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Detection of Tilapia Lake Virus (TiLV) in Tilapia (Oreochromis niloticus) by Semi-Nested Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Method

Arif Bimantara[1*], Nosa Septiana Anindita[1], Taofani Rizal Al Amin[1], Wantonoro[2], Heni Setianah[1]

[1]Biotechnology Studies Programme, Faculty of Science and Technology, Universitas 'Aisyiyah Yogyakarta, Jl. Siliwangi Jl. Ringroad Barat No.63, Area Sawah, Nogotirto, Kec. Gamping, Kabupaten Sleman, Daerah Istimewa Yogyakarta 55592, Indonesia

[2]Nursing Study Program, Faculty of Health, Universitas 'Aisyiyah Yogyakarta, Jl. Siliwangi Jl. Ringroad Barat No.63, Area Sawah, Nogotirto, Kec. Gamping, Kabupaten Sleman, Daerah Istimewa Yogyakarta 55592, Indonesia

bimantara.arif@unisayogya.ac.id

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Abstract

Tilapia productivity is influenced by many factors, one of which is Tilapia Lake Virus (TiLV) disease, which can cause a mortality rate due to infection reaching 90%. The development of nested PCR or semi-nested PCR methods can increase the sensitivity of detection of this virus. This study aims to detect the spread of TiLV attacking tilapia early using the semi-nested PCR method with primers designed in this study. Tilapia with TiLV symptoms were taken from a tilapia farmer in Sleman Regency, Yogyakarta Special Region Province. The fish is dissected, and the kidney and lymph target organs are for RNA isolation. The RNA isolation results were converted into cDNA and then amplified in two stages through the semi-nested RT-PCR method. The detection target is the third segment of the RNA of the TiLV. The first and second amplified amplicons were then electrophorized using agarose gel and observed under UV light. The detection results showed no visible band at the first amplification. In the second amplification, each sample showed positive results with the appearance of bands in the detection target gene. The semi-nested RT-PCR method is proven to increase detection sensitivity. Based on these results, it can be concluded that the semi-nested RT-PCR method in this study is effectively used to detect TiLV. It is necessary to develop research on a field scale to test the effectiveness of this method. This research is expected to provide an alternative detection of aquaculture diseases that are more effective and efficient.

Keywords: semi-nested reverse transcriptase polymerase chain reaction (RT-PCR); tilapia; Tilapia Lake Virus (TiLV)

1. Introduction

Tilapia is a type of fish originating from the African Nile River basin waters. Tilapia belongs to the phylum Chordata, superclass Gnathostomata, class Actinopterygii, order Perciformes, family Cichilidae. Some tilapia species that are susceptible to TiLV disease include Mango Tilapia (Sarotherodon galilaeus), Nile Tilapia (Oreochromis niloticus) and Hybrid Tilapia (O. niloticus X O. aureus) (Eyngor et al. 2014; Bacharach et al. 2016; del-Pozo et al. 2017). Tilapia is a popular fish consumed in Indonesia because it has high socioeconomic value and an affordable price so that various circles of society can easily obtain it. Indonesia is one of the largest tilapia-producing countries in the Asia-Pacific region besides Egypt and China, with production figures reaching 1.2 million tons (FAO 2022).

Tilapia productivity is influenced by many factors, including the Tilapia Lake Virus Disease (TiLVD). Since the last few years, there have been several cases of mass deaths of tilapia in several places of cultivation, such as in Israel and Ecuador, with a mortality rate of 90%. The mass death of such fish is caused by the pathogenic agent Tilapia Lake Virus (TiLV), which belongs to RNA viruses in a new genus of the family Orthomyxoviridae (Eyngor et al. 2014). TiLV is a virus that causes

Syncytial Hepatitis of Tilapia (SHT) with symptoms of diseases such as erosion of the skin, shrinkage of the eyes, pale skin color, loss of appetite, and so on. According to Tsofack et al. (2017), factors triggering the onset of TiLV disease outbreaks are water temperature and high population density. The water temperature that causes the outbreak occurs above 25 °C, the temperature that does not cause death is below 20 °C, and the peak of mass death occurs at 30 °C. Clinical symptoms of TiLV infection include lethargy, blackened body, endophthalmitis, skin erosion, congestion in the kidneys, ocular degeneration, encephalitis, decreased appetite, anemia, and congestion in the brain and skin (Bacharach et al. 2016)(Dong et al., 2017). According to Eyngor et al. (2014), TiLV disease can infect farmed fish and other animals in nature. The spread of the disease has been reported to Southeast Asian countries such as Thailand and Malaysia, causing mass deaths of tilapia (Oreochromis niloticus) and red tilapia Hybrid (Oreochromis spp.). It does not rule out the possibility that the virus has reached Indonesia.

