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# DETECTION OF MEGALOCYTIVIRUS IN CLOWNFISH (*Amphiprion ocellaris*)

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## ABSTRACT

This study aimed to detect the presence of Megalocytivirus in clownfish (*Amphiprion ocellaris*) obtained from the Ambon Marine Aquaculture Center (BPBL). The research used a descriptive laboratory, non-experimental approach, with molecular detection performed through Polymerase Chain Reaction (PCR) and nested PCR techniques. Five clownfish specimens (approximately 3 cm) were sampled, and spleen tissue was used as the target organ for analysis. The extracted DNA was assessed for concentration and purity before amplification by PCR and nested PCR. The amplified products were analyzed using agarose gel electrophoresis to identify the presence of viral DNA bands. The results showed no Megalocytivirus-specific DNA bands in any of the samples, whereas the positive control produced the expected result, confirming the validity of the testing procedure. The study concluded that all clownfish samples from BPBL Ambon were negative for Megalocytivirus infection and were considered free from the virus at the time of sampling.

***Keywords***: *Amphiprion ocellaris, BKHIT Maluku, Megalocytivirus, nested PCR, PCR*

## INTRODUCTION

Clownfish (*Amphiprion ocellaris*) are one of the marine ornamental fish commodities with high economic value and strong demand in both domestic and international markets. Indonesia, including the Maluku region, is one of the distribution centers for marine ornamental fish because of its high biodiversity. The continuously increasing market demand has encouraged the development of aquaculture activities at the Ambon Marine Aquaculture Center (BPBL). However, increased production has also been accompanied by a higher risk of infectious diseases that may threaten the sustainability of aquaculture operations.

One important disease that requires attention is viral infection caused by the genus Megalocytivirus, family Iridoviridae, which is known to cause systemic infections and mass mortality in cultured fish. This virus can cause cell enlargement (megalocytosis) in the spleen, kidney, and liver and may result in high mortality, reaching 20–60% within a short period (Kurita & Nakajima, 2012). Prasetyo et al. (2022) stated that Megalocytivirus frequently infects marine

ornamental fish and causes clinical signs such as enlargement of internal organs, body lesions, and sudden mortality. In Indonesia, Megalocytivirus has been reported to infect various fish species, including grouper, snapper, and several other marine ornamental fish (Abidin, 2013; Santoso et al., 2022). Based on Ministerial Decree of the Ministry of Marine Affairs and Fisheries No. 81 of 2015, this virus has been detected in several regions, including North Sumatra, Lampung, Bali, and the Riau Islands.

Nevertheless, data on viral infection in clownfish, particularly in the Maluku region, remain limited. Clownfish are among the major commodities cultured in Maluku; therefore, the potential for disease transmission is considerable. Early detection is necessary to determine fish health status and prevent disease spread in both aquaculture environments and ornamental fish trade chains. The Fish, Animal, and Plant Quarantine Center (BKHIT) Maluku plays an important role in conducting diagnostic testing to determine the health status of fish entering and leaving the region.

Molecular detection methods such as Polymerase Chain Reaction (PCR) and nested PCR are highly sensitive and specific techniques for detecting Megalocytivirus in fish (Hidayat et al., 2023). The spleen was selected as the target organ because it is a major site of viral replication and can show significant pathological changes during infection. This study aimed to determine the presence of Megalocytivirus in clownfish (Amphiprion ocellaris) and to provide scientific information regarding the occurrence of Megalocytivirus in clownfish, thereby supporting disease prevention and control efforts in marine ornamental fish aquaculture in Maluku.

## MATERIALS AND METHODS

The study was conducted in September 2025 at the Laboratory of the Fish, Animal, and Plant Quarantine Center (BKHIT) Maluku, located in Kate-kate Village, Teluk Ambon District, Ambon City. The research location is presented in Figure 1.




*Figure 1. Research Location Map*

This research was a descriptive laboratory study using a non-experimental approach. It was conducted to describe the presence of Megalocytivirus in clownfish (*Amphiprion ocellaris*) without applying treatments or manipulating variables in the samples. The data obtained were analyzed descriptively based on the results of molecular laboratory testing. The detection methods used were Polymerase Chain Reaction (PCR) and nested PCR, in accordance with Indonesian National Standard (SNI) 8231-2:2018 concerning Megalocytivirus detection using conventional nested PCR. Sampling was conducted by collecting spleen tissue from the fish. The spleen was selected as the target organ because it is a major site of viral replication, considering its role in the immune system

and blood filtration. In addition, the spleen may show characteristic pathological signs of infection, facilitating observation.

