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Isolation, Identification, and Pathogenicity Characterization of a Largemouth Bass Ranavirus from Fujian, China

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Abstract

This study aimed to isolate and characterize the pathogenic agent of a suspected viral disease of largemouth bass (*Micropeterus salmoides*), which broke out in an aquatic farm in Fujian, China. The diseased bass had ulcer skin and muscles, bleeding fin, lesion liver, and enlarged spleen. Histopathological analysis showed multifocal necrosis on the gill, hepatopancreas spleen, and kidney. In the epithelioma papulosum cyprinid (EPC) cell line, stable cytopathic effects (CPE) were observed. Electron microscopy analysis showed icosahedral virus particles in the cytoplasm with a 150-160 nm diameter. PCR amplification revealed that the isolated virus belongs to largemouth bass ranavirus (LMBV) and was designated as LMBV FJ_22109. Further phylogenetic analysis determined that LMBV FJ_22109 is a variant of European and American strains. The intraperitoneal injection of LMBV FJ_22109 resulted in a regress infection, demonstrating its ability to induce pathogenicity in largemouth bass. The cumulative mortality rate was 100% at a culture temperature of 29°C, and 47% at a culture temperature of 17°C. Thus, it confirmed that LMBV FJ_22109 is indeed the causative agent of the suspected viral disease, and its virulence could be influenced by temperature, which will be beneficial for preventing and managing the disease.

Introduction

Largemouth bass, *Micropeterus salmoides*, belongs to the family *Centrarchidae* and the genus *Micropterus*. Since its introduction to China in 1983, this fish species has been extensively cultivated on a large scale because of its robustd environmental tolerance, rapid growth rate, and delicious meat (Li & Wang, 2008). In 2022, Chinese total largemouth bass production reached 800,000 tons, with an economic value exceeding 3.5 billion USD (Ministry of Agriculture and Rural Affairs Fisheries Administration Bureau, 2023). However, epizootic outbreaks of lethal diseases, especially viral diseases, were observed frequently in stocked fish ponds, causing substantial economic losses to farmers (Deng *et al.*, 2010; Ma *et al.*, 2013; Dong *et al.*, 2017).

Common pathogens of largemouth bass include bacteria such as *Flavobacterium columnare* (Bebak *et al.*, 2009) and *Nocardia seriolae* (Wang *et al.*, 2009), parasites such as *Trichodina spp*. (Nastou *et al.*, 2012) and *Apiosoma blanchar*d (Li *et al.*, 2007), and viruses such as *Iridoviridae* (Whittington *et al.*, 2010) and *Rhabdovirus* (Gao & Chen, 2018). Nevertheless, viral diseases exhibit more severe consequences than parasitic and bacterial diseases due to its higher mortality and lack of treatment methods (Deng *et al.*, 2010). Largemouth bass ulcerative disease (LBUD) is one of the main viral diseases of largemouth bass (Chen & Liang, 2022). The disease was first reported in the United States in 1991 (Grizzle *et al.*, 2002) and spread to other regions such as Europe and Asia (George *et al.*, 2015). In 2008, largemouth bass ranavirus (LMBV) was

first isolated and determined to be the pathogen of this disease in China (Deng *et al.*, 2009). Epidemiological investigations revealed that LBUD had a pervasive occurrence with high fatality rate (Jin *et al.*, 2020; Chen & Liang, 2022).

In October 2022, an acute infectious disease broke out among the largemouth bass cultured in Zhangzhou, Fujian, China. This study aimed to determine the pathogen resulting in this outbreak. The isolation and identification of LMBV FJ_22109, was accomplished by utilizing cell culture, transmission electron microscopy observation, PCR detection, and phylogenetic genetic analysis. Subsequently, a challenge assay verified its role as the causal agent of the disease.

Materials and Methods

Clinical Diagnosis

10 Diseased largemouth bass (weighing about 200±22.3 g) were brought to our laboratory and subjected to standard clinical diagnosis, including viral isolation. Light microscopy was used to examine the skin, mucus, and gill tissues for parasites and fungi, and an anatomical examination was conducted to assess any anomalies in the bod cavities.

Histopathological Observation

The gills, hepatopancreas, spleen, and kidney tissues of the diseased bass were dissected and fixed in Bouin's fixative. Paraffin tissue sections (5 µm) were made and stained with hematoxylin-eosin (HE) and observed under a Nikon Eclipse E100 microscope. The nuclei and cytoplasm were stained with hematoxylin (blue) and eosin (red), respectively, allowing for a sharp contrast for observing pathological changes. CaseViewer (3DHISTECH Ltd., Version 2.0) imaging system was employed for image acquisition and analysis.

