Animal Diseases

CASE REPORT

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Non-O1/O139 *Vibrio cholerae* causes severe intestinal disease in bullfrogs (*Rana catesbeiana*)

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Abstract

Bullfrogs (*Rana catesbeiana*) are amphibians with high economic value, but in recent years, bullfrog farming has encountered serious threats of bacterial diseases, and the "bullfrog economy" is facing a continuous decline. In this study, the dominant strain was isolated from diseased bullfrogs in a bullfrog farm in Nanning, Guangxi, and based on its morphological, physiological, and biochemical characteristics and analysis of 16S rRNA gene sequences, the strain was identified as a non-O1/O139 group *Vibrio cholerae* and named TC1. Three virulence factors were identified in this strain, including hemolysin, outer membrane protein, and toxin-coregulated pili. Drug susceptibility testing showed that the strain resisted gentamicin, florfenicol, nitrofural, oxytetracycline, neomycin, penicillin, amoxicillin, doxycycline, and sulfamonomethoxine. The results of artificial infection experiments showed that TC1 caused serious pathologies such as abdominal swelling and anal prolapse in bullfrogs, especially severe intestinal bleeding. Histopathological observations revealed that the bullfrog intestine exhibited obvious pathological lesions. These results provide an essential epidemiological basis for controlling *V. cholerae* infections in aquatic animals and demonstrate the promise of bullfrogs as an amphibian model for studying the pathogenesis of *V. cholerae*.

Keywords *Rana catesbeiana*, *Vibrio cholerae*, Identification, Pathogenicity

Introduction

The bullfrog (*Rana catesbeiana*) belongs to the Ranae family and Rana genus. As one of the most important economic aquatic animals, it is native to North America and Mexico (Wang et al. 2020). Bullfrog has the advantages of delicious meat, rich nutrition, and low fat, which greatly profits the catering industry. Some of the organs and secretions of bullfrogs can also be used as important raw materials in various industries, such as industry, breeding and medicine (Pahor-Filho et al. 2019). However, in recent years, the high-density aquaculture of bullfrogs has led to various diseases, which has seriously hindered the development of this industry (Saucedo et al. 2019). In frog disease prevention and control, there also exists the phenomenon of farmers using drugs indiscriminately, overdosing, or even using prohibited drugs, which has caused tremendous pressure on the environment and led to serious food safety problems (Rana et al. 2011). The infectious agents of frog diseases are bacteria, fungi, and viruses, among which bacteria are the main infectious agents (Bie et al. 2020). In recent years, researchers from various countries have identified bacterial infectious agents in various frogs, mainly including *Vibrio* (Han et al. 2017), *Aeromonas* (Lee et al. 2009), *Pseudomonas*

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(Lekshmipriya et al. 2021), *Elizabethkingia* (Wei et al. 2023a), *Morganella* (Wei et al. 2023b), and *Streptococcus* (Sabagh and Rocha 2014). Bacterial diseases of frogs are extremely harmful to the bullfrog farming industry due to their many pathogenic species, complex and diverse causes, rapid spread, and high morbidity and mortality, and have been the focus of prevention and control in the bullfrog farming process (Trimpert et al. 2021).

The danger of zoonotic pathogens in aquaculture should not be underestimated. These pathogens break the barrier between species and are highly likely to be transmitted to humans through natural contact or the food chain as humans come into closer contact with the aquatic environment and ingest more and more species (Tuševljak et al. 2012). *V. cholerae* is a human-mammal-fish commensal, globally widespread, fast-spreading, highly destructive conditional pathogenic bacteria. As Gram-negative bacteria, *V. cholerae* can survive between 25–40 °C, widely distributed in the water environment (Fraga et al. 2007). The bacterium is a curved arc or rod-shaped, with a single flagellum and bacterial pilus. Some have pods and extremely active movement (Constantin de Magny et al. 2008). According to the bacterium O antigen, *V. cholerae* is divided into more than 200 O serogroups, and pathogenicity has a great difference (Huang et al. 2009). Various animals, such as copepods (Almagro-Moreno and Taylor 2013), shellfish (Baron et al. 2017), fish (Halpern and Izhaki 2017), shaker mosquitoes (Halpern and Senderovich 2015), flies (Paz 2019) and waterfowl (Halpern et al. 2008) have been confirmed as hosts for *V. cholerae*. *V. cholerae* can survive in the host or environment long, even if the environmental conditions are beyond the suitable range for *V. cholerae* growth. *V. cholerae* will not die but will go into a dormant state, i.e., a viable but nonculturable state (VBNC) (Alam et al. 2007), and will be revived when the environmental conditions become suitable (Asakura et al. 2007). Such resilience has allowed *V. cholerae* to cause outbreaks worldwide for many years, including seven global human cholera outbreaks caused by *V. cholerae* of serogroups O1 and O139 (Mukhopadhyay et al. 2014). Nowadays, due to the improvement of urban water supply systems, reports related to infections caused by *V. cholerae* of serogroups O1 and O139 are not common. However, non-O1/O139 *V. cholerae* are widely distributed in the aquatic environment and can cause disease outbreaks and mass mortality in fish, shrimp, and other aquatic animals (Petsaris et al. 2010). For example, *V. cholerae* can infect Japanese marsh shrimp, causing redness and mass mortality (X. Li et al. 2019), and can cause mass mortality in *Litopenaeus vannamei*, which can reach 90% (Cao et al. 2015). *V. cholerae* can also cause ulcer disease in loach. The main clinical signs are surface bleeding, muscle ulcers, and high mortality (Zhang and Liang 2012). *V. cholerae* can also infect green shrimp and cause mortality with an LC_{50} of 4.09×10^4 CFU/mL (X. Li et al. 2019).