After clownfish (*Amphiprion ocellaris*) specimens larger than 3 cm were obtained from BPBL for transport through BKHIT Maluku, the target organ, the spleen, was separated and stored fresh. The samples were then extracted using an RNA/DNA Silica Extraction Kit to obtain purified DNA. The extracted DNA was measured for concentration and purity using a spectrophotometer and then used as a template for the first PCR. If no target was detected, nested PCR was performed to increase sensitivity. PCR products were visualized by agarose gel electrophoresis, and the results were determined based on the presence or absence of DNA bands compared with positive and negative controls.

The study was designed to identify and detect Megalocytivirus in clownfish (*Amphiprion ocellaris*) using PCR and nested PCR techniques. The research was conducted at the BKHIT Maluku Laboratory following molecular standard protocols. Five clownfish specimens measuring 3 cm were obtained from BPBL Ambon. In this study, sample preparation and documentation were conducted carefully before molecular analysis. Each clownfish specimen was subjected to biometric measurements to quantitatively document its size, generally by measuring total length or standard length using a precision measuring instrument. The measurement results were recorded together with the fish identification code. Clinical signs were then examined through behavioral observation (such as lethargy or loss of balance) and physical/morphological examination to detect signs that might be associated with Megalocytivirus, including hyperpigmentation, hemorrhage of the skin, or eye edema.

Following clinical documentation, the fish were euthanized and necropsied using sterile instruments to collect the target organ. The spleen was selected because of its potential to contain a high concentration of virus. Approximately 25–50 mg of spleen tissue was collected. The sample was then extracted using an RNA/DNA Silica Extraction Kit until purified DNA was obtained. The DNA concentration and purity were subsequently measured using a spectrophotometer. The extracted DNA was used as the template for the first PCR, and if negative, nested PCR was performed. PCR products were visualized through agarose gel electrophoresis and interpreted by comparing sample DNA bands with positive and negative controls. Virus identification was conducted in accordance with SNI 8231-2:2018 concerning Megalocytivirus detection using the conventional nested Polymerase Chain Reaction (PCR) method at the BKHIT Maluku Laboratory. The research consisted of the following stages:

**Sample Collection**

Clownfish samples were obtained from BPBL Ambon and were scheduled for transport through BKHIT Maluku. The necessary equipment was prepared beforehand to ensure that the samples arrived in appropriate condition and complied with applicable testing procedures. Five fish measuring 3 cm were collected. The samples were placed in sterile plastic bags, labeled with the sampling location code, placed in a cool box, and transported to the laboratory.

**Necropsy (Sample Preparation)**

The spleen was selected as the target organ during necropsy because it is a major site of Megalocytivirus replication, contains a high concentration of virus, and shows clear pathological changes such as megalocytosis and enlargement. This selection is also consistent with international standards such as the WOAH Aquatic Manual (2022), ICTV (2023), and studies such

as Kurita & Nakajima (2012), which indicate that the spleen and kidney are appropriate organs for molecular detection of Megalocytivirus using PCR and nested PCR.

**1. DNA Extraction (RNA/DNA Silica Extraction Kit)**

a) Add 20–30 mg of sample to 900 µL GT Buffer (homogenize the sample using a pestle/tissue ruptor), then centrifuge at 12,000 rpm for 3 minutes.
b) Prepare a new tube and add 40 µL of Silica solution (mix the silica solution before use).
c) Transfer 600 µL of the centrifuged sample into the tube containing 40 µL Silica solution (vortex until homogeneous).
d) Centrifuge at 12,000 rpm for 15 seconds (not more than 20 seconds), then discard the supernatant.
e) Add 500 µL GT Buffer (homogenize by pipetting until the pellet dissolves, then vortex until homogeneous).
f) Centrifuge at 12,000 rpm for 15 seconds and discard the supernatant.
g) Add 1 mL of 70% ethanol (dissolve the pellet by pipetting, then vortex until homogeneous).
h) Centrifuge at 12,000 rpm for 15 seconds (not more than 20 seconds), discard the ethanol, and remove residual ethanol with a pipette.
i) Add 1 mL DEPC ddH2O (mix by pipetting, then vortex until dissolved).
j) Incubate at 55 °C for 10 minutes, then centrifuge at 12,000 rpm for 2 minutes.
k) Transfer 500 µL of the supernatant to a new tube (DNA/RNA is ready for use).

