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Full Length Research

Identification of iridovirus based on molecular and imunohistochemistry studies on the grouper fish (*Epinephelus* sp.) in Lombok, Indonesia

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Grouper cultivation in floating net cages in Lombok is growing well in Indonesian. Problem of grouper fish production is high mortality rates caused by Iridovirus infection. Aim of study is to determine the incidence of Iridovirus infection in grouper fish based on polymerase chain reaction (PCR) and immunohistochemistry test in Lombok Island. A total of 25 fish grouper with size ± 10 cm length infected naturally iridovirus was collected from floating net cages. Internal organs were fixed with 10% of phosphate-buffer formalin for immunohistochemistry examination and in absolute ethanol of solution for molecular study. The positive result of polymerase chain reaction the bright band at 570 bp. Positive results of Iridovirus showed that 9 from 10 samples of cantang grouper in East Lombok were positif iridovirus infection, and 13 from 15 samples were positive in Central Lombok. The same results were also shown using immunohistochemistry test with antibody from rabbit. The strong immunolabellings stained brown colour were obtained from the spleen and liver. This indicated that the causative agent of high mortality in grouper fish in Lombok was caused by Iridovirus.

Key words: Iridovirus, PCR, immunohistochemistry, Lombok.

INTRODUCTION

Virus of the family iridoviridae are double-standed DNA viruses, icosahedral symmetry and measures 120-300 nm in diameter [1,2]. Iridovirus caused several serious systemic disease in fish, characterized a high mortality rate typically between 20 and 60% and sometimes reaches 100% on the production and welfare of fish aquaculture [3]. Clasification of iridoviruses were divided into five genus: Iridovirus, Chloriridovirus, Ranavirus, Lymphositivirus and Megalocytivirus [1,4,5]. Ranavirus, Lymphositivirus and Megalocytiviruses have been described to infections in a variety of freshwater spesies and marine fish species [5,6,7]. The outbreak of iridoviruses occurred in Southeast Asia and infected more than 50 species of freshwater and marine [8]. The disease was called as infectious spleen and kidney necrosis virus (ISKNV) found in mandarin fish, red sea bream iridovirus (RSIV) in red sea bream fish, rock bream iridovirus (RBIV) in rock bream fish, large yellow croacker iridovirus (LYCIV) in large yellow croaker fish, Taiwan grouper iridovirus (TGIV) in groupers and orange spotted grouper iridovirus (SGIV) [9,10,11,12,13,14,20].

Iridovirus infection is a deadly disease in aquaculture, the economic loss is very high because the virus infected at the stage of larvae until the age of harvest. First case of iridovirus infection of grouper in Indonesia was reported in North Sumatra [15]. Furthermore, viral infection was also found in the BBRPBL Gondol on coral grouper and caused 70% mortalities rates [16]. In Tanjung Pinang infection was also found on grouper and pomfert star and caused 97% loss [17]. Fish appeared swim abnormality, lethargic, severe anemia, anorexia, the body become darker, gills ptechiae and enlargement of spleen [8,6,18,19].

A number of diagnostic methods for iridovirus infection

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have been reported in Indonesia, such as polymerase chain reaction (PCR) [21], co-agglutination method [17], histophatological and electron microscopic changes [22,23,24]. The aim of study is to identify iridovirus disease in grouper (*Epinephelus* sp) in floating net cages in Lombok based on polymerase chain reaction and immunohistochemistry studies.

MATERIAL AND METHODS

Sampling

Number of twenty five fish with clinical signs such a swim abnormality, anorexia, anemia and enlargement of spleen were collected from floating net cages in East and central Lombok. Internal organ were fixed with 10% phosphate-buffer formalin for histopathological examination and in absolute ethanol of solution for molecular study.

