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Research Article

Changes in *miR-462* gene expression of rainbow trout in response to cerebral hemorrhage caused by viral hemorrhagic septicemia (VHS)

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Abstract

This study investigates the expression of *miR-462* in rainbow trout (*Oncorhynchus mykiss*) during viral hemorrhagic septicemia (VHS) infection to understand its role in disease pathogenesis and immune defense. MicroRNAs (miRNAs) are small, non-coding RNAs that regulate gene expression at the post-transcriptional level. VHS is a highly contagious and economically significant viral disease affecting farmed rainbow trout. In this study, 300 rainbow trout were divided into three groups: a negative control group (uninfected), a positive control group (infected without intervention), and an experimental group (infected via intraperitoneal injection with VHS virus). Brain tissue samples were collected over 14 days, and *miR-462* expression levels were quantified using molecular techniques. The results showed a significant upregulation of *miR-462* in the experimental group, with a 3.5-fold increase compared to controls ($p<0.05$), indicating its involvement in the immune response to VHS infection. These findings suggest that *miR-462* plays a role in the host's response to viral infection. The study highlights the need for further investigation into the specific pathways regulated by *miR-462* and its potential as a biomarker for VHS. Limitations include sample size variability, and future studies should explore the functional implications of *miR-462* upregulation and its utility in disease management strategies.

Introduction

Viral hemorrhagic septicemia virus (VHSV) is a highly infectious pathogen affecting a wide range of freshwater and marine fish species, including rainbow trout (*Oncorhynchus mykiss*), a species of substantial economic significance in global aquaculture (Jang *et al*., 2024). Recognized by the World Organization for Animal Health (WOAH) for its high lethality, VHSV has caused severe outbreaks, leading to considerable economic losses in the aquaculture industry (Joiner and Teixeira, 2023). VHSV, a member of the Rhabdoviridae family and Novirabdovirus genus, contains a single-stranded RNA genome encoding six proteins essential for its replication and pathogenicity. The glycoprotein within the viral envelope serves as a critical antigen, facilitating host-virus interactions (Yusuff *et al*., 2019).

VHSV primarily affects salmonids, especially juveniles, with mortality rates reaching up to 100%. Infected fish exhibit severe hemorrhages in vital organs, leading to high mortality rates and economic repercussions for aquaculture operations. The clinical symptoms include exophthalmia, ascites, and lethargy, which are associated with vascular damage and immune system activation (Standish *et al*., 2016). The host immune response to VHSV involves the activation and regulation of immune cells, processes that are tightly controlled by microRNAs (*mi*RNAs). These small, non-coding RNAs modulate gene expression post-transcriptionally, either by promoting mRNA degradation or inhibiting translation. In fish, *mi*RNAs like *miR-462* and *mi*R-731 regulate antiviral immune responses, with a significant role in interferon (IFN)-mediated antiviral pathways, enhancing defense against viral infections. The expression of *miR-462* during VHSV infection in rainbow trout influences the immune response by modulating these pathways, which are crucial for controlling viral replication and reducing vascular damage. This regulation may mitigate cerebral hemorrhage, a severe VHSV symptom, by limiting viral-induced endothelial disruption and inflammation. Recent research has emphasized the crucial role of *mi*RNAs in modulating host-pathogen interactions, with *miR-462* emerging as a key player due to its upregulation during viral infections. Studies have demonstrated that *miR-462* expression significantly increases following interferon (IFN) stimulation, suggesting its involvement in antiviral mechanisms. This *mi*RNA's role in directly targeting viral RNAs or regulating immune responses highlights its potential as a biomarker for disease resistance in aquaculture species (Bela-Ong *et al*., 2014).

