Detection of Fish Disease Caused by Iridovirus on Grouper (*Epinephelus* sp.) and Pomfret Star (*Trachinotus blochii*) with Co-agglutination Method in Tanjungpinang, Indonesia

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Abstract: Iridovirus infection often causes death and considerable economic losses in the aquaculture industry. This research applies the co-agglutination method that is fast, cheap and accurate in confirming the diagnosis of the cause of an outbreak of illness caused by iridovirus in the field, so that remedial action can be taken quickly and appropriately to minimize the impact of wider losses. Samples were taken from the grouper and pomfret star farms that are experiencing outbreaks of infectious diseases in the months from May to August 2015, Tanjungpinang, Indonesia. The sick and allegedly attacked by iridovirus samples showed abnormal swimming clinical symptoms, weakness and the swollen spleen. The swollen spleen of sick fish created suspension in phosphate buffered saline (PBS) with pH 7.2, and then centrifuged at 8,000 rpm for 10 min. The supernatant after centrifuge was used as the test sample. On a clean object glass, 50 μ L of the supernatant was treated with 50 μ L kit co-agglutination pre-prepared. The positive results were shown by the agglutination reaction after 10-15 min, while as a negative control, PBS was reacted with co-agglutination kit that looked homogeneous (no agglutination). It was showed that the grouper (*Epinephelus* sp.) on four farms and pomfret star (*Trachinotus blochii*) on one farm that experienced an outbreak of infectious disease in Tanjungpinang showed positively infected iridovirus. The same positive iridovirus result was also demonstrated by examination using polymerase chain reaction (PCR) at 570 bp. So, the causative agent of plague on grouper and pomfret star was iridovirus. In addition, the co-agglutination test based on serology is more quick, cheap and accurate.

Key words: Iridovirus, grouper, pomfret star, co-agglutination and polymerase chain reaction.

1. Introduction

Iridovirus, known as a systemic disease with a high fatality rate (90%) on farmed fish species both in sea and fresh water, has a wide geographic distribution including Africa, America, Asia, Australia and Europe [1]. Iridovirus disease in marine fish was reported to have occurred in several Asian countries, including Taiwan, Singapore, Japan, Korea, Thailand and Hong Kong [2-6]. While the first iridovirus infection cases in Indonesia were reported in 1993 by Rukyani et al. [7]. From the results of environmental monitoring and

fish disease in the years 2011-2013, iridovirus are pathogenic microorganisms that are commonly found throughout the province of Riau Islands (Tanjungpinang, Bintan, Batam, Lingga and Natuna) [8]. This disease has caused economic losses in large scale. Stricken fish swims abnormally and lethargically, but there are no visible external lesions detected, and the body tends to be darker with enlargement of spleen and die suddenly [9]. The disease is a limiting factor on the optimization of production, not only cause of the decline in production but also often the cause of reducing the competitiveness of fishery products. This, of course, will influence the direction of government policy that

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is currently being aggressively launched a productivity improvement program through industrialization aquaculture fisheries, especially grouper and pomfret star that became the leading commodity. The iridovirus rapidly transmit with a high mortality rate, and there is lack of effective treatment on fish populations attacked. Thus, it becomes a threat to the development of grouper (*Epinephelus* sp.) and pomfret star (*Trachinotus blochii*) in Indonesia [8, 10]. Therefore, a method is needed to be fast and accurate in detecting early emergence of this disease, so that the fast action and precise handling can be taken to minimize the impact of wider loss.

A number of diagnostic methods have been developed to detect diseases caused by viruses, including cell culture [11], fluorescent antibody technique (FAT) [12] and polymerase chain reaction (PCR) [13]. But all these methods require special equipment and expertise, as well as longer periods of time. A method based on serology, co-agglutination which is simpler, faster, more accurate and inexpensive, has been developed [14]. This method can be used to detect bacteria and viruses [15]. Co-agglutination equivalent of FAT, however, does not require a fluorescence microscope which is expensive, so it is suitable for use in the field [16].

The co-agglutination method is fast, accurate and cheaper in confirming the diagnosis of the cause of an outbreak of illness caused by iridovirus [17]. In this research, it was applied to detect the fish disease caused by iridovirus on grouper (*Epinephelus* sp.) and pomfret star (*Trachinotus blochii*) in Tanjungpinang, Indonesia.