Cells and Virus Isolation

The EPC cell line (strain: ATCC CRL-2872) was commercially purchased (Wangmuchun, China) and propagated in our laboratory with 40 passages. The cells were cultured in Leibovitz's L15 medium (HyClone) supplemented with 10% fetal bovine serum (FBS, HyClone) at 27°C. For viral isolation, the liver, spleen, and kidneys of diseased basses were homogenized with Dulbecco's phosphate-buffered saline (D-PBS) containing streptomycin - penicillin (100 U/mL) and amphotericin B (5 µg/mL). The tissue samples were centrifuged at 10,000 × g at 4°C for 10 minutes. The resulting supernatant was then collected and passed through a syringe filter with a pore size of 0.45 µm. Subsequently, the filtrates were administered to EPC cells and incubated at 27°C using a cell maintenance medium (L-15 supplemented with 2% FBS). The cells were examined daily using an inverted light microscope (Nikon, Japan). Following a three-day of incubation period, the supernatants from the incubated cells were collected and utilized for a subsequent round of incubation. The infected EPC cells exhibited a consistent cytopathic effect (CPE) in the third to fifth subculture passages (P3 to P5). Infected cell supernatants were collected and stored at -80 ℃ for further use.

Electron Microscopy

The infected EPC cells were collected when 40-50% of the cells showed CPE and centrifuged at 300 × g for 10 minutes to pellet down the cells. The cells were initially pre-fixed with 1 mL of 2.5% (v/v) glutaraldehyde in PBS buffer (pH 7.3, 0.1 M) at 4°C. Subsequently, the cells were sequentially submerged in different ratios of ethanol-resin solutions for embedment. Then subjected to polymerization for 24 hours at a temperature of 70°C. The ultrathin slices with 50 nm in thickness were dissected and stained using 1% uranyl acetate and lead citrate. The morphology and distribution of the virions were then observed and photographed using a Hitachi HT7700 transmission electron microscope.

PCR Amplification and Phylogenetic Analysis

The infected EPC cells were collected and used for DNA extraction with a virus DNA/RNA extraction kit according to the manufacturer's instructions (TIANGEN). As the disease was highly suspected to LBUD, the MCP-F/MCP-R primer pair (Table 1) was designed based on the sequence of the major capsid protein (MCP) gene of the LMBV reference strain (GenBank accession number: GU256635). PCR amplification was performed with the extracted DNA, using the EPC cells and sterile water as the control samples. Then, the PCR amplicon was cloned and sequenced (Sangon Biotech). The DNA sequence was used to retrieve sequences of other representative iridoviruses by BLAST, and a phylogenetic tree was constructed using the MEGA-X software utilizing the Neighbor-Joining (NJ) method.

Table 1. Primers used for PCR amplification

Primer	Primer sequences (5'-3')	Predicted size (bp)
MCP-F	TCTCGCCACTTATGACAGCC	1557
MCP-R	TGACCAGTCCCCTGATGGAA	
Rana-F	TATGTGCTCAACTCTTGGCTGGTC	475
Rana-R	CCACGATGGGCTTGACTTCTCC	

Viral Titer Determination

To assess viral titer, EPC cells were seeded in 96-well cell culture plates with 10^4 cells/0.1 mL per well. Ten-fold serial dilutions of the virus were made in L-15 supplemented with 2% FBS and then applied to the EPC cells. Each dilution was applied to four wells and repeated three times. The appearance of CPE was observed five days post-incubation. The 50% tissue culture infective dose ($TCID_{50}$) was calculated using the Reed-Muench method, and the plaque forming unit (PFU) was calculated based on the empirical formula, PFU = $TCID_{50}$ × 70%.

Pathogenicity of the Virus

Largemouth basses that had never experienced similar diseases were obtained from a breeding farm in Zhangzhou, Fujian, China. A total of sixty largemouth basses between 100-150 g were randomly assigned into two groups. The challenged group's fishes (n = 30) were intraperitoneally injected with 1 mL diluted cell culture supernatants containing 10^4 PFU of LMBV FJ_22109, respectively. Fishes in the control group (n=30) were injected with 1 mL diluted uninfected EPC cell supernatants. The fishes were cultured at 29±2°C and fed with commercial pellet feed. Clinical signs and mortality were recorded daily. To understand the pathogenicity of the virus under relatively low culture temperatures, a similar experiment was conducted at 17±3°C.

