STUDY PROPAGATION OF GSDIV (GROUPER SLEEPY DISEASE IRIDOVIRUS) IN CULTURED GF (GRUNT FIN) CELL

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ABSTRACK

Grouper Sleepy Disease Iridovirus (GSDIV, genus *Megalocityvirus*) remain unsolved in Indonesian aquaculture. A GSDIV propagating study had been conducted to elaborate the capability of GSDIV replication in GF cell line. GSDIV isolate derived from spleen of diseased grouper fish were infected to GF cells line. GSDIV-infected GF cells were characterized by enlargement cells as CPE (cytophatic effect) which floated in cultured-medium. CPE-GSDIV was positively confirmed by PCR test. GSDIV-viral concentrations were ranging between $10^{2.80}$-$10^{5.80}$ $TCID_{50}$/mL. The newly passage GF cells were more sensitive to GSDIV which was shown by the abundance of CPE. GSDIV derived from GF cells were caused 10%-100% mortality of humpback grouper, *Cromileptes altivelis*.

KEYWORDS: GSDIV, grunt fin cells, CPE, *C. altivelis*

INTRODUCTION

In Indonesia, iridovirus-like infection was first reported to infect orange spotted grouper in net cage in Sumatra Island (Owen, unpublished data). Subsequently, iridovirus infection found in orange spotted grouper cultured in pen cage in Lampung, Sumatra Island. It infection was confirmed to be iridovirus same as red sea bream iridovirus (RSIV) base on PCR analysis (Koesharyani *et al.*, 2000). Histopathological analysis of this infection revealed formation of enlarged cells and necrotic cells mainly in the spleen and kidney tissues (Mahardika *et al.*, 2001). Under electron microscopy, these enlarged cells revealed enlarged inclusion bodies containing many virus particles which surrounded by thin membranous boundary from host-cell cytoplasm (Mahardika *et al.*, 2004). Virus particles were hexagonal in shape with diameter 150-200 nm. Based on the pathognomonic signs in which showed sleepy behaviour, the virus infection designated as 'grouper sleepy disease iridovirus (GSDIV)' to mark the distinctive between ranavirus in Malabar grouper (GIV) (Sudthongkong *et al.*, 2002). GSDIV in Indonesia has high similarity with the members of *Megalocytivirus* (Chinchar *et al.*, 2005; Mahardika *et al.*, unpublished data).

Recent economic loses in Indonesian aquaculture due to GSDIV infection is remain unsolved. Optimizing culture condition is still the major prevention method. However, it will be hard to optimize the culture condition when fish had been reared in cage. Therefore, studying the character of GSDIV is necessary to gain the prevention method.

Cell line is a reliable means for isolating and propagating many viruses including marine fish viruses (Fryer & Lannan, 1994). It also a powerful tool for studying parameters in fish virology such as ultrastructural features, pathogenecity, and molecular genomic (Mahardika *et al.*, 2008; Tsai *et al.*, 2005; Chou *et al.*, 1998). To date, more than 150 cultured cells have been developed, mostly are derived from fresh water fish species (Fryer & Lannan, 1994). However, few cell lines are sensitive to marine fish viruses including iridovirus.

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GF (grunt fin) cell is one of many cell line which derived from fin tissue of adult salt water blue striped grunt. It characterized by fibroblast like cell with adhere growth mode (Anonymous, 1961). GF cell is suitable for propagating red sea bream iridovirus (RSIV, genus *Megalocityvirus*) (Mahardika & Miyazaki, 2009). Other cell lines were also known susceptible for propagating several isolat of *Megalocytivirus*. However, GSDIV found in Indonesia was not cultured in any cultured cell lines, yet. This research was focused on the propagated features of GSDIV virus in cultured GF cells for further virus availability stock to vaccine.