A serious bacterial disease outbreak recently occurred in bullfrog farms in Nanning, Guangxi. The diseased bullfrogs had swollen abdomens, detached anuses, and rotten feces, and dissection revealed red and swollen intestines and congested internal organs. Our group conducted a pathogenic study of bullfrogs with typical symptoms in the farms and found that these bullfrogs were infected with *V. cholerae*. Several studies have confirmed the threat posed by *V. cholerae* to fish and shrimp (Zhou et al. 2021). However, reports of *V. cholerae* infection in amphibians are relatively rare, and data from related studies are scarce. The present study is the first time that non-O1/O139 group *V. cholerae* was found and isolated from diseased bullfrogs in a bullfrog farm in Guangxi. The results of the current study suggest that *V. cholerae* is equally threatening to amphibians. The researchers performed physiological and biochemical assays and drug-sensitive characterization of the isolated TC1 strain, conducted phylogenetic tree analysis, identified the virulence genes of the strain, and performed artificial infection tests to determine the pathogenic characteristics of the bacterium for bullfrogs. This study provides a reliable pavement for future in-depth studies on the species-spanning mechanism of *V. cholerae* infection in amphibians and develop the methods that can be targeted for the prevention and treatment of cholera in the aquatic industry.

Case presentation

Case history

Diseased bullfrogs were sampled from a bullfrog farm in Guangxi, China. The diseased bullfrogs exhibited obvious clinical signs, such as swollen abdomen, large amounts of fluid accumulation in the abdominal cavity, and congestion of internal organs. The autopsy revealed severe intestinal inflammation with red and swollen intestines and heavy bleeding (Fig. 1a-f). After the isolate was inoculated with culture plates for 12 h at 37 °C, it formed moist, round, elevated, yellow-green colonies on TCBS culture plates (Fig. 1g). The isolated strains were stained red by Gram's stain, indicating that the strain was Gram-negative (Fig. 1i).

Clinical findings

Physiological and biochemical characteristics of the isolates and serotype identification

TC1 isolate is a typical Gram-negative bacterium, and its physiological and biochemical characteristics are listed in Supplementary Table 2. TC1 isolate was positive for ONPG, Lysine decarboxylase, Omithin decarboxylase,

