Expression of Hsp70 and β-actin Genes as The Immune Response against Viral Nervous Necrosis that Infected Asian Seabass (*Lates calcalifer*)

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Abstract

The viral nervous necrosis (VNN) is betanodavirus of the family Nodaviridae with acute infection and associated with high levels mortality up to 100% of numerous larval-stage marine and freshwater fish species. The danger signals of viral infection that are capable of activating APCs (Antigen Presenting Cells), furthermore produced molecules protein receptors such as interferon and heat shock proteins. Stimulating interferon type I (IFN I) induce several antiviral molecules, further binding the actin cytoskeleton to reach the site of infection. This study demonstrated a correlation between increased levels of Hsp70 (heat shock proteins) and actin filamentous (β-actin) within invasion wild-type isolate of ssRNA VNN from Asian Seabass (*L. calcalifer*) juvenile. Furthermore, expression of Hsp70 and actin as an indicator or biomarker of stressed states in fish. The reverse transcriptase polymerase chain reaction (RT-PCR) method used to finding expression Hsp70 and β-actin. Whereas nested RT-PCR used for VNN genes. The up-regulation of Hsp70 was observed on the brain tissue higher than eye tissue of sample positive infected VNN. Whereas different expression of β-actin, β-actin receptor expression tends to be stable in the organs of the brain and eye, both invaded VNN or normal, while on the eye, the ratio slightly increased based on the intensity of the band. The research shows that altered expression of heat shock protein 70 and beta-actin gene receptors in the target organs that response to invasion VNN at Asian seabass (*L. calcalifer*) juvenile. Alteration expression of heat shock protein 70 and beta-actin can be used as an indicator or biomarker of stress cells, especially from VNN invasion at fish.

Keywords: Asian seabass, β-actin, Hsp70, Immune response, Viral nervous necrosis.

INTRODUCTION

Since outbreak at 1997 in Indonesia sea bass (*Lates calcalifer*) aquaculture [1] and for past three decades, the Viral Encephalopathy and Retinopathy (VER) or Viral Nervous Necrosis (VNN) has been frightening spectre to the sea bass aquaculture industry in Asian Marine Culture [2,3]. VER is Betanodavirus of the family Nodaviridae with acute infection [4], and they are virus associated with high levels mortality up to 100% of numerous larval-stage marine fish species [5,6]. In every cases of VER, fingerlings and juvenile fish affected exhibit erratic swimming patterns (whirling) with up swimbladder, skin darkness, anorexia (poor appetite), solitary or clusters near the side of a pool, lordosis or scoliosis, exophthalmia, and a range of neurological abnormalities, including vacuolization and cellular necrosis in the central nervous system and retina [6,7,8].

Based on phylogenetic analysis, including host range, optimum temperature, and geographic distribution gene of nervous necrosis virus (NNV) can be categorized into four genotypes: Red-Spotted Grouper Nervous Necrosis Virus (RGNNV), Barfin Flounder Nervous Necrosis Virus (BFNNV), Tiger Puffer Nervous Necrosis Virus (TPNNV), and Striped Jack Nervous Necrosis Virus (SJNNV). The phylogenetic analysis on the similarity variable T4 region (partial RNA2) which encode the Cp (coat protein) genes. RGNNV was found in warm water fish, TPNNV and BFNNV were found in cold water fish, and SJNNV were found in warm and cold water fish. The optimal range temperatures for viral replication in the different strains such as RGNNV: 25–30°C, TPNNV: 20°C, BFNNV: 15–20°C, and SJNNV: 20–25°C [9].

In some cases of viral infections, viral proteins can target effector molecules by encoding homologous cytokines or cytokine receptors, having acquired such genes through modification or capture of host cellular genes, or inhibit the complement-mediated killing of virus-infected cells [6]. An external pathogen (such as virus) is first recognized by pattern-recognition receptors

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(PRRs), and this induces diverse intracellular signals that provide pathogen defense [10].

