DISTRIBUTION ANALYSIS OF ENLARGED CELLS DERIVED FROM GROUPER SLEEPY DISEASE IRIDOVIRUS (GSDIV) INFECTED HUMPBACK GROUPER *Cromileptes altivelis*

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ABSTRACT

Characteristic of *Megalocytivirus* infection has been known to produce formation of inclusion body bearing cells (IBCs) on internals organs of fish predominantly on spleen and kidney. *Megalocytivirus* that infected grouper is known as Grouper Sleepy Disease Iridovirus (GSDIV). This study was conducted to answer the effect of entry sites of GSDIV on distribution of enlarged cells formed on the internal organs of humpback grouper *Cromileptes altivelis*. Enlarged cells were observed histologically under the light microscope on spleen, head kidney, trunk kidney, liver, gill, heart, stomach, intestine, muscle and brain. Entry sites were designated to intramuscularly injection, intraperitoneally injection, dipped gill and inoculum added feed. Enlarged cells were formed on spleen, head kidney, trunk kidney, liver, gill, heart, stomach, muscle, except on intestine and brain. All the entry sites resulted in formation of enlarged cells on spleen, head kidney, trunk kidney, liver, heart. Spleen and head kidney were the most frequent observed organ. These results suggested that distribution of enlarged cells were not affected by the entry site of GSDIV.

KEYWORDS: *Megalocytivirus*, enlarged cells, distribution, internal organs

INTRODUCTION

Megalocytivirus which infect grouper is known as Grouper Sleepy Disease Iridovirus (Sudthongkong *et al.*, 2002). International Committee on Taxonomy for Viruses (ICTV) had classified this viral isolate based on sequence of key viral genes eg. ATPase and major capsid protein/MCP into a new genus named *Megalocytivurus*. ICTV also considered its capability to form Inclusion Body Bearing Cells (IBCs) as the distinguishing factor from other genus within *Iridoviridae* family (Chincar *et al.*, 2005). Under the light microscopy, IBCs as well as necrotized cells were also the diagnostic features that observed as enlarged cells (Miyazaki, 2007).

The ultrastructure study of enlarged cells on GSDIV infected fish revealed that IBCs contained replication site of the virus (Mahardika *et al.*, 2004; 2008; Sudthongkong *et al.*, 2002). Enlarged cells are widely distributed among the internals organ of infected fish predominantly on spleen and head kidney (Chao *et al.*, 2004; Le *et al.*, 2009; Mastuti *et al.*, 2010; Wen *et al.*, 2006). Previous study by Chao *et al.* (2004) suggested that the viral particles distributed trough circulatory system, where spleen and head kidney were the first site of viral replication.

Megalocytivirus had frequently been reported to cause severe economic losses in aquaculture industry. Ambient water and trash

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fish feeding might be the sources of viral particles in fish farming (He *et al*., 2002; Choi *et al*., 2006). It was suggested that the gill and digestive tract were the most probably entry sites of the viral particles. This paper will discuss the effect of entry sites of GSDIV on the distribution of enlarged cells formed in the internal organs of humpback grouper *C. activelis* as a model.

MATERIALS AND METHODS

This study was held in September to December 2010 at Research Institute for Mariculture Gondol-Bali, Indonesia. The experimental fishes were healthy humpback grouper (total length 8-12 cm) collected from private hatchery at Gondol-Bali. A total of 75 fishes were kept in five 100 L aquarium with 70-80 L ambient sea water and aeration. An experiment was divided to five groups that were artificially infected with different routes of given GSDIV inoculum through: intramuscular injection (IM), intraperitonial injection (IP), gill immersion for 5 minutes, artificial feed per day for 7 days, and control without any treatment (Table 1). Fish were anaesthetically using FA-100 (10 mg/L) before treatment. A day after challenge test, all treated fish were given commercial fed twice per day. Daily water exchange (about 40%-50%) was done with bottom cleaning of the aquarium. To prohibit viral transmission, we relocated the water excess in other tank and added 10% chlorine for stand a day, particularly when experiment was started. Moribund and fish that had just died were removed from the tanks and dissected. At the end of the experiments, all surviving fish from all groups were removed and dissected for further observation.

Viral inoculum were made by homogenized the infected spleen, kidney, and liver of humpback grouper which were positively PCR tested. The organs were homogenized in 9 folds volume of 2% fetal bovine serum (FBS) supplemented with Eagle's minimum essential medium (EMEM-2). Then, the homogenate was centrifuged at 3,000 *g* for 15 minutes at 4°C and supernatant was filtered (450 nm). The filtered supernatant as virus inoculum was stored at 80°C until used for experiment.

