Comparison of RT-PCR and rRT-PCR Methods in Detection of Viral Hemorrhagic Septicemia Virus (VHSV) in Marine Ornamental Fish

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Abstract. Reverse Transcription PCR (RT-PCR) and Real Time Reverse Transcription PCR (rRT-PCR) are methods that can be used to detect VHSV (Viral Hemorrhagic Septicemia Virus) on freshwater and seawater fish. rRT-PCR is more widely used than the RT-PCR method because of several advantages such as requiring shorter time and fewer stages. Both methods have their own advantages and disadvantages, so it is necessary to compare the two methods in detecting the presence of VHSV in marine ornamental fish. The research was carried out with 3 steps: sensitivity test, specificity test, and sample test. The sensitivity test showed that the smallest dilution concentration that could be detected by both methods was the same at 10^{-3} with 100 copies in the 77bp band. Specificity tests on both methods showed that VHSV primers used are specific. Sample tests in both methods showed the same results in each of the samples tested. The results of sensitivity, specificity and sample tests conducted on RT-PCR and rRT-PCR methods showed the same results (positive) thus both methods can be used to detect the VHSV in marine ornamental fish.

Keywords— Marine Ornamental Fish, RT-PCR, rRT- PCR, VHSV.

1 Introduction

Viral Hemorrhagic Septicemia Virus (VHSV) is a virus that causes Viral Hemorrhagic Virus (VHS) disease, which is one of the important diseases that can cause mortality in fish [1]. In recent years, this virus has been frequently found in free-living marine fish species [2]. More than 48 species of fish caught from northern hemisphere waters including North America, Asia and Europe have been detected positive for VHSV [2]. The waterborne transmission of VHSV causes the rapid spread of this virus [3]. Detection of VHSV virus in fish is important to avoid a VHS pandemic in aquaculture and open water.

Reverse Transcription PCR (RT-PCR) may be used to detect the presence of viruses on fish samples. This method allows researchers to detect the presence of the virus in a sample as done by Dhar at 2008 [4] who conducted detection of Infectious Hematopoietic Necrosis Virus (IHNV) in *Oncorhynchus mykiss* using RT-PCR. The same method was also conducted by Koutna et al, 2003 [5] to detect the presence of Spring Viraemia of Carp Virus (SVCV) in carp and Dong et al, 2017 [6] to detect the presence of Tilapia Lake Virus (TiLV) in tilapia. However, there are several research institutions that use the more modern Real Time Reverse Transcription PCR (rRT-PCR) method. [7] Hodneland et al, 2011 used this method to detect the presence of Betanodavirus in Sea Bass (*Dicentrarchus labrax*). rRT-PCR is also being utilized to detect the presence of VHSV in freshwater and seawater fish as done by Warg et al in 2014 [8]. The presence of Viral Nervous Necrosis (VNN) was also successfully identified using the same method in milkfish by Suryani et al, 2022 [9].

In general, testing using rRT-PCR is more widely used than the RT-PCR method because of several advantages such as requiring shorter time and fewer stages. Both methods have their own advantages and disadvantages, so it is necessary to compare the two methods in detecting the presence of VHSV in the kidneys and spleen of fish because these two organs are where most viruses are found [2][10]. In addition, the detection of VHSV in seawater ornamental fish has not been widely practiced, especially in Indonesia.

2 Materials and Methods

In this study, a comparison between the two methods was carried out using several tests, namely sensitivity test, specificity test, and sample test. Sample preparation was carried out on five fish sample from three different species. Before necropsy is carried out, the process of measuring the length of the fish body using a ruler followed by measuring the weight of the fish using a scale, then necropsy is carried out during this stage where the ornamental seawater fish fry are slashed in the ventral part of the fish and then the spleen, and kidneys are taken. Then the organs were removed, placed in a 1.5ml microtube.

RNA extraction in this study was carried out using Tri Reagent, a monophasic solution of phenol and guanidinium isothiocyanate that simultaneously dissolves biological material and denatures proteins [11]. The main stages of RNA extraction consist of 3 stages, namely homogenization, RNA precipitation, and RNA washing [12].