Virus Re-isolation

he liver, spleen, and kidney tissues were extracted from largemouth basses injected with LMBV FJ_22109. These tissues were then homogenized and inoculated with EPC cells to isolate the virus. The virus was subcultured for three subsequent generations and identified using PCR with a primer pair Rana-F/Rana-R specific to LMBV (Table 1) (Wang *et al.*, 2013).

Results

Clinical Symptoms

First, we aimed to characterize the pathological features of the diseased fish. The diseased largemouth basses showed letharagy and darken body color, with multiple ulcerative lesions on its muscles and skin (Figure 1A). Inflammatory lesions, bleeding, swelling, and scales shedding were observed on the pectoral, anal, and caudal fins (Figure 1B). The gill structure remained intact but exhibited localized congestion and necrosis (Figure 1C). Large ulcerous lesions could reach up to 2 × 3 cm inside the muscle layer. Additionally, adhesion was observed between visceral organs in the abdominal cavity, and yellowish-pale and brittle marble-like lesions were observed in the liver. The spleen appeared enlarged with thickening of outer membrane (Figure 1D). Microscopic examination showed no parasitic or fungal infections in the skin, mucus, and gills. Together, these symptoms indicate that a viral infection may be the causative pathogen of the disease.

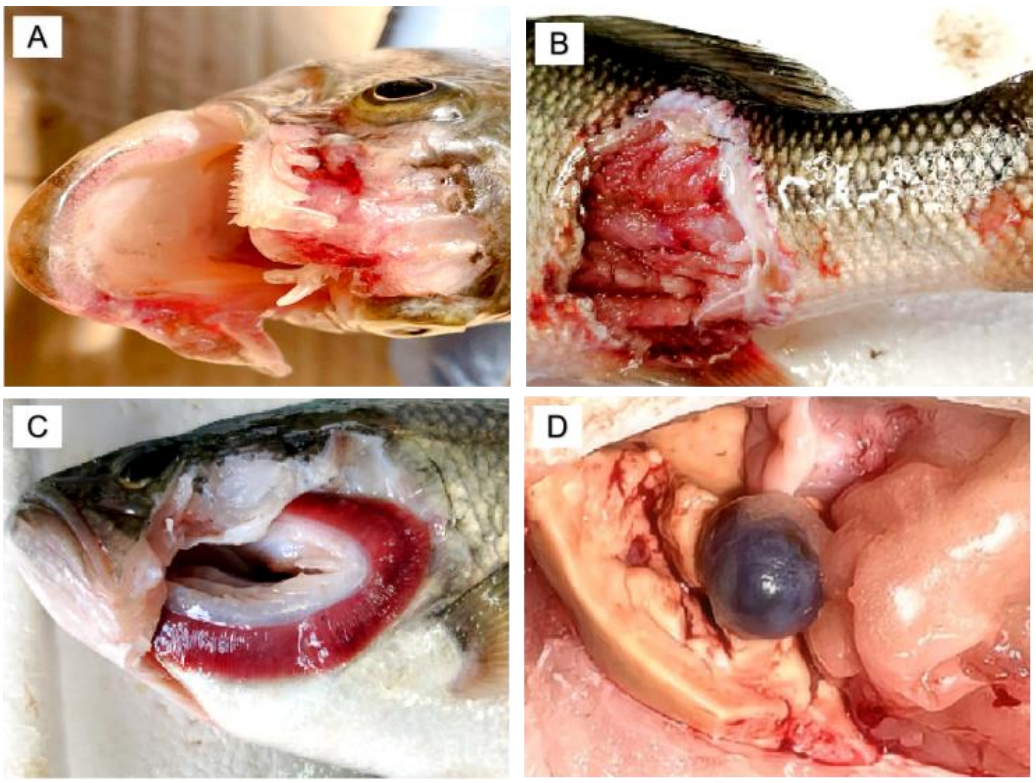

Figure 1. Clinical signs of diseased largemouth bass. (**A**) Mucosal ulcer coloboma. (**B**) Lesion of somatic skin and muscle ulcer. (**C**) Gill lesions with congestion and (d) pale liver and enlarged spleen.