Preventive measures to prevent the spread of disease due to TiLV can be taken by early detection of its presence to control its spread. Molecular detection methods such as Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) can be a solution to the detection method against the disease. The PCR process for amplifying RNA is known as the Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) (Hewajuli and Dharmayanti 2014). The principle of this method is to convert viral genetic material in the form of RNA into cDNA and then propagate through amplification reactions by DNA polymerase. Nested PCR is a modification of PCR designed to improve sensitivity and specificity. Nested PCR uses two pairs of primers and 2 PCR reactions, respectively. The first pair of primers (Outer primers) will stick (annealing) at the end of the sequence of the second pair of primers and is used to initiate the PCR reaction. The amplicon produced from the first PCR reaction will be used as a mold for the second pair of primers (Inner Primers) for the second amplification process. In the presence of the Double Step, This technique will minimize the non-specific Generated target sequence (Carr, Williams, and Hayden 2009). One of the modified results of nested PCR, i.e., semi-nested PCR.

The difference between the two techniques is the amount of primer used. In semi-nested PCR, there are Outer primers, which consist of a pair of primers, forward and Reverse, which will amplify the first reaction (First round), and there is one Inner primer that will pair with the outer primer to perform amplification in the second reaction. Semi-nested RT-PCR combines the RT-PCR technique with a semi-nested PCR (Ongagna-Yhombi et al. 2013). The majority of molecular detection of TiLV that has been done previously is by nested-PCR method (Bacharach et al. 2016)(del-Pozo et al. 2017)(Hewajuli and Dharmayanti 2014). In addition, some existing primaries also show the presence of secondary structures (Dong, Siriroob, et al. 2017)(Eyngor et al. 2014). Applying TiLV detection in tilapia using the semi-nested RT-PCR method is expected to be the first preventive step in tackling the spread of the virus. In this study, experiments will be carried out using semi-nested RT-PCR with a combination of primers designed to detect TiLV in symptomatic tilapia.

2. Research Methods

2.1.Tools and Materials

Tools used include thermocycler machines (LABNET Multigene™), refrigerated centrifuge (Hermle), electroforators (blueGel™), scalpels, surgical scissors and surgical tweezers. Materials used for the RNA extraction process include Direct-zol™ RNA Miniprep Kits (Zymo Research) and tilapia kidneys and lymph with clinical TiLV symptoms. The material used for the cDNA synthesis process is ReverseTra Ace -α- ® (TOYOBO). The materials used for the nested RT-PCR process are GoTaq® Green Master Mix M712 (Promega) and primer. The primers designed in this study consisted of F1 forward primers (5'- TTG-GGC-ACA-AGG-CAT-CCT-AC-3') and R1 reverse primers (5'-TTT-CCC-TGC-CTG-AGT-TGT-GC-3') for the first amplification as well as R2 reverse primers (5'- CGT-GCG-TAC-TCG-TTC-AGT-3') paired with F1 primers for the second amplification. The first amplification

will produce amplicons along 415 bp, while the second amplification will produce amplicons as long as 250 bp. The materials used for electrophoresis consist of agarose, FluoroVue™ Nucleic Acid Gel Stain (Smobio), 100 bp marker, TiLV positive control, and nuclease-free water (NFW).

2.2. Sampling and Necropsy

Test samples were taken from tilapia farming ponds in Sleman Regency, Special Region of Yogyakarta. Samples are selected based on clinical symptoms due to TiLV virus infection, such as changes in body color, damage to the eyes, and erosion of the skin. Furthermore, tilapia is put into polyethylene (PE) plastic filled with water and added oxygen. Then, the PE plastic is tied using rubber and taken to the laboratory for testing. In the laboratory, fish are dissected using a section kit and taken to the kidneys and lymph, where the TiLV virus replicates. The target organ is weighed as much as 100 mg and put into a sterile microtube.