Additionally, Woldemariam *et al*. (2020) indicated that miRNAs are involved in fine-tuning both immediate immune responses and subsequent inflammatory processes, making them essential components of the host's defense against viral infections. They highlighted that most differentially expressed *mi*RNAs belong to conserved families known to respond to viral infections in teleosts, such as *miR-21*, *miR-146*, and *miR-462*, while also identifying some species-specific miRNAs. This finding underscores the evolutionary conservation of miRNA functions in immune responses across different species. The importance of these molecules in

combating infectious hematopoietic necrosis virus (IHNV) in rainbow trout has been further supported by research identifying five key miRNAs that target critical immune genes, including TRIM25, DHX58, STAT1, TLR7/8, and HSP90A1. This suggests that those *mi*RNAs play a regulatory role in modulating the immune response by targeting multiple immune-related genes (Zhao *et al*., 2022).

Despite advancements, the mechanistic link between *miR-462* expression and specific disease symptoms, such as cerebral hemorrhage in VHSV-infected trout, remains underexplored. Understanding this connection is critical for developing effective disease management strategies. Therefore, this study aims to investigate the expression of *miR-462* in rainbow trout during VHSV infection, particularly focusing on its association with cerebral hemorrhage, to elucidate its role in immune defense and its potential application in improving aquaculture health management.

Materials and methods

Ethical approval

The study was conducted following the ethical guidelines established by the Animal Support and Protection Committee of Shahid Beheshti University of Tehran, ensuring compliance with animal welfare standards.

Viral strain and cultivation

The VHS virus strain DK-3593B, originally isolated from infected rainbow trout, was transported under controlled conditions to the cell culture laboratory at Shiraz University. We ensured that it represented a strain typically found in the local aquaculture environment (Fars, Chaharmahal and Bakhtiari, Kohgiluyeh and Boyer-Ahmad Provinces) to maintain ecological and epidemiological relevance. The strain's genotype was confirmed through sequencing, and its characteristics were compared to regional isolates to ensure its appropriateness for the study. Upon arrival, the virus was prepared for cultivation and downstream analyses to support experimental requirements.

Chinook Salmon Embryo (CHSE) cells were obtained from the Disease Diagnosis Center of the Veterinary Organization and transported to the virus culture laboratory at Shiraz University. The cells were maintained in 50 mL flasks with a 10 cm^2 surface area and cultivated in a medium comprising 90% Eagle's Minimal Essential Medium (EMEM), 10% Fetal Bovine Serum (FBS; Gibco), and antibiotics (penicillin G at 100 IU/mL and streptomycin at 100 µg/mL). All components of the medium were filtered using a 0.22 µm filter for sterility. The flasks were incubated at 21°C for 3–4 days, during which the cells reached confluence. Following this, trypsinization was performed for passaging. Two milliliters of a trypsin-EDTA solution were filtered and added to the flask containing adherent cells. After 10–12 minutes of incubation, the cells were detached by gentle agitation, and a 1:3 passage ratio was maintained. The health and morphology of the cells were monitored throughout the incubation period using an inverted microscope.

Twelve hours after the VHS virus was inoculated into the cell culture medium, the cell cultures were examined under a microscope at 40× and 100× magnifications

to identify cytopathic effects (CPE). To avoid cross-contamination during the viral inoculation, strict protocols were followed. All virus handling, cell culture procedures, and inoculations were performed in designated biosafety cabinets, and contamination control was implemented by using sterile equipment and working in separate areas for different experimental phases. Virus inoculations were conducted in a designated inoculation area with personal protective equipment (PPE), and all materials and surfaces were decontaminated following each procedure. Additionally, appropriate controls were included in each experimental setup to ensure the accuracy and integrity of the viral inoculation process (Weiskirchen *et al*., 2023).

The culture media from flasks exhibiting CPE were collected and centrifuged at 1000 RPM for 5 minutes. The resulting supernatant was used for subsequent molecular analysis using reverse transcription polymerase chain reaction (RT-PCR). For virus titration, the method described by Reed and Muench (1938) was employed to determine the median tissue culture infectious dose (TCID50).

