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EVALUATION OF RESISTANCE AND GENE EXPRESSION OF BARRAMUNDI, *Lates calcarifer* POST-INFECTION OF NERVOUS NECROSIS VIRUS

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ABSTRACT

The most common problem in barramundi *Lates calcarifer* seedling production is the high mortality (>90%) caused by nervous necrosis virus (NNV) infection. This research aims to evaluate the resistance and gene expression of barramundi challenged by NNV. Two populations were used in this study, i.e., Australian, and Situbondo-originated barramundi populations. The immune-related gene expression levels in the liver, head kidney, and spleen were observed at 48 and 96 hours after post-infection (hpi). Barramundi's survival and blood parameters were evaluated post-NNV infection. The results showed that the highest survival was revealed in Situbondo's barramundi (42.0±4.47%) compared to Australian barramundi (20.0±7.07%) and no mortality was observed in the control without NNV infection. The higher survival rate in barramundi from Situbondo was in line with the blood profile. The number of red blood cell from Situbondo barramundi post-NNV infection (ST) at 96 hpi was higher ($P<0.05$) than Australian barramundi post-NNV infection (AT). The number of white blood cell of ST at 48 hpi was higher ($P<0.05$) than AT, but started to decrease at 96 hpi in ST barramundi. The total white blood cell in AT barramundi increased from 48 to 192 hpi. TNFα and IL1-β gene expression levels were significantly higher in the liver, head kidney, and spleen of Situbondo compared to Australian barramundi at 48 hpi, while MHCIIα gene expression in Situbondo's was significantly higher compared to Australian barramundi at 96 hpi. These results indicate the important roles of all the genes in the barramundi's immune responses against viral infection. Based on the results of the research, Situbondo's barramundi has the potential to be used as a candidate for generating broodstock of disease-resistant strain.

KEYWORDS: barramundi; viral infection; fish mortality; blood parameter; gene expression

INTRODUCTION

Barramundi (*Lates calcarifer*) is one of the economic and commercial aquaculture commodities (Ye *et al*., 2017). Based on production data from the Directorate General of Aquaculture (Ministry of Marine Affairs and Fisheries), 20 provinces in Indonesia produce barramundi in 2021 with a total production of 6247.47 tons. These fingerlings are produced from small-scale hatcheries in Bali, Lampung, and East Java (Jerry, 2014). The barramundi fingerling that was nursed at small-scale hatcheries came from Australian and Indonesian broodstock, including Situbondo.

Barramundi culture still depends on the fingerlings derived from wild-caught broodstock and selected Australian broodstocks with fast growth characteristics. The market demand is quite high, causing mass natural spawning to be carried out without following the breeding procedure so that the inter-batch variability in growth and survival is high (Domingos *et al*., 2013). Their heritability level also decreased significantly, phenotypic variation was high at the larvae rearing stages (D21-D80), susceptible to stress, decreased immunity level, and susceptibility to disease (Ye *et al*., 2017; Fu *et al*., 2013). This has been an obstacle and challenge in the barramundi nursery until now.

Pathogenic infections in marine fish culture may cause production failures and economic losses (Abdullah *et al.,* 2017). The main problem of disease

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infection for barramundi is caused by Nervous Necrosis Virus (NNV). NNV also called as Viral Encephalopathy Retinopathy (VER). This disease infected several fishes in hatchery and fish culture on floating net cages in Indonesia, India, Malaysia, and Taiwan (Sharma *et al.,* 2019; Sembiring *et al.,* 2018; Abdullah *et al.,* 2017, Liu *et al.,* 2016; Wu *et al.,* 2013). NNV infects 10 marine fish families from the Indo-Pacific and the Mediterranean (Toffan *et al*., 2017). This virus infects the central nervous system, causing cellular vacuolation in the brain in larvae, the retina of the eye, and reproductive organs in adult fish (Yang *et al*., 2022). VNN disease is characterized by abnormal swimming behavior and high mortality (>90%), especially infecting almost all stages of fish growth (larvae and juveniles) (Sembiring *et al*., 2018; Shetty *et al*., 2012).