**2. Measurement of DNA Concentration and Purity**

a) Measure DNA concentration (ng/µL) using a DNA concentration measuring instrument at a wavelength of 260 NM. Calculate DNA concentration using the following formula:
b) DNA concentration = A260 × 50 × DF
c) where: A260 = absorbance value at 260 NM; DF = dilution factor.
d) Dilute the sample if the concentration obtained is higher than required.
e) Measure DNA purity by calculating the ratio of absorbance at 260 NM to 280 NM (A260/A280).
f) Store the DNA solution at −20 °C if it is not used immediately.

**3. Amplification**

a) Thaw the DNA template, master mix, primers, and NFW by placing them on an ice block rack.
b) Prepare the cocktail according to Table 1. Prepare 10% more cocktail volume than required.
c) Homogenize all cocktail components and distribute them into 0.2 ml micro tubes for the step-one master mix.
d) Add 2 µL of DNA template (10–100 ng) from the test sample, positive control (Megalocityvirus-positive DNA), and negative amplification control (NTC).
e) Perform amplification using a thermal cycler according to the amplification conditions/program in Table 2.
f) If the first-stage PCR result is negative, proceed with nested PCR using the master-mix formulation in Table 3.
g) Transfer 2 µL of the first-stage PCR product into the master mix for nested PCR.
h) Perform nested PCR amplification according to Table 4.

Table 1. Cocktail composition for first-stage PCR

| No. | Component | Volume | Cycle |
|---|---|---|---|
| 1 | 2× Master mix | 12.5 | 1× |
| 2 | Forward Primer 1105 (10 µM) | 1 | 0.4 |
| 3 | Reverse Primer 1106 (10 µM) | 1 | 0.4 |
| 4 | Nuclease-Free Water (NFW) | 8.5 | – |
| 5 | DNA Template | 2 | 10–100 ng |
| | Total volume | 25 | |

*Table 2. First-stage amplification program*

| Process | Temperature (°C) | Time | Cycles |
|---|---|---|---|
| Initial denaturation | 95 | 15 min | 1 |
| Amplification | 94 | 30 s | 30 |
| | 55 | 30 s | |
| | 72 | 60 s | |
| Final extension | 72 | 7 min | 1 |
| Storage | 12 | Until next stage | |

*Table 3. Cocktail composition for nested PCR*

| No. | Component | Volume | Cycle |
|---|---|---|---|
| 1 | 2× Master mix | 12.5 | 1 |
| 2 | Forward Primer 1105 (10 µM) | 1 | 30 |
| 3 | Reverse Primer 1106 (10 µM) | 1 | |
| 4 | Nuclease-Free Water (NFW) | 8.5 | |
| 5 | DNA Template | 2 | 1 |
| | Total volume | 25 | |

*Table 4. Nested PCR amplification program*

| Process | Temperature (°C) | Time | Cycles |
|---|---|---|---|
| Initial denaturation | 95 | 15 min | 1 |
| Amplification | 94 | 30 s | 30 |
| | 55 | 30 s | |
| | 72 | 60 s | |
| Final extension | 72 | 7 min | 1 |
| Storage | 12 | Until next stage | |

**4. Electrophoresis**

a) Place the prepared gel in the electrophoresis chamber.
b) Fill the chamber with 1× TAE buffer until the gel is submerged.
c) Load 5 µL of marker or DNA marker (100 bp DNA ladder).
d) Load 8 µL of PCR product into the wells of the agarose gel using a micropipette.
e) Close the electrophoresis chamber and apply an electrical voltage of 100–150 V for 30 minutes.
f) Stop electrophoresis when the bromophenol blue dye reaches approximately two-thirds of the agarose gel length.

**Data Analysis**

The data were analyzed descriptively to systematically and factually describe the phenomenon under investigation. The resulting data were obtained from observations in the form of images.

