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by Sang Ayu Made Putri Suryani

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Vertical Infections and Prevalence of Viral Nervous Necrosis (VNN) in Milkfish (*Chanos chanos*)

Sang Ayu Made Putri Suryani[1*], Ni Made Darmadi[1], Made Goura Premananda[2] and Gusti Ngurah Permana[3]

[1]Water Resources Management Study Program, Faculty of Agriculture, Warmadewa University, Jl. Terompong, Denpasar 80239, Bali, Indonesia

[2] Magister Programme of Environment Science, Udayana University, Denpasar 80232, Bali, Indonesia

[3] Center for Research and Development of Marine Cultivation, Buleleng 81105, Bali, Indonesia

*Correspondence :
suryanip@rocketmail.com

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Abstract

Milkfish juvenile is the leading fishery species in Bali with an export value in 2019. The increase in demand is related to the intensification of aquaculture which causes various diseases, especially Viral Nervous Necrosis (VNN). VNN infection in juveniles has been through water media. The VNN infection in milkfish may occur vertically. This study aims to determine the vertical infection and prevalence of VNN in milkfish. The VNN examination was carried out using the reverse transcription real-time Polymerase Chain Reaction (rRT-PCR) method. This research was conducted at BKIPM Denpasar. Samples were milkfish eggs taken from 15 spawning ponds in Gerokgak District, Buleleng Regency. The results showed 3 positive samples from the 15 samples tested. A4 VNN positive sample with cycle threshold (CT) 39.29 showed a virus concentration of 146 copies, Sample F positive for VNN with CT 38.79 showing the 1978 copies of virus concentration, Sample G was positive for VNN with CT 39.16 showing a virus concentration of 1588. Positive results of VNN examination on milkfish eggs with real-time PCR indicate the presence of vertical transmission of VNN. It is also supported by the measurement of water quality in milkfish spawning ponds shows appropriate standard conditions. The results of the observation of clinical symptoms also show the milkfish in a healthy state seen from swimming behavior that does not show symptoms of VNN infection. There are 3 positive samples in sample A4 with a virus concentration of 1,466 copies, sample F with a virus concentration of 1,978 copies and sample G with a virus concentration of 1,588. Detected VNN in eggs milkfish showed vertical VNN infection. Prevalence of infection. Milkfish egg against VNN shows a value of 20% which is included in the frequent infection category.

INTRODUCTION

Milkfish seeds have been exported to various countries in Asia. The main export destination country for Indonesian milkfish seeds is the Philippines with an export value of 2.12 billion seeds. Other milkfish export destinations are Taiwan,

Singapore, Thailand, Sri Lanka, Malaysia, Hong Kong, China, Colombia, East Timor, and Vietnam (Kementerian Kelautan dan Perikanan, 2020). The increasing demand for milkfish seeds is in line with the intensification of milkfish cultivation. Intensification of cultivation can lead to various diseases caused by viruses, bacteria, and parasites. The type of virus that often causes losses in aquaculture is Viral Nervous Necrosis (VNN) (Manan and Fitriatin, 2015; Lee and Bahaman, 2012). VNN in Indonesia was first identified in a snapper hatchery in East Java in 1997.

Transmission of VNN in grouper can occur if healthy fish come into contact with fish infected with VNN within 4 days of contact (Lestari and Sudaryatma, 2014; Gilda *et al.*, 2009). VNN transmission through water media comes from infected fish feces. VNN infection in grouper larvae is also known to be transmitted through parents who are positive for VNN infection, resulting in vertical VNN infection (Sudaryatma *et al.*, 2012). VNN in grouper was detected in the gonads, intestines, kidneys, and liver. VNN can spread in the ovaries of the parent fish, so the eggs can be a medium for vertical transmission of VNN (Gomez *et al.*, 2006; Bandín and Souto, 2020).

Common clinical symptoms in VNN-infected milkfish showed uncoordinated movements such as vertical and circular swimming behavior, sinking to the bottom of the water, and stomach facing upwards when the milkfish were resting. Infected milkfish seeds show swelling of the swim bladder, changes in skin color that become dark and pale. Milkfish VNN-infected generally shows vacuolization (swelling) and necrosis in the brain, spinal cord, and retina of the eye (Sethi *et al.*, 2018).