Amplification by RT-PCR method was performed with a total reaction volume of 12.5µl. All reagents used in amplification by RT-PCR method were reagents from Bioline (Meridian Bioscience, USA) consisting of 2X My Taq HS Red Mix as much as 6.25 µl, VHSVqF Primer as much as 0.5 µl, VHSVqR Primer as much as 0.5 µl, and NFW as much as 4.1 µl and used AMV Enzyme (Promega, USA) as much as 0.125 µl.

Amplification by the rRT-PCR method was performed with a total reaction volume of 12.5µl. All reagents used in amplification by RT-PCR method were reagents from Go

Taq® Probe 1-Step RT-qPCR System (Promega, USA) consisting of Go Taq® Probe qPCR Master Mix with dUTP as much as 6.25 µL, VHSVqF Primer as much as 0.5 µL, VHSVqR Primer as much as 0.5 µL, Probe as much as 0.025 µL, RT Enzym as much as 0.125 µL, and NFW as much as 4.1 µL.

The primers used are VHSVqF primers and VHSVqR primers with N gene targets [13] and produce PCR products of 77bp obtained by calculating the number of nucleotide bases of the target gene. These primers were designed from the VHSV sequence taken from GenBank with access number MK829675.1.

For the sensitivity test, 1µl of positive control dilution was added to the master mix so that the final reaction volume was 12.5µl, in the specificity test, 1µl of positive control of VNN, Megalocytivirus, IHNV and EHNV viruses in different tubes was added to the master mix so that the final reaction volume was 12.5µl, while for the sample test, 1µl of template was added to the master mix so that the final reaction volume was 12.5µl.

Sensitivity tests were conducted to validate the method used to test the sample and measure the lowest concentration limit range that can still be detected using the same method [14].The sensitivity test was carried out by performing a tenfold dilution of the stock. The composition of the material used in this dilution is 1µl VHSV positive control and 9µl Nuclease Free Water (NFW).

Specificity tests were performed to verify that the primers used do not produce amplicons of the expected size for other gene target lines [14]. This specificity test was performed using VHSV primers to amplify positive controls of Viral Nervous Necrosis (VNN), Megalocytivirus, Infectious Hematopietic Necrosis Virus (IHNV), and Epizootic Haematopoitic Necrosis Virus (EHNV) of the expected size for other gene target lines [12]. This specificity test was performed using VHSV primers to amplify positive controls of Viral Nervous Necrosis (VNN), Megalocytivirus, Infectious Hematopietic Necrosis Virus (IHNV), and Epizootic Haematopoitic Necrosis Virus (EHNV).

Sample tests were conducted to determine whether the marine ornamental fish samples are infected with VHSV or not [15]. The sample test is carried out by inserting 1µl of template into the master mix so that the final volume of the reaction is 12.5µl.

Temperature protocols for amplification by the RT-PCR and rRT-PCR method can be examined in Table 1 and Table 2 below:

Table 1. Amplification temperature protocol of RT-PCR method

Stages	Time	Temperature	Cycles
Reverse Transcription	10 minutes	55°C	1 Cycles
Pre Denaturation	2 minutes	95°C	
Denaturation	30 seconds	95°C	40 Cycles

Table 2. Amplification temperature protocol of rRT-PCR method

Stages	Time	Temperature	Cycles
Reverse Trancription	30 minutes	50°C	1 Cycles
Pre Denaturation	15 minutes	95°C	
Denaturation	15 seconds	95°C	40 Cycles
Annealing	60 seconds	60°C	
Extention	60 seconds	72°C	

Detection of amplification products by RT-PCR method was carried out using agarose gel with a concentration of 2% and then electrophoresed for 25 minutes with a power of 110 volts.

3 Results

The sensitivity test was carried out by performing a multilevel dilution multiplied by ten on the positive control. The purpose of the sensitivity test is to validate the method which will be used to test the sample, in addition it is used to determine the smallest possible concentration that is able to be amplified [14].