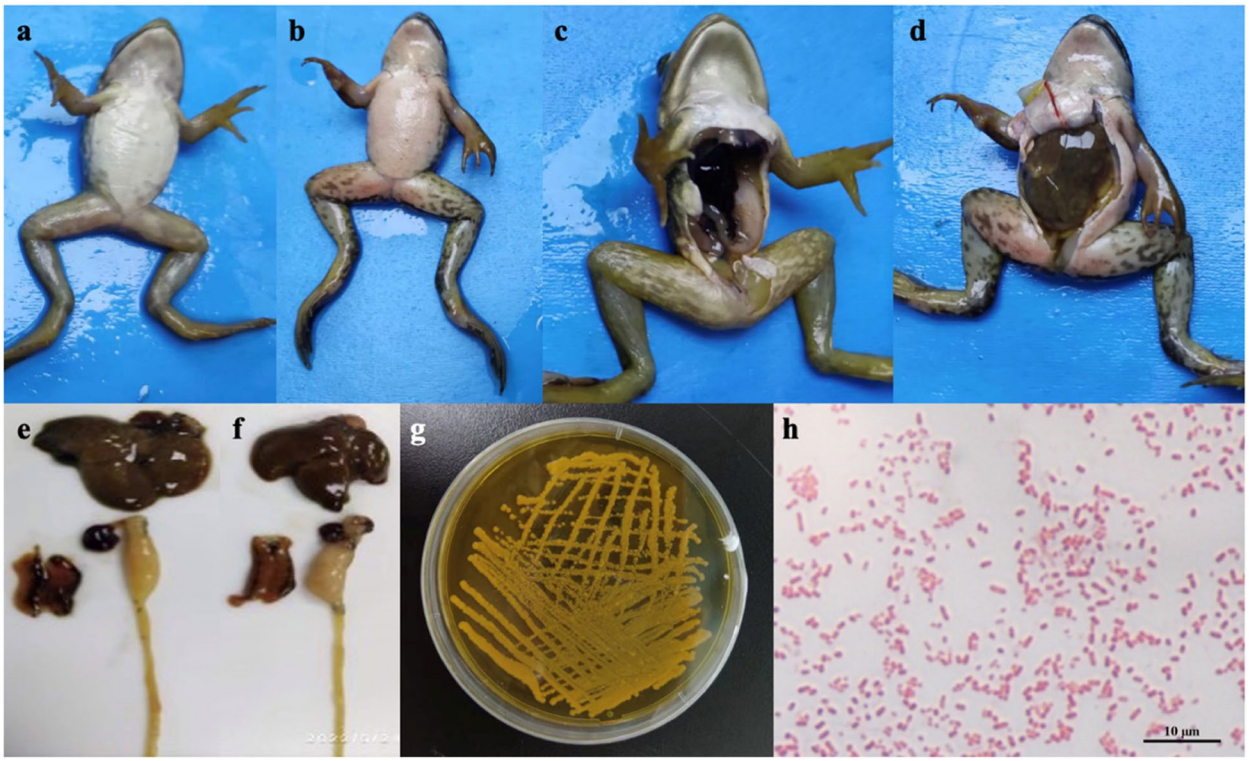

Fig. 1 Clinical signs of diseased bullfrogs and morpho-molecular identification of the clinical isolate. **a** Abdomen characteristics of healthy bullfrogs. **b** Abdomen characteristics of diseased bullfrogs. **c** Visceral morphology of healthy bullfrogs. **d** Visceral morphology of diseased bullfrogs. **e** Liver, spleen, kidneys, and intestines of healthy bullfrogs. **f** Liver, spleen, kidneys, and intestines of diseased bullfrogs. **g** Colony morphology on TCBS plate. **h** Morphology of individual colonies under a scanning electron microscope. **i** Gram-stained bacteria

Citrate-sodium, Indole production, Voges-Prokaver Gelatinase, Glucose, Mannitol, Sucrose, Oxidase, NO_2, MOB, McC, OF-O, OF-F, but negative for arginine decarboxylase, H_2S production, urease, tryptophan deaminase, inositol, sorvose, rhamnose, melibiose, amygdalin, arabinose, and N_2 were negative (Supplementary table 2). The isolates did not agglutinate with any of the *V. cholerae* O1/O139 diagnostic sera by O-antigen slide agglutination test and were further identified as non-O1/O139 type *V. cholerae*.

16S rRNA phylogenetic analysis of the isolates

The results showed that the strain had the highest homology with *V. cholerae*. The 16S rRNA gene sequences of several *Vibrio* species and other important pathogenic bacteria were selected to construct a phylogenetic tree, as shown in Fig. 2. The results showed that the isolate was clustered into a branch with *V. cholerae*. Therefore, combined with this isolate's morphological-molecular identification and physiological and biochemical characteristics, the TC1 isolate was confirmed as *V. cholerae*.

Drug susceptibility testing of the isolate

The results of drug susceptibility tests for 12 antibiotics showed that strain TC1 was sensitive to enrofloxacin and mequindox, moderately sensitive to rifampicin, and resistant to gentamicin, florfenicol, nitrofural, oxytetracycline, neomycin, penicillin, amoxicillin, doxycycline, and sulfamonomethoxine (Supplementary table 3).

Virulence gene testing of the isolate

PCR amplification assays showed that TC1 had virulence-related genes such as *hlyA*, *ompW*, and *tcpA1*, but not *O1-rfb*, *O139-rfb*, *ace*, *chxA*, *ctxAB*, *NAG-ST*, *T3SS (vcsC2)*, *T3SS (vcsV2)*, and *zot*, which also corroborates the results of the O-antigen slide agglutination test, and the isolates belong to non-O1/O139 type *V. cholerae*. (Fig. 3).