Polymerase chain reaction (PCR) test

Total genomic DNA was extracted from spleen using Genomic DNA purification kit (Promega Wizard) following the manufacturer protocol. The pair 1F (5'-CTCAAACACTCTGGCTCATC-3') and 1R (5'-GCACCAACACATCTCCTATC-3') were designed from the nucleotide sequence 959 PstI restriction fragment area DNA target 570 bp - DDBJ accession number AB006954 [10]. The PCR was performed in a total reaction volume 25 µL containing 12 µL mastermix (GoTaq Green), 1 µL of each primer (10 pMol), 9 µL of Nuclease free water and 2 µL DNA template. The mixture was incubated in a automatic thermal cycler programmed for 30 cycles, 1 cycles of pre denaturation 94°C for 2 min, denaturation 94°C for 30 s, annealing 57°C for 60 s, extention 72°C for 60 s and final extension 72°C for 5 min.

Primary antibody production

Three male of rabbits were immunized by iridovirus vaccines. Rabbits were intraperitoneally injected with graded dosages from 0.5 cc, 1 cc, 2 cc, 3 cc and 3 cc every week. The serum was collected in the sixth weeks [17,27]. The serum was inactivated at 56°C in water bath for 30 min. Serum anti-iridovirus was prepared by ammonium sulfate $(NH4)_2SO_4$ precipitation and purified by dialysis membrane MWCO 6-8 kDa using PBS buffer pH 7.2, then the purified IgG antibodies was stored at 4°C.

Immunohistochemistry test

The spleen and liver were dehydrated in ethanol (70, 80, 90, 95%, absolute), cleared in xylol and embedded in paraffin. Embedding tissue were sectioned microtome then processed for immunohistochemistry. Tissue sections for immunohistochemistry were deparafinated in xylene, then rehydrated in ethanol (absolute, 90, 80 and 70%) and washed with water for 2 min. Slides were treated in citrate buffer, heated in microwave oven for 5 min. The preparation is neutralized using peroxidase block for 5 min and washes it 2 times each 5 min. The next preparation was incubated with protein block for 5 min and washed in PBS 2 times each 5 min. The primary antibody from rabbit was polyclonal antibody anti-irido was incubated for 30 min, washed in PBS 2 times each 5 min, then incubated with post primary for 30 min and dripped with novo link polymer for 30 min. The slides were washed in PBS 2 times each 5 min and incubated using DAB working solution. Slides were rinsed with water, counterstained using haematoxyline. After dehydration process using serial graded of ethanol, cleared with xylene, and mounted with entellan to be examined under light microscope.

RESULTS AND DISCUSSION

Iridovirus outbreaks were very rapidly occurred from January to February 2018. A total of 25 fish samples in two location of outbreak area consisted of 10 grouper fish from East Lombok and 15 grouper fish from Central Lombok. Grouper fish showed clinical symptoms such as abnormalities swim, weakness, dark coloration of body, decreased appetite and dwells at the bottom of the net cage. The macroscopically changes appeared hemorrhages of gills and enlargement of spleen and liver (Figures 1A and B).

The presence of swollen spleen indicated the main target of this virus is the hematopoietic organs and its replication involving the nuclear and cytoplasmic compartment [3]. Hematopoietic disorder caused by iridovirus infection caused the clinical symptoms in grouper fish almost identical [8,17,25,24].

The PCR results of spleen and liver of infected fish showed that 9 from 10 grouper in East Lombok were positif iridovirus and, 13 from 15 samples from Central Lombok were positif iridovirus (Table 1). The positive result of rDNA amplification of Iridovirus showed a bright band at 570 bp of DNA fragment compared to positive and negative control (Figure 2).

This study using spesific primer for detection Genus Megalocytiviruses resulted in 570 bp amplicon (Figure 2). This primer set 1F and 1R was designed by Kurita *et al*. [10] recommendation from OIE [27]. This primer was used to amplify the 570 bp gene sequences of the 959 bp *Pst*-I rectriction fragment for detection Red Sea Bream Iridovirus (RSIV) and Infection Spleen and Kidney Necrosis Virus (ISKNV).