Based on the description above, it is necessary to research milkfish to determine vertical VNN infection as occurs in groupers. Examination of the presence of VNN in fish was carried out using the real-time polymerase chain reaction (RT-PCR) method. The results of the study are expected to provide information on the vertical transmission of VNN in milkfish and infection prevention and control can be carried out more precisely and quickly.

METHODOLOGY

Place and Time

This study was conducted in July 2021. Sampling locations were carried out in spawning ponds in six locations, namely Patas Village, Penyabangan Village, Sanggalangit Village, Gerokgak Village, Gondol Village, and Musi Village, Gerokgak District, Buleleng Regency.

Research Materials

Research materials include gel ice, chloroform, 95% ethanol, 75% ethanol, RNA extraction solution, isopropanol, DPEC ddH2O, aquades, primer R (20M), primary F (20M), probe (10M), RNA templates, quantities probe RT PCR master mix, RNAse free water, quantities RT mix. The tools used include egg collector, micropipette, microtip, surgical scissors, petri dish, paper mat, tweezers, pastel, PCR workstation, mini spin down, qPCR machine unit, centrifuge, vortex, 1.5 ml Eppendorf, DO meter, PH meter, Microtube, Micropipet, Microtube rack, Freezer, and refrigerator.

Research Design

VNN examination with rRT-PCR used 3 positive control VNNs, namely VNN 104, 105, and 106 and 1 negative control. The data are in the form of a graph of the cycle threshold (CT) value of the rRT-PCR examination results, a graph of the test curve, and the value of the VNN concentration. A positive test is indicated by a CT graph that exceeds the threshold line. Furthermore, the number of VNN is presented in concentration.

Work Procedure

RNA Extraction

Extraction is the process of separating the nucleic acid (RNA) of a pathogen from its host cell. The steps of RNA extraction are homogenization and sample preparation (Walsh *et al.*, 2001). Milkfish eggs were crushed using sterile

scissors then the sample was put into a 1.5 ml Eppendorf tube containing 0.5 μl of RNA extraction solution. The sample was ground with pestle, then 100 μl of chloroform was added to maximize the work of the RNA extraction solution. Chloroform functions as an organic solvent. The sample was vortexed at high speed for 20 seconds. The goal is to dissolve the chloroform. The next step is centrifugation at 12,000 rpm for 15 minutes at a temperature of 2-8 °C to produce the supernatant. Precipitation is a coagulation process of RNA that dissolves in the liquid phase into a solid. The resulting solution is divided into three layers. The supernatant which was the top layer was transferred as much as 200μl into a new 1.5 ml Eppendorf tube which already contained 200 μl of 2-Propanol (Isopropanol).

The next stage was vortexed and centrifuged at 12,000 rpm for 10 minutes. At the stage of giving isopropanol is an RNA precipitation process. Isopropanol will bind to RNA and agglomerate in the liquid phase to become solid or form pellets attached to the wall of the Eppendorf tube after centrifugation. Isopropanol also functions to remove or clean the chloroform residue left in the previous extraction stage. After the centrifugation process, the supernatant contained in the Eppendorf tube was removed slowly so that the RNA pellet was not wasted (Barber *et al*., 2006; Kuniawati *et al*., 2019)

Washing RNA by removing the supernatant, then washing the RNA pellet by adding 500 μl of 75% alcohol, centrifuging at 9,000 rpm for 5 minutes, discarding the alcohol and then drying it. It aims to remove alcohol residues from RNA pellets. Alcohol residue removal is performed by evaporation means because alcohol is volatile. After the RNA pellet was air-dried, then buffer was added. RNA pellets are dissolved with 200 μl of DPEC ddH2O and kept at a temperature of -20 °C. DPEC is also used to disable RNA enzyme work so that no degradation to RNA structures and storage occurs at -20°C temperatures so that extraction results can be stored for a long time sequentially (Pertiwi *et al*., 2016).

PCR Real Time Amplification

The amplification process using the real-time PCR method serves to multiply the DNA material. At the beginning of the process occurred Reverse transcription which served to convert the genetic material of the RNA virus into DNA. RNA virus amplification using real-time PCR method has 4 stages i.e., pre-denaturation, denaturation, annealing, and extension. the pre-denaturation process constitutes the reverse transcription process of RNA (Sulandri and Zein, 2003). Reverse transcription constitutes a process of copying RNA into DNA, whereby a single strand of RNA is to be converted into complement DNA (cDNA) with a temperature setting of 50 °C for 30 minutes for the first stage, continuing with a PCR initial activation stage with a temperature of 95 °C for 15 minutes.