2.3. RNA Isolation and cDNA Synthesis

RNA isolation was carried out according to the protocol of the Direct-zol™ RNA Miniprep Kits (Zymo Research). RNA isolation results are stored at -20ºC. The cDNA synthesis process is carried out according to the protocol of ReverseTra Ace -α- ® (TOYOBO). The results of cDNA are stored at -20ºC.

2.4. Semi-nested RT-PCR

The amplification process is carried out according to the GoTaq® Green Master Mix M712 (Promega) protocol. The composition of the PCR reaction for the first amplification consists of 12.5 ul PCR Master Mix, 9.5 ul nuclease-free water, 1 ul forward and reverse primers, and cDNA as a template of 1 ul. The primers used in this first amplification were F1 and R1 primers. Furthermore, amplification was carried out using a thermal cycler for 30 cycles with reaction conditions by the results of primary optimization that had been carried out previously, namely pre-denaturation temperature 95°C for 2 minutes, denaturation 95°C for 30 seconds, annealing 54°C for 30 seconds, extension 72°C for 30 seconds and final extension 72°C for 5 minutes. The amplicon results from the first amplification are used as templates in the second amplification process. The composition and conditions of the second amplification reaction are the same as the first, but they differ only in the primary pairs, namely the F1 and R2 primers.

2.5. Electrophoresis

The first and second amplification results were visualized through agarose gel electrophoresis. 5 ul of each amplicon was put into a 2% agarose gel well, then electrophoresis at 110 volts for 40 minutes. The amplicon in the first amplification has a length of 415 bp, while the second amplification amplicon has a length of 250 bp.

2.6. Data Analysis

Data analysis was carried out descriptively based on the visualization of bands that appeared after the electrophoresis process of both amplicons in agarose gel.

3. Results and Discussion

Tilapia samples with illness or clinical symptoms were found in as many as six tails. Examination of clinical symptoms during the study was conducted on fish suspected of being infected with the virus by observing their physical condition. The characteristics of the fish's physical condition include

damage to the eyes and skin erosion (**Figure 1**). Tilapia attacked by TiLV will show symptoms of decreased appetite, slow movement, and inactivity (Husna et al. 2020).

Figure 1. Tilapia samples showing clinical symptoms of TiLV infection. The arrows in the image indicate eye damage and skin erosion.

After the fish sample is brought to the laboratory, the first step is an autopsy to take the target organ. The organs that are the target organs of TiLV examination in this study are the kidneys and lymph. Both organs are places where blood circulation occurs, so pathogens such as TiLV will infect many of these two organs. Kidneys and lymph are transportation places to remove substances useful for the body of fish and sources of disease, such as bacteria, viruses, and parasites (Surfianti, Soewarno, and Mahasri 2016).

After the target organ is obtained, then an RNA isolation process is carried out. This isolation process aims to bring pure RNA. The isolation process consists of the destruction of cell membranes, RNA separation, and RNA precipitation (Ariyanti and Sianturi 2019). After the RNA sample is obtained, the cDNA synthesis process is carried out using reverse transcriptase. The synthesis of cDNA is carried out because the genetic material of the TiLV virus is RNA, so it needs to be processed reverse transcriptase to convert RNA into cDNA. Field Prayitno et al. (2020) state RT PCR uses reverse transcriptase enzymes to convert RNA into cDNA. RT PCR requires primers that function as DNA chain synthesis in polymer chain reactions. cDNA is synthesized by reverse transcription using total RNA templates and random Primary (Muzuni et al. 2014). The success of cDNA synthesis is influenced by the purity of RNA free from contamination of proteins, polysaccharides, DNA, and RNA integrity (Okanti, Putri, and Fathoni 2020).

The cDNA results obtained are then used as a template in the first amplification process with the PCR method. The F1 and R1 primers used in this study had a target band of 415 bp. The results of amplicon electrophoresis produced by this first amplification process are shown in **Figure 2**.