Histopathological Changes

Then, we attempted to assess the diseased tissues by histopathological examination. Swelling and denaturation of epithelial cells were observed in gill filaments (Figure 2A). In hepatopancreas, erythrocytes were aggregated in clusters, cytoplasmic steatosis and necrosis with the light staining of hepatocytes (Figure 2B). Phagocyte aggregation and monocytopenia were observed in the spleen (Figure 2C). The renal papilla (Rp) showed signs of moderate erythrocyte coagulation, and the renal tubules' (Rt) epithelial cells underwent degeneration, necrosis, and disintegration, which resulted in narrowing and blockage of the Rt lumen (Figure 2D). Histopathological alterations indicated organic damage in the kidney, spleen, gills, and hepatopancreas, which led to physiological and metabolic abnormalities in the infected fish and ultimately led to their mortality.

Virus Isolation

EPC cells were utilized to isolate the virus using the supernatants of homogenized tissues from the diseased largemouth bass. A typical viral infection CPE was observed after two subculture passages (P2) and tended to be stable after three passages (P3). The EPC cells underwent a notable transformation within 24 hours post-infection, transitioning from a semi-suspended spherical state to a shrunken and translucent state. By the fourth day post-infection (d.p.i.), this change progressed further, causing most infected cells to become suspended and form distinctive plaques (Figure 3A). The control cells maintained an adherent epithelial-like morphology (Figure 3B). Then, the cells were collected and observed under transmission electron microscopy after ultrathin sectioning. A large number of latticed and scattered viral particles with a diameter of 150-160 nm, icosahedral, and symmetrical structures were observed in the cytoplasm (Figure 3C). Wherein, mature virions have a high electron density and appear as deeply stained cores, while immature virions have a low electron density and light staining (Figure 3D). These characteristics are similar to the morphological structure of iridovirus.

Viral Identification

NFurther, PCR was used to amplify the conserved gene MCP of iridovirus. A specific DNA band of approximately 1600 bp was obtained from P5-infected EPC cells , while no band was amplified from the controls (Figure 4). The sequence shared high homology with the reference strains of LMBV in GenBank. Accordingly, the isolated virus was identified as an LMBV strain of the *Santee Cooper ranavirus* species and named LMBV FJ_22109.

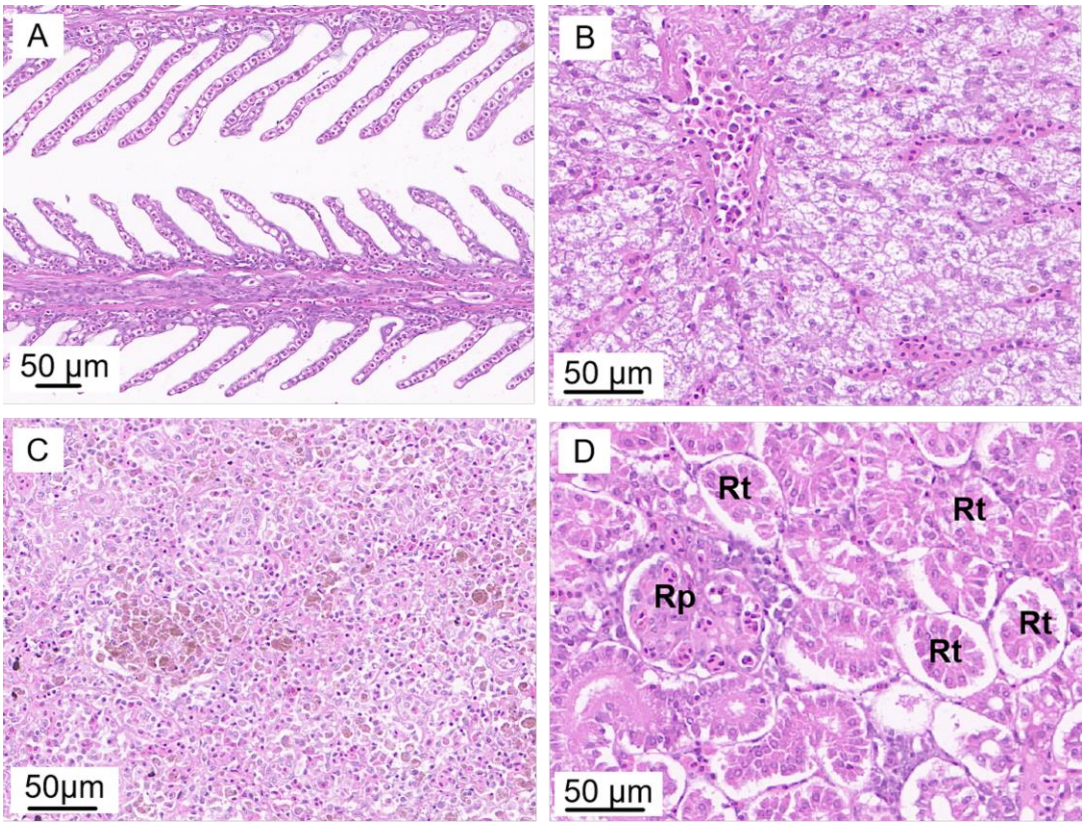

Figure 2. Histopathological examination on gill (**A**), hepatopancreas (**B**), spleen (**C**), and kidney (**D**). Rp and Rt represents the renal papilla and the renal tubules, respectively.

