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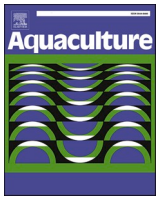

CRISPR/CasRx: A novel antiviral approach to combat largemouth bass (*Micropterus salmoides*) Rhabdovirus infections

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ABSTRACT

The Largemouth bass (*Micropterus salmoides*), a freshwater species of considerable economic significance native to North America, is currently facing a formidable challenge from the *Micropterus salmoides* rhabdovirus (MSRV). This viral pathogen has emerged as a primary agent of mortality within the species, with its recurrent outbreaks posing a substantial impediment to the sustainable and ecologically sound cultivation of Largemouth bass. The absence of a viable control strategy has underscored the urgent need for innovative approaches to mitigate the impact of MSRV on the aquaculture industry, thereby preserving the integrity and health of this valuable fishery resource. This study presents the development and validation of a CRISPR/CasRx-based approach to target and reduce MSRV replication. This study designed specific CRISPR/CasRx RNAs (crRNAs) to target the MSRV's five structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and large protein (L). The construction of a CasRx-MSRV plasmid system was achieved and demonstrated potent antiviral activity against MSRV in both *in vitro* and *in vivo* models. Our initial evaluation focused on the CRISPR/CasRx system's ability to inhibit MSRV replication in GCO cells, with a particular emphasis on the L protein. The CasRx-MSRV-L system achieved a significant 97.79 % inhibition rate, corresponding to a five-log reduction in viral titer. This finding prompted us to further explore the therapeutic and prophylactic potential of the CasRx-MSRV-L system against MSRV infection in largemouth bass. While therapeutic application modestly extended the survival of infected fish, prophylactic administration significantly improved the survival rate of juvenile fish by 79.22 %. Collectively, these results underscore the promise of the CRISPR/CasRx system as a novel and potent tool for conferring resistance against MSRV, with broader implications for combating RNA viruses. Furthermore, our findings contribute to the burgeoning body of evidence supporting the application of CRISPR/CasRx technology as a strategic approach to combat RNA viruses, thereby laying a robust theoretical foundation for future research and development in this field.

1. Introduction

The Largemouth bass (*Micropterus salmoides*), colloquially referred to as the California bass, represents a commercially significant species in global aquaculture(Sun et al., 2023). Afflicted by the *Micropterus salmoides* rhabdovirus (MSRV), a negative-strand RNA virus classified within the Rhabdoviridae family, this species faces a considerable pathogenic challenge. The MSRV genome, encompassing approximately 11 kilobases of nucleotides, encodes for five essential proteins: the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and the polymerase or large protein (L)(Li et al., 2023). Notably, MSRV exhibits age-specific virulence, predominantly impacting juvenile Largemouth bass, with reported infection rates leading to nearly 80 to 90 % mortality among the fry population. These fatalities have precipitated substantial economic losses and pose a significant barrier to the advancement of the aquaculture industry(Li et al., 2023). Consequently, the imperative for the development and implementation of efficacious and secure disease management strategies to mitigate the impact of

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MSRV on this valuable fishery resource is both apparent and pressing.

The escalation in the aquaculture output of Largemouth bass (*Micropterus salmoides*) has been accompanied by an upsurge in disease-related challenges, particularly those posed by *Micropterus salmoides* rhabdovirus (MSRV). Consequently, research into MSRV and its mitigation strategies has intensified annually, albeit with a relatively narrow focus on host immune responses, vaccine development, and antiviral drug discovery. Studies on the MSRV-induced immune response have employed RNA sequencing, transcriptomics, immune parameter analysis, intestinal microbiome profiling, RNA interference, and single-virus tracking techniques. These investigations have elucidated that the intestinal tract is the principal portal of MSRV entry into the host, with the head kidney identified as a sensitive viral target tissue. Additionally, it has been observed that MSRV migrates to the external mucosal tissues during the later stages of infection, facilitating horizontal transmission (Yi et al., 2023). MSRV infection has been shown to activate host signaling pathways, including *RLR*, *NLR*, *NF-κB*, *Jak-STAT*, and apoptosis pathways, suggesting that an early interferon and pro-inflammatory response are instrumental in the host's resistance to MSRV (Gao and Chen, 2018; He et al., 2022). Furthermore, the intestinal microbiota's composition may correlate with the expression of immune-related genes in response to MSRV infection (Fei et al., 2022). The virus's entry mechanism into cells has been characterized as clathrin-dependent and pH-sensitive, proceeding *via* clathrin-mediated endocytosis and following the classical endosomal/lysosomal pathway post-internalization (Lu et al., 2023).

Vaccination stands as a pivotal strategy in the armamentarium against viral infections in fish, offering a prophylactic measure to safeguard these aquatic species from debilitating diseases. In the realm of *Micropterus salmoides* rhabdovirus (MSRV), advancements in vaccine development have been marked by the creation of various formulations, including immersion and oral vaccines (Lyu et al., 2019a; Xu et al., 2022), DNA vaccine (Ma et al., 2022; Yang et al., 2022)and live attenuated vaccine (Lijuan et al., 2018). Notably, innovative delivery systems have been explored, such as the utilization of single-walled carbon nanotubes (SWCNTs) (Guo et al., 2020; Liu et al., 2022) and bacterial nanocellulose (BNC) (Li et al., 2022a), to enhance the efficacy of the MSRV G protein subunit vaccines. These developments have laid a foundational theoretical framework for MSRV prevention and control.

Paralleling the progress in vaccine development, antiviral drug discovery has been propelled forward through the application of structure-activity relationship analysis and pharmacophore modeling. This systematic approach has yielded a repertoire of potential anti-MSRV compounds, encompassing arctigenin and its derivatives (Hu et al., 2022; Shen et al., 2020), coumarin and its derivatives (Hu et al., 2021), quinoline and its derivatives (Li et al., 2022b), magnolol and its derivatives (Jin et al., 2022), ribavirin (Yang et al., 2021a), ursolic acid (Li et al., 2023), prodigiosin (Song et al., 2023), neem amide (Zhang et al., 2023b), mangiferin (Dai et al., 2023), taurine, water extract of *Gentiana macrophylla* (Zhou et al., 2024a, 2024b), chlorogenic acid (Niu et al., 2023), sophoretin, epigoitrin, and limonin. Despite the identification of these promising candidates, it is imperative to recognize that none have yet transitioned to commercial availability for MSRV prevention and control. This underscores an urgent need for continued innovation and research in the domain of antiviral drug development.

In the contemporary landscape of molecular biology, an array of gene-editing technologies has emerged, with the CRISPR/Cas system at the forefront, offering a precise and sequence-specific means of manipulating DNA to rectify mutations(Gaj et al., 2013; Sander and Joung, 2014). CRISPR/Cas system is categorized into two primary modalities: multi-effector complex-mediated interference and single-effector-mediated interference, with the latter gaining significant attention due to its simplicity and efficiency (Makarova et al., 2020). Among the class II systems, the Cas13 family, a type VI system, has been identified as a unique RNA-targeting enzyme, distinguishing itself from the DNA-targeting Cas9 and Cas12 proteins (Tong et al., 2023). Notably, within the Cas13 family, CasRx (RfxCas13d) has been recognized for its compact size, minimal toxicity, and superior specificity and cleavage efficiency, positioning it as a promising candidate for RNA-targeting applications (Keng et al., 2023; Nguyen et al., 2020; Perez-SanJose et al., 2021). The CasRx system's potential as an antiviral tool has been extensively explored, with applications ranging from human immunodeficiency virus (HIV) (Nguyen et al., 2021) to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and its Delta and Omicron variants (Cui et al., 2022; Hussein et al., 2023; Liu et al., 2023; Zeng et al., 2022). Additionally, the system has demonstrated efficacy against hepatitis E virus (HEV)(Zhao et al., 2024), Seneca Valley virus (SVV) (Zhang et al., 2022)and a variety of plant viruses (Mahas et al., 2019; Zhan et al., 2023; Zhang et al., 2022), as well as the grouper nervous necrosis virus (RGNNV) (Wang et al., 2021a). Collectively, these studies substantiate the CasRx system's robust antiviral capabilities against a spectrum of RNA viruses, heralding a new era in antiviral research and therapeutics.

