, Liu X, Zhao RH, Wang ZJ, Xi HY, Ou HD, Han WY, Guo ZM, Gu JM, Zhang L: The bacteriophage vB-CbrM-HP1 protects *Crucian* carp against *C. braakii* infection. *Front Vet Sci,* 9:888561, 2022. DOI: 10.3389/fvets.2022.888561

50. Lin DM, Koskella B, Lin HC: Phage therapy: An alternative to antibiotics in the age of multi-drug resistance. *World J Gastrointest Pharmacol Ther,* 8 (3): 162-173, 2017. DOI: 10.4292/wjgpt.v8.i3.162