Immunohistochemistry was performed to illustrate the presence of viral distribution in the tissue. Organs such

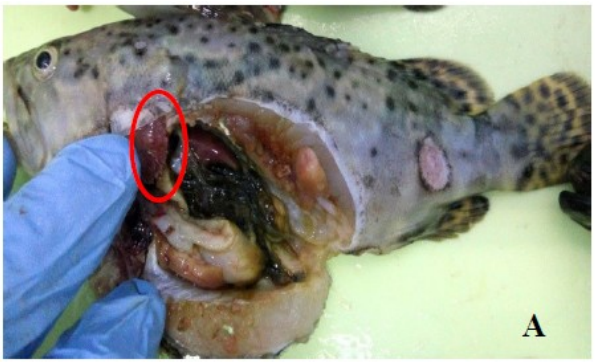

Figure 1. Clinical sign of infected grouper fish. A) Dark coloration body and hemorrhage of gills, B) Enlargement of spleen and liver.

Table 1. Result of PCR and immunohistochemistry studies of groups from Lombok.

No.	Location	Comodity	PCR	Immunohistochemistry
1.	East Lombok	Cantang grouper	+	+
2.			-	-
3.			+	+
4.			+	+
5.			+	+
6.			+	+
7.			+	+
8.			+	+
9.			+	+
10			+	+
11.	Central Lombok	Cantang grouper	+	+
12			+	+
13			+	+
14			+	+
15			-	-
16			+	+
17			+	+
18			+	+
19			+	+
20			+	+
21			+	+
22			+	+
23			-	-
24			-	-
25			+	+

(+) positive result, (-) negative result.

as spleen and liver were collected from positive iridovirusinfection. The strongest immunolabellings of iridovirus appeared in spleen, the specific antibody and antigen reaction stained by chromogen showed brown color (Figure 3A). Positive reaction found in sinusoid of liver (Figure 3B).

This is indicated that iridovirus infection can spread systemically in several organs of fish. The virus can enter the host through the gills or injured skin [28]. The most important pathological changes seen in hematopoietic tissue caused similar lesson in all host [3]. This study illustrated the incidence of iridoviruses in East and Central Lombok, Indonesia based on PCR test and immunohistochemistry in infected organs and has never been reported. The detection of iridovirus infection in grouper based on PCR test and immunohistochemistry