In our investigation, we harnessed the CRISPR/CasRx (RfxCas13d) system, an innovative RNA-targeting tool, to explore its capacity to interfere with RNA viruses. Specifically, we tailored CRISPR/CasRx-derived RNAs (crRNAs) to target the five structural proteins-nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and large polymerase protein (L)-of *Micropterus salmoides* rhabdovirus (MSRV). Subsequently, we engineered CasRx-MSRV plasmids to evaluate the system's antiviral efficacy against MSRV both *in vitro* and *in vivo*. Our findings demonstrated that the CasRx-MSRV system exerted a potent antiviral effect, indicating its potential as a therapeutic intervention against MSRV infections.

2. Materials and methods

2.1. Virus,cell and fish

The strain of MSRV-FJ985 (NCBI: MT818233.1) was isolated from MSRV-infected *Micropterus salmoides* and preserved in our laboratory (Yang et al., 2021b). MSRV was proliferated in GCO (Grass carp ovary cell lines) cells (kindly provided by Zhejiang Institute of Freshwater Fisheries, Huzhou, Zhejiang, China) as previously described (Lyu et al., 2019b; Yang et al., 2021b).

Virus proliferation: The frozen MSRV virus suspension was taken out from the liquid nitrogen tank and added to the monolayer GCO cells growing to 80 % - 90 % by volume ratio of 0.1 %. After 48 h of culture at 25 °C, the GCO cells were observed by inverted microscope. When 80 % of the cells showed cytopathic effect (CPE), the cell suspension containing MSRV was collected, centrifuged at 4 °C, 12000 rpm for 10 min, and then packed into a 1.5 mL centrifuge tube. Store in a refrigerator at −80 °C or in liquid nitrogen.

The experimental fish of largemouth bass comes from our experiment to breed by ourselves and we detected common pathogens of largemouth bass, including MSRV, Largemouth Bass Virus (LMBV), *Nocardia spp.* and *Aeromonas spp.*, and also detected surface parasites. All tests showed that the test fish did not carry common pathogens of largemouth bass. The average body length and body weight of largemouth bass ($n = 60$) were 4 ± 0.5 cm and 1.7 ± 0.2 g, respectively. Before the start of the experiment, the fish were acclimated at 25 °C for 14 days in a pond with a flowing water circulation system. During this period, commercial feed (Fuxing Biological Feed Co., Ltd.) was fed twice a day. The animal use program was approved by the Animal Health and Use Committee of Northwest A & F University (IACUC).

2.2. Design of MSRV crRNAs

The crRNA design of MSRV referred to previous studies (Guo et al., 2021; Wessels et al., 2020). The genomes of MSRV was downloaded from the NCBI database, the Cas13d guide RNA design website (https://cas13design.nygenome.org/) was used to design the guide RNA of

the five proteins of MSRV. A total of 1282 MSRV-N crRNA, 845 MSRV-P crRNA, 641 MSRV-M crRNA, 1450 MSRV-G crRNA and 6024 MSRV-L crRNA were designed (See supplementary material for details). According to the score given by the Cas13d guide RNAs tool, five MSRV crRNAs with the highest activity were selected and named as MSRV-N-crRNA, MSRV-P-crRNA, MSRV-M-crRNA, MSRV-G-crRNA, and MSRV-L-crRNA, respectively. The sequence information is shown in Table 1. The position of crRNA in the MSRV genome is shown in Fig. 1A.

2.3. Generation of plasmids

2.3.1. CRISPR/CasRx plasmid

The CRISPR/CasRx plasmid purchased in HedgehogBio Science and Technology Ltd. (Shanghai, China). At the same time, we modified the original plasmid and deleted the CRISPR RNA (*Nhe* I, *Sal* I restriction sites) as an empty plasmid (CasRx-NT). The CRISPR / CasRx plasmid and CasRx-NT plasmid are shown in Fig. 1. B; The crRNA restriction site is shown in Fig. 1C.

2.3.2. MSRV crRNA sequence primers synthesize

According to the CRISPR/CasRx plasmid restriction site, the crRNA-F (crRNA sequence) and crRNA-R (crRNA reverse complementary sequence) sequences were synthesized by adding a linker localization sequence to the crRNA. The sequence was synthesized by Sangon Biotech (Shanghai) Co., Ltd. and purified by PAGE (Polyacrylamide gel electrophoresis). The primers of crRNA synthesis sequence are shown in Table 2.

Each pair of crRNA-F and crRNA-R was diluted with water to a concentration of 50 μM, 1 μL each, and ddH_2O was added to 10 μL. The PCR instrument was used for annealing reaction (95 °C, 5 min). After the annealing reaction is completed, it is cooled to room temperature, and the connection reaction is carried out immediately or stored at −20 °C.

2.3.3. Plasmid enzyme-cut and link up

The CRISPR/CasRx plasmid was digested according to the *Bbs* I (NEB) restriction endonuclease instruction. The reaction system was 1 μg plasmid, 5 μL 10 × NEBuffer, 1 μL *Bbs*I restriction enzyme, and ddH_2O was added to 50 μL. The digestion reaction solution was gently mixed with a pipette gun, incubated at 37 °C for 5–10 min, 65 °C for 20 min, after the reaction, agarose gel electrophoresis of nucleic acid was performed, and the digested products were recovered. The recovered product was immediately subjected to a ligation reaction or stored at −20 °C for later use.

According to the T4-Ligase (Takara) ligase instruction, the crRNA double-strand annealing product was ligated to the digested plasmid. The reaction system consisted of 2 μL 10 × ligation buffer, 2 μL enzyme digestion plasmid, 6 μL double-stranded annealed crRNA, 2 μL T4 DNA Ligase, and ddH_2O to 20 μL. The reaction system was gently mixed with a pipette and reacted at 16 °C for 1–5 h. The ligation products were immediately transformed or stored at-20 °C.

The ligation product was transformed according to the instructions of Stbl3 competent cells (AngYuBio, China). The specific steps are as follows: The Stbl3 competent cells were placed in ice to melt. When the cells were in a mixed state of ice and water, the target DNA (plasmid or ligation product) was added and gently mixed with the bottom of the EP tube by hand, and stood on ice for 25 min. 42 °C water bath heat shock 45 s, quickly put back on the ice and stand for 2 min; add 0.9 mL room temperature S.O.C. medium or LB medium to the centrifuge tube, resuscitate at 30 °C 225 rpm for 90 min; centrifuged at 5000 rpm for one minute, the supernatant was taken, and 100 μL of the supernatant was gently blown and resuspended on the S.O.C.medium containing the corresponding antibiotics. The plate was inverted and placed in an incubator at 30 °C for overnight culture; a single colony of the plate was picked and cultured in a liquid medium containing ampicillin.