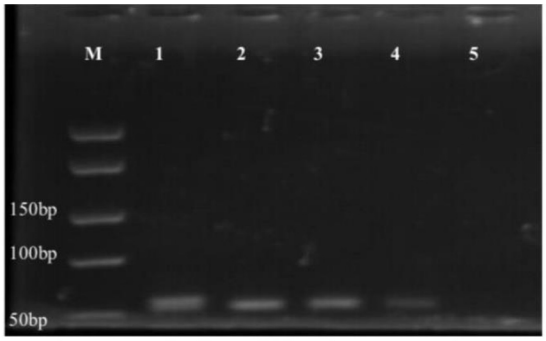

Fig. 1. Results of Sensitivity Test with RT-PCR Method. Description: (M : 50bp marker, 1: C(+), 2 : C(+)10^{-1}, 3 : C(+) 10^{-2}, 4 : C(+) 10^{-3}; 5: C(-))

The results of sensitivity testing using the RT-PCR method (Figure 1) show that the VHSV positive control is amplified at 77bp, which means that the result of amplification with an annealing temperature of 60° is in accordance with the target. The RT-PCR product of 77bp was obtained by counting the number of nucleotide bases of the target gene. No contamination occurred in the negative control as well as in the stock positive control, positive control 10^{-1} dilution, positive control 10^{-2} dilution, positive control 10^{-3} dilution.

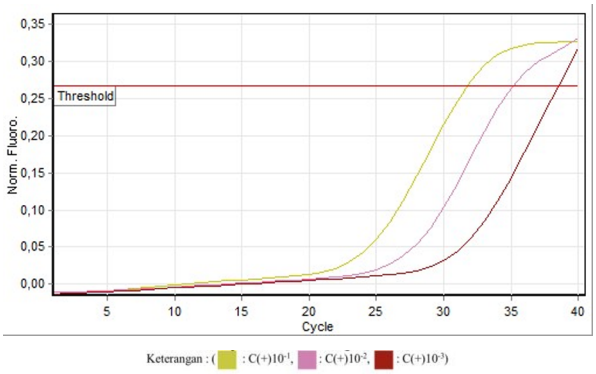

Fig. 2. Sensitivity Test Results with the rRT-PCR Method.

The sensitivity test using the rRT-PCR method (Figure 2) indicates that the Ct value that appears ranges from 31.73 with a copy number of 10,000 for dilution 10^{-1}. For dilution 10^{-2}, the Ct value appeared to range from 35.15 with a copy number of 1,000, and for dilution 10^{-3}, the Ct value appeared to range from 38.57 with a copy number of 100.

Figure 2 shows the results of the sensitivity test with the RT-PCR method that the positive control of the stock, positive control with dilution of positive control of dilution 10^{-1}, positive control of dilution 10^{-2}, positive control of dilution 10^{-3} amplified on the band with a size of 77bp, while the results of the sensitivity test in Figure 2 above can be seen that the sensitivity test on the RT-PCR method obtained Ct values that appear ranging from 31.73 with the number of copies 10,000 for dilution 10^{-1}. For dilution 10^{-2}, the Ct value appears to range from 35.15 with a copy number of 1,000, and for dilution 10^{-3}, the Ct value that appears ranges from 38.57 with a copy number of 100.

Based on Figure 2 above, the slope value is obtained, namely -3.422, the value of R^2 is 1.000. The equation obtained from the graph in Figure 2 is y = -3.422x + 45.413. The graph obtained is in the expected interval because the R^2 value obtained is 1.000 which means> 0.980, this is in accordance with the literature which states that the graph is in the expected interval must have an R^2 value> 0.980 [16].

According to the sensitivity test results that were obtained, it can be stated that the two methods used are able to detect the smallest concentration of VHSV positive control dilution results and are valid, this is in accordance with the purpose of this sensitivity test according to [14] that this sensitivity test is carried out to determine the smallest concentration that can still be amplified. The smallest concentration that can be used in testing VHSV with RT-PCR and rRT-PCR methods according to the results of the research conducted is a 10^{-3} dilution with a total of 100 copies.

Specificity tests were performed by amplifying VHSV primers with positive controls of Viral Nervous Necrosis, Megalocytivirus, Infectious Hematopietic Necrosis Virus, and Epizootic Haematopoitic Necrosis Virus. This specificity test is carried out to verify that the primers that have been prepared do not produce amplicons of the expected size for other gene target lines [14].