After the RT-PCR process is continued the PCR amplification process where the complement DNA (cDNA) is to be multiplied using two pairs of primers i.e., RNA 2 FOR with the base sequence CAA-CTG-ACA-RCG-AHC-ACA-C. The real-time PCR method has the principle of breaking down double-stranded to single-stranded, this process is affected by its temperature and timing. The division of the double strand into single strands (denatures) takes 15 seconds at 94 °C. Annealing is a primary pasting process on a single strand DNA of at 55 °C with a time of 45 seconds. The primer will adhere to the base (forward) and tip (reverse) of each single DNA. The next stage is DNA extension, the temperature is raised to 55 °C for 45 seconds, and the temperature is the optimum temperature for the activity of the polymerization enzyme. The process of denaturation, annealing, and extension will occur in 40 repetitive cycles (Kurniawati *et al*., 2019).

Data Analysis

The result data from the VNN examination is presented in the form of images, curves, and tables. The collected data will be analyzed descriptively and calculated on the prevalence of VNN. Prevalence is calculated by the formula:

$$\text{Prevalence} = \frac{\text{infected sample}}{\text{total infected sample}} \times 10$$

RESULTS AND DISCUSSION

VNN examination with rRT-PCR

Of the 19 test samples consisting of 3 positive controls, 1 negative control, and 15 samples of milkfish eggs were tested. Real-time testing lasts for 40 cycles that occur repeatedly. The determination of positive samples can be seen if the curve crosses the threshold line and negative samples if the curve does not cross the threshold line. In Figure 2, if the concentration of virus in the positive control increases, then CT will decrease and otherwise. The virus concentration in the positive control of VNN in this study was used in as many as 3 positive controls with concentrations of 10^4,10^5 and 10^6.

Table 1. Test results with PCR method.

No	Color	Sample	Organ	CT	Final concentration (copy)	Test results
1		NTC	Egg			Negative
2		VNN 10^4	Egg	34.59	24.750	Positive
3		VNN 10^5	Egg	30.87	247.500	Positive
4		VNN 10^6	Egg	26.98	2.475.000	Positive
5		A1	Egg			Negative
6		A2	Egg			Negative
7		A3	Egg			Negative
8		A4	Egg	39.29	1.466	Positive
9		B1	Egg			Negative
10		B2	Egg			Negative
11		C1	Egg			Negative
19		C2	Egg			Negative
20		D1	Egg			Negative
21		D2	Egg			Negative
22		D3	Egg			Negative
23		E1	Egg			Negative
24		E2	Egg			Negative
25		F	Egg	38.79	1.978	Positive
26		G	Egg	39.16	1.588	Positive

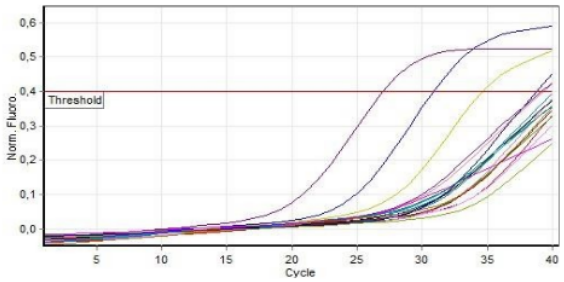

Figure 1. PCR threshold graph.

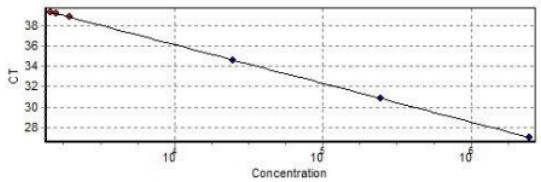

Figure 2. Standard curve PCR method.