## RESULTS AND DISCUSSION

Based on the test results from clownfish (*Amphiprion ocellaris*), the general mechanism of Megalocytivirus infection is characterized by the entry of the virus into the fish through epithelial tissues, followed by dissemination through the bloodstream to target organs such as the spleen and kidney. In these organs, the virus infects host cells and replicates in the cytoplasm, causing cell enlargement (megalocytosis) and tissue damage. However, based on observations in this

study, there was no indication that this infection mechanism occurred. Clinically, the fish appeared normal, without signs such as changes in body coloration, hemorrhage, or behavioral disturbances. Necropsy results also showed that the internal organs, particularly the spleen, did not exhibit enlargement or pathological changes suggestive of viral infection. Detection using PCR followed by nested PCR showed that all clownfish samples were negative for Megalocytivirus. This was indicated by the absence of target DNA bands at approximately 430 bp in the first-stage PCR and approximately 167 bp in the nested PCR. The absence of these DNA bands indicates that viral genetic material was not detected in the spleen tissue, suggesting that the infection and viral replication mechanism described for Megalocytivirus did not occur in the tested samples.

Electrophoresis results showed that the positive control produced a DNA band at the expected target size, whereas the negative control showed no DNA band. Therefore, the test results were considered valid and reliable.

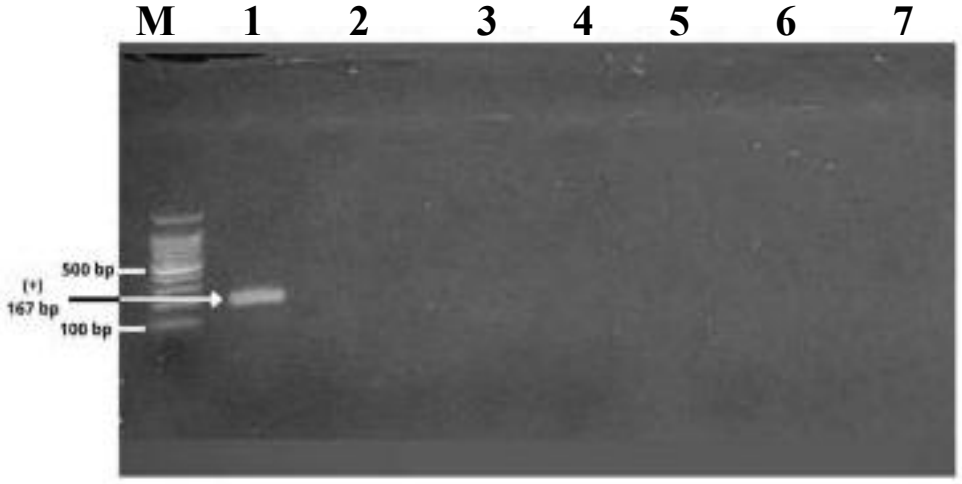



*Figure 2. Results of Megalocytivirus testing in clownfish*

Legend:
Lane M : Marker
Lane 1 : Positive control (+)
Lane 2 : Negative control (−)
Lanes 3–7 : Clownfish samples (Megalocytivirus-negative)

Megalocytivirus is a virus belonging to the family Iridoviridae and genus Megalocytivirus, which is known to cause serious disease in various cultured fish species in both freshwater and marine environments. The virus is characterized by cell enlargement in the spleen, kidney, and liver and can cause systemic infections leading to mass mortality (Kurita & Nakajima, 2012). In this study, detection was conducted using PCR and nested PCR. The first-stage PCR was intended to detect a target DNA fragment of 430 bp, whereas nested PCR was used to increase detection sensitivity with a target DNA fragment of 167 bp. Nested PCR is particularly useful when the amount of viral DNA in a sample is very low or cannot be detected in the first-stage PCR. The sequential use of two primer pairs serves to increase detection sensitivity and specificity, thereby providing more reliable results, particularly in studies of latent or carrier infections.

Electrophoresis results from PCR and nested PCR showed no DNA bands in any of the test samples, whereas the positive control showed a DNA band of the appropriate size. This indicates that amplification proceeded properly and that the PCR reagents functioned according to the standard, so the negative sample results can be considered reliable. The validity of these results is supported by the World Organisation for Animal Health (2022), which states that a PCR test is valid when the positive control shows amplification and the negative control shows no DNA band. In

addition, the absence of DNA bands in the samples indicates that the target viral DNA was not detected in the tested samples (Nugroho et al., 2019; Xuan et al., 2021).