Table 1
The MSRV crRNA sequences.

crRNA sequence name	Sequence
MSRV-N-crRNA	CAAATGTTGACAGAGCTGCACAG
MSRV-P-crRNA	ATCATCATCAAAGATCGGCCCAG
MSRV-M-crRNA	GCACTCATAAGAGATGGCAACCG
MSRV-G-crRNA	TTTTCAAAAGATATGGCAAGCAT
MSRV-L-crRNA	ATCAATAAATGGATGCCCCCAGT

2.3.4. Plasmid sequencing identification

Using the bacterial plasmid extraction kit (TIANGEN, Beijing, China), the expanded bacteria were subjected to plasmid extraction. Using 2 × Rapid Taq Master Mix (Vazyme, Nanjing, China), U6 Primer (5' -ATGGACTATCATATGCTTACCGTA-3') and crRNA-R as primers for PCR amplification. The reaction system was 0.5 μL U6 primer, 0.5 μL crRNA-R, 11 μL PCR Master Mix, and ddH_2O was added to 20 μL, and the reaction procedure is 95 °C 5 min, 30 cycles of 95 °C 30 s, 60 °C 30 s and 72 °C 60 s, 72 °C 10 min. The amplified products were sequenced and identified, and the correctly sequenced bacteria were preserved and expanded, and the plasmid was extracted using the endotoxin-free plasmid extraction kit (TIANGEN, Beijing, China). According to the above operations, five plasmids targeting the five structural proteins of MSRV were constructed, which were CasRx-MSRV-N, CasRx-MSRV-P, CasRx-MSRV-M, CasRx-MSRV-G and CasRx-MSRV-L, respectively.

2.4. Cell transfection and CasRx antiviral effect

2.4.1. Plasmid transfection detection and optimal transfection time

According to the instruction of Lipo8000™ transfection reagent (Beyotime, Shanghai, China), 6 smids were transfected first. After 12, 24, 36, 48, 60 and 72 h of transfection, inverted fluorescence microscope (OLYMPUS CKX53, Jepen) was used to observe whether the plasmid could be successfully transfected and normally expressed in GCO cells. The optimal virus infection time after transfection was determined by fluorescence quantity and fluorescence intensity. After determining the peak time of plasmid transfection expression, the cells were collected for flow cytometry analysis (BD FACSAria™ III, USA) to determine the transfection efficiency.

The specific operation of plasmid transfection is as follows: about 2–7 × 10^5 cells per well were inoculated into six-well plates for culture on the day before transfection (18–24 h), so that the cell density reached about 70–80 % on the second day; before transfection, the six-well plate with cells was replaced with 2 mL fresh culture medium per well. A clean sterile centrifuge tube was taken. For each well in the six-well plate to be transfected, 125 μL of Opti-MEM ® Medium serum-reduced medium without antibiotics and serum was added to each well of the cells to be transfected. Add 2.5 μg plasmid DNA, and gently blow and mix with a pipette gun; add 4 μL Lipo8000 ™ transfection reagent, gently blow and mix; the prepared Lipo8000 ™ transfection reagent-DNA mixture, 125 μL per well, was evenly dropped into the well and gently mixed. After 24 h of continuous culture, the transfection effect was observed by inverted fluorescence microscope.

2.4.2. Anti-MSRV effect of CRISPR/CasRx in vitro

Cell transfection was divided into 8 treatment groups, N, P, M, G, L, A, MSRV infection positive control group and negative control group. Group A was the transfection group mixed with five plasmids, and the MSRV infection positive control group and the negative control group were transfected with CasRx-NT plasmid.

Cell culture and transfection were carried out according to the above operation, with 6 replicates in each group. MSRV diluent with a concentration of 10^3 $TCID_{50}$ was added at the best time after transfection (virus mother titer was 10^7 $TCID_{50}$/mL (48 h), diluted with M199 medium), and incubated at 25 °C for 2 h; after 2 h of virus incubation, the virus solution was aspirated, and the cells were rinsed with M199 medium for 2–3 times. Fresh medium was added and cultured at 25 °C for

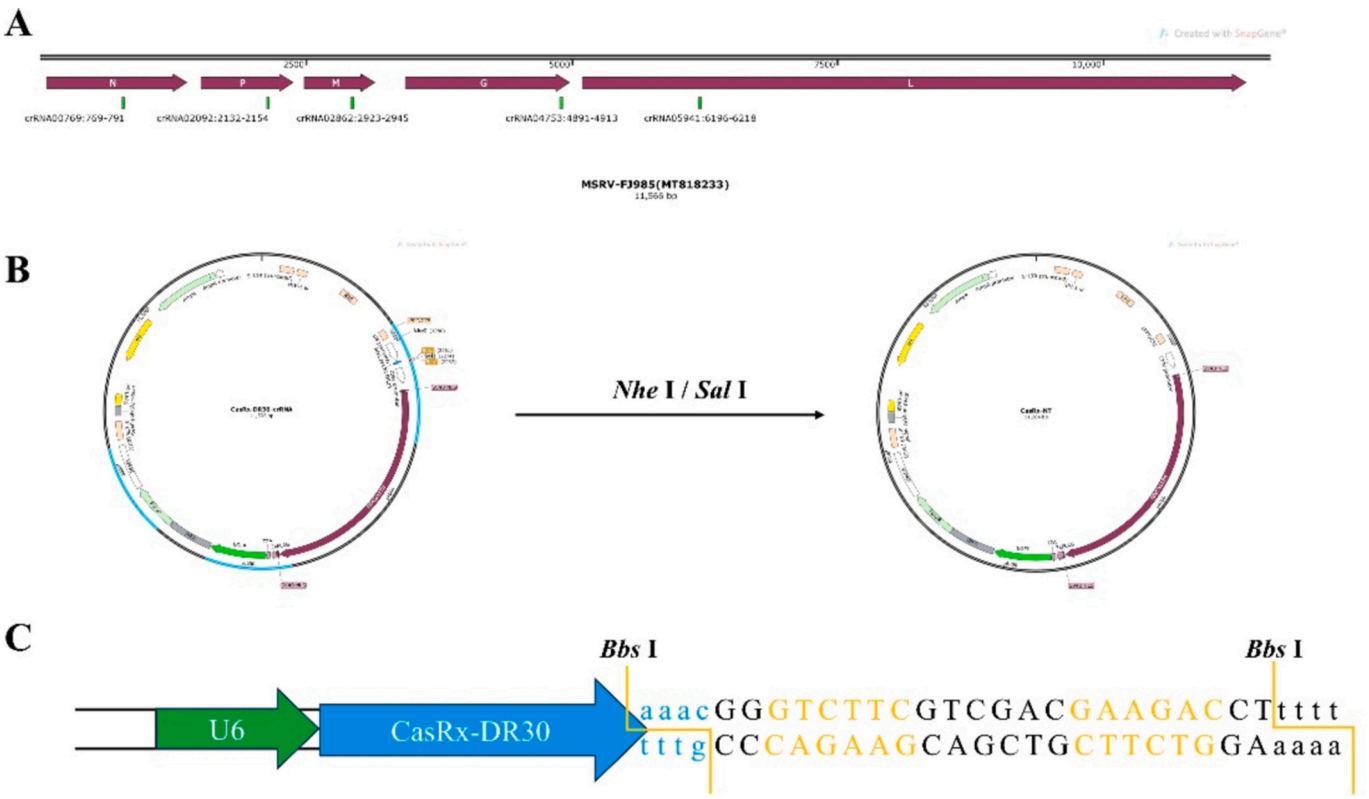

Fig. 1. A: The location of crRNAs in MSRV genome; B: The backbone of CRISPR/CasRx plasmid was digested with *Nhe* I and *Sal* I, and the DR30-crRNA structure was removed to obtain CasRx-NT plasmid.; C: The CRISPR/CasRx plasmid crRNA restriction site map. Plasmid map produced using Snapgene software.