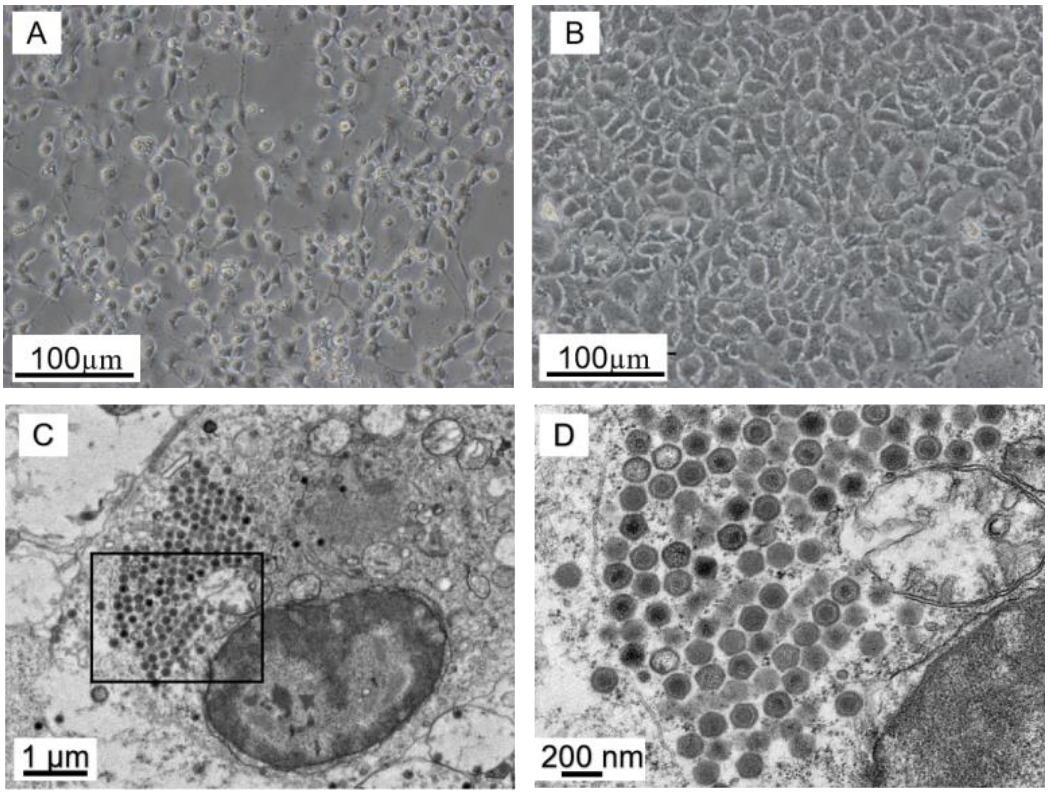

Figure 3. Cytopathic effect of cells and transmission electron microscopic observation of virus. (**A**)EPC cells infected with viruses. (**B**)Uninfected EPC cells. (**C**)Virus particles in infected EPC cells. (**D**) Enlarged morphology of virus particles**.**

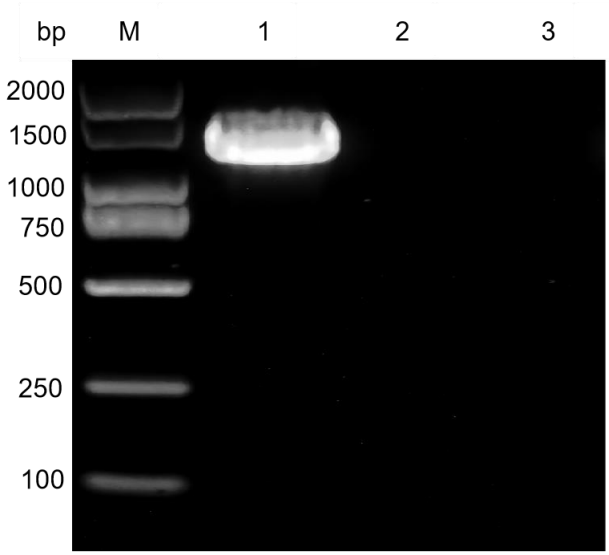

Figure 4. PCR identification of the isolated virus. M: DL2000 DNA Marker; 1: EPC cells infected with virus at generation P5. 2: EPC cells. 3: Blank control using H_2O as a template.