Evaluation on the blood profiles can be useful in monitoring the health of fish, such as catfish infected with *Aeromonas hydrophila* (Suprapto *et al*., 2017). Gene expression analysis is one method for measuring the immune response at the molecular level. Immune-related genes have a crucial role in preventing disease infection. Cytokines, which include interleukin 1 (IL1), interleukin 8 (IL8), tumor necrosis factor-alpha (TNF), CXC2-and CC-chemokines, interferons (IFN), and myxovirus resistance (Mx), are immune modulators that regulate immune responses and effector phases in innate and adaptive immunity (Xu *et al*., 2013). IL1β in early response is secreted when pathogens infect the body (Angosto *et al*., 2012). Induction of TNFα, IL1β was reported to play an important role in the immune response in grouper, gilthead sea bream, and pompano NNV-infected (Wang *et al*., 2019; Chiang *et al*., 2017; Poisa-Beiro *et al*., 2008), and induction of MHC-IIα expressed was reported in rainbow trout *Oncorhynchus mykiss* infected by novirhabdovirus (IHNV) (MacKenzie *et al*., 2008). This research aims to evaluate the resistance to NNV and immune-related gene expression of Situbondo and Australian barramundi. Evaluation of the resistance of the barramundi populations post-infection of NNV virus has not been reported.

MATERIALS AND METHODS

Fish and Experimental Condition

The fingerling barramundi, (body length: 0.76 ± 0.09 cm) was obtained from the Situbondo brackish-water Aquaculture Development Center, for the Situbondo population, and the Lampung Mariculture Development Center for the Australian population. The fingerling then was reared for 60 days by flowthrough system consisting of similar tanks (1000 L capacity) with a flow rate of 4 L min^{-1}. The temperature of water rearing was 29.2 ± 1.2°C with a salinity of 33.0 ± 1.0 g L^{-1}.

NNV Preparation and Challenge Test

The NNV inoculum for the challenge test originated from infected barramundi's eyes and brain organs, which had been confirmed by PCR method. The organs were homogenized and crushed in sterile phosphate buffer saline (PBS). The dose was obtained from the LD70 test for each fish population. The homogenate was centrifuged at 7500 rpm for 10 minutes at a temperature of 4°C. The supernatant was filtered with a 0.45 µm millipore filter, then the NNV homogenate was stored at -80°C until it was used.

A total of 240 barramundi (BL: 7.2 ± 0.3 cm; BW: of 5.1 ± 0.11 g) was used in the challenge test. The challenge test was divided into 4 groups, namely AT (Australian fish population, infected with NNV); AK (Australian population without challenge test, injected with PBS); ST (Situbondo population infected with NNV); and SK (Situbondo population without NNV infection), each consisting of 10 fish, with six replications. The fishes were anesthetized using 4-Allyl-2-methoxy phenol (Eugenol) by Merck (200 µL L^{-1} seawater, for 1 minute) before being injected with NNV and PBS. AT and ST fish were challenged by injecting 100 µL $fish^{-1}$ of NNV using a sterile syringe by intraperitoneal (IP) route. AK and SK fish were injected with 100 µL $fish^{-1}$ PBS. Fish are challenged in aquaria (60 x 50 x 40 cm) with strong aeration. The fish were fed once a day at apparent satiation. Fish mortality was observed every day for 14 days.

Furthermore, the replacement of water in the rearing tanks of as much as 30% of the total volume was conducted every day to maintain water quality, whereas the water quality was maintained in the normal range for barramundi culture.

Response and Clinical Symptoms Observation

Clinical symptoms were observed every day for 14 days of observation. The data is interpreted descriptively. Parameters observed included swimming activity, feeding response, and changes in the body color of the treated fish (Sembiring *et al*., 2018).