Experimental design and fish treatment

A total of 300 healthy rainbow trout (average weight: 1000±100 grams) were purchased from Dasht Sabz Firouz Koh Company in January 1401, with a valid health certificate from the country's Veterinary Organization. The fish were transported to the Khajir Agricultural Research Center using a specialized vehicle for transporting fish. Upon arrival, the fish were divided into three disinfected tanks, each containing 100 fish, ensuring uniform conditions across all tanks.

The water conditions in the tanks were monitored throughout the experiment, with oxygen content ranging from 10–7 mg/liter, pH between 6.8 and 6, and temperatures maintained between 12–14°C. The water used in the tanks was filtered and chlorine-free, with approximately 50% of the water replaced daily. The fish were fed three times a day with dry pellets from the Aquatic Feed Factory.

After a 10-day acclimatization period, the virus inoculation began. The VHS virus was injected intraperitoneally into the experimental group at a concentration of 10^4 TCID50. A negative control group was maintained, with no intervention, while a positive control group received an intraperitoneal injection of normal saline free from any pathogens.

Sampling and tissue collection

Brain tissue samples were collected from both experimental and control groups on days 1, 2, 4, 6, 8, 10, 12, and 14. For each time point, three fish were randomly selected from each group, anesthetized using a clove powder solution, and then sacrificed. Their brain tissues were carefully dissected and pooled into a single sample per group. These pooled samples were placed in labeled microtubes and transferred to a nitrogen tank for storage.

Safety and waste disposal

After each sampling session, all equipment was disinfected, and the bodies of the infected fish were disposed of by incineration in a pit containing quicklime to prevent further contamination. Clinical

symptoms of the disease were first observed 15 days post-injection, consistent with VHS infection.

RNA extraction for real-time PCR

For RNA extraction, 10 mg of brain tissue from each sample was thoroughly homogenized and mixed with 400 µL of lysis buffer, following the GENALL nucleic acid extraction protocol (Made in Taiwan). The RNA extraction was carried out according to the manufacturer's instructions provided with the kit. After the extraction process, the RNA quality and integrity were assessed using a Nanodrop spectrophotometer and an Agilent Bioanalyzer. The RNA integrity number (RIN) was determined, and only samples with an RIN value of 7 or higher were used for downstream applications. The 260/280 nm absorption ratio was measured to ensure RNA purity, with a ratio between 1.8 and 2 indicating high-quality RNA. The extracted RNA samples were then aliquoted into microtubes and stored at -70°C for further use in Real-Time PCR analysis.

cDNA synthesis

The synthesis of viral complementary DNA (cDNA) was carried out using the reverse transcription method. A total of 1 µL of extracted viral RNA was combined with 1 µL of 1.25 mM random primer and 2.2 µL of nuclease-free water. To the reaction mixture, 4 µL of 5X RT buffer (containing 375 mM KCl, 250 mM Tris-HCl, 15 mM MgCl2, and 50 mM dithiothreitol, pH 3.8; Promega), 20 U of RNasin (Promega), and 200 µM of each dNTP were added, bringing the total volume to 20 µL. The reaction was carried out in a thermal cycler at 45°C for 1 hour to allow reverse transcription, followed by a 10-minute incubation at 72°C to complete the cDNA synthesis.

Real-time quantitative PCR reaction for gene expression analysis

For the quantitative measurement of *miR-462* expression, 1 µg of extracted RNA was converted into complementary DNA (cDNA) using the Real-Time PCR Master mix (SYBR Green 2X, Pars Tos, Iran). The PCR reaction was prepared in duplicate to ensure accuracy. The PCR reactions were conducted using the Stratagene MX3000P Real-Time PCR machine according to the manufacturer's instructions. To normalize *miR-462* expression, GAPDH and U6 snRNA were used as internal control genes, selected for their stable expression under the experimental conditions. The relative expression levels of *miR-462* were calculated using the comparative Ct method (ΔΔCt), with the internal controls serving as references for normalization (Tables 1 to 3).

Table 1: Composition of real-time PCR reaction for gene expression analysis.