MATERIALS AND METHODS

Virus Stock

GSDIV suspicious fishes were collected prior to confirmation with PCR analysis. The PCR analysis was conducted based on a pair primer set of 1-F and 1-R which were derived from DNA sequence of the *PstI* 1 fragment of RSIV. For about 570 bp of amplification product were generated (Kurita *et al.*, 1998). DNA templates were extracted from about 10 mg of spleen tissue using isogen (Wako Nippon Gene, Osaka, Japan) according to the manufacturer's protocol.

Virus was took from the targeted organ (spleen) and homogenized in 9x volume of EMEM-2. The homogenate was centrifuged at 3,000 rpm, 4°C for 15 minutes. Supernatant were filtered using 0.45 µm filter membrane. The filtrate was stored at -80°C and used for viral inoculum.

Cell Culture

GF and KF-1 (koi fin) cells were grown in 25 cm^2 sterile cultured-flask. The cell was seeded into Eagle's minimum essential medium (EMEM, Nissui, Japan) supplemented with fetal bovine serum (FBS, Sigma, USA), L-glutamin (Nissui, Japan), and penstrep-kanamycin (Sigma, USA). FBS supplementation was adjusted into 2% (EMEM-2) prior to GSDIV inoculation and 10% (EMEM-10) prior to cell line maintenance. The optimal pH of the medium (7.2-7.4) was buffered with $NaHCO_3$. The GF cells were incubated at 25°C.

Routine passages were done, when the cells reached in 95% confluent according standard trypsinization method. Briefly, cells were washed using 3 mL EDTA-PBS then dissociated using 5 drops of trypsin-EDTA for about 5 minutes at room temperature with shaking the flask if needed. When the cells were dissociated, 3 mL of EMEM-10 was added immediately to inactivate the trypsin. The cells suspension was divided into 3 flask and grown in 5 mL of EMEM-10.

GSDIV Propagation on GF Cell Monolayer

Two methods of GSDIV infection were conducted in this experiment. The experiment one, 40%-50% of confluent GF monolayer was inoculated 0.2 mL of viral inoculum and incubated for one hour at 25°C. Then, EMEM-2 added to the monolayer. While the experiment two, 40%-50% of confluent GF cells monolayer was double inoculated with 0.1 mL of viral inoculum after 1 hour passage and 24 hour after passage. Susceptibility of GF cells were studied by observing the cytophatyc effect (CPE) produced by GSDIV. When maximum CPE was obtained, the monolayer was harvested and used for further analysis.

The concentration of virus were enumerated by titration. A volume of 50 µL of each serial diluted CPE (10^0-10^{-10}) were inoculaled into a row of 96 well microplate which was contained GF cells. The inoculation was repeated 4 times at the rows bellow. Titer value was determined according the $TCID_{50}$ Method as described by Reed & Muench (1938) and reported as $TCID_{50}$/mL. We also determined virus titer of the spleen filtrate as a comparison.

GSDIV Detection in GF Cells

GF cells monolayer with CPE were confirmed for GSDIV by PCR test. DNA templates were extracted using Trizol-LS. (Invitrogen, USA) according to the manufacturer's protocols. As well as confirmation of virus isolate, the amplification was performed using 1-F, 1-R primer set according to Kurita *et al.* (1998).

Pathogenecity Test to Humpback Grouper (*C. altivelis*)

GSDIV on GF cells were tested for pathogenecity to humpback grouper. CPE producing GF cells were harvested and injected intramuscularly to humpback grouper juveniles. The infection dose was 0,1 mL/fish. Infected fishes were observed from the onset of infec-

tion to the caused mortality. The moribund or dead fish also analyzed by PCR and histopathologycally.

RESULTS

Inoculation of 0.2 mL viral inoculum into 40%-50% confluent GF cells monolayer resulted in few CPE (Figure 1C). While double inoculation of 0.1 mL into freshly passage GF cells resulted in many CPE instead (Figure 1D). At the onset of formation, CPE was appeared as enlarged rounding cells. Then the cells increased in number and detached from the layer. However, with the time of incubation the enlarged rounding cells stopped to form while the normal remaining cells continued to grow. On the other hand, KF-1 cells was susceptible for GSDIV in which CPE did not occured in monolayer of KF-1 inoculated-GSDIV.