The fish with a response and clinical symptoms of NNV infection was verified molecularly using the RT-PCR method. Five dead barramundis' eyes and brain organs were mixed into one microtube for VNN detection (Mahardika *et al.,* 2020). According to the protocol, RNA from the NNV virus was extracted using Trisure (Bioline, UK). Synthesis of cDNA using a reverse transcription system (Promega) kit according to the procedure. The cDNA samples were kept

at -20 °C until they were used. NNV detection was carried out following the procedure of Novriadi *et al*. (2015) and using a pair of primers F2 (5'-CGTGTCAGTCATGTGTCGCT-3') and R3 (5'-CGAGTCAACACGGGTGAAGA-3') (Koesharyani & Novita, 2006).

Measurement of Hematological Parameters

Blood samplings were collected at 0, 48, 96, 144, and 192 hours post-infection and it was done by random sampling of three individuals per treatment. Firstly, the fish were anesthetized using *4-Allyl-2-methoxy phenol Eugenol* by Merck (200 µL L^{-1} seawater, for 1 minute) and the blood collection was carried out at the bottom of the lateral line in the caudal fin, namely through the caudal vein using a 1 mL sterile syringe. The sterile syringe had previously been rinsed using heparin as an anticoagulant. The blood profiles observed were red blood cells (RBC) and white blood cells (WBC). Total RBCs and WBCs were calculated using a hemocytometer (Johnny *et al*., 2003).

MHCIIα, TNFα, and IL1-β Gene Expression Levels

Samples of fish head-kidney, spleen, and livers from each group were isolated at 0, 48, and 96 hours post-NNV infection (n= 3 for each time point). Before organ target collection, the fish were anesthetized using 4-Allyl-2-methoxy phenol Eugenol by Merck (200 mL L^{-1} seawater, for 1 minute). The organs were collected and stored in Trisure reagent (Meridian Bioscience Asia Pte Ltd, Singapore) and kept at 80°C before the gene expression analysis. Total RNA was extracted using Trisure reagent (Meridian Bioscience Asia Pte Ltd, Singapore) following the manufacturer's instructions. The spectrophotometry method measured RNA concentration at 260 and 280 nm. The cDNA synthesis was carried out from 20 ng mL^{-1} total RNA using the sensiFAST™ cDNA synthesis kit (Bioline, UK) following the manual procedure. Gene expression analysis was performed using the quantitative real-time polymerase chain reaction (qPCR) method. The qPCR process was carried out on an ABI PRISM 7500 machine (Applied Byosystems, Inc. USA) using the 5x Hot Firepol Evagreen qPCR mix (Rox) kit (Solis biodyne, Estonia). The reaction volume for cDNA amplification was 20.0 mL with a final concentration of 1x hot master mix (Rox), 10 pmol F/R primer pair (Table 1) each 250 nM, NFW (Nuclease Free Water) was added to a volume of 20 mL and cDNA (0.01 ng mL^{-1}). The qPCR program was set as follows: initial denaturation of 95°C for 15 minutes, followed by 40 cycles: 95°C for 15 seconds, the annealing temperature of 60°C for 30 seconds, and a final extension temperature of 72°C for 20 seconds. Gene expression levels compared to the AT or ST fish at 0 h. The expression levels of all the genes were analyzed according to the $2^{-\Delta\Delta ct}$ method (Livak & Schmittgen, 2001) after normalization with the elongation factor 1α (EF1α) gene.

Survival Rate Observation

Survival rate (SR) was calculated at the end of the challenge test with the following equation:

$$SR\ (\%) = \left[\frac{(Nt)}{(No)} \times 100 \right]$$

where:

Nt = is the number of fish that live at the end of the study (individual)

No = is the number of fish at the beginning of the study (individual)

Data Analysis

The data were analyzed using the SPSS version 20 program (SPSS, Inc., USA) to see differences between treatments using the One-way ANOVA method followed by Duncan's posthoc test analysis.

