Oral Presentation (PAT-2)

Rapid Diagnostic Test of Red Sea Bream Iridoviral Disease (RSIVD) in Grouper *Epinephelus* sp. Based on Serological Co-Agglutination and Molecular Study

Dwi Sulistiyono[1], Surya Amanu[2], Kurniasih[2]*, Yuli Purwandari Kristianingrum[2]

[1]Post Graduate Student Faculty of Veterinary Medicine, Gadjah Mada University, Indonesia
[2]Faculty of Veterinary Medicine, Gadjah Mada University, Indonesia

Key words: Co-agglutination , RSIVD, Grouper, PCR, Sequencing

INTRODUCTION

Red sea bream iridoviral disease (RSIVD) is caused by red sea bream iridovirus (RSIV), a double stranded DNA of Icosahedral virus with a diameter of 120-240 nm [1]. RSIV is one of the species of the Megalocytivirus, Genus of the Iridoviridae Family, first reported to infect red sea bream (Pagrus major) fish, at Sikoku Island, Japan 1991, and since then it has been noted to cause considerable economic losses to fisheries in Singapore, Taiwan, Thailand, Korea, Philippines, Malaysia and also in Indonesia [2,3,4]. Rapid transmission with high mortality rates in fish populations infected becomes a serious threat to the aquaculture fishery business. Stained imprints or tissue sections [1], monoclonal antibody technique (MAb), Immunofluorescent Antibody Tests (IFAT) [5], Polymerase Chain Reaction (PCR) [6] Electron Microscope and Multiplex PCR [2] methods have been introduced. Although it is very effective for detecting RSIVD in infected fish, but requires training and specialized equipment at a high cost.

Co-agglutination test is a diagnostic method, used both in humans and animals in detecting bacterial or viral diseases [7], this method is fast, easy to use, and does not require special equipment. Test results from co-agglutination are easily seen macroscopically, so it is suitable if developed in RSIVD detection in the field case. This study aims to create and conduct RSIVD co-agglutination kit field tests supported by molecular studies and diagnostic analysis of the sensitivity and specificity of the accuracy and reliability of the kit. Then the test results will be compared from the pooling and individual samples.

MATERIALS AND METHODS

Sample of grouper fish is taken from fish culture center in Bintan Regency of Riau Islands, as many as 40 samples. The grouper samples were then tested using individual co-agglutination and PCR kits and pooling.

Production of antibodies

Immunization was performed on rabbits with RSIV vaccine through intraperitoneally where successive doses each week was 0.5 cc, 1 cc, 2 cc and 3 cc. Repeated immunizations on a weekly dose each day are intended to increase antibody titers [8]. On the fifth week the serum is harvested from rabbits with 10 cc of blood per rabbit, blood was in incubator at 37°C until serum anti RSIV (Imunoglobulin) was separated with sediment. Anti-serum was collected in a 15 ml conical tube, inactivated at 56° C for 30 minutes [8].

Purification of Immunoglobulin

Purification of immunoglobulins were done by precipitation of ammonium sulfate. Theresulting precipitation solution is placed in a 15 ml conical tube, then centrifuged at a 6000 rpm for 30 minutes. The supernatant was removed by the pellet resuspended with physiological NaCl (0.85% NaCl) up to the initial volume, the anti-serum is then precipitated up to 3 times. The dialysis process was carried out to remove the salt content contained in anti serum by means of an anti-serum suspension, was inserted into a 15 ml volume membrane of MWCO 6-8 kDa and was immersed in PBS pH 7.2 in a 300 ml beaker glass for 36 hours, with PBS replacement pH 7.2 every 12 hours. The results of the dialysis process obtained immunoglobulin solution as the material of co-agglutination kits [9].

Preparation of A *Staphylococcus aureus* protein

The pure culture isolates of *Staphylococcus aureus* (ATCC 12598) were inoculated evenly on the TSA (*Tryptic Soy Agar*) media and incubated at 37°C for 24 hours. *Staphylococcus aureus* was harvested using inoculation loop and PBS pH 7.2, collected in a 15 ml conical tube and washed 3 times. The final wash pellet was added with 0.5% formalin in PBS pH 7.2 incubated overnight at room temperature (Amanu *et al*., 2015). The pellet was heated on a waterbath with a temperature of 80°C for 15 minutes and cooled rapidly, then washed 3 times with PBS pH 7.2, the final wash made 10%

Staphylococcus aureus suspension in PBS pH 7.2 [9].

Binding of Immunoglobulin with Protein A *Staphylococcus aureus*

The 10% suspension of *Staphylococcus aureus* is added to the same volume as the immunoglobulin immunization result and is incubated overnight in room temperature. This mixture was then centrifuged at 3000 rpm for 10 minutes, the supernatant was discarded, it was resuspended back to the initial volume with PBS pH 7.2 (obtained RSIV co-agglutination kit) [9].