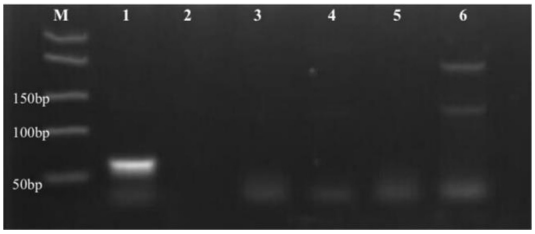

Fig. 3. Results of Specificity Test with RT-PCR Method. Description: (M: Marker; 1: C(+) VHSV; 2: C(-); 3: C(+) VNN; 4: C(+) Mg; 5: C(+) IHNV; 6: C(+) EHNV.

Figure 3 above shows that VHSV primers cannot be used to amplify positive controls of Viral Nervous Necrosis, Megalocytivirus, Infectious Hematopietic Necrosis Virus, and Epizootic Haematopoitic Necrosis Virus, this is shown by the absence of bands in accordance with the VHSV target formed in wells 3, 4, 5, and 6. This confirms that the VHVS primer used in this study is specific so that only the band appears in well number 1 which is the positive control of VHSV, while the positive controls for VNN, Megalocytivirus, IHNV, and EHNV viruses do not produce bands that are parallel to the VHSV positive control.

Specificity test of rRT-PCR showed the same results as the RT-PCR method, namely VHSV primers cannot be used to amplify positive controls of Viral Nervous Necrosis, Megalocytivirus, Infectious Hematopietic Necrosis Virus, and Epizootic Haematopoitic Necrosis Virus indicated by the Ct value of 40 which means a weak reaction, This is in accordance with the literature which states that the Ct value between 38-40 indicates a weak reaction that can result from the contamination of the external environment, so that although there is fluorescence but in theory no cDNA amplification of the target is produced [17].

Specificity tests showed that VHSV primers did not produce amplicons of the expected size for other gene target lines. The primers used in this study target the N gene, this is in accordance with research conducted by [11] VHSV primers used in the research conducted target the N gene. This VNN virus targets a different gene from VHSV, VNN targets coat protein (CP) which encodes RNA2 [18]. The target gene for megalocytivirus and EHNV is Major Capsid Protein [19][20]. Similar to VNN and megalocytivirus, IHNV also targets a different gene from VHSV, the target gene of IHNV is the G gene [21]. Because of the differences in gene targets of VNN, megalocytivirus, IHNV and EHNV viruses, VHSV primers did not produce amplicon for the target pathways of these viruses.

Sample test is done to determine whether the samples of marine ornamental fish tested in RT-PCR and rRT-PCR methods are positive for VHSV or not [15]. The samples used in this study were Chromis viridis species with sample codes 1467 and AFB10.2, *Pomacentrus coelestis* species with sample code LSR 10.2 and *Pomacentrus sp*. species with sample codes BC 10.4 and BA 10.4.

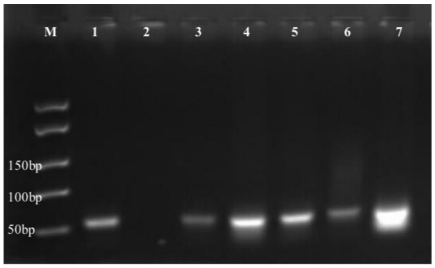

Fig. 4. Sample Test Results with RT-PCR Method. Image description: (M : 50bp marker, 1: positive control, 2: negative control, 3: sample (+) *P.coelestis,* 4: sample (+) *Ch.viridis*, 5: sample (+) *Pomacentrus sp.*, 6: sample (+) *Ch.viridis*, 7: sample (+) *Pomacentrus sp.*

Figure 4 shows that in each of the samples LSR 10.2, 1467, BC 10.2, AFB 10.2, and BA 10.4, a band at 77 bp appears, which is the same as the band that appears in the positive control. The negative control/NTC did not show any band at all.

Sample test results using the rRT-PCR method can be reviewed in fig 5.