RESULTS AND DISCUSSION

Visual Response

Visual response of barramundi post NNV infection are shown in Table 2. The results of clinical signs observed in barramundi post-NNV infection showed that fish that were suspected to be positively infected with NNV decreased appetite to eat at 48 hpi, started to be solitary at 96 hpi, darkened body color at 72 hpi, and often showed abnormal swimming behavior at 120 hpi for the AT barramundi. The body color is darker for the ST barramundi at 96 hpi.

Confirmation of the presence of the virus in the treatment of NNV infection was carried out by the RT-PCR method (Figure 1). The fish that died during the study indicated that the barramundi was positively infected with VNN, as shown in Figure 1 sample number 1 and 2.

The clinical symptoms of various fish species infected by NNV are different. The barramundi NNV-infected showed clinical symptoms; the fish looked lethargic and floated on the surface with hyperinflation of the swim bladder according to Munday *et al*. (2002). The clinical symptoms of barramundi in this study have shown the swim bladder of this fish is not enlarged. VNN disease can infect the brain. It can cause fish to swim around in circles, loss of appetite, hyperactivity, and sporadically swimming (Sudaryatma *et al*., 2012; Sembiring *et al*., 2018).

Table 1. Primer sequences used in the study

Gene	Sequence (5' – 3')	Tm (°C)	Reference
MHCIIα	F: CTGGATGGTGAAGAGAAGTG R: TGGAAGGAGGATCATTGTC	60	Mohd-Shaharuddin *et al*. (2013)
TNFα	F: CGAGGGCAAGACTTTCTTTG R: GCACTGCCTGTTCAGCTACA	60	Poisa-Beiro *et al.* (2008)
IL1β	F: CAGGACTCCGGTTTGAACAT R: GTCCATTCAAAAGGGGACAA	60	Poisa-Beiro *et al.* (2008)
EF1α	F: GCTTCGAGGAAATCACCAAG R: CAACCTTCCATCCCTTGAAC	60	Geay *et al*. (2011)

Table 2. Response of barramundi post-NNV infection

Population	Movement activity	Feeding response	Discoloration
AT	A total of 1-4 individuals begin solitary and weakening at 96 hpi and 1-3 individuals swim abnormally at 120 hpi.	Loss of appetite at 48 hpi	A total of 1-2 individuals' pigmentation in the body at 72 hpi
AK	Always active swim	Response to feed	The body color silver
ST	A total of 1-2 individuals begin solitary and weakening at 96 hpi and 1-2 individuals swim abnormally at 120 hpi.	Loss of appetite at 48 hpi	A total of 1-3 individuals' pigmentation in the body at 96 hpi
SK	Always active swim	Response to feed	The body color silver

Description: AT = Australian fish population infected with NNV)
AK = Australian population injected with PBS without a challenge test)
ST = Situbondo population infected with NNV)
SK = Situbondo population not infected with NNV)