The danger signals of viral infection that are capable of activating APCs (antigen presenting cells), in this model are either molecules protein receptor produced by stressed cells such as interferon and heat shock proteins [11,12]. The Hsp (Heat shock protein) genes are highly conserved and have been marked in a wide range of organisms. The heat shock response is an evolutionarily conserved mechanism for maintaining cellular homeostasis following sublethal noxious stimuli. The heat shock protein affects diverse a part of fish physiology, belonging growth and aging, stress physiology and endocrinology, immunology, environmental physiology, stress endurance and acclimation [13]. The heat shock proteins have constitutive functions in unstressed cells, and that is necessary for protein metabolism [14].

Viral infection causes stressed aquatic organism, and as a consequence of such as cellular stress of protein synthesis, particularly causing defect and disruption in protein homeostasis. The defect in protein homeostasis can trigger deviate protein aggregation. As an antigen, viral infection stimulates expression of intracellular Hsp (Heat shock protein) especially Hsp70. The previous study explained that Hsp and MHC (Major Histocompatibility Complex) are two part of peptide binding protein in immune response [18]. Peptide non-native from the viral antigen, bound by Hsp70. Hsp70 polypeptide binding and release is regulated through cycles of ATP hydrolysis and exchange. Misfolding protein caused by viral infection is managed, in part, through the action of Hsp70 molecular chaperones. However, a non-native polypeptide is bound by an Hsp40 co-chaperone (co-factor of Hsp70) for degradation by the ubiquitin-proteasome system (*poly-ub*) [39]. The Hsp70 chaperones are present in most cell compartments and are essential in several processes, folding of polypeptides, refolding of misfolded proteins, translocation of intracellular proteins, and binding of misfolded proteins for degradation [15]. The folding of nascent polypeptide chains acts as a molecular chaperone and mediates the repair and degradation of altered or denatured proteins facilitated by Hsp70 [16].

Fish are an eminent vertebrate specimen to inquiry the physiology, function, and arrangement of Hsps. It is because in their natural environment fish are unprotected to thermal and other stressors. The connection between Hsp synthesis and the establishment of thermotolerance has been investigated by some researcher [17]. Studies on Hsp70 are the most extensive and have demonstrated that the regulation of Hsp70 gene expression occurs mainly at the transcriptional level [14]. The modifications in environmental salinity, disease, and chemical exposure are known to differ the expression and the arrangement of Hsps. *Vibrio anguillarum* acute infection induced an increase in Hsp70 (Heat shock protein 70) in rainbow trout liver and head kidney as examined on immunoblots [19]. Enhancement of hepatic Hsp70 expression in sea bream caused by the *Vibrio alginolyticus* infection at one-day subsequent pathogen administration [14,18].

At the mechanism of innate immune response, viral infection causes stimulating Interferon Type I (IFN I) and induce IFN-Stimulated Gene (ISG). ISG has several antiviral molecules, such as ISG56 or as known protein 56 (P56). P56 protein binds to the actin cytoskeleton component to reach the site of infection for viral elimination [40]. Actin is the others receptor-mediated immune response. The actin cytoskeleton plays pivotal roles in cell shape, cell migration and signaling [20]. Functionally, actin determines the shape and replacement of cells, as well as phagocytosis, intracellular communication and the distribution of organelles [21].

The actin cytoskeleton is important for lymphocyte antigen receptor signaling. The actin cytoskeleton regulates a number of cellular functions via polymerization and depolymerization. Polymerization and depolymerization are a process that includes dynamic changes between monomer Globular (G-actin) and Filamentous or polymerized actin (F-actin) [38]. At the immune response, especially for B-cell activation depends on the polymerization of the actin cytoskeleton [22]. Actin is associated with all three eukaryotic RNA polymerases and it is directly involved in gene transcription [23].

This study has demonstrated a correlation between increased levels of Hsp70 (heat shock proteins) and actin filamentous (β-actin) within invasion wild-type isolate of ssRNA VNN from Asian Seabass (*L. calcalifer*) juvenile. We highlight some of the interactions that are promoted by viral proteins which redirect the function of the actin cytoskeleton and Hsp70. Furthermore, the author use of Hsp70 and actin as an indicator or biomarker of stressed states in fish that infected viral ssRNA VNN.