Components for Reaction	Volume (µL)	Final Concentration
D.W.	6	-
2X Real-Time PCR Master Mix (SYBR Green)	10	-
Primer-F	1	10 pM
Primer-R	1	10 pM
cDNA	2	-
Total	**20**	-

Table 2: The primers used in the study.

Sequence	Primers
miRNA Forward	GTCAGCCGCATCTTCTTTTG
miRNA Rear	CGTTGACTCCGACCTTCAC

Table 3: Thermal cycling conditions for real-time quantitative PCR.

Temperature (°C)	Time	Cycles
95	10 min	1
95	15 sec	40
Gapdh: 60	30 sec	
Mir-462: 70	30 sec	
72	30 sec	
95	30 sec	1 melting curve
60	20 sec	
95	20 sec	

Data analysis

Statistical analysis was conducted using descriptive statistics to summarize gene expression data for the various experimental treatments and sampling times. The normality of data distribution was assessed with the Kolmogorov-Smirnov test, which indicated that the data did not follow a normal distribution (p<0.05). As a result, non-parametric tests were applied for further analysis. The Kruskal-Wallis test, followed by Tukey's post-hoc test, was used to compare gene expression between the experimental groups and across different sampling times. All statistical analyses were performed using SPSS software (version 22.0), and results were considered statistically significant at p<0.05.

Results

Confirmation of viral cytopathic effect and VHS virus detection

Signs of cytopathic effect (CPE) were observed 24 hours after inoculation of the virus into the cell culture medium compared with normal morphology of CHSE cells (Fig. 1). The CPE intensity increased over time, reaching full coverage of the flask surface after 3 days.

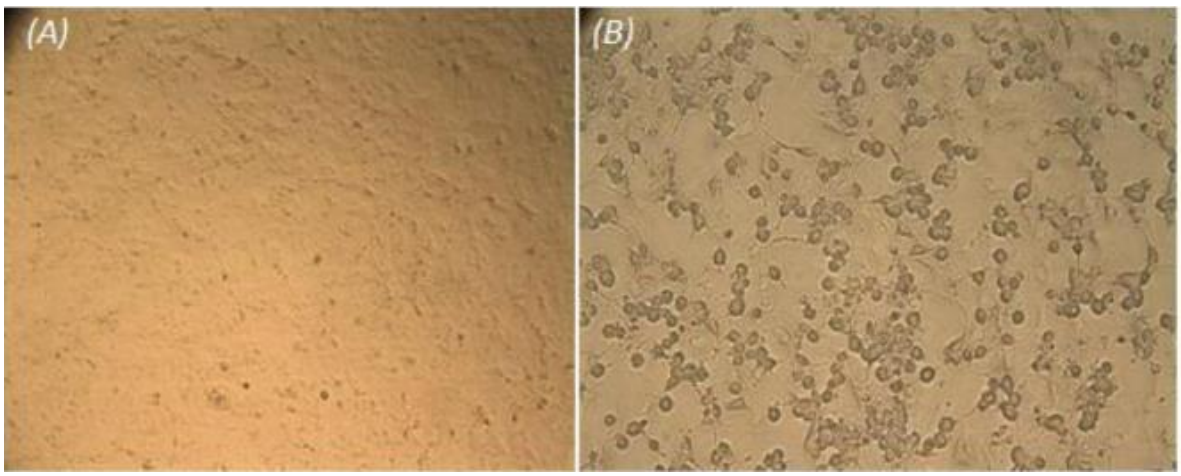

Figure 1: The normal morphology of CHSE cells in a cell culture flask (A), and cytopathic effect symptoms of VHS virus in CHSE cells (B).