Histological sections were made according to previous standard methods. A portion of organs were dehydrated in serial dilution of alcohol (70%, 80%, 90%, 95%, and 100%). Sections with 0.4–0.6 µm thickness were stained with Mayer's hematoxylin-eosin. Observation to 10 pcs of fish focused in presentation of enlarged cell within the internals organ (spleen, head kidney, trunk kidney, liver, stomach, intestine, gill, heart, muscle at injection site and brain).

PCR test to confirm the moribund and dead fish infected with GSDIV were undertaken using primers 1-F and 1-R (Kurita *et al.*, 1998). DNA templates were extracted from about 15 mg of spleen and liver tissue using ISOGEN (Wako Nippon Gene, Osaka, Japan) according to the manufacturer's protocol. The PCR amplification was performed under conditions described previously by Mahardika *et al.* (2008).

RESULTS AND DISCUSSION

Enlarged cells mostly present in the spleen particularly at the stroma. Necrotized and degenerated cells also observed in the spleen (Figure 1a). Study which was led by Chao (2004), suggested that 120 hours after *Megalocytivirus* infection was the fist time of activated macrophage, at this time the number of necrotized cells became higher. Highly concentration of virus caused the cells hypertrophied, necrotized and degenerated, it impact to swollen spleen (Go & Whittingthon, 2006). Enlarged cells also present in high number in the head kidney (Figure 1b). Previous studies

Table 1. Treatment methods of given GSDIV inoculum to humpback grouper *C. altivelis*

Treatment	Dosage of GSDIV inoculum
Intramuscular injection	0.1 mL/fish
Intraperitonial injection	0.1 mL/fish
Gill immersion (dipped gill)	0.1 mL/both gill/fish for 5 min.
Inoculum added feed	2 mL/10 g feed/15 fish/day for 7 days
Control	No treatment