Experimental infection of the isolate

Strain TC1 artificially infected bullfrogs with clinical signs such as abdominal swelling and anal prolapse similar to those of naturally infected bullfrogs. At doses of 5×10^7 and 5×10^8 CFU, the bullfrogs started to die on the second day, and all died on the fourth day. At doses of 5×10^6 and 5×10^5 CFU, the bullfrogs started to die

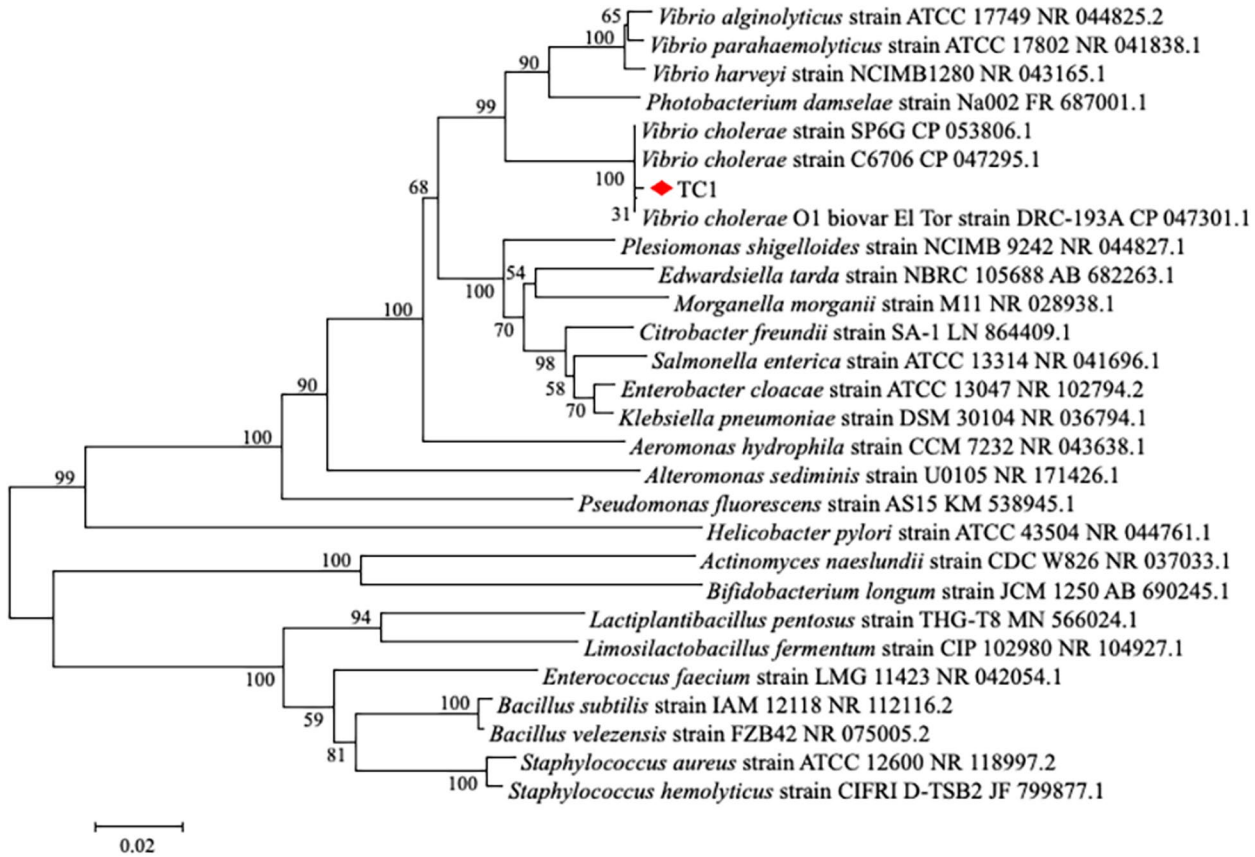

Fig. 2 The phylogenetic tree of 16 s rRNA sequence. The evolutionary history was inferred using the neighbor-joining method. Sequence accession numbers are shown adjacent to bacterial titles

on the third day, with a final mortality rate of 80% and 70%, respectively. At a dose of 5×10^4 CFU, the bullfrogs started to die on the fourth day, and the final mortality rate was 70%. In contrast, bullfrogs in the control group injected with PBS showed no mortality during the test (Fig. 4). The LD_{50} of TC1 strain was calculated as 5062.1 CFU using Karber's method.

Pathological findings

The experimentally infected bullfrogs showed obvious pathological lesions of the intestine. In the intestine, compared with the control group (Fig. 5A), the experimentally infected bullfrogs' submucosal connective tissue was loose and inflammatory cells infiltrated the muscular layer and serosa (Fig. 5B).