Table 2
Sequences of primers for this study.

Primer name	Primers sequence(5′ - 3′)
MSRV-N-crRNA	F: aaacCAAATGTTGACAGAGCTGCACAG R: aaaaCTGTGCAGCTCTGTCAACATTTG
MSRV-P-crRNA	F: aaacATCATCATCAAAGATCGGCCCAG R: aaaaCTGGGCCGATCTTTGATGATGAT
MSRV-M-crRNA	F: aaacGCACTCATAAGAGATGGCAACCG R: aaaaCGGTTGCCATCTCTTATGAGTGC
MSRV-G-crRNA	F: aaacTTTTCAAAAGATATGGCAAGCAT R: aaaaATGCTTGCCATATCTTTTGAAAA
MSRV-L-crRNA	F: aaacATCAATAAATGGATGCCCCCAGT R: aaaaACTGGGGGCATCCATTTATTGAT
MSRV-N (RT-qPCR)	F: GCCCACATCGCATCATTCAC R: GTGGCAGAGTAAGGGGACAC

48 h (MSRV infection positive control group almost completely cytopathic effect). Three replicates in each group were used for crystal violet staining for EPC observation, determination of virus titer in supernatant (the $TCID_{50}$ was determined as described by Reed and Mench), cell morphology observation and the remaining three replicates were used for viral nucleic acid extraction using a viral nucleic acid extraction kit (TransGen Biotech, Beijing, China) for subsequent RT-qPCR experiments.

2.4.3. Anti-MSRV effect of CRISPR/CasRx in vivo

We will use the CasRx plasmid with the best antiviral activity *in vitro* for *in vivo* antiviral test. Each gram of fish was injected with 1 μg CasRx plasmid, and CasRx-NT plasmid was injected into the positive and negative control groups of virus infection. Two days after plasmid injection, MSRV infection was performed as a virus infection prevention test. At the same time, MSRV infection was first performed , plasmid injection was performed at 8 h after infection as a viral infection therapy group. MS-222 was used to anesthetize largemouth bass before all injection operations. The cumulative number of deaths was recorded every day after virus infection, and the 14-day survival curve was drawn.

2.5. Construction of absolute fluorescence quantitative PCR standard curve

2.5.1. Construction of standard plasmid pMD19T-MSRV-N

The MSRV virus RNA was extracted and reverse transcribed to obtain cDNA. The N protein gene fragment was used as the target gene for PCR amplification to obtain an 182 bp length amplification fragment. The amplification system is the same as described above. The primers are shown in Table 2 (Shen et al., 2020). The reaction procedure is 95 °C 5 min, 95 °C 30 s, 55 °C 30 s and 72 °C 60 s, 72 °C 10 min, 30 cycles. The PCR products were electrophoresed using 1 % agarose gel, and the bands consistent with the size of the target fragment were cut and the gel was recovered.

The MSRV-N fragment amplified by PCR was ligated according to pMD ™ 19-T Vector Cloning Kit (Takara) and transformed into DH5α competent cells (AngYuBio, China). After the monoclonal colony was expanded and cultured, the bacterial plasmid was extracted and sequenced. The sequencing was correct, and the standard plasmid pMD19T-MSRV-N was obtained.

2.5.2. Construction of standard curve

The DNA concentration of pMD19T-MSRV-N plasmid was measured by ultramicro spectrophotometer (NanoDrop OneC, Thermo) and converted into plasmid copy number. Diluted with sterile water to 1×10^9, 1×10^8, 1×10^7, 1×10^6, 1×10^5, 1×10^4, 1×10^3, 1×10^2 eight gradient copy number, Each copy number was repeated 8 times using Taq-HS SYBR ® Green qPCR Premix (Yugong Biotech, China) for qPCR to construct an absolute quantitative standard curve for MSRV-N. The reaction system was 10 μL qPCR Premix, MSRV-N Forward (10 μM) and

MSRV-N Reverse(10 μM) each 0.4 μL, 1 μL pMD19T-MSRV-N, and Nuclease-Free Water to 20 μL. The reaction program was 95 °C 5 min, 95 °C 15 s, 60 °C 30 s, 40 cycles.

3. Results

3.1. Plasmid transfection detection and optimal transfection time

To ascertain the expression kinetics and transfection efficiency of six recombinant plasmids in GCO cells, we implemented a temporal analysis coupled with fluorescence microscopy and flow cytometry. Post-transfection, cells were examined at intervals of 12, 24, 36, 48, 60, and 72 h using an inverted fluorescence microscope. Fluorescence microscopy at 48 h post-transfection revealed a peak in expression for all plasmids(analysis by ImageJ software), as evidenced by both the number and intensity of fluorescent signals (Fig. 2).

Subsequently, at the 48-h mark, cells from each transfection group were subjected to flow cytometry to quantify transfection efficiency. The proportion of EGFP-positive cells, indicating successful transfection, was measured as follows: 7.02 % for the N group, 6.99 % for the P group, 7.03 % for the M group, 6.99 % for the G group, 6.85 % for the L group, 6.41 % for the A group, and 8.05 % for the NT group. These results highlight a moderate yet consistent transfection efficiency across all groups, with the NT group demonstrating the highest rate (Fig. 3).

3.2. MSRV-N absolute fluorescence quantitative curve

The standard plasmid pMD19T-MSRV-N was sequenced correctly and successfully constructed. The standard plasmid pMD19T-MSRV-N was subjected to qPCR, and the absolute quantitative standard curve of MSRV-N was constructed according to the quantitative results and virus copy number. The obtained absolute quantitative standard curve equation was $y = -3.2627\times + 36.71$, the correlation coefficient $R^2 = 0.99$, and the amplification efficiency $E = 2.03$, indicating that the standard plasmid pMD19T-MSRV-N absolute quantitative curve was successfully constructed and had high linearity. The graphical representation of our qPCR data includes the amplification curve (Fig. 4A), which illustrates the cycle threshold (Ct) values against the dilution series of the plasmid; the melt curve (Fig. 4B), providing insight into the specificity of the qPCR assay by showing a single peak corresponding to the expected Tm; and the standard curve (Fig. 4C), which is a testament to the assay's quantitative capabilities.

3.3. Anti-MSRV effect of CRISPR/CasRx in vitro

Following the transfection of plasmids into GCO cells, an interval of 48 h was allowed before initiating MSRV infection to ensure adequate expression of the transfected constructs. In the positive control group, cells were infected with MSRV for a duration of 48 h. Post-infection, both the morphological changes in the cells and the presence of fluorescence were meticulously observed to assess the cytopathic effects (CPE) induced by MSRV. To further characterize the infected cells, crystal violet staining was conducted, which not only facilitates the visualization of CPE but also provides a means to quantify the extent of infection.

The experimental findings revealed a significant outcome: GCO cells that had been transfected with the CasRx plasmid, specifically designed to target MSRV, demonstrated a marked reduction in the cytopathic effects typically associated with MSRV infection. This observation suggests that the CasRx plasmid can effectively counteract the detrimental effects of the virus on cell morphology. Moreover, the cells exhibited an increased level of resistance to MSRV infection, implying a potential protective effect conferred by the CasRx plasmid.

In addition to the observed reduction in CPE, fluorescence observation at 96 h post-transfection revealed the persistent presence of EGFP fluorescence, albeit at reduced levels. This finding indicates that the transfected plasmid was not only successfully taken up by the cells but also maintained its expression over an extended period. The maintenance of fluorescence provides a visual confirmation of the plasmid's continued influence within the cells.