2. Materials and Methods

2.1 Anti-iridovirus Collection and Purification of Rabbit Serum

Iridovirus antibodies were produced from rabbits immunized with the iridovirus vaccine. Immunization process followed the method described in Ref. [18]. Immunization should be as much as four times the

distance once a week between the first and subsequent injections. The doses injection was 0.5 mL in the first week, 1 mL injection of the second week, 2 mL injection of the third week and 3 mL injection of the fourth week. Seven days after the last injection, blood was taken from the marginal ear vein, and serum was collected in a tube and then inactivated at a temperature of 56 °C in waterbath for 30 min. Immunoglobulin of anti-iridovirus was separated from rabbit serum by ammonium sulfate precipitation method [18].

2.2 Preparation Protein A

Staphylococcus aureus (ATCC 12598) was cultured on trypticase soy agar (TSA) and incubated at 37 °C for 24 h. Isolates harvested from the TSA were collected in tubes and washed three times with PBS (pH 7.2). Isolates added 0.5% formalin PBS solution (pH 7.2), left overnight at room temperature, then heated at 80 °C for 15 min and cooled quickly. After, it was centrifuged at 6,000 rpm for 15 min and the supernatant was exhausted. Pellet was made 10% in PBS with pH 7.2, and this suspension as an ingredient in the test [19].

2.3 Immunoglobulin Binding Protein A

The same volume of *Staphylococcus aureus* antiserum iridovirus was added into cell suspension in PBS (pH 7.2) and left overnight at room temperature. The suspension is then centrifuged at 3,000 rpm for 15 min, and the supernatant was discarded, PBS (pH 7.2) was added to return to the initial suspension volume and is used as a test kit for the co-agglutination test [19].

2.4 Sample Collection

Sampling was performed in four locations of grouper and one location of pomfret star farm from three villages in Riau Islands cultivation centers, Tanjungpinang city, Bintan district, namely Pangkil village (three locations of grouper farm KJA 1, KJA 2,

KJA 3), Mantang village (one location of grouper farm) and Madong village (one location of pomfret star farm). Infected samples, which showed clinical infection symptoms from visual observation, were collected from fishes from each location. Organ was tested by co-agglutination kit and also tested with PCR.

2.5 The Testing Procedure

Spleen organ in fishes that shows clinical symptoms of illness is made of crushed 4× to 9× volume suspension in PBS (pH 7.2). Suspension is centrifugated at 8,000 rpm for 10 min, and the supernatant was used as the test sample. Above the object glass, the 50 µL kit co-agglutination iridovirus in drop was added into 50 µL sample (supernatant), and homogenized by shaking the object glass. Observations were made after 10-15 min with a contrasting background. Positive case shows clotting/agglutination (such as sand grains that do not come late with homogenates), and negative if there is no clumping/agglutination (homogenates remain homogeneous) [19].

3. Results and Discussion

In May to August 2015, there has been an outbreak of mass mortality on fish farming of grouper (*Epinephelus* sp.) and pomfret star (*Trachinotus blochii*) in Riau Islands cultivation centers, Tanjungpinang city, Bintan district. Accumulation of dead fish reached 97% and resulted in significant economic losses. The numbers of grouper and pomfret star deaths from all three regions are presented in Table 1 and Fig. 1.

The affected fish showed clinical symptoms of

abnormal swimming, weakness, darker body color and the swelling of spleen (Fig. 2). Samples were taken from the area of the outbreak. The infected fishes were taken, examined with rapid test kit which is a co-agglutination iridovirus, and then confirmed with PCR.

Based on test results with co-agglutination kit in a completion time of 15 min, samples of grouper and pomfret star taken from the outbreak area showed positive iridovirus. This is evidenced by the formation of agglutination reaction after iridovirus co-agglutination kit reacted with the sample (Fig. 3). This agglutination reaction is the bonding that occurs between the fragment of antigen binding (FAB) part free of immunoglobulin G with iridovirus (antigen homolog), while the fragment crystallizable (FC) portion of immunoglobulin G binding protein A *Staphylococcus aureus* causes macroscopically visible agglutination reaction. Both of these principles make co-agglutination kits accurate, fast and do not require special equipment in the observation results, so this method is very cheap and suitable for use in the field [19]. Test with co-agglutination kit previously has been developed to detect the disease in fish caused by bacteria and viruses [15], such as for the detection of *Vibrio vulnificus* [20], infectious hematopoietic necrosis virus (IHNV) [14], *Pseudomonas fluorescens* infection in fishes [21], viral nervous necrosis (VNN) [19] and iridovirus [17]. Co-agglutination method is fast, accurate and inexpensive and equivalent to FAT [16].

Test results with co-agglutination kits of the samples taken from all the outbreak locations were

Table 1 Total deaths from the plague iridovirus infection on the farm.