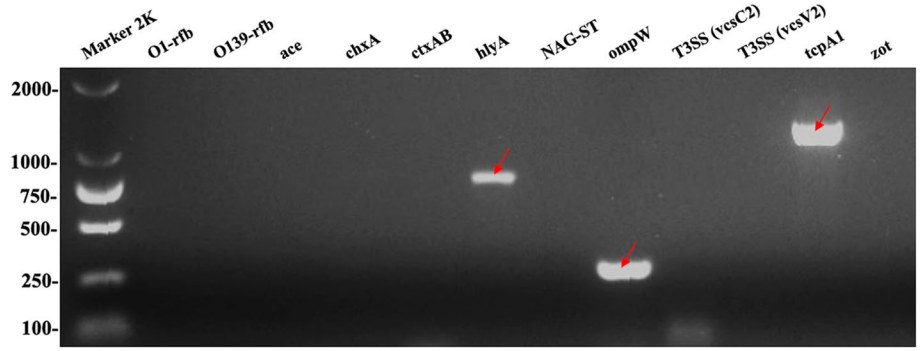

Fig. 3 Virulence Genes of TC1. The clear electrophoretic bands of *hlyA*, *ompW*, and *tcpA1* amplified sequences demonstrated that the TC1 genome contains these virulence genes. A molecular weight marker (base pair/bp) is indicated on the left of the gel

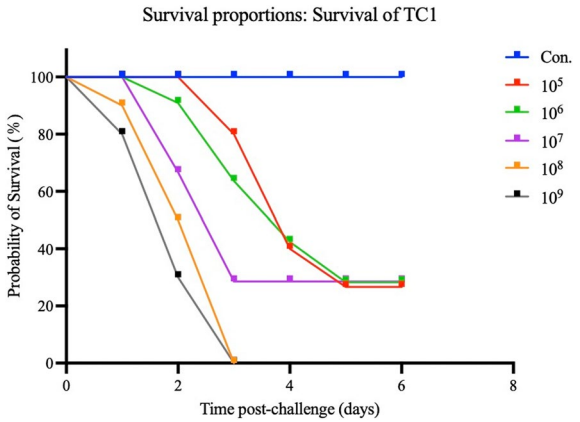

Fig. 4 Kaplan–Meier survival curve of bullfrogs experimentally challenged with *V. cholerae* and determination of LC_{50} value. Each group gave 10 bullfrogs, the abscissa is the number of experimental days, and the ordinate is the mortality rate

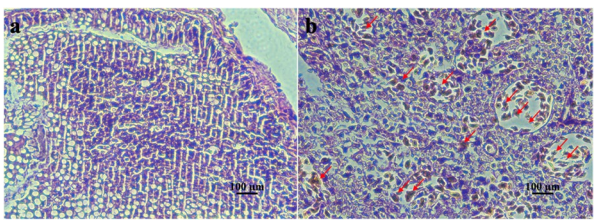

Fig. 5 Intestinal histological lesions of a diseased bullfrog. **a** The healthy bullfrogs. No pathological changes in intestinal cells. **b** The experimentally challenged bullfrogs. Submucosal connective tissue was loose and inflammatory cells infiltrated the muscular layer and serosa. Red arrows show pathological lesions

Specimen preparation

Bacterial isolation

Bullfrogs with typical clinical signs and near death were selected for isolation of pathogenic bacteria, dissected with 75% alcohol by volume, removed the bullfrog intestine placed, the dissected tissues were in a sterile homogenizer, added an appropriate amount of sterile water, and homogenized until there was no lumpy tissue. The homogenate was diluted 10 times, and 100 μl was applied to TCBS plates and incubated overnight at 28℃. The single dominant colony was picked according to colony morphology and scribed on TCBS plates until a single cultured strain, TC1, was obtained. Single colonies were picked for Gram staining and observed under a microscope for morphological characteristics of the bacteria. The TC1 strain was dehydrated by ethanol for 30 min, then dried, gold-plated, and observed using a Hitachi s-3400N (Hitachi, Tokyo, Japan) scanning electron microscope.

Physiological and biochemical tests

The isolated and purified individual colonies were subjected to Gram staining and microscopic examination. The purified strain TC1 was activated by shaking in a constant temperature shaker, and then the physiological and biochemical properties of the bacterial solution, including sucrose, glucose, mannitol, sorbitol, and citrate-sodium, were determined using an API® 20E (bioMérieux, Marcy l'Etoile, France) bacterial identification system. The steps were performed according to the reagent instructions, and the results were recorded.

Serotype identification of the isolate

The serotypes of the isolated bacteria were identified according to the procedure of the *V. cholerae* diagnostic serum kit, group O1/O139 (Tianrun Biological Company, China). Firstly, put drops of saline (control) and the bacterial suspension to be tested on clean slides respectively, then add drops of the diagnostic serum and mix well, and after standing for 2 min, it was observed whether the liquid on the slide became turbid, and the one that became turbid was positive.