Figure 2. The result of amplicon electrophoresis from the first amplification process. M : marker 100 bp; + : positive control; - : negative control; 1-6: tilapia cDNA sample

Based on the visualization results shown in Figure 2, no band appears at the first amplification. The amplification process went well, indicated by the emergence of bands at positive controls along 415 bp and the absence of bands appearing in negative controls. The next process is to perform a second amplification (nested PCR) to ensure the results of the first amplification electrophoresis. Process nested PCR is the propagation of target DNA fragments using two specific pairs of primers using the two-step PCR method. The result of the first amplification will be a Template to nested PCR. According to

Ngaliyatun et al. (2013), nested PCR is carried out to improve accuracy and sensitivity and reduce contamination in PCR products.

The second amplification process in this study used F1 and R2 primer pairs. Both primers produce amplicones as long as 250 bp. This second amplification target is shorter than the first and is inside the first amplification result. The results of the second amplified electrophoresis are shown in **Figure 3**.

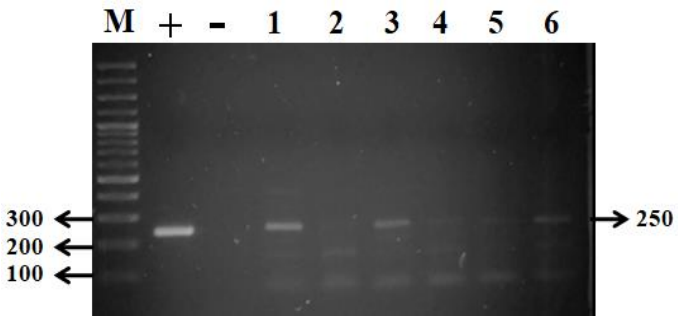

Figure 3. The result of amplicon electrophoresis from the first amplification process. M : marker 100 bp; + : positive control; - : negative control; 1-6 : First amplification results

Based on the results of electrophoresis visualization in Figure 3, all tilapia samples were infected with TiLV. This is evidenced by the appearance of a band with a length of 250 bp in each test sample. According to Carr et al. (2009) and Wanger et al. (2017), the semi-nested PCR method can increase sensitivity and specificity and reduce the number of non-specific bindings. This is because in the second reaction, there are many amplicons, and the first reaction only contains the target sequence without an off-target sequence. Husna et al. (2020) also perform TiLV detection using the method of nested RT-PCR; however, it produces very thin amplicon bands on electrophoresis visualization. The primers used in this study can be an alternative to TiLV detection using the method of semi-nested RT PCR because it can produce good electrophoresis visualization. Based on research by Taengphu et al. (2022), the results of this study show that the method used can have a sensitivity and specificity that is 100% equivalent to the qRT PCR method. This indicates that the combination of primers used can work effectively and efficiently.

Saragih & Junaidi (2021) convey that TiLV can attack tilapia from the age of fry based on the semi-nested RT PCR test results. According to Mugimba et al. (2018), TiLV can grow optimally at a temperature of 28 ° C, so at a temperature of > 25 ° C, it can increase TiLV replication and accelerate infection in tilapia. Clinical symptoms of tilapia taken as samples in this study indicate TiLV infection. This is by the statement of Dong et al. (2017) that the clinical symptoms of TiLV infection in tilapia are characterized by decreased appetite, blanched body color, fish flocking at the bottom of the tub, fish movement becomes sluggish, inactive, and ultimately mass death.

The results of this study showed that tilapia samples that had symptoms of TiLV infection were indeed infected with the virus. The number of viruses results in the absence of bands in the results of the first amplified electrophoresis. This can lead to false negative results. The use of semi-nested RT PCR can be a solution to this problem. It is necessary to conduct field-scale testing to determine the effectiveness of the primers used in this study to detect TiLV using the semi-nested PCR method. This method can be applied to the effective and efficient molecular detection of pathogens that attack farmed fish.

4. Conclusion

The semi-nested RT PCR method used in this study effectively detected TiLV. This method has been proven to increase the sensitivity of detection of this virus. The primers designed in this study

were also confirmed to detect TiLV well. This research can be an alternative detection of aquaculture diseases, especially TiLV. Furthermore, research is needed on field tests of the effectiveness of the semi-nested RT PCR method to detect TiLV.

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