No.	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
1	VHS 10^1	Standard	31,73	10.000	10.000	
2	VHS 10^2	Standard	35,15	1.000	1.000	
3	VHS 10^3	Standard	38,57	100	100	
4	NTC	Unknown				
19	LSR 10.2	Unknown	20		7.42	
20	1467	Unknown	25		5.96	
21	BC 10.2	Unknown	28		5.08	
22	AFB 10.2	Unknown	30		4.5	
23	BA 10.4	Unknown	35.56		2.87	

Fig. 5. Sample Test Results with rRT-PCR Method.

Figure 5 shows that the sample with code LSR 10.2 has Ct values that appear to range between 20 with 7.42 copies. For the sample with code 1467, the Ct value appears to range between 25 with a copy count of 5.96. For the sample with the code BC 10.2 the Ct value appears to range between 28 with a copy count of 5.08. In the test sample with code AFB 10.2 the Ct value appears in the range of 30 with the number of copies 4.5. And samples with code BA 10.4 Ct values appear to range from 35.56 with a copy count of 2.87. For the negative sample / NTC (No Template Control) the Ct value does not appear.

The results of the sample tests carried out using the RT-PCR method showed that all five samples were positive for VHSV. This is indicated by the appearance of a band at 77bp which is the same as the band that appears on the positive control. In the negative control/NTC, no band appeared at all, indicating that the negative control/NTC was not contaminated by VHSV DNA. The test results using rRT-PCR on the five samples showed the presence of sample contamination characterized by the appearance of the Ct value. The Ct value is inversely proportional to the number of target DNA findings in the sample as stated by [22]. The absence of Ct value in the negative/NCT sample indicates that the NTC is not contaminated. The negative control serves to determine whether or not there is contamination when mixing the master mix into the sample [23].

In the amplification process with the rRT-PCR method, there is no need to do the electrophoresis process because in this method, using a probe molecule that can be measured every reaction. The Ct value is the value of the rRT-PCR reaction determined by the threshold line set in the exponential phase of the logarithmic scale amplification plot [24]. Based on the results of this test, the RT-PCR method can be used as an alternative to detect VHSV in remote fish quarantine centers.

4 Discussion

Based on the sample tests that have been carried out with RT-PCR and rRT-PCR methods, the comparison results can be examined in Table 3

Table 3. Comparison of VHSV Detection Results with RT-PCR and rRT-PCR Methods

No.	Kode Sampel	Spesies	RT-PCR	rRT-PCR
1	LSR 10.2	*Pomacentrus coelestis*	+	+ (Ct : 20)
2	1467	*Chromis viridis*	+	+ (Ct :25)
3	BC 10.2	*Pomacentrus sp.*	+	+ (Ct : 28)
4	AFB 10.2	*Chroma viridis*	+	+ (Ct : 30)
5	BA 10.4	*Pomacentrus sp.*	+	+ (Ct : 35.56)

The test results on the same 5 samples of marine ornamental fish with the RT-PCR method found that all samples were confirmed positive for VHSV, while for the sample test results with the rRT-PCR method the samples were confirmed positive with Ct values of 20,25,28,30, and 35.56.

According to the Government Regulation No.85 of 2021, for one test per sample using the RT-PCR method, the price to be paid by service users is IDR 250,000, while sample testing using the rRT-PCR method costs IDR 325,000. From this economic point of view, it can be said that sample testing using the RT-PCR method is more affordable than the rRT-PCR method.

The advantages of the RT-PCR method are that it is quite accurate and relatively cheaper, while the disadvantage of this method is that it takes a long time to test samples or is less efficient in terms of time [25].

The advantage of the rRT-PCR method is that this method is able to detect PCR products from the initial phase to the end of the reaction so that it is efficient in terms of time and of course has high sensitivity, while the disadvantage of the rRT-PCR method is that the price is less affordable or expensive [26].

5 Conclusion

The results of sensitivity, specificity and sample tests conducted on RT-PCR and rRT-PCR methods showed the same results (positive) thus both methods can be used to detect the VHSV in marine ornamental fish samples.

6 Acknowledgement

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