The results of the VNN examination process with the Reverse Transcription Real-Time PCR method. Through reading the threshold graph has been valid which is indicated by the amplification of 19 test samples, namely 3 positive VNN examples (VNN 10^4, 10^5, and 10^6) CT threshold curve of each positive VNN sample is higher than the threshold line and 1 negative sample CT threshold curve below the threshold line. Three of the 15 samples of the CT threshold curve are above the threshold line. This shows that there are 3 positive samples, namely samples A4, F, and G. Table 1 shows that the VNN concentrations of positive samples are A4 = 1,466 copies, F = 1,978 copies, and G = 1,588 copies. Based on the number of positive VNN samples as many as 3 samples among 15 samples, the prevalence of VNN was 20%.

The results of the VNN examination on milkfish eggs using the reverse transcription real-time PCR method were positive, indicating the presence of vertical VNN transmission. Vertical transmission of VNN occurs in groupers. As reported by Gomez *et al.* (2006) that VNN infection in grouper larvae is also known to be transmitted through mothers who are positively infected with VNN, resulting in vertical VNN infection. VNN in grouper was detected in the gonads, intestines, kidneys, and liver. VNN can spread in the ovaries of the mother fish, so the eggs can be a medium for vertical transmission of VNN. Eggs are an important viral reservoir.

Water Quality Measurement

The results of water quality measurements on the sampling of the maintenance media include the parameters of dissolved oxygen, temperature, and pH are described in Table 2. The standard used is the optimum value for milkfish maintenance, so if one of the three parameters measured shows a value that is not up to standard. The optimum value can be tolerated if the other parameters reach the optimum value.

Table 2. Results of water quality measurement.

Parameters	A1	A2	A3	A4	B1	B2	C1	C2	Standard
Temperature (°C)	29.85	29.65	30.75	29.35	30.55	29.45	29.75	29.55	29
pH	7.5	7.95	7.65	7.45	7.55	8.15	7.45	7.75	7.5-8.5
DO (ppm)	6.55	6.45	6.75	6.55	6.85	6.55	6.55	6.65	>5

Parameters	D1	D2	D3	E1	E2	F	G	Standard
Temperature (°C)	30.85	31.15	29.95	29.75	31.25	30.85	28.65	29
pH	7.75	7.75	8.05	7.85	7.65	8.05	7.45	7.5-8.5
DO (ppm)	6.8	6.85	6.65	6.55	6.95	6.85	6.15	>5

Note:
A : location in Patas Village
B : location in Gerokgak Village
C : location in Gondol Village
D : location in Penyabangan Village
E : location in Penyabangan Village
F : location in Sanggalangit Village
G : location in Musi Village

The results of temperature measurements shown in Table 2 are ponds that have the code D2 with a temperature of 31.15 °C and E2 with a temperature of 31.25 °C which are not the optimum temperature for milkfish cultivation which has a value of 28-30 °C (Syahid *et al.*, 2006; Sustianti *et al.*, 2014). Tropical fish can still live normally in the range of 31-35 °C if the dissolved oxygen concentration is high enough (Ghufron *et al.*, 2007). The dissolved oxygen content (DO) shown in Table 2 ranges from 6.1 – 7 ppm, indicating that the dissolved oxygen content in all ponds meets the optimal requirements for dissolved oxygen content for milkfish cultivation where the oxygen content for optimal milkfish cultivation is greater than 5. The pH values shown in Table 2 have 3 pools whose pH values have not reached the optimum value, namely in pools A4, HD, and C1 with a pH value of 7.45, but this value is not too far from the optimum value, which is at a pH value of 7.5- 8.5 and it can be said that the pH value of 7.45 is still suitable for milkfish cultivation (Sustianti *et al.*, 2014).

Observation of Clinical Symptoms

The results of observations of clinical symptoms of milkfish in each sampling location did not show clinical symptoms because milkfish were not a specific host for the spread of the VNN virus. VNN can also be detected in fish without clinical signs of infection. Grouper is a specific host for the spread of the VNN virus and is a source of virus for its larvae with symptoms of circular swimming behavior (Sethi *et al.*, 2018; Lestari and Sudaryatma, 2014). Milkfish positive infected with VNN can be seen in Table 2. The positive milkfish samples have a higher CT value than the positive control. The higher the concentration of the VNN virus, the lower the CT value.

CONCLUSION

In milkfish vertical infection occurs as happened in grouper. VNN prevalence of 20 % is included in the frequent category. This needs to be watched out for efforts to prevent and control the incidence of infection, including through ozonation of eggs, and mapping of the brooders that are the origin of VNN-infected eggs until vaccination is carried out.

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