16S rRNA gene sequence determination and phylogenetic tree analysis

After extracting the nucleic acids of strain TC using a bacterial genomic DNA kit (QIAGEN, Germany), the universal primers 27F: 5'-AGAGTTTGATCATGGCTCAG-3' and 1492R: 5'-TACGGTTACCTTGTTACGACTT-3' were used to amplify the 16S rDNA gene of strain TC. The amplification program was: 95℃ for 5 min; 30 cycles of 94℃ for 1 min, 56℃ for 1 min, and 72℃ for 1 min; and 72℃ for 10 min. The amplification products were detected using 1.5% agarose gel electrophoresis (Bio-Rad). The 16S rRNA fragment of TC1 was about 1500 bp long, consistent with the expected size. The positive amplification products were recovered and sent to Aoke Dingsheng Biotechnology Co., Ltd. (Wuhan, China) for sequencing. The obtainedsequence (GenBank accession number: OP824765) was subjected to BLAST analysis on the NCBI website (http://blast.ncbi.nlm.nih.gov) for comparison, and a phylogenetic tree was established using the neighbor-joining method in the MEGA 11.0 software package (Yu et al. 2018).

Antibiotic susceptibility test

A standard NCCLS antimicrobial susceptibility test was performed using the paper diffusion method for gentamicin (10 μg/tablets), rifampicin (5 μg/tablets), enrofloxacin (10 μg/tablets), florfenicol (30 μg/tablets), nitrofural (30 μg/tablets), mequindox (10 μg/tablets), oxytetracycline (1 μg/tablets), neomycin (30 μg/tablets), penicillin (6 μg/tablets), amoxicillin (30 μg/tablets), doxycycline (30 μg/tablets) and sulfamonomethoxine (30 μg/tablets). Briefly, 100 μl of each bacterial isolate (10^8 CFU/mL) were plated on LB agar plates. Antibiotic tablets were placed on the surface of the agar plates and

cultured anaerobically for 24 h at 37℃, and the diameters of inhibition halos (mm) were measured and recorded.

Screening of virulence genes

Twelve virulence genes (*O1-rfb*, *O139-rfb*, *ace*, *chxA*, *ctxAB*, *hlyA*, *NAG-ST*, *ompW*, *T3SS (vcsC2)*, *T3SS (vcsV2)*, *tcpA1*, *zot*) were screened by conventional PCR. Primers used to amplify these genes are shown in Supplementary Table 1. PCR amplification products were electrophoresed on a 1% agarose gel stained with ethidium bromide, and the imager took pictures and recorded the results. (Supplementary Table 1).

Artificial infection test

Sixty healthy bullfrogs were randomly divided into six groups, each containing ten bullfrogs. Five bacterial concentrations of TC1 isolates were prepared: 1×10^5, 1×10^6, 1×10^7, 1×10^8, and 1×10^9 CFU/mL. Each of the five groups of bullfrogs was injected anally with five bacterial concentrations at a dose of 500 µl/each, and the remaining control group was injected with PBS. All bullfrogs were kept at 25 °C for 7 d to observe and record pathological symptoms. The onset of disease clinical signs and morbidity were also recorded. Bacteria from the gut of the test-infected bullfrogs were re-isolated.

Histopathological observation

Bullfrogs selected from infected and dying bullfrogs with typical symptoms were dissected, and the intestines were separated and washed with a phosphate-buffered solution (PBS). They were fixed in 4% paraformaldehyde fix solution fixative for 24 h, dehydrated in ethanol and cleared in xylene, embedded in paraffin blocks, sectioned at 4 µm and stained with hematoxylin and eosin (H&E) for histopathological observation.