Once CPE was complete, the flask was transferred to a -70°C freezer for storage. To confirm the validity of the CPE results obtained from the VHS virus, 1 ml of the solution from the flasks containing CPE was centrifuged at 1000 RPM for 5 minutes, and the supernatant was used for RT-PCR analysis. For definitive detection of the VHS virus, specific primers targeting the *16sRNA* gene were employed. The primer sequences used were: Forward5: TGCATGAAGCTTCAGTCCCCAGGGA TGATGNCC3′ and R-5′ACACCTGAGCTCTTCTTTGGAGGG

CAAACNATY3′, which produce a 1523 base pair band (Thiry *et al*., 1991).

The miR-462 relative expression assay

The mean relative expression level of *miR-462* was 0.785 with a standard deviation of 0.906, indicating variability in expression across the samples. The minimum and maximum expression levels observed were 0.25 and 4.44, respectively, based on a total of 27 measurements. These findings highlight the range of *miR-462* gene expression within the study.

The descriptive statistics for *miR-462* gene expression across experimental treatments are summarized in Table 4, highlighting differences among the groups. Additionally, Table 5 presents a comparative analysis of *miR-462* gene expression across various sampling times, illustrating the temporal fluctuations in expression levels.

Table 4: Descriptive statistics of relative gene expression by experimental groups.

Treatment	Sample size (n)	Mean ± SE	95% Confidence Interval (Lowercase – Uppercase)
No Injection	9	0.254 ± 0.000	-
Normal saline Injection	9	0.580 ± 0.078	0.399 – 0.762
Pathogen Injection	9	1.519 ± 0.427	0.534 – 2.504

Table 5: Descriptive statistical results of relative gene expression by sampling times.

Time (days)	Sample size (n)	Mean ± SE	95% Confidence Interval (Lowercase – Uppercase)
1st day	3	0.301 ± 0.027	0.182 – 0.419
2nd day	3	0.432 ± 0.112	-0.049 – 0.914
3rd day	3	1.163 ± 1.163	-1.675 – 4.001
4th day	3	0.478 ± 0.143	-1.137 – 1.093
6th day	3	1.919 ± 1.282	-3.597 – 7.436
8th day	3	0.563 ± 0.202	-0.309 – 1.436
10th day	3	0.552 ± 0.194	-0.285 – 1.389
12th day	3	0.672 ± 0.281	-0.539 – 1.885
14th day	3	0.981 ± 0.516	-1.240 – 3.203

The data presented in the tables demonstrate significant variation in the relative expression of the *miR-462* gene across different treatments. The lowest expression level was observed in the group without injection (0.254±0.00), whereas the pathogen injection group exhibited the highest expression (1.519 ± 0.427). In terms of temporal variation, *miR-462* expression reached its lowest point (0.301 ± 0.027) on the first day prior to injection and peaked (1.919±1.282) 16 days post-injection. These fluctuations over the sampling period highlight the dynamic response of *miR-462* expression to the treatments applied.

The results of the Kolmogorov-Smirnov test indicated that the significance level for the measured gene expression was less than 0.05 (p-value=0.030; Z=1.450), suggesting that the data distribution deviates significantly from normality. This non-normal distribution necessitated the use of non-parametric statistical methods for analysis. Consequently, the Kruskal-Wallis test, a non-parametric alternative to parametric variance analysis, was employed to compare the mean values

between treatments for the evaluated parameters.

The analytical test results demonstrate a significance level of less than 0.05 in the comparison of experimental groups, signifying a statistically significant variation in *miR-462* gene expression among them (p=0.000; Chi^2=20.398). Conversely, as presented in Table 5, the significance level for comparisons across sampling times exceeds 0.05, indicating no significant temporal differences in *miR-462* gene expression (p=0.896; Chi^2=3.537).

The results depicted in Figure 2 indicate that, based on the post-hoc test, the relative expression of the *miR-462* gene was significantly higher in fish injected with the VHS pathogen compared to the non-injection and physiological serum injection groups throughout the 14-day experimental period (p<0.05; X = 1.519±0.427). Additionally, the highest relative expression level of *miR-462* was observed on the sixth day following VHS virus injection; however, the post-hoc test revealed that this difference was not statistically significant (p>0.05; X=1.282±1.919) (Fig. 3).