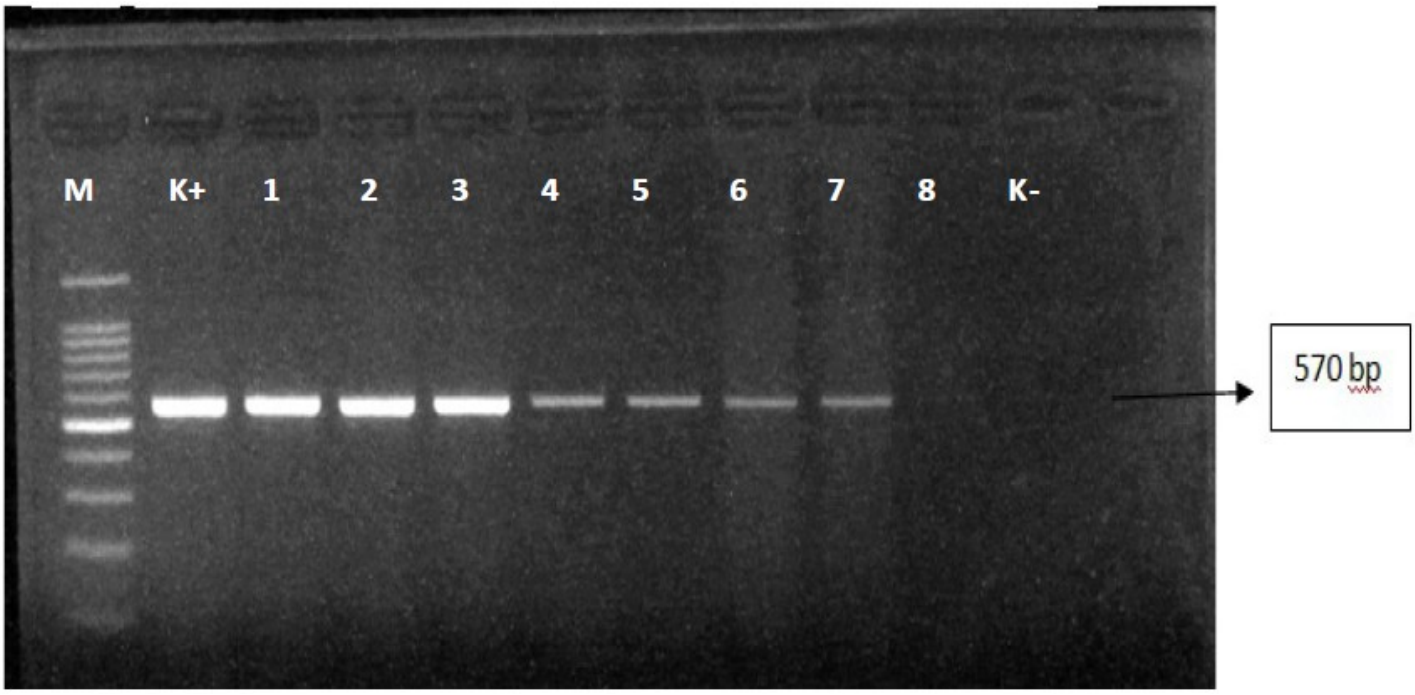

Figure 2. The result of PCR from East and Central Lombok, amplification of PCR product 570 bp. (M) Marker, (K+) positive control, (1) fish sample 1, (2) fish sample 2, (3) fish sample 3, (4) fish sample 4, (5) fish sample 5, (6) fish sample 6, (7) fish sample 7, (8) fish sample 8, (K-) negative control.

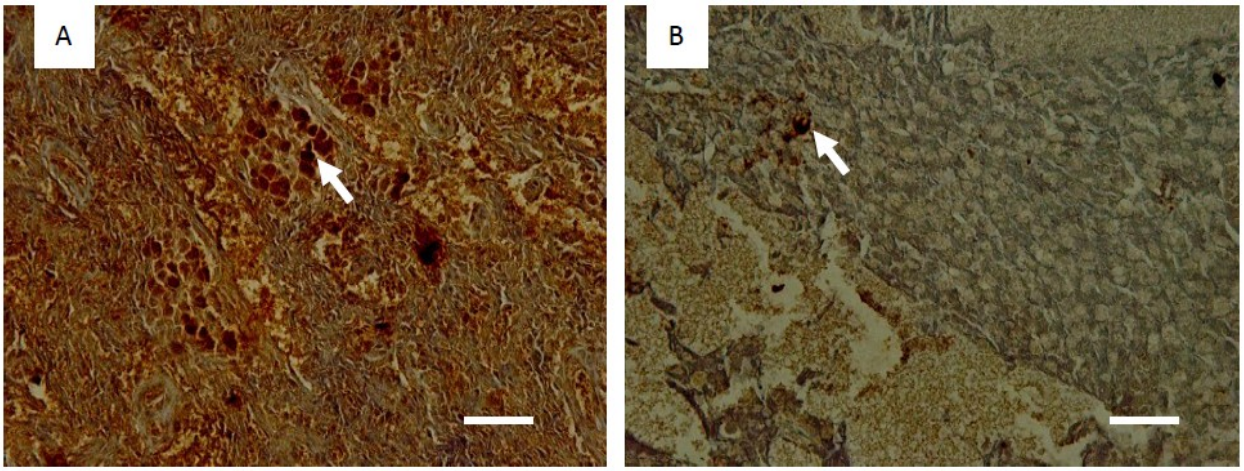

Figure 3. Immunohistochemistry results of the spleen (A), liver (B) inclusion body is brown (arrow).

showed the similar result test. This virus can replicate in spleen and liver.

Conclusions

The result showed the rDNA of Iridovirus had abright band at 570 bp. The immunohistochemistry showed that distribution of virus can be found in spleen and liver. The high mortality of grouper fish in floating net cages in East and Central Lombok was caused by iridovirus infection.

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