This method is a highly sensitive and specific molecular approach for detecting viruses through amplification of specific target DNA fragments. In this study, the target gene produced a fragment of approximately 167 bp, which served as an indicator of the presence of Megalocytivirus DNA when fish were infected. The electrophoresis gel contained seven lanes, each with a different function. The first lane, M (Marker), represented the DNA molecular-size marker (DNA ladder). The marker served as a reference for identifying the size of amplified DNA bands. In the figure, the DNA marker bands were clearly visible at several positions, including one around 167 bp, which served as a reference for determining whether the amplified products from the fish samples contained a DNA fragment corresponding to the Megalocytivirus target gene.

Lane 1 was the positive control, consisting of a sample known to contain Megalocytivirus DNA. A single DNA band was clearly visible at 167 bp. The presence of this band indicates that the PCR process proceeded properly and successfully amplified the viral target gene. It also indicates that the PCR reagents, primers, and thermal conditions used in the thermal cycler functioned optimally. The presence of a band in the positive control confirms that the detection method applied was valid and reliable.

Lane 2 was the negative control, consisting of a reaction containing all PCR components except the target DNA, which was replaced by nuclease-free water. No DNA band was visible in this lane, indicating that no amplification occurred. The absence of a band in the negative control was expected because it indicates that no DNA contamination occurred during the PCR process. According to the World Organisation for Animal Health (2022), the negative control should show no amplification to ensure test validity. If a DNA band appears in the negative control, this indicates possible cross-contamination and the test results cannot be considered reliable (Nugroho et al., 2019; Xuan et al., 2021).

Lanes 3 to 7 represented the five clownfish (Amphiprion ocellaris) samples tested. No DNA bands were observed at 167 bp in any of these lanes. The absence of these bands indicates that Megalocytivirus DNA was not detected in any of the tested clownfish samples. Thus, all samples were declared negative for Megalocytivirus infection. These negative results indicate that the clownfish obtained from the study site, namely the Ambon Marine Aquaculture Center (BPBL), and tested at the Laboratory of the Fish, Animal, and Plant Quarantine Center (BKHIT) Maluku, were in healthy condition and free from detectable Megalocytivirus infection. The failure to detect viral DNA using PCR indicates the absence of detectable active infection in the tested samples. According to the World Organisation for Animal Health (2022), a negative molecular test result can be used as an indicator that the tested organism is not infected with the target pathogen.

This is an important finding because it indicates that the fish population at the study site was not exposed to a virus known to cause systemic infection and mass mortality in cultured fish (Kurita & Nakajima, 2012; Qin et al., 2023). However, a negative result does not completely rule out the possibility of a latent infection with a very low viral load below the detection limit of the method used. In addition, the negative result may have been influenced by good aquaculture environmental conditions. Factors such as stable water quality, appropriate stocking density, and nutritious feed can improve fish resistance to viral infection. As stated by Prabowo et al. (2023), environmental factors play an important role in influencing viral infection levels in fish; therefore, optimal environmental management is an important component of disease prevention.

Based on the electrophoresis results shown in the figure, it can therefore be concluded that the tested clownfish (Amphiprion ocellaris) were free from detectable Megalocytivirus infection. This condition indicates that aquaculture and fish health management at BPBL Ambon were functioning well and in accordance with applicable biosecurity standards. According to the World Organisation for Animal Health (2022), good biosecurity practices and routine fish health monitoring are important measures to prevent the introduction and spread of disease in cultured

fish. Nevertheless, considering that Megalocytivirus can persist in the environment and may spread through ornamental fish trade activities, routine PCR testing should continue to be conducted periodically to ensure that fish populations remain free from the disease (Qin et al., 2023; Prasetyo et al., 2022).

## CONCLUSION

Based on the results of the study, it can be concluded that Megalocytivirus was not detected in clownfish (Amphiprion ocellaris) originating from BPBL Ambon. These results indicate that the fish were in healthy condition and suitable for transport. Follow-up is recommended so that fish disease detection is conducted periodically to ensure that the health status of cultured fish is maintained. Biosecurity principles and environmental quality monitoring should continue to be strengthened, particularly in the marine ornamental fish aquaculture sector, which has high economic value. Similar studies should also be developed to detect the possible presence of other viruses that may infect ornamental fish in the Maluku region, so that disease prevention efforts can be implemented more effectively.

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