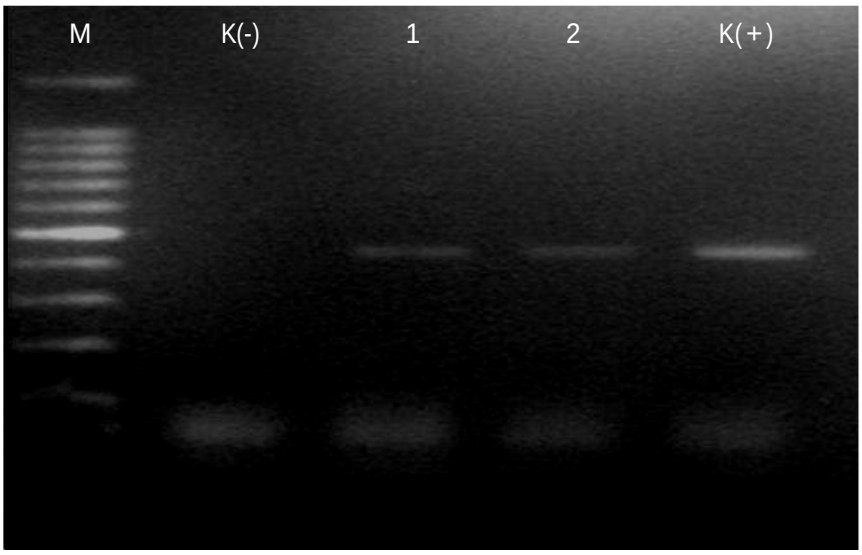

Figure 1. Confirmation of NNV in barramundi *L. calcalifer* after challenge. Description M: marker (100 bp); 1 and 2: NNV-infected barramundi (n= 5, pool). K is for non-infected barramundi (PBS); K+ is for NNV positive control. Positive NNV is represented by a line (band) of 426 bp.

Fish Blood Profiles

The blood profiles of AT and ST barramundi post-NNV infection was presented in Table 3. The number of red blood cells decreased at 48–96 hpi, then started to increase again at 144 hpi for the ST barramundi, while for AT barramundi an increase in red blood cells was observed at 192 hpi. The red blood cell count of ST barramundi at 96 hpi was higher ($P < 0.05$) than AT barramundi. The number of white blood cells in ST barramundi at 48 hours was higher ($P < 0.05$) than in AT but started to decrease at 96 hpi in ST fish, while the total white blood cells count in AT barramundi increased from 48 to 192 hpi.

The decrease in the number of RBCs at 48 hpi corresponded to the clinical symptoms of the test fish, loss of appetite at 48 hpi; the expression of TNFá and IL1â increased, while the expression of MHCIIá decreased. This is following the results of research by Bailone *et al*., (2010), who reported that a decrease in the number of erythrocytes and hemoglobin levels of tilapia *Oreochromis niloticus* could be caused by infection with the bacterium *Aeromonas hydrophila*.

The decrease in the number of RBCs causes the fish to become hypoxic, and anemic, and the metabolic system is disturbed, which is characterized by decreased appetite. Disruption of the metabolic system causes the body's immunity to decrease, making it susceptible to disease and causing death.

An increase in the WBCs count indicates a fish body response to NNV infection. The decreased WBC count indicated that the recovery of WBC in ST fish was faster than in AT fish. The number of WBCs increased in the African catfish *Clarias gariepinus* against an *Aeromonas hydrophila* bacterial infection (Suprapto *et al*., 2017). The WBCs count is used as an indicator of the health status of several fish species because it provides information related to the tolerance of infectious diseases such as viruses and bacteria or stressors in fish (Daneshvar *et al*., 2012; Santos *et al*., 2012; Sayed & Moneeb, 2015).

Immune Gene Expression

The gene expression of MHCIIα, TNFα, and IL1β of ST and AT barramundi population post-NNV infection are shown in Figure 2. ST barramundi had a higher MHCIIα gene expression level than AT barramundi at 96 hpi ($p < 0.05$). The highest level of MHCIIα expression at 96 hpi occurred in the head kidney of fish from the local population of Situbondo (6.44 ± 1.12-fold change), followed by the liver (4.63 ± 1.36-fold change) and spleen (2.29 ± 0.23-fold change). The MHCIIα gene plays a role in the specific immune response and resistance of barramundi after NNV infection. In general, the level of gene expression in specific immunity shows a low value at the beginning of infection.

The ST barramundi had a higher TNFα expression than the AT barramundi ($p < 0.05$). The results of this study showed that the gene expression level of ST barramundi was significantly higher than that of AT barramundi at 48 hpi. This high level of expression is a response to an NNV infection that triggers the immune system. The highest induction of TNFα expression at that hour occurred in the head kidney of ST barramundi (18.13 ± 4.19-fold change), followed by the liver (10.84 ± 1.78-fold change) and spleen (8.27 ± 0.52-fold changes).