The visual and staining results, encapsulated in Fig. 5, offer a compelling visual representation of the experiment's outcomes. The figure is expected to include images of cell morphology post-MSRV infection, fluorescence microscopy images to depict EGFP expression,

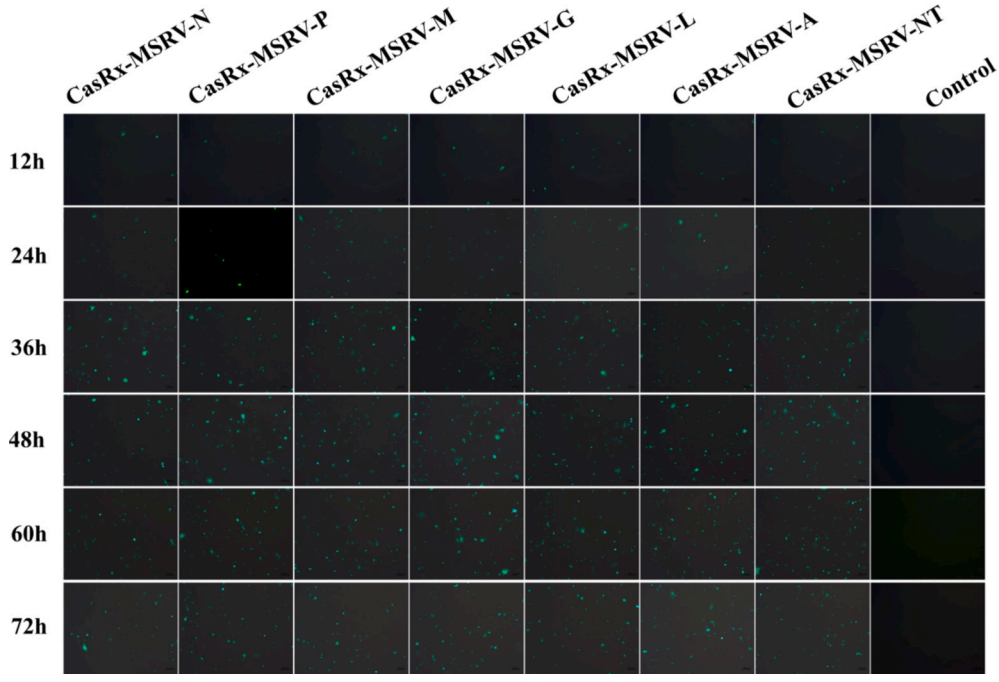

Fig. 2. The expression of EGFP fluorescence in GCO cells at 12, 24, 36, 48, 60 and 72 h after transfection in each plasmid transfection group. The results showed that GCO cells peaked at 48 h after transfection.

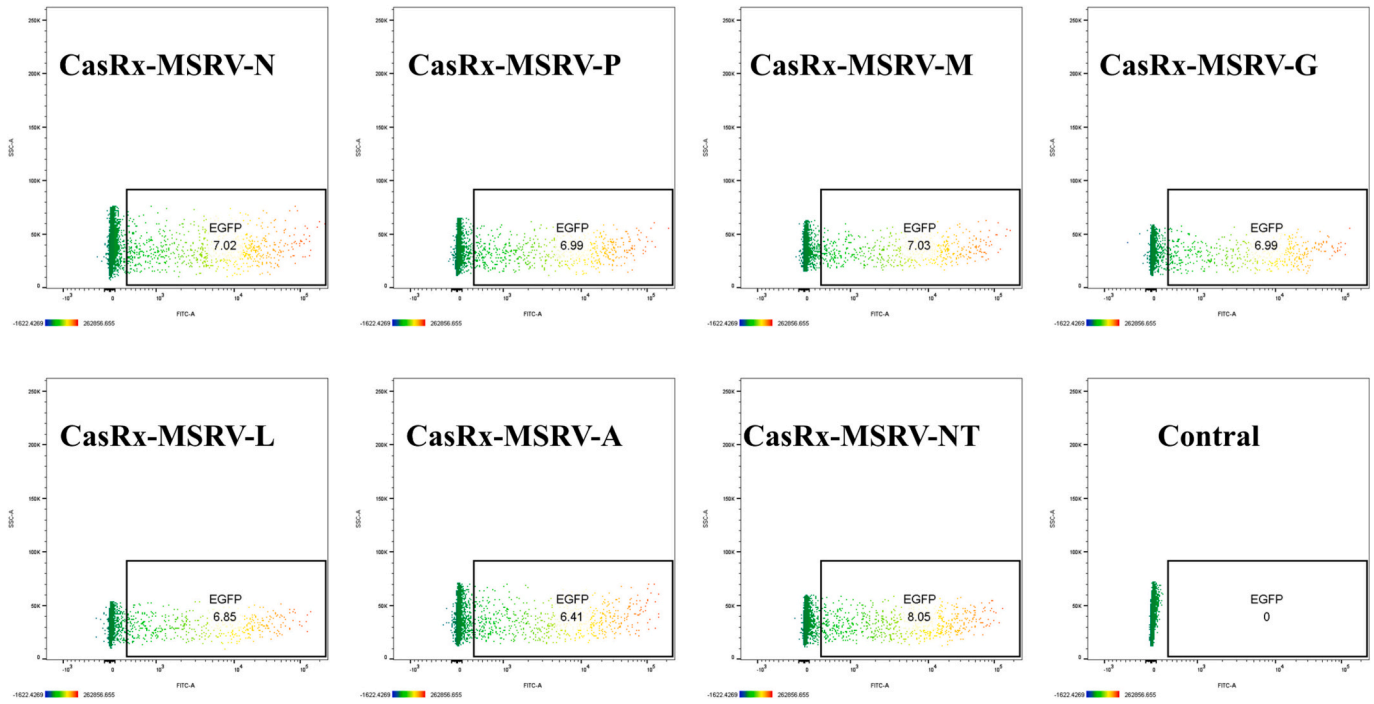

Fig. 3. The transfection efficiency of each group the results of EGFP sorting by flow cytometry showed that the transfection efficiency of each group was 7.02 % in group N, 6.99 % in group P, 7.03 % in group M, 6.99 % in group G, 6.85 % in group L, 6.41 % in group A and 8.05 % in group NT, respectively.