Further phylogenetic analysis using the MCP sequences showed that the isolated virus LMBV FJ_ 22109 shared 100% homology with LMBV strains isolated from Asia and clustered into same evolutionary branch (Figure 5), and shared a range of 98.17% to 98.61% identity with LMBV strains isolated from Europe and America. Notably, the genetic differences between LMBV FJ_22109 and members of the *Rana grylio virus* (RGV) were significant.

Viral Pathogenicity

he viral suspension of P5 collected on the fifth d.p.i. was diluted to 10^4 PFU and intraperitoneally injected into largemouth bass. Under normal temperature conditions (29±2°C, the largemouth bass challenged with LMBV FJ_ 22109 resulted in death 3rd d.p.i. and reached a peak mortality at the 5th d.p.i., with a cumulative mortality rate of 100% within seven days

(Figure 6A). Under low temperature culture conditions (17±3°C), clinical symptoms were observed in the challenged fishes from 3rd d.p.i. with weakened vitality, dull body color, and black spots. The fish started to die at the 17th d.p.i., with a cumulative mortality rate of 47% in the 30-day observation period (Figure 6B). No symptoms or deaths were observed in the control group. The dying fishes exhibited red, swollen, and ulcerated lesions on the base of the pectoral fin, black spots on the body, as well as local scale shedding around the anus and tail stalk. They also exhibited typical ulceration on the skin and muscles, all consistent with the symptoms of naturally infected basses (data not shown). The visceral tissues of the challenged largemouth basses were collected and inoculated with EPC cells for virus re-isolation. The infected EPC cells showed similar CPE (Figure 7A), and the DNA band with the expected size of 475 bp was amplified explicitly from the supernatants of cultured cells (Figure 7B), indicating that the virus could be re-isolated from the challenged basses. These results confirmed that LMBV FJ_22109 is the pathogen of the diseased largemouth basses.