Table 3. Red blood cell (RBC) and white blood cell (WBC) of barramundi *Lates calcarifer* post-NNV infection

Parameters	Treatments	Time after infection (hours)				
		0	48	96	144	192
RBC (x106 cell mm-3)	AT	3.44 ± 0.39^{a}	2.43 ± 0.05^{b}	1.44 ± 0.06^{a}	1.84 ± 0.09^{a}	2.28 ± 0.06^{a}
	AK	3.49 ± 0.11^{a}	3.40 ± 0.11^{a}	3.58 ± 0.08^{c}	3.36 ± 0.12^{c}	3.52 ± 0.10^{c}
	ST	3.47 ± 0.06^{a}	2.40 ± 0.05^{b}	1.91 ± 0.09^{b}	2.22 ± 0.03^{b}	2.52 ± 0.05^{b}
	SK	3.44 ± 0.09^{a}	3.25 ± 0.10^{a}	3.36 ± 1.14^{c}	3.31 ± 0.23^{c}	3.54 ± 0.15^{c}
WBC (x106 cell mm-3)	AT	$1.12 \pm 0{,}03^{a}$	1.23 ± 0.03^{b}	1.73 ± 0.08^{c}	1.70 ± 0.05^{c}	1.63 ± 0.03^{c}
	AK	1.13 ± 0.08^{a}	1.12 ± 0.06^{a}	1.12 ± 0.08^{a}	1.12 ± 0.03^{a}	1.13 ± 0.12^{a}
	ST	1.12 ± 0.03^{a}	1.68 ± 0.28^{c}	1.50 ± 0.05^{b}	1.55 ± 0.15^{b}	1.40 ± 0.05^{b}
	SK	1.12 ± 0.06^{a}	1.12 ± 0.03^{a}	1.12 ± 0.08^{a}	1.10 ± 0.10^{a}	1.12 ± 0.08^{a}

Descriptions: The same letters in the column bar on the same parameter and hour (mean ± SD, n = 3) indicated no significant differences ($P > 0.05$). AT (Australian fish population infected with NNV); AK (Australian population injected with PBS without a challenge test); ST (Situbondo population infected with NNV); and SK (Situbondo population not infected with NNV)