Test procedure

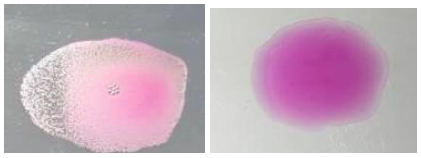
The pooling sample is a sample of organ mixture of 5 fishes used as 1, while the individual sample is the sample of each fish. The crushed spleen organ is made into a 1: 2 suspension in PBS pH 7.2. Suspension of sample is centrifuged at 8000 rpm for 10 minutes, supernatant is t h e dissolved antigen used as test sample. 50 µl supernatant sample and 50 µl of RSIV co-agglutination kit was dropped on an object glass and homogenized by wiggling it. Positive control using vaccine and negative control using PBS pH 7.2. Observations were made after 10 minutes against a contrasting background. Thye result is positive if agglutination or grain-like sand occurs and negative if agglutination does not occur.

***Polymerase Chain Reaction* (PCR)**

The positive and negative reaction results in the sample were confirmed by *Polymerase Chain Reaction* (PCR) examination. The primer used was the sequence of RSIV DNA genome, with the forward 1F: '5-CTCAAACACTCTGGCCC-3 and reverse primer 1R: '5- GCACCAACACATCTCCTATC-'3. Target DNA measuring 570 bp [6]. Extraction uses the Dnesy protocol. Amplification was carried out by mixing several reagents from the QIAGEN DNA-kit and the Iridovirus DNA template (sample). The mixture is then introduced to the thermocycler with a denaturation temperature of 94°C for 30 seconds; annealing 57°C for 60 seconds; and extension 72 ° C for 60 seconds. This cycle lasted 30 times. The next positive result will be sent to Standard Fish Quarantine Test Center (BUSKI) for sequencing.

RESULT AND DISCUSION

The positive result of the test using coagglutination kit is indicated by the presence of aglutinate as shown in Figure 1a and the negative test results of the sample appear homogeneous as shown in Figure 1b, from the test results as many as 40 individual samples obtained 14 positive samples while 26 negative samples, similar results were also demonstrated by tests using PCR. Positive test results on PCR assay were shown with tire emergence at 570 bp as shown in Figures 2 and 3. The test results are tabulated in table 1 below.



a b

Figure 1. Positive results a) and negative result b) testing with co-agglutination kit

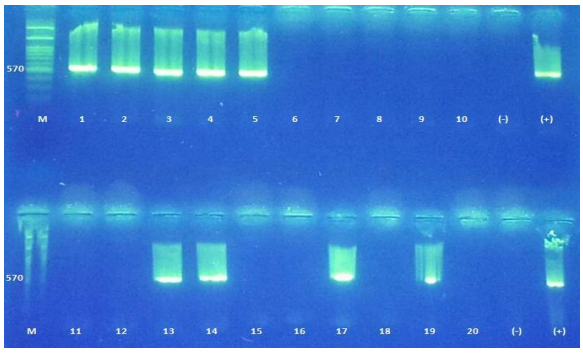


Figure 2. Results of PCR test. Sample number 1-5, 13, 14, 17, and 19 were positive

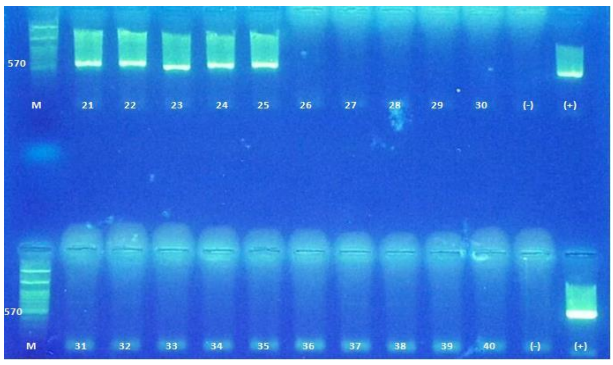


Figure 3. Results of PCR test sample 21 – 40. Sample number 21, 22, 23, 24, 25 were positive.

Table 1. Results of pooling and individual tests

No	Test result			
	Pooling		Individual	
	Co-agglutination	PCR	Co-agglutination	PCR
1			+	+
2			+	+
3	+	+	+	+
4			+	+
5			+	+
6			-	-
7			-	-
8	-	-	-	-
9			-	-
10			-	-
11			-	-
12			-	-
13	+	+	+	+
14			-	-
15			-	-
16			-	-
17			+	+

18	-	+	-	-
19			+	+
20			-	-
21			+	+
22			+	+
23	+	+	+	+
24			+	+
25			+	+
26			-	-
27			-	-
28	-	-	-	-
29			-	-
30			-	-
31			-	-
32			-	-
33	-	-	-	-
34			-	-
35			-	-
36			-	-
37			-	-
38	-	-	-	-
39			-	-
40			-	-

The test results above are then included in the Thorner-Remain Screening Test formula for diagnostic sensitivity and specificity diagnostic calculations (Table 2).