Virus titer analysis of the CPE producing monolayer showed that the abundance of CPE was correlated to the virus titer. GF cells with CPE appeared had titer virus as $10^{2.80}$ $TCID_{50}$/mL, whereas GF cells with many CPE appeared had titer virus as $10^{3.55}$-$10^{5.80}$ $TCID_{50}$/mL (Table 1). However, concentration of virus of the infected cells had lower value than virus inoculum which was derived from spleen filtrate ($10^{6.30}$ $TCID_{50}$/mL).

PCR based confirmation of CPE producing GF-monolayers showed that the monolayers were infected by GSDIV. About 570 bp of amplicon were produced from 6 CPE producing GF-monolayer (Figure 2). Thin band was found in GF-infected GSDIV which low viral titer (Figure 2, lane 4 and 5), but thick band was occured in GF-infected GSDIV which high viral titer. This cultured GSDIV were pathogenic to grouper, *C. altivelis*. It caused 10%-100% mortality according to the abundance of CPE (Table 2). Lower mortality (10%-27.6%) were occured when fish injected with viral inoculum containing a few CPE (lower viral titer). On the other hand, higher mortality (60%-100%)

Table 1. Viral titer of GF-GSDIV inoculum

Culture flask	Days harvested of CPE (dpi)		Virus titer (TCID 50/mL)
1	A few CPE appeared	4 dpi	$10^{2.80}$
2	Many CPE appeared	5 dpi	$10^{3.55}$
3	Many CPE appeared	6 dpi	$10^{5.80}$
4	A few CPE appeared	6 dpi	$10^{2.80}$
5	Many CPE appeared	2 dpi	$10^{4.30}$
6	Many CPE appeared	5 dpi	$10^{4.30}$
7	Many CPE appeared	5 dpi	$10^{4.05}$

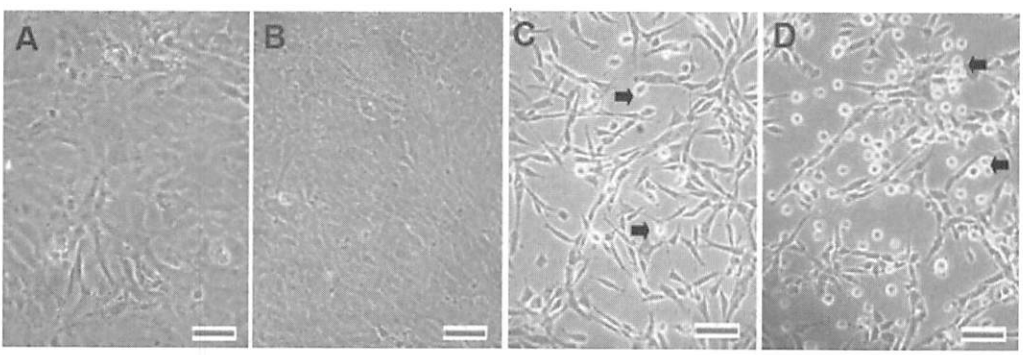

Figure 1. Cultured cells. A) Normal GF cells, B) Normal KF-1 cells, C) Monolayer of GF cells infected with spleen-GSDIV inoculum shows a few number of enlarged rounding cells as CPE (representative as +), D) Monolayer of GF cells infected with spleen-GSDIV inoculum shows many enlarged rounding cells (representative as ++) (scale = 25 µm)