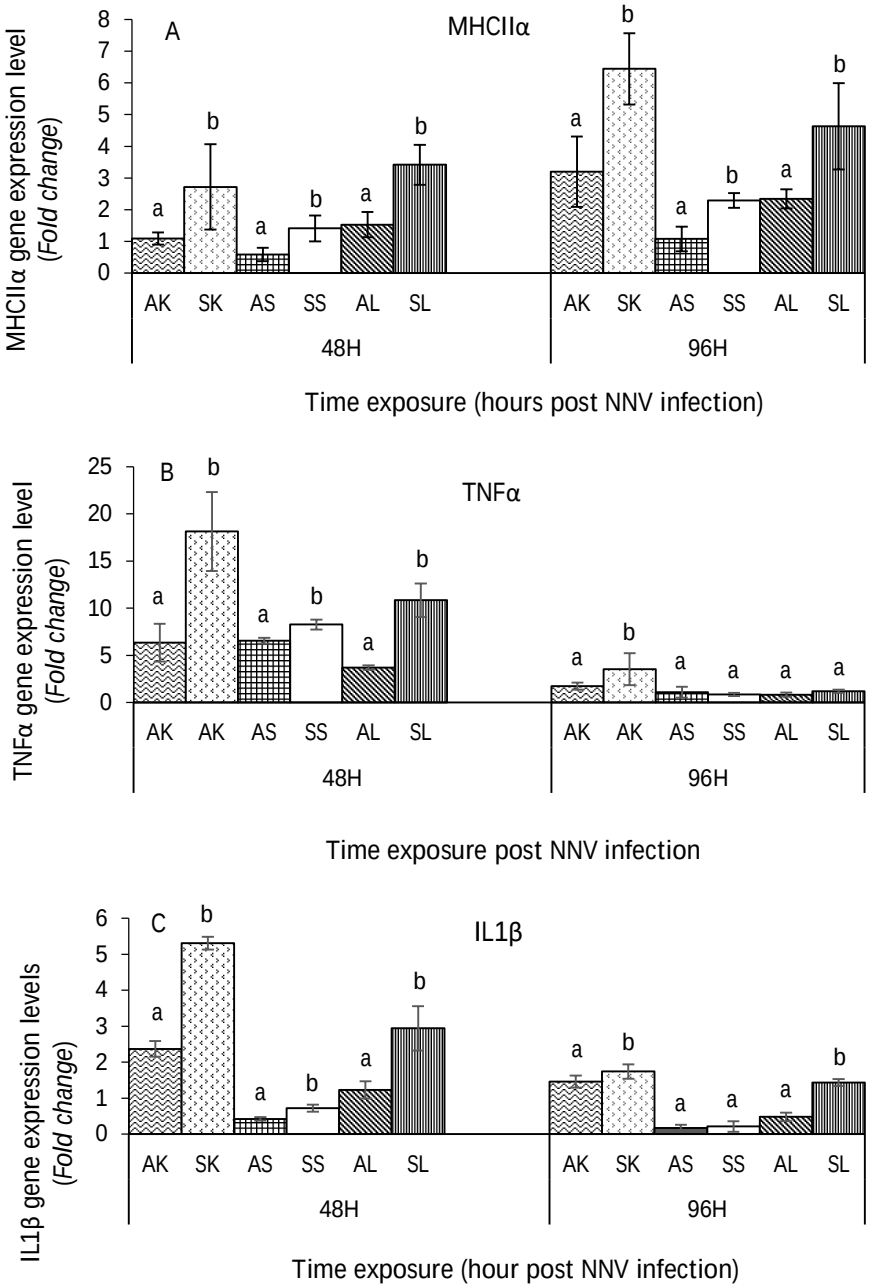

Figure 2. Gene expression data are shown as mean ± SD (n=3) after normalization with the elongation factor 1α (EF1α) gene, the expression levels of all the genes were compared to the expression levels at 0 hpi. Values in the same bar and population followed by the same letter in different populations and at the same hour showed no significant difference (P>0.05). AK (Head kidney AT barramundi); SK (head kidney ST barramundi), AS (Spleen AT barramundi), SS (Spleen ST barramundi), AL (Liver AT barramundi), SL (Liver ST barramundi), Major histocompatibility complex class IIα (MHCIIα), tumor necrosis factor alpha (TNFα) and interleukin-1-beta (IL1β).

ST barramundi had a higher IL1β gene expression level than AT ($p < 0.05$). The expression level of this gene was highest at 48 hpi. The highest induction of IL1β expression at that hour occurred in the head kidney of ST barramundi (5.31 ± 0.18-fold change), followed by the liver (2.94 ± 0.62-fold change) and spleen (0.72 ± 0.10-fold change). TNFα and IL1β gene expression were higher at 48 hpi and decreased at 96 hpi for both populations. TNFα and IL1β are the non-specific immune system. Thus, it is most likely that the non-specific immune system responds early in the infection, and then the specific immune system works after the non-specific immune system. This is because the immune system has recognized the NNV that has infected barramundi and given it a cytokine signal.

This study's results align with Elbahnaswya & Elshopakey (2020) that when the immune system recognizes the pathogen, it will signal cytokines, activate TNFα and provide an immune response. The body can eliminate the pathogen without causing inflammation or tissue damage. Poisa-Beiro *et al*. (2008) also reported that *Dicentrarchus labrax* after being infected with a nodavirus showed an increased expression pattern at 72 hpi, then it decreased at 120 hpi for TNFα and IL1β. The results of this study showed a decrease in TNFα and IL1β gene expression at 96 hpi.