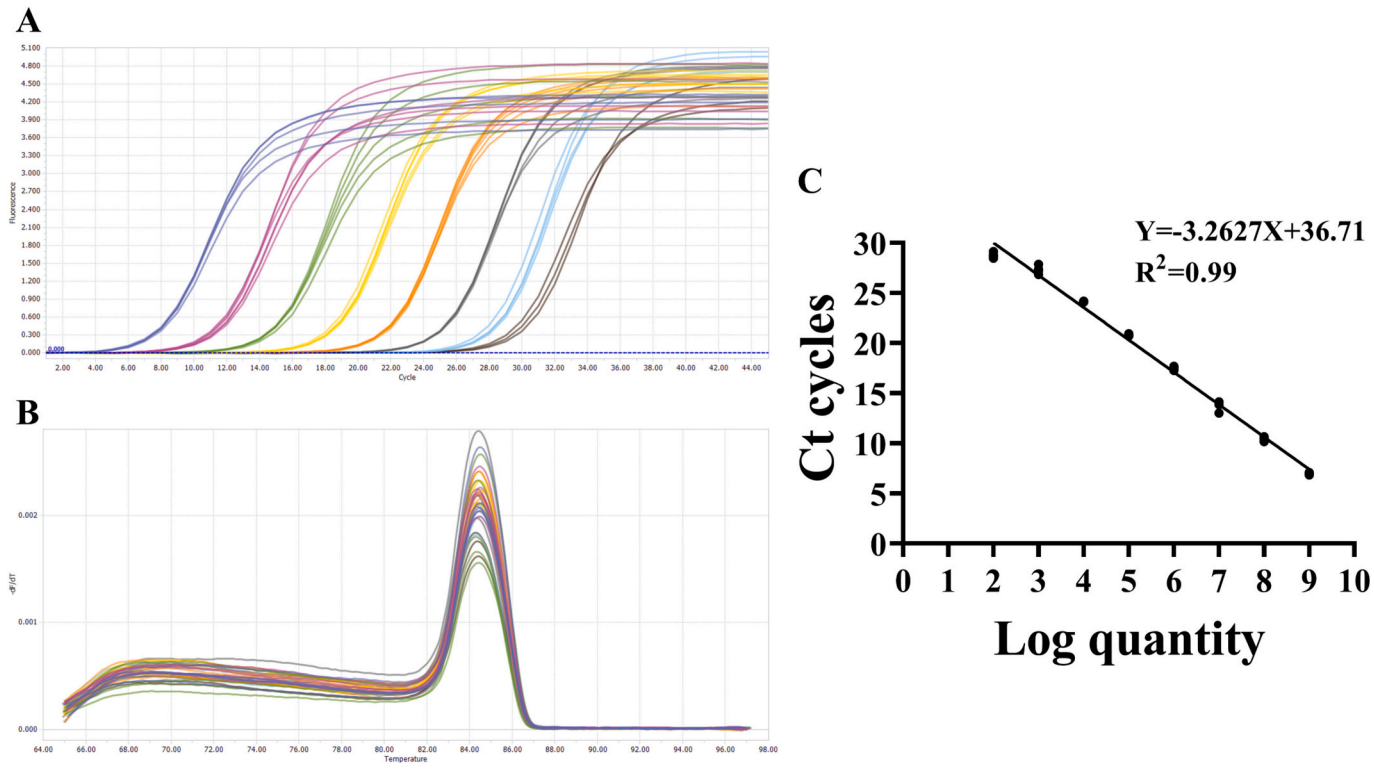

Fig. 4. The quantitative curve and dissolution curve of pMD19T-MSRV-N showed good amplification effect. The obtained absolute quantitative standard curve equation was $y = -3.2627\times + 36.71$, the correlation coefficient $R^2 = 0.99$, and the amplification efficiency $E = 2.03$, indicating that the standard plasmid pMD19T-MSRV-N absolute quantitative standard curve was successfully constructed and had a high linear relationship. A: Absolute quantitative amplification curve of MSRV-N; B: MSRV-N absolute quantitative dissolution curve; C: MSRV-N absolute quantitative standard curve.

and crystal violet staining to assess the extent of CPE and cell viability.

The outcomes of the reverse transcription quantitative polymerase chain reaction (RT-qPCR) assay are graphically represented in Fig. 6A. This assay was conducted to evaluate the impact of CRISPR/CasRx plasmids, each targeting a distinct structural protein of the MSRV, on viral replication within GCO cells. Post-transfection, a significant

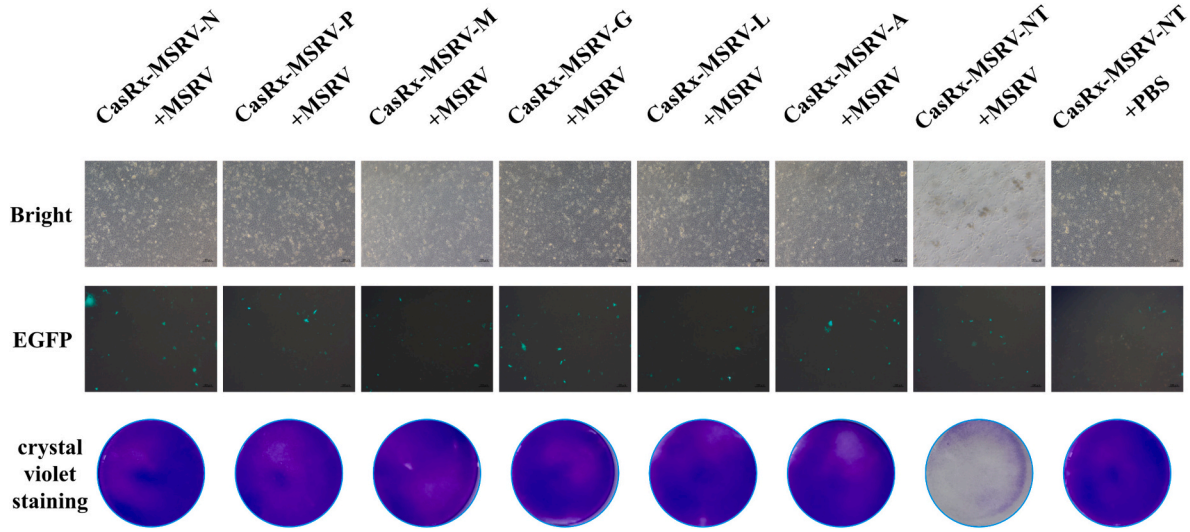

Fig. 5. The GCO cells transfected with each group of plasmids were infected with MSRV for 48 h. The results of light microscopy and crystal violet staining showed that the CasRx system had a good antiviral effect on MSRV, maintaining the normal morphology of the cells and avoiding cell death. At the same time, it showed that the expression of green fluorescent protein EGFP could still be observed in 96 after transfection. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