Table 2. Evaluation Diagnostic Sensitivity and Diagnostic Specificity

Status Sample Test		**PCR**		**Total**
		Positive	**Negative**	
Test result	**Positive**	14	0	14
Co-agglutination	**Negative**	0	26	26
Total		14	26	40

Diagnostic Sensitivity: $\frac{14}{14+0} \times 100\% = 100\%$

Diagnostic Specificity: $\frac{26}{0+26} \times 100\% = 100\%$

Calculation of sensitivity diagnostic value obtained 100% and diagnostic value spesivisitas obtained 100% thus can be concluded that co-agglutination have a high accuracy value. Antibodies have very specific reactivity properties against antigens that stimulate the formation of antibodies. The interaction of antigens with antibodies done outside the body is a very useful diagnostic tool for various diseases, and this approach is called "serology" [9]. This reaction can not always be observed either macroscopically or with the naked eye especially on viral antigens, so secondary reaction observations are needed. In testing with co-agglutination kits utilizing the properties of protein A in *Staphylococcus aureus* that have the ability to bind parts Fc of IgG [10] leaving the Fab part free immunoglobulin to bind antigen. When the antibody-coated cells are mixed with homologous antigens, the Fab part of the antibody specifically binds the antigen, causing coaglutination cells, so it can be observed macroscopically.

Sequencing results of Iridovirus showed a similarity level of 99% with *Megalocytivirus* strains RSIV. This suggests that the co-agglutination kit can be used to detect RSIV. Testing with co-agglutination kit can not be performed for pooling samples, because there is a reduction in antigen concentration due to dilution, so antigens and antibodies are not equivalent.

CONCLUSION

Co-agglutination kit RSIVD can be used as rapid diagnostic test for RSIV. It has a high diagnostic sensitivity and diagnostic specificity value, fast, easy and cheap. Kit is only better for individual sample than pooling samples based on PCR results

ACKNOWLEDGEMENT

Research was sponsored by PUPT-Indonesian Education Ministry

REFERENCES

[1.] Inouye K, Yamano K, Maeno Y, Nakajima K, Matsuoka M, Wada, Sorimachi M, Jeong JB, Park KH, Kim HY, Hong SH, Chung JK, Komisar JL, Jeong HD. 2004. Multiplex PCR for the diagnosis of red sea bream iridoviruses isolated in Korea. *Aquaculture*, 235, 139–152.

[2.] Oseko N, Chuah TT, Palamisamy V, Maeno Y, Kurita J. 2004. Iridovirus isolated from diseased sea bass *Lates calcarifer* and red drum *Sciaenops ocellatus* causing mass mortality in Malaysia. *In:* 7th Asian Fisheries Forum 04 Abstracts, Penang, Malaysia, 127.

[3.] Koesharyani I, Roza D, Mahardika K, Johnny F Zafran, Yuasa K (2001) Marine fish and crustacean diseases in Indonesia. In: Sugama K, Hatai K, Nakai T (eds) Manual for fish disease diagnosis. II. Gondol Marine Research for Mariculture and Japan International Cooperation Agency, Bali, p 5–7

[4.] Nakajima K, Maeno Y, Fukudome M, Fukuda Y, Tanaka S, Matsuoka S, Sorimachi. 1995. Immunofluorescence test for the rapid diagnosis of red sea bream

[5.] Kurita J, Nakajima K, Hirono I, Aoki T. 1998. Polymerase chain reaction (PCR) amplification of DNA of red sea bream iridovirus (RSIV) *Fish Pathol*., **33**, 17–23.

[6.] Del Río. ML, Gutie´rrez. CB, Rodríguez EF. 2003. Value of Indirect Hemagglutination and Coagglutination Tests for Serotyping *Haemophilus parasuis. Journal of Clinical Microbiology*, 41 (2) 880–882.

[7.] Garvey JS, Cremer NE, Sussdorf DH. 1977. Methods in Immunology: A Laboratory test for Instruction and Research, 3rd ed.. Massachusetts: W. A.Benjamin Inc.

[8.] Amanu S, Rifai AB, Yuliana L, Dwi Wahyu P. 2015. "Evaluation of Coagglutination Test Kit for Red Sea Bream Iridovirus." *Journal of Agricultural Science and Technology* B 5 (6): 437-40.
[9.] Nakajima K, Sorimachi M. 1994. Biological and physico-chemical properties of the iridovirus isolated fromcultured red sea bream, Pagrus major. *Fish Pathology.* 29:29–33. 27.
[10.] Fudenberg HH, Stites DP, Caldwell JL, Wells JV. 1980. *Basic Clinical Immunology 3edition*. Maruzen Asean PTE.LTD. Singapore. 606