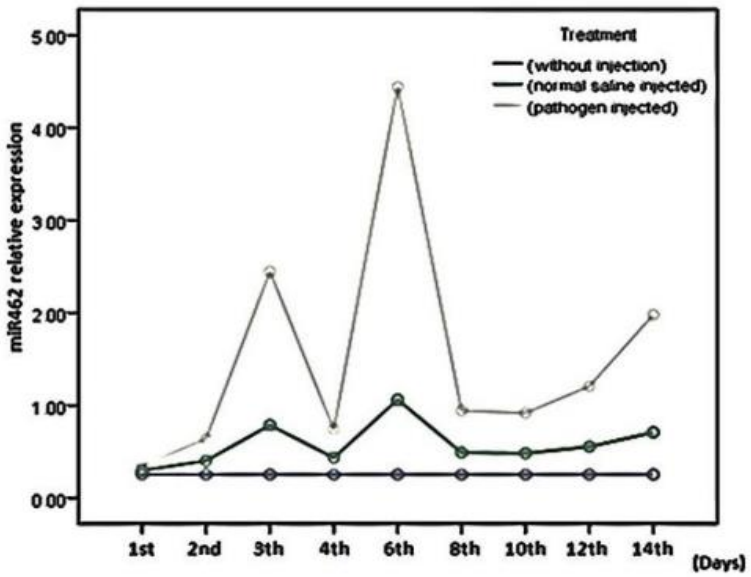

Figure 2: Comparison of the mean relative expression of the miR-462 gene in different groups exposed to the VHS pathogen.

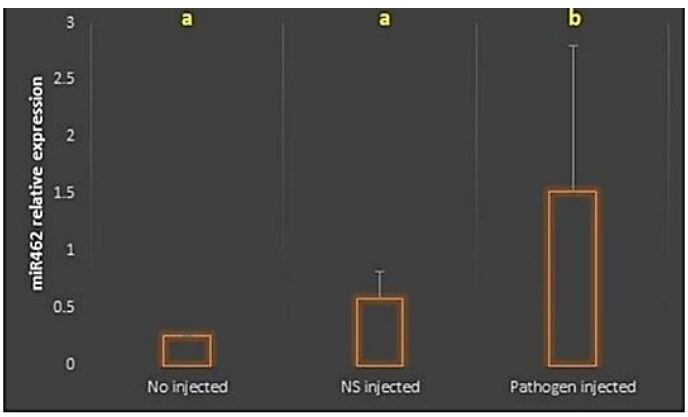

Figure 3: Comparison of the mean relative expression of the miR-462 gene across different sampling times.

Discussion

Viral Hemorrhagic Septicemia (VHS), caused by the VHS virus (VHSV), remains one of the most detrimental infectious diseases in rainbow trout (*Oncorhynchus mykiss*), responsible for high mortality rates and significant economic losses in aquaculture (Joiner and Teixeira, 2023). Despite efforts to develop effective treatments, the management of VHS continues to rely heavily on preventive measures. In this context, immune modulation offers a promising alternative approach for controlling VHS outbreaks, as stimulating the host's immune system can enhance its resistance to infection.

MicroRNAs (*mi*RNAs), particularly miR-462, have emerged as important regulators of immune responses in fish, including antiviral immunity. miRNAs function at the post-transcriptional level, modulating gene expression to influence immune pathways, including the regulation of interferon (IFN)-mediated immunity (Bela-Ong *et al*., 2014). In our study, the expression of miR-462 was significantly elevated in VHSV-infected rainbow trout, indicating its potential role in the antiviral immune response. This finding aligns with prior research, including that of Schyth *et al*. (2019), who also reported upregulation of miR-462 in response to VHSV infection in fish liver, suggesting that *miR-462* participates in regulating immune responses during viral infections.