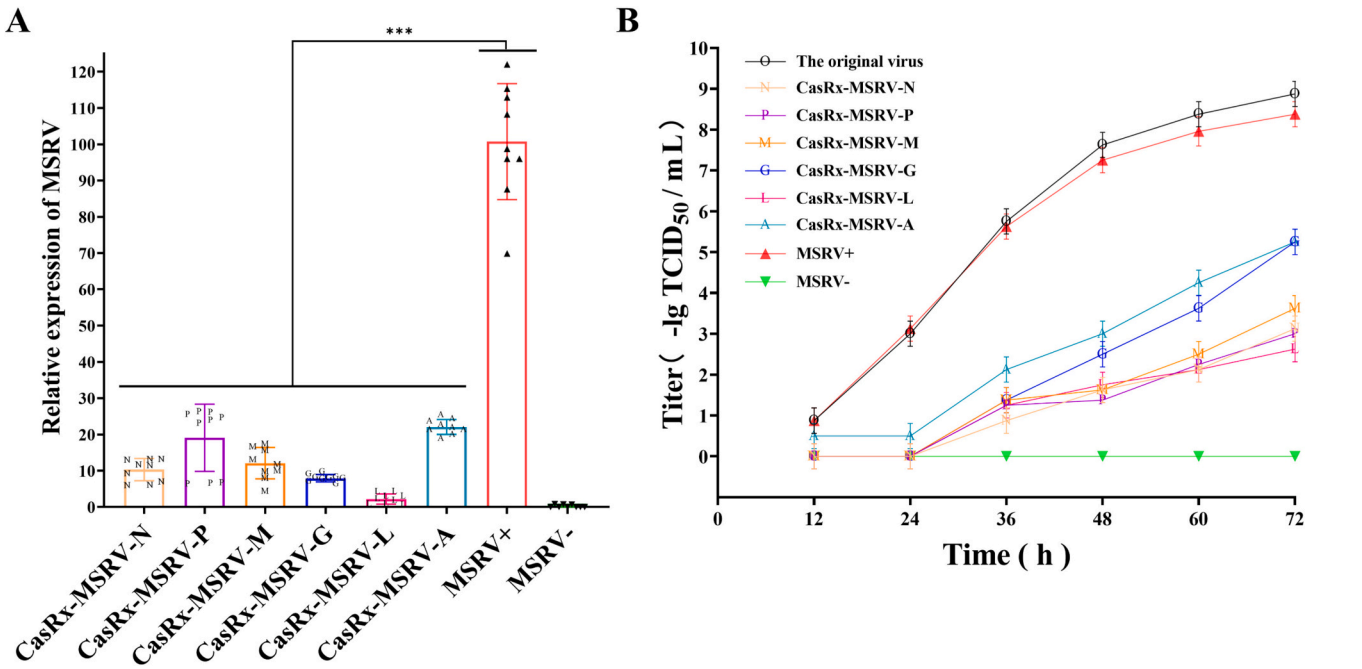

Fig. 6. The results of *in vitro* virus inhibition rate and cell culture supernatant titer determination showed that the virus inhibition rates of N, P, M, G, L and A groups on MSRV were 89.70 %, 80.91 %, 87.91 %, 92.08 %, 97.79 % and 77.93 %, respectively. The virus titers of the cell culture supernatant of each group at 72 h were $1 \times 10^{3.125}$ $TCID_{50}$/mL in group N, 1×10^{3} $TCID_{50}$/mL in group P, $1 \times 10^{3.625}$ $TCID_{50}$/mL in group M, $1 \times 10^{5.25}$ $TCID_{50}$/mL in group G, $1 \times 10^{2.625}$ $TCID_{50}$/mL in group L, $1 \times 10^{5.25}$ $TCID_{50}$/mL in group A and $1 \times 10^{8.25}$ $TCID_{50}$/mL in group MSRV infection positive control, respectively. A: Relative expression of MSRV-N gene; B: Virus titer of cell culture supernatant. Values are presented as mean ± SD. ***, $p < .001$.

reduction in MSRV copy numbers was observed when compared to the positive control group, which was infected with MSRV only. Specifically, the N, P, M, G, L, and A plasmid transfection groups exhibited inhibition rates of 89.70 %, 80.91 %, 87.91 %, 92.08 %, 97.79 %, and 77.93 %, respectively. These figures underscore the potency of these plasmids in curtailing MSRV replication, with the CasRx-MSRV-L plasmid demonstrating the highest inhibition rate. Complementary to the RT-qPCR data, virus titer assessments were carried out on the cell culture supernatant from each plasmid transfection group at 72 h post-infection. The results, depicted in Fig. 6B, revealed substantial variation in virus titers among the groups. The titers were as follows: $1 \times 10^{3.125}$ TCID50/mL for group N, 1×10^{3} $TCID_{50}$/mL for group P, $1 \times 10^{3.625}$ $TCID_{50}$/mL for group M, $1 \times 10^{5.25}$ TCID50/mL for group G, $1 \times 10^{2.625}$ TCID50/mL for group L, $1 \times 10^{5.25}$ $TCID_{50}$/mL for group A, and a notably higher titer of $1 \times 10^{8.25}$ $TCID_{50}$/mL for the MSRV infection positive control group. The CasRx-MSRV-L plasmid transfection group stood out with the lowest virus titer, indicative of its superior antiviral activity.

Given the outstanding performance of the CasRx-MSRV-L plasmid in inhibiting MSRV replication, as evidenced by both the RT-qPCR and virus titer assays. Therefore, we decided to use CasRx-MSRV-L plasmid

to verify the antiviral effect of MSRV in largemouth bass.

3.4. Anti-MSRV effect of CRISPR/CasRx in vivo

Based on the promising results from the *in vitro* antiviral assays, which indicated that the CasRx-MSRV-L plasmid exhibited superior antiviral activity, we proceeded to evaluate its efficacy in an *in vivo* model. The *in vivo* tests were designed to compare the survival rates of different groups following the administration of the CasRx-MSRV-L plasmid and subsequent MSRV infection. The survival curve, depicted in Fig. 7, illustrates a stark contrast between the negative control group, which remained free of MSRV infection and thus experienced no mortality within the 14-day observation period, and the positive control group, where all individuals succumbed to the virus within 7 days post-infection. This comparison underscores the severity of MSRV infection and the urgency of effective intervention.

Among the test groups, the preventive administration of the CasRx-MSRV-L plasmid demonstrated a significant protective effect, with a survival rate of 79.22 % against MSRV infection. This rate is markedly higher than that of the positive infection control group and indicates a substantial enhancement in the survival outcome due to the plasmid's antiviral activity. However, the therapeutic application of the CasRx-MSRV-L plasmid, initiated after MSRV infection, did not yield a significant difference in survival rate compared to the positive control group. The therapeutic group exhibited only a modest delay in death time by two days, suggesting that the timing of plasmid administration may be critical in determining its protective efficacy.

These findings, while highlighting the potential of the CasRx-MSRV-L plasmid as a prophylactic agent, also underscore the need for further research to optimize its therapeutic application. The results emphasize the importance of developing effective antiviral strategies that can be administered both preventively and therapeutically in the context of MSRV infection.

4. Discussion

Our research delineates the profound antiviral efficacy of the CRISPR/CasRx system against the largemouth bass rhabdovirus, evidencing marked suppression in both *in vivo* and *in vitro* experimental paradigms. Confronted with the quintessential challenge of low transfection efficiency in fish cell lines, as previously elucidated by Goswami et al. (Goswami et al., 2022), our application of flow cytometry revealed a transfection rate of GCO cells at a mere 7 %. Nonetheless, the CRISPR/CasRx system, and particularly the construct tailored for the MSRV L protein, demonstrated unparalleled potency in curtailing MSRV replication, an outcome that was corroborated within our *in vivo* assays.The antiviral efficacy of the CRISPR/CasRx system surpasses that of a plethora of conventionally screened pharmaceuticals and RNA interference (RNAi) modalities, as per a meticulous review of extant literature(Hu et al., 2022; Hu et al., 2024; Jin et al., 2022; Li et al., 2023; Li et al., 2022; Liu et al., 2024; Niu et al., 2023; Shen et al., 2020; Yang et al., 2021b; Yang et al., 2024; Yuan et al., 2023; Zhang et al., 2023a; Zhang et al., 2024; Zhou et al., 2024a, 2024b). The CRISPR/CasRx system distinguishes itself through its capacity to directly cleave viral RNA genomes and mRNA transcripts, a mechanism that is both innovative and highly specific (Keng et al., 2023). The L protein, pivotal in the MSRV life cycle, is integral not only to the formation of the rhabdovirus nucleocapsid through interactions with the N and P proteins and viral RNA, but also to the essential enzymatic activities of transcription, replication, polyadenylation, capping, and methylation of viral mRNA, as characterized by Wagner (Wagner, 1987).