IL1β is a proinflammatory cytokine that plays a role in controlling infection homeostasis, the mediator of inflammation and tissue repair. This gene plays an important role in the inflammatory response as the initiator of the other cytokines during inflammation. As the results from the study of barramundi post-NNV infection, it is assumed that NNV infection triggers inflammation in the tissue and causes brain damage. Inflammation is a response of the immune system to maintain fish survival during infection.

Fish Mortality

The mortality of Situbondo (ST) and the Australian barramundi (AT) that were challenged for 14 days is presented in Figure 3. ST and AT barramundi began to die on the 5th day, at 4% and 6%, respectively. There were no deaths in the ST barramundi after the 10th day, while the AT barramundi did not die after the 12th day. The peak of mortality for both ST and AT barramundi occurred at 168 hpi. The survival of ST barramundi was 42.00 ± 4.47% at the end of the observation, two times higher ($P < 0.05$) than AT barramundi, with a survival of 20.00 ± 4.47%. The fish injected with PBS were all alive.

Based on observations, it was shown that barramundi from both populations began to die at 120 hpi. This virus can be transmitted to healthy fish within 4 days. An increase in the white blood cell count indicates a response from the fish body to NNV infection. The number of deaths began to decrease at 192 hpi for ST barramundi, while the decrease in mortality for AT barramundi occurred after the 10th day. These results are consistent with a significant increase in the RBC count between populations at 144 to 192 hpi and a decrease in the white blood cell count from 96 to 192 hpi between populations.

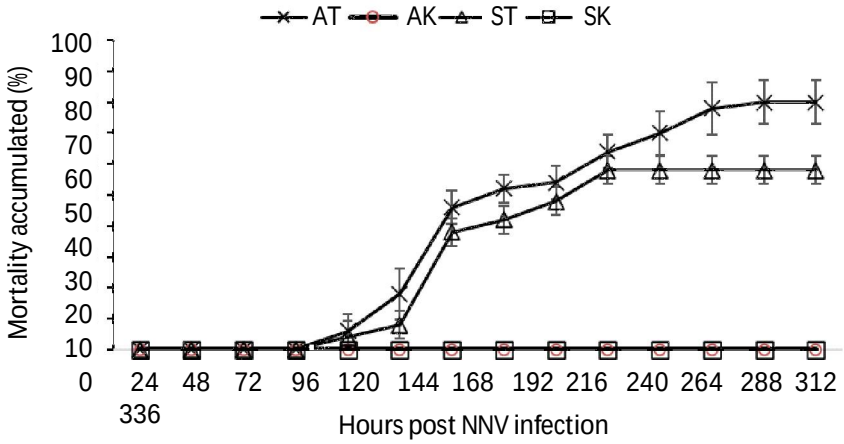

Figure 3. Mortality of barramundi post-NNV infection. Data is shown as fish survival mean ± SD (n = 3). AT (Australian fish population infected with NNV); AK (Australian population injected with PBS without a challenge test); ST (Situbondo population infected with NNV); and SK (Situbondo population not infected with NNV).

These results indicate the important roles of genes in barramundi's immune response against viral infection. These results are a response to NNV infection that triggers the immune system, so the Situbondo barramundi is more resistant to NNV infection than the Australian barramundi. Based on these results, Situbondo barramundi (ST) has the potential to be used as a candidate for generating broodstock of disease-resistant varieties.

CONCLUSIONS

Overall, the number of red blood cells, and white blood cells, also MHCIIα, TNFα, and IL1β gene expression values of Situbondo barramundi (ST) population were higher than those of the Australian barramundi (AT) population. The Situbondo barramundi is more resistant to NNV infection than the Australian barramundi.

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