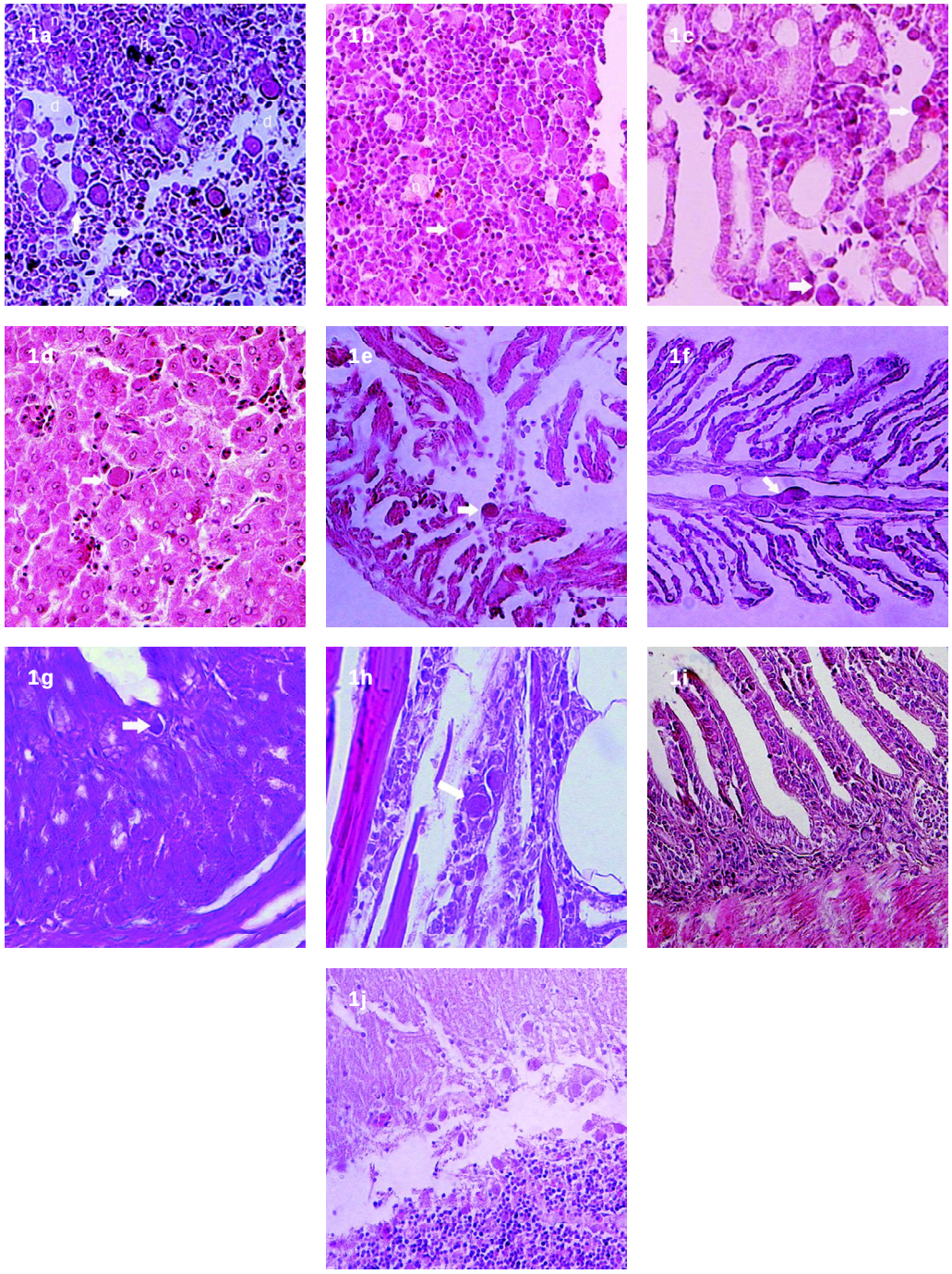

Figure 1. Histological section of the internal organs of diseased *Cromileptes altivelis*. Enlarged cells were observed in spleen (1a), head kidney (1b), trunk kidney (1c), liver (1d), heart (1e), gill (1f), stomach (1g), and muscle (1h). Necrotic (n) and degenerated (d) cells also formed in spleen and head kidney. On the other hand, no enlarged cells were detected in intestine (1i) and brain (1j)

concluded that iridovirus infections caused severe anemia as the enlarged cells mainly present in spleen and head kidney which are the haematophoetic organs (Mahardika *et al.*, 2004; 2008; Wen *et al.*, 2006; He *et al.*, 2002). Formation of enlarged cells replaced the site of haematophoitic cells as well as necrotized cells which both caused decreasing blood production and finally causing anemia. In the trunk kidney, enlarged cells found in the hematophoetic tissues among the renal tubules (Figure 1c). Less enlarged cells formed in liver, heart, gill, stomach and muscle. In the liver, enlarged cells formed at hepatic sinusoid (Figure 1d). In the heart, enlarged cells were seen at the epicardium or among the myocardium (Figure 1e). In the gill, enlarged cells formed at the epithel of secondary lamella or near the bone at the core of gill lamella (Figure 1f). Formation of enlarged cells in gill and heart might be contributed to mortality as it clogged the capillary (Chao *et al*., 2004; Mahardika *et al*., 2009). With very low frequency of enlarged cells were seen at the mucosal layer in the stomach. Enlarged cells were also found at the connective tissue among the subdermal striated muscle in the site of injections (Figure 1g).

No enlarged cells formed in the intestine and brain (Figure 1i, 1j). The study led by Le (2009) detected the viral particles between durameter and arachnoid of the outer membrane of brain by *in-situ hybridization* (ISH) method. Other study led by Shimmoto *et al.* (2009) detected viral particles in brain and muscle by *quantitative* PCR (qPCR) method. However, in infected fish with asymptomatic case had lower concentration of *Megalocytivirus* in the spleen than in the heart, stomach, muscle, eye and gill. Thus, they are potential as the carrier of *Megalocytivirus* (Choi *et al.*, 2006).

Figure 2 showed that the viral infection through intramuscular (IM) and intraperitonial (IP) injections resulted in formation of enlarged cells in spleen and head kidney in every observed fish with slight different. The result of this study was similar to the result of experimental infection of Taiwan Grouper Iridovirus by intraperitonial injection to *Epinephelus* hybrid (Chao *et al.*, 2004), they observed the formation of enlarged cells mostly in the spleen, head kidney. Infection through feeding did not resulted formation enlarged cells in the gill, however enlarged cells were found in the stomach and target organs with similar than those in IP method. This indicated that the viral particles were entered digestive tract and induced some enlarged cells within stomach before transmitted to spleen and the other organs. Enlarged cells in muscle were resulted only from IM method. These enlarged cells were resembled surrounding injection site. Taken together, fish infected with GSDIV viral particles were produced enlarged cells as well as necrotic cells mainly in spleen and kidney as the target organs, and there is few or no effect of entry point of GSDIV.

Figure 3 showed the survived fish were recovered from infection at 14 days post infection (dpi). Intramuscular and intraperitonial