In recent years, CRISPR / Cas system has been used as a new antiviral strategy for anti-virus (Mahas and Mahfouz, 2018). This innovative approach was concurrent with the development of diverse Cas proteins, each offering unique capabilities within the realm of genetic manipulation. The subsequent exploration by researchers to harness the CRISPR/Cas system as an antiviral instrument has led to a proliferation of studies targeting a spectrum of viral species (Freije et al., 2019). Notably, the Cas13 family has emerged as a pivotal RNA virus interference tool, with various Cas13 variants demonstrating potent and specific antiviral activity through transient and stable overexpression experiments (Kushawah et al., 2020; Mahas et al., 2019). Among these, CasRx has garnered particular interest due to its compact size, which facilitates the use of adeno-associated viruses (AAV) as vectors for gene therapy. This attribute renders the CRISPR/Cas13d system a promising candidate for a new generation of simple, flexible, and rapid therapeutics and preventatives against RNA virus infections (Nguyen et al., 2020). The prospect of integrating such a system into clinical

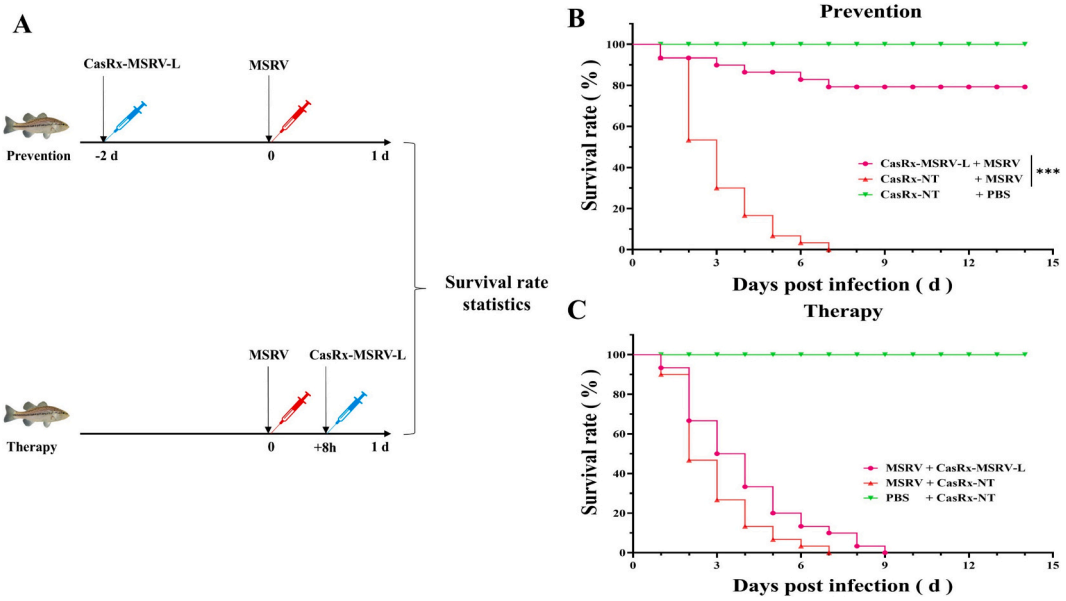

Fig. 7. The CasRx-MSRV-L plasmid system was used to prevent and treat MSRV infection in largemouth bass. The results showed that the CasRx-MSRV-L plasmid system had a significant preventive effect on MSRV and could therapy MSRV-infected fish to delay the death time. A: A schematic diagram of the anti-MSRV effect of CRISPR/CasRx *in vivo*; B: Survival rate curve of prevention group; C: Survival rate curve of therapy group.

applications heralds a transformative era in the management of viral diseases.

In the realm of aquatic animal research, the application of CRISPR/CasRx technology for antiviral intervention has been notably sparse, with a seminal study by Wang et al. (Wang et al., 2021b) reporting its successful deployment in Epinephelus. This work demonstrated the efficacy of a tailored CasRx system to impede the red-spotted grouper nervous necrosis virus (RGNNV), achieving significant interference with viral infection in both *in vitro* and *in vivo* settings. Wang et al. designed and constructed crRNAs for the two genes CP and RdRp of RGNNV, respectively. *In vitro* experiments, both single crRNA and combined crRNA significantly reduced the CPE phenomenon and viral RNA copy number of cells, and reduced the infectivity of RGNNV by 200–300 times, and the virus titer was reduced by about 3 logarithmic levels. *In vivo* experiments, intracranial injection of CasRx system significantly reduced the level of viral mRNA, reduced brain tissue lesions, and increased the survival rate of grouper by 50–60 %. The findings corroborate the potential of CRISPR/CasRx to engineer interference against fish RNA viruses, aligning with the outcomes of our own investigations.However, our attempt to employ plasmid injection as an antiviral therapeutic in MSRV-infected juvenile fish yielded null therapeutic effects, merely extending the survival of the treated group by two days. The advantage of plasmid DNA-mediated CRISPR/Cas system is that plasmid DNA is easy to construct and the production cost is low (Wang, 2018). However, Cas proteins need to be produced through transcription and translation pathways, and gene editing time is delayed. This shortfall in therapeutic response is hypothesized to stem from the suboptimal expression levels or kinetics of the injected CasRx plasmid system, which were outpaced by the rapid infection and replication dynamics of the virus, thereby failing to manifest the prophylactic antiviral efficacy observed in preventive applications(Zhou et al., 2024a, 2024b). Consequently, we postulate that the utilization of CasRx RNA and crRNA complexes or ribonucleoprotein (RNP) complexes for antiviral therapy may engender superior outcomes, a notion supported by the collective findings within the field(Freije et al., 2019; Singsuksawat et al., 2021). The exploration of these advanced delivery modalities is imperative for optimizing the CRISPR/CasRx system's utility in combating viral pathogens in aquatic species.

The advent of cutting-edge technologies in gene editing and vector delivery has marked a significant paradigm shift in the therapeutic landscape, as delineated by Yahya and Alqadhi (Yahya and Alqadhi, 2021). Gene therapy has emerged as a potent modality in the arsenal against intractable conditions, including genetic disorders and malignancies, with a burgeoning body of evidence attesting to its efficacy (Arabi et al., 2022; Ma et al., 2020; Sayed et al., 2022). The CRISPR/Cas system, with its precision in targeting and editing genetic sequences, has been proposed as a gene therapy strategy for the clearance of virally infected cells or as a preventative measure against viral infection and replication, as indicated by Chen et al. and Stone et al. (Chen et al., 2017; Stone et al., 2016). Nevertheless, the application of the CRISPR/Cas system in antiviral therapies is not without its challenges, sharing the same hurdles of biosafety and efficient delivery mechanisms as traditional gene therapy approaches, as noted by Koujah et al. (Koujah et al., 2019). Despite these impediments, the promise of the CRISPR/Cas system in the prophylactic and therapeutic domain against pathogenic viral infections remains a beacon of hope, offering innovative avenues for future research and clinical applications, as envisioned by Soppen and Lebbink (Soppe and Lebbink, 2017).

5. Conclusions

In summary, our study underscores the remarkable antiviral efficacy of the CRISPR/CasRx system against largemouth bass rhabdovirus, demonstrating its potency across both *in vivo* and *in vitro* experimental models. The *in vitro* component of our investigation revealed that cells transfected with CRISPR/CasRx can significantly suppress the replication and dissemination of the virus. Mirroring these findings, our *in vivo* experiments provided compelling evidence that the CRISPR/CasRx system not only impedes the virus's capacity to invade the host but also enhances the survival rate of the infected organisms.These results collectively highlight the therapeutic potential of the CRISPR/CasRx system as a robust intervention strategy against viral infections in aquatic species. The ability to restrict viral activity and bolster host survival underscores a promising avenue for future research and applications in both prophylactic and therapeutic contexts within aquaculture and beyond.

CRediT authorship contribution statement

Kechen Yang: Writing – original draft, Methodology, Formal analysis. **Baoxia Ma:** Investigation, Data curation. **Zhenmin Wu:** Investigation, Data curation. **Yao Wang:** Investigation, Data curation. **Sen Yang:** Investigation, Data curation. **Fei Ling:** Methodology, Investigation. **Tianqiang Liu:** Methodology, Investigation. **Kun Xu:** Supervision, Project administration. **Gaoxue Wang:** Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2025.742189.

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