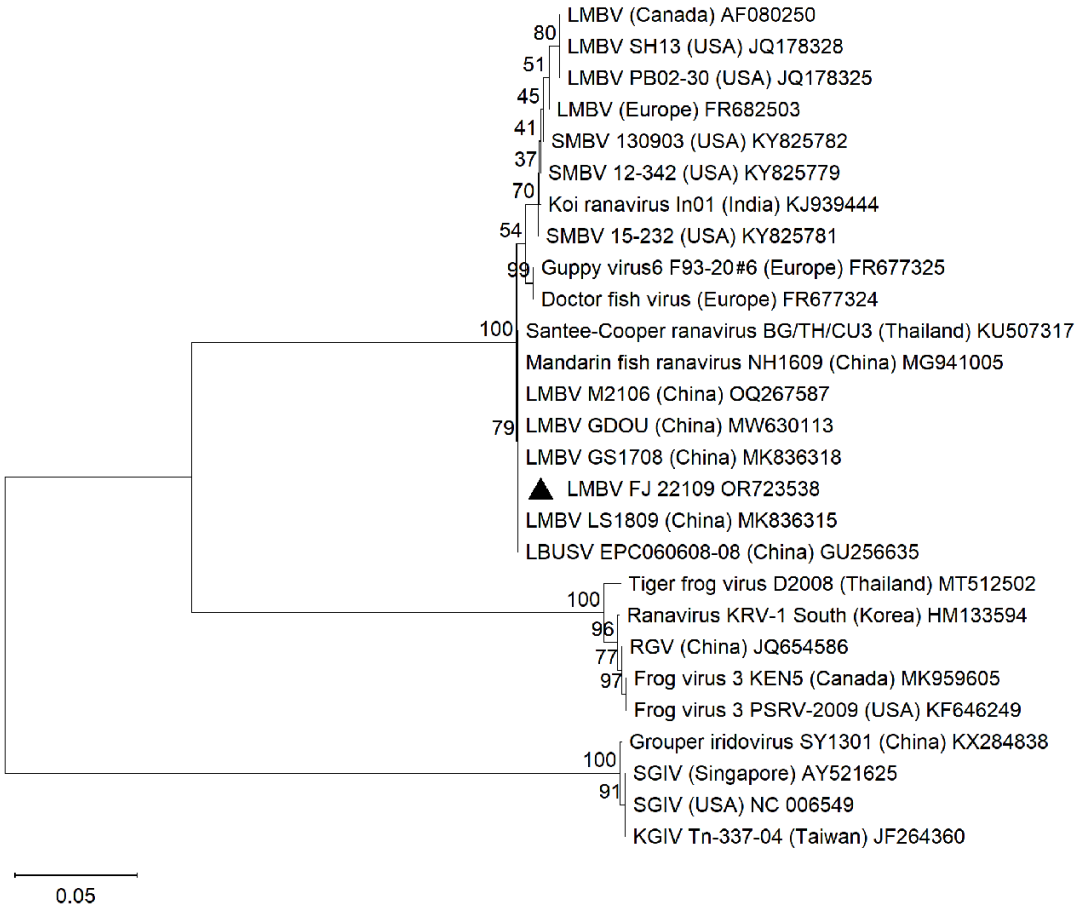

Figure 5. Phylogenetic analysis of the isolated virus.

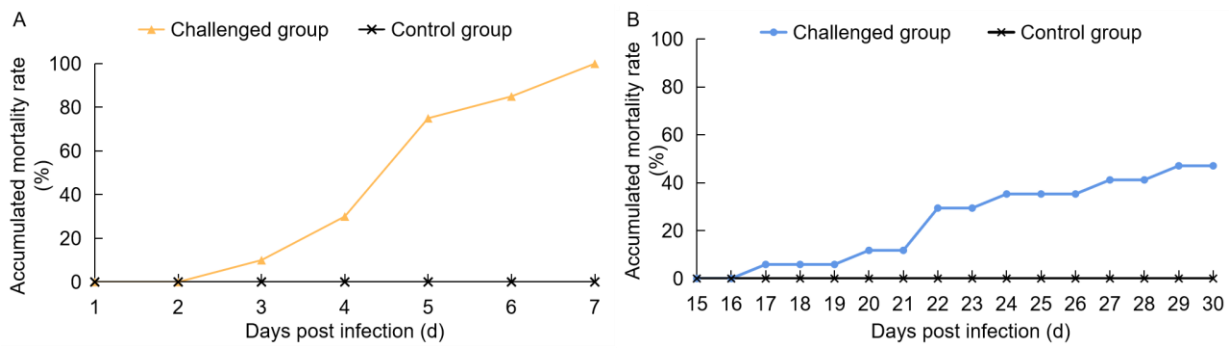

Figure 6. The accumulated mortality rate of largemouth bass challenged with LMBV FJ_22109. (A) Regress infection by the virus under normal temperatures (29±2℃). (B) Regress infection by the virus under low temperature (17±3℃).

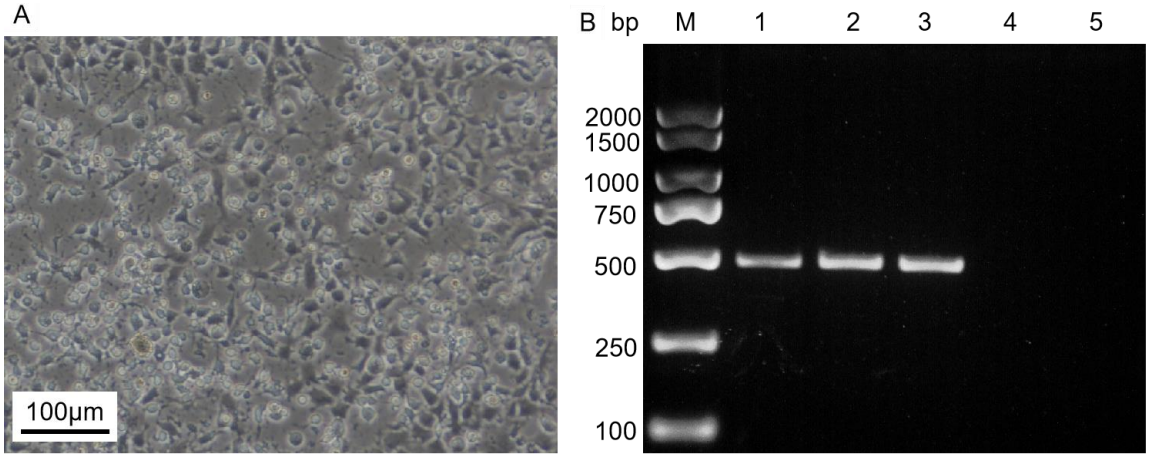

Figure 7. Identification of the re-isolated virus. (**A**) EPC cells infected with viruses. (**B**) PCR identification. M: DL2000 DNA Marker. 1,2,3: EPC cells infected with viruses at generation P1, P2 and P3. 4: EPC cells. 5: Blank control using H_2O as a template.