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MATERIALS AND METHODS

Fish and Materials

The Asian seabass juvenile (*Lates calcalifer*) approximately 10-12 cm in length and 15-20 g in weight were obtained from the fish hatchery Situbondo Brackishwater Aquaculture Development Center, Ministry of Marine Affairs and Fisheries. The lethal sampling method was used for fish sampling. The visual methods were corresponding to the appearance of clinical signs. The type of clinical sign of viral nervous necrosis (VNN) such as blackening, solitary, anorexia, whirling, and exophthalmia. Ten Asian Seabass juvenile were collected randomly from tanks with 32-35 ppt salinity and 28-30°C temperature where the outbreak occurred.

The Asian Seabass juvenile were preserved in live condition. Further, the sample was carried to the laboratory for shortly processing and extraction of RNA. At the laboratory, the live fish were killed and dissected by sharp scissors to severance of spinal cord. Brain and eye were collected as the tissues target for infection of VNN, frozen immediately and stored at -25°C until RNA extraction.

Isolation of VNN, Heat shock protein (Hsp) and β-actin cDNA

The total RNA was isolated from the samples of all untreated fish (based on clinical sign; healthy and infected fish) : 25 mg of tissue from each the brain and eye of *L. calcalifer* was used for extraction of total RNA by using Trizol (Invitrogen, USA) and treated with RNase-free DNase I (Qiagen, USA) to remove any remaining genomic DNA, according to the manufacturer's instructions.

The reverse transcription was carried utilizing the MyTaq $^{(TM)}$ One-Step RT-PCR kit (Bioline UK Cat.No. Bio-65049), following the manufacturer's manual. The reaction system at a final volume of 20 µL contained 7 µL H_2O (NFW), 12,5 µL GoTaq®Green Master Mix, 0.5 µL Tetro reverse transcriptase enzyme, and 4 µL total RNA (500 ng). The reaction was carried out at 45°C for 45 min, and 94°C for 2 min.

Nested RT-PCR and RT-PCR amplification and Measurement of the VNN, Heat Shock Protein (Hsp) and β-actin

Lates calcalifer VNN, Hsp70, and β-actin were amplified using primers designed based on sequences in GenBank and analysis by using the NCBI BLAST. The primer at Table 1. The PCR amplification for VNN genes was carried out under the following conditions: pre-denaturation at 94°C for 2 min; 25 cycles of 94°C for 40 s, 55°C for 40s, extension at 72°C for 40s, and final extention at 72°C for 5 min. The second nested RT-PCR for VNN genes under the following condition : pre-denaturation at 94°C for 2 min; 25 cycles of 94°C for 40s, 50°C for 40s, extension at 72°C for 40s, and the final extention at 72°C for 10 min. Furthermore, the PCR amplification for HSP 70 and β-actin genes was carried out under the following conditions: pre-denaturation at 94°C for 2 min; 35 cycles of 94°C for 30s, 54°C for 30s, extension at 72°C for 30s, and final extention at 72°C for 10 min. The PCR product was examined by electrophoresis with 1.5% agarose gels containing sybersafe. The ImageJ software were used for semi quantitative digital analysis DNA band PCR [24,25].

Table 1. List Primers for amplification RT-PCR

No	Gene	Primer sequences	size (bp)
1.	Actb F	TACCACCGGTATCGTCATGGA	150
	Actb R	CCACGCTCTGTCAGGATCTTC	
2.	Hsp70 F	CGTAAGAGGTGGAAACGCCA	562
	hsp70 R	CAGCGTTGGACACCTTTTGG	
3.	VNN F2	CGTGTCAGTCATGTGTCGCT	420
	VNN R3	CGAGTCAACACGGGTGAAGA	
4.	VNN F'2	GTTCCCTGTACAACGATTCC	294
	VNN R'3	GGATTTGACGGGGCTGCTCA	

Source: from various scientific journals such as : 1. [26]; 2. NCBI get primer menu; 3 and 4 [27].

DNA Sequencing

Positive template of PCR product contains cDNA VNN, Hsp70, and β-actin packed for sequencing. Sequencing done at First Base Laboratories (molecular biology service) Malaysia.

RESULTS AND DISCUSSION

VNN Infection in Asian Seabass

The previous survey of Betanodavirus illustrated that the clinical sign mass mortality of the Asian Seabass (*L. calcalifer*) at 18–21 dph (days post-hatch) typical clinical manifestations such as anorexia, blackening, lack of swimming coordination and settling to bottom [28]. According to the visual observations of VNN that infected juvenile Asian Sea Bass (*Lates calcalifer*) show the same typical clinical manifestation with the previous survey, in addition fish that infected VNN show exophthalmia, solitary or clusters near the side of a pool, lordosis or scoliosis, and erratic swimming patterns (whirling) (Fig.1).