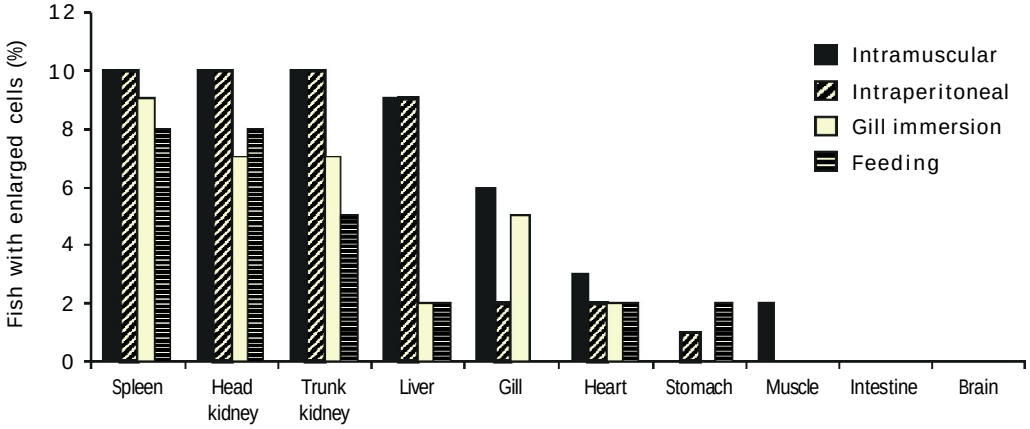

Figure 2. A total of diseased *Cromileptes altivelis* contains enlarged cells in the observed organs/tissue

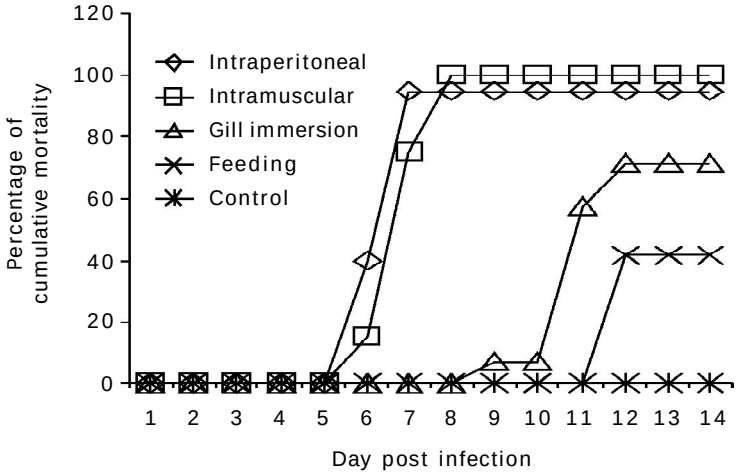

Figure 3. Cumulative mortality of the infected *Cromileptes altivelis* after infected with GSDIV through various methods of exposure

methods resulted in mortality 3 and 6 days earlier than gill immersion and feeding methods. At 5 dpi, IP group had 100% mortality days post infection and IM group had 95% mortality. Gill immersion method resulted in mortality at 8 dpi with 23.57% lower than IM group, while infection trough feeding resulted in mortality at 11 dpi with 53.45% lower than intramuscular method.

The exposure by injected resulted higher mortality rate (Cullen & Owens, 2002). Injection methods gave more possibility to allow the precise dose of infection (0.1 mL/fish) than that of gill immersion or feeding method. However, either gill immersion or feed methods promise the efficiency of vaccination method in large fish farming.

Reisolation of *Megalocytivirus* using PCR test confirmed that fish positively infected after artificial infection. About 570 bp DNA fragment were amplified by primer 1-F and 1-R. Spleen and kidney homogenate from all of experimental fish group contained viral particles (Figure 4). In refer to Choi *et al.* (2006) *Megalocytivirus* detection using spleen homogenate are suitable for acutely infected fish.

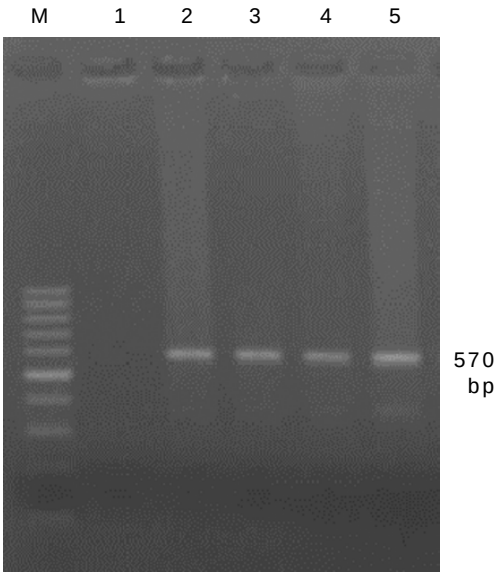

Figure 4. PCR photograph of experiment *Cromileptes altivelis* showed that fish without any treatment were negative of GSDIV which shown no band in agarose gell in lane 1, while fish entered with GSDIV through: IM (lane 2), IP (lane 3), dipped gill (lane 4) and added feed (lane 5) were positive containing gen of virus GSDIV which shown a single band of 570 bp as predictive size within each lane. M: marker 100 bp

CONCLUSION

Infection of GSDIV resulting the formation of enlarged cells in the spleen and head kidney of *C. altivelis* without affected by the entry site of infection. Various infection meth-

ods showed different capabilities causing mortality.

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