Discussion

In this study, the dominant pathogenic bacterium was isolated from diseased bullfrogs on a farm in Nanning, Guangxi, China, and identified as non-O1/O139 *V. cholerae* by morphological observation and molecular identification, physiological and biochemical identification, and molecular biology methods. *V. cholerae* is a pathogenic bacterium dangerous to the global aquaculture industry (Sheikh et al. 2022). *V. cholerae* has been reported to cause morbidity and mortality in aquaculture species such as *Carassius auratus*, *Ctenopharyngodon idllus*, *Fugu obscurus*, and *Misgarnus anguillicaudatus* to date, causing huge economic losses to the aquaculture industry (Rehulka et al. 2016). However, from the available literature, there are few detailed studies on *V. cholerae* infecting bullfrogs, all of which are limited to the study of infection clinical signs (Yang et al. 2022). In this study, the strain was identified as *V. cholerae* by comparison of 16S rRNA sequence amplification analysis, and the sequence homology was greater than 99% with *V. cholerae* type O1 and non-O1/O139 isolated from humans on Gen Bank, as well as non-O1/O139 isolated from the aquatic environment. A phylogenetic tree was constructed, and the pathogenic bacterium was also found to have close homology with *V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus*, and *Photobacterium damselae*. The drug sensitivity test found that the strain was resistant to most antibiotics, indicating that the strain carried more drug-resistance genes, probably due to the over-administration of antibiotics in culture ponds. The virulence test found that it had at least three virulence genes, *hlyA*, *ompW*, and *tcpA1*, encoding hemolysin, outer membrane protein, and cholera toxin co-regulated bacterial pilus, respectively (Ramamurthy et al. 2020). The regression infection experiment revealed that bullfrogs infected with *V. cholerae* showed very typical clinical signs (e.g., intestinal redness, intestinal vascular rupture, anal prolapse), confirming the strong pathogenicity of this *V. cholerae* strain to bullfrogs and also confirming that *V. cholerae* can cause bullfrog enteritis, indicating that the aquaculture industry should strengthen the prevention and control of *V. cholerae*, and also advancing the pathogenetic research process of bullfrog enteritis.

Although the TC1 isolated in this experiment was a non-O1/O139 type *V. cholerae* and could not encode cholera toxin (CT), which was the most important virulence factor of *V. cholerae* (Hsiao and Zhu 2020), it was still highly pathogenic to bullfrogs. According to the present study, we detected that TC1 contains three virulence genes, *hlyA*, *ompW*, and *tcpA1*, capable of encoding hemolysins, outer membrane proteins, and toxin co-regulatory pilus (TCP). HLY is a toxin that causes the lysis of red blood cells, and the gene encoding *hly* is a vital virulence determinant cluster in animals infected with vibriosis, causing hemolytic sepsis and enteritis in the host (Kanoktippornchai et al. 2015). According to Halder et al., the *hly* gene is the dominant gene in the virulence gene of *V. cholerae*, which is carried by the vast majority of *V. cholerae* (Halder et al. 2022). OMP is the major structure of the bacterial outer membrane and is a protein specific to Gram-negative bacteria, and past studies have shown that it is not only associated with adhesion but also closely related to the virulence of the strain (Morgan et al. 2019). Many experiments confirm that OMP is an important virulence factor involved in host-cell interaction and recognition and is essential for the survival of pathogenic *Vibrio* (H. Li et al. 2018). OMP is also closely related to the iron uptake process of *Vibrio* and is an essential pathogenic factor (Bari et al. 2012).

TCP mediates the colonization of the small intestinal mucosa by *V. cholerae*. Because those pilus and CT are co-regulated by the Tox R regulatory system, it is referred to as a toxin co-regulated pilus (Herrington et al. 1988). Some studies have reported that TCP mediates the adhesion of the bacterium over long distances during *V. cholerae* pathogenesis, but the mechanism is not yet clear (Silva and Benitez 2016). According to previous studies, in *V. cholerae*, the *tcpA* gene is usually expressed first, synthesizing the TcpA protein and adhering to small intestinal epithelial cells before the ctxAB gene encoding CT initiates expression (Taylor et al. 1987). The synthesis of TCP is a prerequisite for CT secretion by *V. cholerae*. TCP is still poorly understood, and further studies are needed regarding its composition and protective effects on the bacterium. Interestingly, for non-O1/O139 type *V. cholerae*, strains capable of synthesizing TCP seem rare (Daboul et al. 2020; F. Li et al. 2014). The TC1 isolated in this experiment with a TCP virulence factor is a topic worthy of further exploration, and we need to determine further which type this *tcpA* gene is.

Fish challenged with *V. cholerae* typically exhibit pale livers, abdominal hemorrhages, nasal gill congestion, and acute or subacute ocular lesions (Hossain et al. 2018). Clinical signs of *V. cholerae* infected fish show some similarity to other *Vibrio* species (including *V. harveyi*, *V. algolyticus*, and *V. parahaemolyticus*), and typically, affected fish show signs of sepsis (Hernández-Cabanyero et al. 2022). Dark body color, lethargy, anorexia, bleeding at the base of fins, liver enlargement, petechial hemorrhage, and skin ulcers are the most common clinical signs of *Vibrio* diseases in fish (Mohamad et al. 2019). Adhesins, hemolysins, proteases, and DNA enzymes are virulence components associated with the pathogenicity of *Vibrio*, allowing them to invade their hosts and cause tissue damage (Baffone et al. 2001). Unfortunately, studies on the pathogenicity of *Vibrio* on bullfrogs have been scarce so far, and the aquaculture industry has not given sufficient attention to them.