Another study explaining that clinical symptoms are not always altered in infected fish [7]. Based on the nested RT-PCR test, the results on the test showed that two of six samples

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identified positive VNN did not show clinical sign (Fig.1 and 2). To make the infection and replication, virus requires access to the host intracellular environment such as the capacity of the host to develop a proper immune response, the velocity of virus replication, cytopathogenicity and the spread of infection within and between organs, which again may or may not depend on the presence of specific cellular receptors [29]. Several studies further elucidate the absence of clinical symptoms of viral attack, the possibility of the virus invading the host cell through the lysogenic cycle [21].

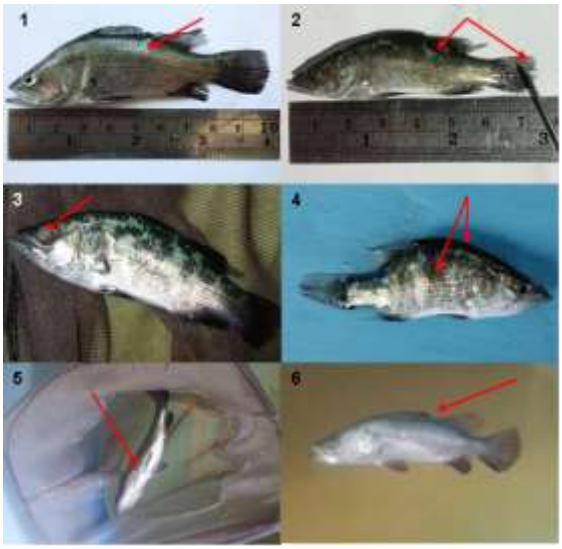

Figure 1. Clinical Symptoms Fish Infected with VNN.
Description: 1. Blackening, 2. Damage fin, 3. Exophthalmia, 4. Petichae, 5. Whirling, 6. Solitary.

Several studies explained that VNN invasion of fish can occur with vertical transmission through broodfish to the eggs in the broodstock offspring [4,28,30] and horizontal transmission of infected fish to non-infected, fellow fish in one water area infected with the virus or contaminated through waters, and through natural live feed [9]. The review found that fish infected through horizontal transmission, from the environmental factor such as the temperature. In these studies, the water temperature of fish tanks between 28-30°C. This temperature is optimal temperature of RGNNV, one type of VNN development in the fish host [7,9]. Optimal temperature of RGNNV development that can cause outbreaks between 25-30°C.

VNN Genes Expression

The Partial cDNA band of VNN genes that infected Asian Seabass (*L. calcalifer*) showed single fragments of 294 base of a nucleotide sequence for VNN genes were determined. The band of all fish samples was compared among genes from healthy and infected fish. In this study, VNN genes were identified in 6 samples which positive infected VNN from 10 observed samples.

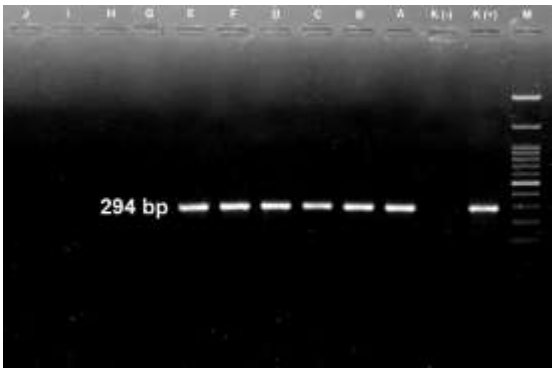

Figure 2. nestedRT-PCR test for VNN that infect Asian Seabass.
Description:
Line M = marker until 1000 bp
K (+) = positive control of VNN genes
K (-) = negative control (nuclease free water)
Line A – J = *L. calcalifer* sample
Line A and F = positive detected VNN genes at 294 bp
Line G – J = negative from VNN genes