No.	Village location	Commodity	Population	Number of death	Total death (%)
1	Pangkil				
Farm 1	KJA 1	Grouper	6,000	4,920	82
Farm 2	KJA 2	Grouper	11,000	6,820	62
Farm 3	KJA 3	Grouper	3,000	2,910	97
2	Mantang farm	Grouper	3,000	2,490	83
3	Madong farm	Pomfret star	28,400	17,608	62

(a) Death of groupers in Pangkil

(b) Death of groupers in Mantang

(c) Death of pomfret star in Madong

Fig. 1 Death of groupers in Pangkil (a) and Mantang village (b), and death of pomfret star in Madong village (c).

(a) Spleen swell on grouper

(b) Spleen swell on pomfret star

Fig. 2 Spleen swell on grouper (a) and pomfret star (b) samples taken from the outbreak area.

positive, as seen in Fig. 3. The samples treated with kit co-agglutination occurs agglutinate which looks like a lump of sand, and it is similar to that seen in the positive control. This shows that anti-iridovirus in the co-agglutination kit reacts with iridovirus in the

samples by antibody and antigen binding homolog. The interaction of antigen-antibody binding is each antibody with the specific antigen, and the interaction is similar to a lock and key. Antibodies have a molecular structure that corresponds perfectly with

Sample KJA 1

Control (-)

Control (+)

(a) Co-agglutination test of grouper sample from farm 1 in Pangkil village

Control (+)

Control (-)

Sample KJA 2

(b) Co-agglutination test of grouper sample from farm 2 in Pangkil village

Control (+)

Control (-)

Sample KJA 3

(c) Co-agglutination test of grouper sample from farm 3 in Pangkil village

Grouper Mantang

Control (-)

Control (+)

(d) Co-agglutination test of grouper sample from farm in Mantang village

Sample pomfret star

Control (-)

Control (+)

(e) Co-agglutination test of pomfret star sample from farm in Madong village

Fig. 3 Results of the co-agglutination test.

Fig. 4 The results of PCR on grouper taken from farm 1 (1), farm 2 (2), farm 3 (3), control (-) and control (+) in Pangkil village.

Fig. 5 PCR results of grouper from Mantang village (1), pomfret star from Madong village (2), control (-) and control (+).

Table 2 Results of the co-agglutination test and PCR.

No.	Commodity	Location village	Test results	
			Co-agglutination	PCR
1	Grouper	Farm 1 in Pangkil	Positive iridovirus	Positive iridovirus
2	Grouper	Farm 2 in Pangkil	Positive iridovirus	Positive iridovirus
3	Grouper	Farm 3 in Pangkil	Positive iridovirus	Positive iridovirus
4	Grouper	Farm in Mantang	Positive iridovirus	Positive iridovirus
5	Pomfret star	Farm in Madong	Positive iridovirus	Positive iridovirus

the antigen, and each type of specific antibodies is against specific type antigens [22]. While in the negative control, the agglutinate was not seen.

To further strengthen the confirmation of the diagnosis, in addition to co-agglutination test, all the

samples later was confirmed by testing using PCR methods. The same results are also shown in tests using PCR. Samples of grouper taken from the farm in Mantang village outbreaks area and pomfret star from the farm in the Madong village outbreaks area both

showed positive marked by approximately 570 bp fragment generated from DNA (Figs. 4 and 5), in parallel to the positive control of the two samples [23], while the negative control did not show band fragments DNA.

To make it easier to analyze the test data using co-agglutination kit and PCR test of all samples taken from the plague, then all the results of test were made in Table 2. As seen in Table 2, the samples of grouper on farm KJA 1-3 in Pangkil village and a farm in the Mantang village and pomfret star in Madong village using co-agglutination and PCR method all showed the same positive iridovirus results, so it can be concluded that the causative agent of mass death against grouper and pomfret star in the village Pangkil, Mantang and Madong is iridovirus.

Thus, it can be concluded that the co-agglutination kit test has the same accuracy as PCR test. But the co-agglutination kit test has the advantage. It is faster in the testing process, only taking 15 min, while PCR takes 6 h, more easily because it does not require special equipment, less expensive because it does not require testing of materials and reagent lots, and more practical because it can be done in the field.

4. Conclusions

From the results in this study, it is known that the grouper (*Epinephelus* sp.) on four farms and pomfret star (*Trachinotus blochii*) on one farm that experienced an outbreak of infectious disease in Tanjungpinang showed positively infected iridovirus. The same positive iridovirus results were also demonstrated by examination using PCR at 570 bp. The final conclusion was that the causative agent of plague on grouper (*Epinephelus* sp.) and pomfret star (*Trachinotus blochii*) was caused by iridovirus. In addition, the co-agglutination test based serological is more quick, cheap and accurate.

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