The drug sensitivity characteristics of *V. cholerae* from different sources are often strain-specific (Igere et al. 2020). For example, some researchers collected 50 non-O1/O139 *V. cholerae* strains from Haitian surface water and tested 16 antibiotics, showing that close to a quarter were sensitive to all 16 antibiotics, most were resistant only to sulfonamides, and a few strains were multi-drug resistant (Baron et al. 2016). Researchers have also tested multiple strains of *V. cholerae* transmitted during a cholera outbreak in the Bay of Bengal, testing 22 antibiotics and finding a higher diversity of resistance in *V. cholerae* type O1 compared to non-O1/O139 types. Of the hundreds of *V. cholerae* strains, the largest proportion was resistant to sulfonamides, nalidixic acid, trimethoprim, and streptomycin, while only four percent were resistant to neomycin (Verma et al. 2019). Compared with the above study, we found that strain TC1 was multi-drug resistant and more resistant than *V. cholerae* isolated in its natural environment, which may be a consequence of bullfrog farming ponds overdosed with antibiotics.

Given the severity of *V. cholerae* damage and the wide range of its hosts, several species have been used as animal models to study *V. cholerae*-host interactions, such as rats, rabbits, fish, and flies (Richardson 2014). Lactating or juvenile rats have often been used to study the pathogenesis of *V. cholerae*, and studies in juvenile rats have identified the basic virulence factors of *V. cholerae* (Klose 2000). since the 1980s, juvenile or adult rabbits have been widely used as models for studying *V. cholerae* (Ritchie et al. 2010). In the last decade, *V. cholerae* has been isolated from more than 30 species of fish, and studies have found that non-virulence-producing *V. cholerae* establish a symbiotic relationship between fish and *V. cholerae* by secreting chitinases and proteases to degrade macromolecules ingested by fish (Senderovich et al. 2010). Based on the association between fish and *V. cholerae*, the zebrafish (*Danio rerio*) has also been developed in the last decade as a natural host model for studying *V. cholerae* (Mitchell and Withey 2018).

Regardless of the animal model, these models have helped researchers to gain a faster and better understanding of the pathogen's mechanism of infection and host sensing of the pathogen and have also facilitated the development of cholera vaccines. However, little attention has been paid to the interactions between *V. cholerae* and amphibian hosts. Amphibians are a key transitional link in the evolution of fish animals to birds and mammals. They are extremely important for studying interspecies evolution, including enhancing organismal immunity (Wake and Koo 2018). Studying the pathogenesis between *V. cholerae* and amphibian hosts is a good entry point for a deeper understanding of *V. cholerae* transmission mechanisms and pathogenesis. The results of this experiment suggest that bullfrogs are a promising amphibian that can be used to study *V. cholerae*, and future attempts to establish a bullfrog model against *V. cholerae* could be made.

Conclusion

In conclusion, non-O1/O139 group *V. cholerae*, named TC1, was isolated from diseased bullfrogs by combining morphological and physiological-biochemical characterization and molecular biology. Pathological tissue observation TC1 can cause systemic histopathological damage in bullfrogs. These results provide pathogenetic support for the prevention and control of cholera disease in bullfrogs.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s44149-023-00092-w.

Additional file 1: Supplementary Table 1. PCR identification primers for *Vibrio cholerae*. **Supplementary Table 2.** Physiological and biochemical characteristics of TC1. **Supplementary Table 3.** Susceptibility of TC1 to antibiotics.

Acknowledgements
Not applicable.

Authors' contributions
W.L conceived and designed research, analyzed data, participated in the experiment, and wrote the manuscript, D.W performed the experiments and analyzed data, M.L performed the experiments and analyzed data, K.K performed the experiments and analyzed data, D.S performed the experiments and analyzed data, B.L contributed the reagents and materials, S.H performed the experiments and analyzed data, J.J contributed the reagents and materials, Q.Y conceived and designed research, P.L designed research and provided funding support for this paper. All authors have read and agreed to the published version of the manuscript.

Funding
This work was supported by grants from the National Natural Science Foundation of China (U20A20102), Agricultural Science and technology project of Guangxi (Z2022167), Guangxi Innovation Team Project of National Modern Agricultural Industrial Technology System (nycytxgxcxtd-2021–08-02), the Basic Research Fund of Guangxi Academy of Sciences (2019YJJ1005) and Guangxi mangrove coastal wetland ecological protection and sustainable use of small highland talents project (BGMRC202104).

Availability of data and materials
The data supporting this study's findings are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
Following the ARRIVE (Animal Research: Reporting in Vivo Experiments guidelines for reporting animal research), procedures involving fish were performed and approved by the Ethical Committee of the Guangxi Academy of Sciences, Nanning, China.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Received: 15 May 2023 Accepted: 19 July 2023
Published online: 05 September 2023

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