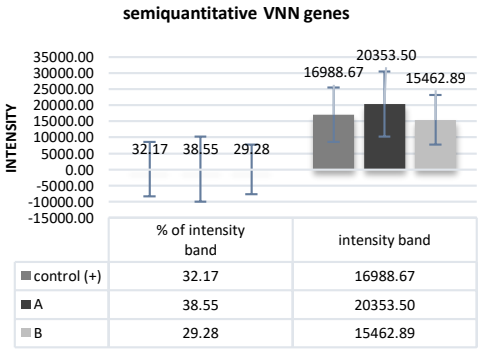

Figure 3. Semiquantitative VNN genes using ImageJ software. Based of fig.2, 3 nested RT-PCR band of VNN genes was analysis using software, and result intensity of band.
Description:
Control (+) = gene template (+) VNN gene
A = the brain tissue positive (+) VNN gene
B = the eye tissue postive (+) VNN gene

The expressions of the VNN genes was observed in brain and eye tissues in infected fish of this study. However, the expressions levels in different tissues varied. The emergence of this band indicates the presence on a copy of VNN genomes in the target sample of organs.

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Whereas the sample that did not appear the PCR band indicated that the target organ had no viral genomes or possibly a very small volume of VNN viral genome that could not be duplicated and visualized by the nested RT-PCR method. The brain tissues showed a higher expressions band level of the VNN genes than eye tissues (based on the semiquantitative analysis using imageJ software Fig.3). The difference in the copy of the viral genome level of VNN may be caused to the distribution and replication of the viral genome on the host's target organs [31].

Profile of β-actin and Hsp70

Reverse transcriptase PCR (RT-PCR) method was used to determine the profile of β-actin and Hsp70 bands in each target organ such as the brain and eye. Effect of VNN invasion on the expressions of the Asian Seabass Hsp70 and β-actin genes (Fig.4).

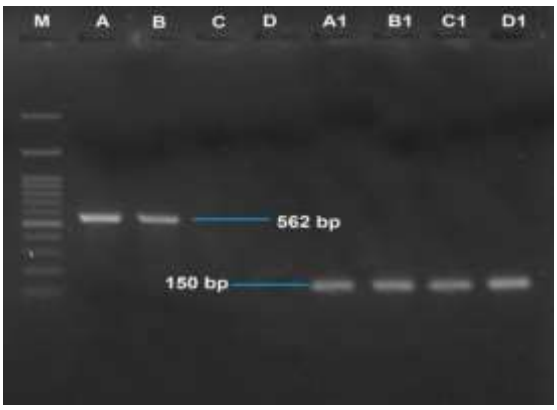

Figure 4. Different light band of RT-PCR test in infected fish and negative fish band of Hsp70 at 562 bp, and β-actin at 150 bp.
Description of Line:
M = Marker until 1000 bp band;
A = Hsp70 at the brain positive (+) sample of VNN genes
B = Hsp70 at the eye positive (+) sample of VNN genes
C = Hsp70 at the brain negative (-) sample of VNN genes
D = Hsp70 at the eye negative (-) sample of VNN genes
A1 = β-actin at the brain positive (+) sample of VNN genes
B1 = β-actin at the eye positive (+) sample of VNN genes
C1 = β-actin at the brain negative (-) sample of VNN genes
D1 = β-actin at the eye negative (-) sample of VNN genes

According to Figure 4, the two target organs of VNN invasion express their respective β-actin and Hsp70 receptors. In a sample detected negative VNN also expresses the β-actin receptor. The interaction between receptors and viruses is an early interaction of virus invasion to the host.

The receptor is a surface molecule acting as a key for initiating the viral infection process, which is capable of directly triggering the penetration of virions attachment on cell membranes (such as endosome and plasma membranes) into specific cellular regions and leading to subsequent infections [32]. Expression of actin as a protein and polymer that form cytoskeleton abundant in eukaryotic cells that have important functions such as motility, growth, cytokinesis, endocytosis and intracellular transport [10,33]. The brain tissues show higher expressions band level of the Hsp70 genes than eye tissues (based on the semiquantitative analysis using imageJ software Fig.5).