Discussion

LBUD is a highly consequential viral disease caused by LMBV, posing a severe danger to the worldwide largemouth bass aquaculture industry (Xia *et al.*, 2018). In the United States, where LMBV was first reported, the infection displayed no characteristic clinical symptoms on the fish body except equilibrium loss due to an enlarged swim bladder (Plumb *et al.*, 1996; Hanson *et al.*, 2001). However, different clinical symptoms of LMBV infection were observed in China, including skin ulcers and muscle necrosis, bleeding at the fin base and tail stalk, enlarged spleen, pale liver, and acute fibrinous peritonitis (Huang, 2017; Xu *et al.*, 2020; Zhao *et al.*, 2020). In this study, skin and muscle ulcers, visceral adhesion, and liver and spleen lesions were found in the diseased bass, which was consistent with LBUD (Whittington et al., 2010; Zhao et al., 2020). Therefore, the suspected disease was initially identified as LBUD.

The iridovirus diseases of largemouth bass include infectious spleen and kidney necrosis virus disease (Zeng *et al.*, 1999), swollen liver and spleen in largemouth bass (Ma *et al.*, 2011), and largemouth bass ulcerative syndrome (Deng *et al.*, 2009). The pathogens of these viral diseases are members of the *iridoviridae* family, but belong to different genus due to differences at pathogenicity. This study successfully isolated a virus from the diseased largemouth bass and identified it as the LMBV strain of the *Santee Cooper ranavirus* species. The MCP gene is a highly conserved gene of iridovirus, which can be used to analyze the evolutionary relationship or as a biomarker for identifying new members of iridovirus (Holopainen *et al.*, 2009). Studies indicated that LMBV isolated from Asia might have evolved into a new independent group due to environmental effects and host interaction (Zhao *et al.*, 2020). According to MCP sequences, LMBV FJ_ 22109 shares 100% similarity with other Asian LMBV strains but differs significantly from European and American strains.

Previous work has shown that the optimal temperature for LMBV replication is 30°C (Piaskoski *et al.*, 1999). This temperature range has been associated with a heightened incidence of LBUD, typically occurring at temperatures between 25°C and 30°C (Bennett *et al.*, 1989; Neal & Lopez-Clayton, 2001), where the cumulative mortality rate of LMBV infection can reach 60-70% (Zhao *et al.*, 2020). Our study revealed notable differences in the virulence of the virus concerning the progression of the infection and the rate of mortality rate at temperatures of 29±2°C and 17±3°C, respectively. The data suggests that breeding temperature does not affect the appearing time of the clinical symptoms, but delaying the death time and reducing the overall mortality rate. Therefore, water temperature may play a vital role in the virulence of LMBV infection (Grant *et al.*, 2003), which can be exploited for LBUD control and prevention.

Conclusion

In this study, a largemouth bass virus LMBV FJ_22109, was isolated from diseased largemouth bass with clinical symptoms of LUBD in China. The isolated virus was determined to be a distinct LMBV strain differing from European and American isolates. The regression infection has been confirmed as the primary cause of the local disease and has exhibited a significant level of pathogenicity. Nevertheless, the breeding temperature can significantly influence the mortality rate of the virus, which will be applicable for effectively preventing and controlling LBUD.

Ethical Statement

All animal experiments were approved by the Animal Ethics Committee of Institute of Biotechnology, Fujian Academy of Agriculture Science (Fuzhou, China) with approval code of BI-AEC-2020041001.

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Author Contribution

Conceptualization, J.X.Y. and J.Q.G.; Methodology, J.X.Y. and J.Q.G.; Formal analyze, X.C., J.X.Y. and H.C.; Investigation, J.X.Y. and H.C.; Writing-Original Draft Preparation, X.C.; Writing-Review and Editing: J.Q.G.; Supervision, J.Q.G.; Project Administration, J.Q.G.; Funding acquisition: J.Q.G.

Conflict of Interest

The authors declare no conflict of interest.

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