The control groups, including those with no injection or treated with physiological serum, showed the lowest *miR-462* expression (0.254±0.00). In contrast, the VHSV-infected group exhibited the highest expression level (1.519±0.427), implying that *miR-462* might be involved in limiting viral replication or modulating the host's immune system in response to the viral challenge. These results support previous studies that have suggested *miR-462*, along with other related *mi*RNAs such as *miR-731*, is upregulated during viral infections, where it plays a role in modulating immune responses to facilitate better control of viral replication (Schyth *et al*., 2019).

In terms of temporal dynamics, our study found that *miR-462* expression peaked on day 6 post-injection (1.919±1.282), consistent with previous reports on fluctuations in *mi*RNA expression following infection (Bela-Ong *et al*., 2014). Notably, while the expression of *miR-462* showed fluctuations across the study period, no statistically significant temporal differences were observed in the *miR-462* expression across different time points. This lack of significance, despite fluctuations, may point to complex regulatory mechanisms underlying *miR-462*'s expression. The peak on day 6 could represent an early immune response, corresponding with the initial activation of antiviral immune pathways, while the lack of sustained temporal changes might suggest that the regulation of *miR-462* occurs mainly during the acute phases of infection.

The role of *miR-462* in immune regulation can be attributed to its interaction with key antiviral genes, such as those involved in the IFN signaling pathway. *miR-462* has been shown to act synergistically with IFN genes to enhance antiviral defenses (Lee *et al*., 2004). The elevated *miR-462* levels observed in our study may reflect its role in promoting immune responses that target

viral replication. Our findings are consistent with those of Zamanejad *et al*. (2018), who demonstrated that *miR-462* regulates immune-related genes to enhance host defenses against viral infections.

Moreover, the study by Schyth *et al*. (2019) provides valuable insights into the potential mechanisms by which *miR-462* mediates antiviral immunity. Their research suggested that miR-462 expression is upregulated in response to IFN and that neutralizing *miR-462* during VHSV infection increases mortality, highlighting its crucial role in mounting an effective immune response. Our results, which show increased *miR-462* expression in VHSV-infected fish, further support the idea that *miR-462* is integral to the immune response and may function to mitigate the effects of viral replication.

The findings of this study contribute to the growing body of evidence highlighting the critical role of *miR-462* in antiviral immunity in fish. The elevated expression of *miR-462* following VHSV infection suggests that this *mi*RNA could serve as a biomarker for viral infections in aquaculture. Furthermore, given that immune stimulation is often more effective than direct treatment in managing viral diseases such as VHSV, enhancing miR-462 expression could represent a viable strategy for preventing or mitigating the impact of VHSV outbreaks in aquaculture systems.

One limitation of this study is the pooling of fish samples for RNA extraction and subsequent miRNA expression analysis. Pooling is commonly done to minimize biological variation, especially when dealing with limited sample sizes; however, it can obscure individual responses, particularly in terms of temporal variation and potential inter-individual differences in immune responses (Shiry *et al*., 2020). Future studies would benefit from examining individual fish samples to better capture the variability in *miR-462* expression and its dynamics within the population. Additionally, the use of pooled samples limits the ability to directly correlate *miR-462* expression with other immune markers at the individual level, potentially leading to a loss of some finer details in immune response pathways.

Conclusion

In conclusion, *miR-462* plays a key role in modulating the immune response to VHSV infection in rainbow trout, particularly by regulating antiviral mechanisms that inhibit viral replication. The upregulation of *miR-462* following infection suggests that this microRNA could serve as an important biomarker for monitoring VHSV in aquaculture. Future research exploring the intricate interactions between *miR-462* and other immune regulators will provide deeper insights into its potential for immune modulation. These findings could pave the way for the development of *miR-462*-based strategies to prevent and manage viral outbreaks, enhancing the sustainability and disease resistance of aquaculture systems. By leveraging *miR-462* as both a biomarker and a tool for immune modulation, we can advance innovative approaches to disease management, offering new solutions to mitigate the impacts of VHS and other viral diseases in aquaculture.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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