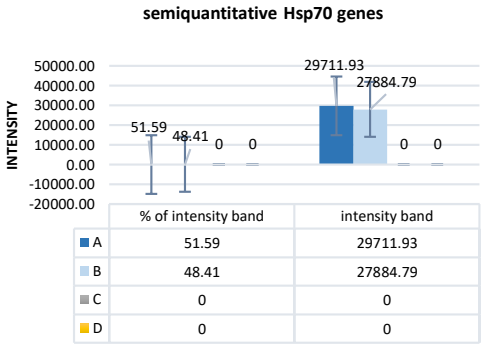

Figure 5. Semiquantitative VNN genes using ImageJ software. Based of fig.4, 2 RT-PCR band of positive (+) Hsp70 genes was analysis using software, and result intensity of band.
Description:
A = Hsp70 at the brain positive (+) sample of VNN genes
B = Hsp70 at the eye positive (+) sample of VNN genes
C = Hsp70 at the brain negative (-) sample of VNN genes
D = Hsp70 at the eye negative (-) sample of VNN genes

VNN invasion of the CNS (Central Nervous System), the brain and the eyes, provides an enhanced immune response of receptor Hsp70 in the brain and eye organ to virus degradation. The Hsp70 up-regulated and expression on tumor cell surface and is not expressed in normal cells [34]. VNN invasion caused an Hsp70 stimulation, plays a role in protein aggregation, refolding damaged proteins, protein degradation, immune signal transduction, converting proinflammation to immune cells (cytokines), and producing variations in immune responses [35]. Study at the tilapia (*Oreochromis niloticus*) shows the

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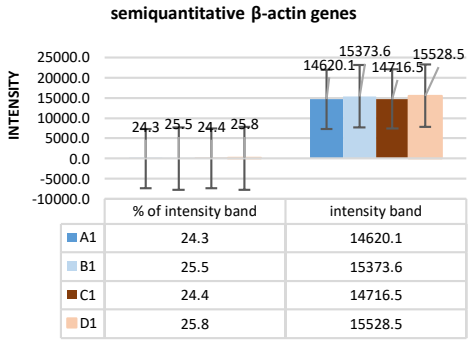
conservative Hsp characteristics of the Hsp70 family, and the introns Hsc70-1, Hsc70-2, and Hsc70-3, have a significant increase of the Hsp family in the liver, kidney (head kidney), lymph, and gills after 6 hours infected with *Streptococcus agalactiae*. According to an *Oreochromis niloticus* Hsp70 research, a significant up-regulation in Hsp70 transcripts was stimulated infection of *Streptococcus agalactiae* [36].



	% of intensity band	intensity band
A1	24.3	14620.1
B1	25.5	15373.6
C1	24.4	14716.5
D1	25.8	15528.5

Figure 6. Semiquantitative VNN genes using ImageJ software. Based of fig.4, 4 RT-PCR band of positive (+) β-actin genes was analysis using software, and result intensity of band.

Description:

A1 = β-actin at the brain positive (+) sample of VNN genes

B1 = β-actin at the eye positive (+) sample of VNN genes

C1 = β-actin at the brain negative (-) sample of VNN genes

D1 = β-actin at the eye negative (-) sample of VNN genes.

Figure 6 explains that β-actin receptor expression tends to be stable in the organs of the brain and eye, both invaded VNN or normal, while the ratio on the eye organ was slightly increased based on the intensity of the band. The propensity for stable actin gene expression is also explained by previous research which elucidate that actin gene expression has the highest stability compared to 7 other encoding gene expressions such as DNA directed RNA polymerase II subunit I (DRP2), elongation factor-1 alpha (EF1α), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), glucose-6-phosphate dehydrogenase (G6PD), hypoxanthine phosphoribosyl transferase (HPRT), ribosomal protein L8 (RPL8), and 18S ribosomal RNA (18S) in the exposed of Cadmium metal at rainbow trout growth phase [37].

CONCLUSION

The research shows altered expression of heat shock protein 70 and beta-actin gene receptors in the target organs that response to invasion VNN at Asian Seabass (*L. calcalifer*) juvenile. Alteration expression of heat shock protein 70 and beta-actin can be used as an indicator or biomarker of stress cells, especially from VNN invasion at fish. The research elucidates that the intracellular stress response is likely to be essential in increasing the survival and health of the infected fish. Measurements of intracellular Hsp70 and β-actin could be a helpful application, particularly when considering minimal invasive methods for diagnostic, acquiring the influence of stress which related to the immune response for ssRNA VNN infection.

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