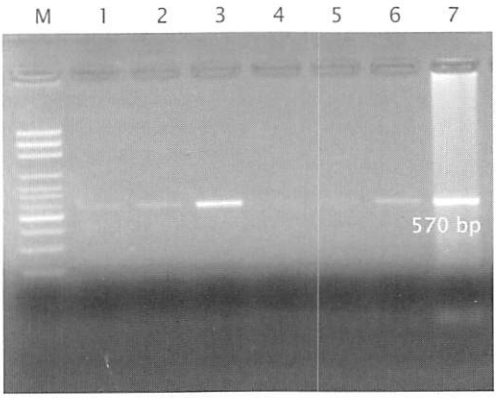

Figure 2. PCR confirmation of GSDIV derived from CPE producing cell. Lane 1; 2 dpi ($10^{4.30}$ $TCID_{50}$/mL), lane 2; 5 dpi ($10^{3.55}$ $TCID_{50}$/mL), lane 3; 6 dpi ($10^{5.80}$ $TCID_{50}$/mL), lane 4; 4 dpi ($10^{2.80}$ $TCID_{50}$/mL), lane 5; 6 dpi ($10^{2.80}$ $TCID_{50}$/mL), lane 6; 5 dpi ($10^{4.05}$ $TCID_{50}$/mL), lane 7; positive control

Table 2. Total mortality of humpback grouper juvenile infected with GF-GSDIV inoculum

Days harvested of CPE (dpi)		Number of fish	Percent mortality
	6 dpi (I)	29	27.6
A few CPE appeared	6 dpi (II)	29	27.6
	2 dpi	30	16.7
Many CPE appeared	5 dpi	5	60
A few CPE appeared	3 dpi	5	30
A few CPE appeared	2 dpi	5	10
Many CPE appeared	2 dpi	5	60
Many CPE appeared	5 dpi (10^{-1})	10	100

shown in fish which injected with viral inoculum containing many CPE (higher viral titer). Diseased fish were loss apetite at 3 dpi and subsequently fish became weak and take a rest on the bottom of aquarium. Fish mortality were firstly accured at 4 dpi and reached a peak at 5 dpi. Some fish were recovery after 6 dpi and were fed well.

DISCUSSIONS

The abundance of the CPE was vary with the age of infection. Many CPE was observed at 2 dpi or 5 dpi, but several flask also showed few CPE at 2 dpi or 5 dpi. While at 6 dpi no flask showed many CPE produced. Based on surveillance system, Ariel *et al.* (2009) found that susceptibility of cell line can vary not only between the cell line and laboratory, but also between lineages of the same cell line. Thus, selection to cell lineages that relative superior in their susceptibility is needed.

Several *Megalocytivirus* had been successfully isolated and propagated in cultured cell such as BF-2 (bluegill fry), KRE (embryonic of kelp and red spotted grouper hybrid), GF and CHSE (chinook salmon embryo), however the concentration of virus was dramatically decreased with the serial passage (Chou *et al.*, 1998; Nakajima & Sorimachi, 1994; Mahardika & Miyazaki, 2009). *Megalocytivirus* propagating method had also been done in new established cell line such as GP (grouper, *Epinephelus tauvina*) (Chew-Lim *et al.*, 1994), SF (Asian seabass fry) (Chang *et al.*, 2001), cultured cell devived from eye, fin, heart, and swim blader of *Epinephelus awoara* (Lai *et al.*, 2003), and GS (grouper spleen, orange spotted grouper) (Qin *et al.*, 2006). Several

Megalocytivirus had been reported to be failed to propagate in comercial cell line, they were DGIV (dwarf gourami iridovirus), TBIV (turbot iridovirus) (Go & Whittington, 2006; Oh *et al.*, 2006).

The result of this research indicated that GSDIV can be propagated in comercial GF cell line although the titer is still unstable. *Megalocityvirus* propagation in GF cell had been studied in RSIV-infected GF cells under the electron microscope (Mahardika & Miyazaki, 2009). RSIV-infected GF cells revealed the presence of two types of enlarged cells: IBCs, and cells allowing virus propagation within an intracytoplasmic virus assembly site (VAS) and having the fragmented or absent nucleus. The ultrastuctural feattures of RSIV infected GF cells was resembled the result of the ultrastructural features of IBCs derived from GSDIV infected cells (Mahardika *et al.*, 2004).

Double infection of GSDIV in which by the time of passage and 24 hours after passage produced more CPE than single infection to 40%-50% confluent monolayers. In this case we believe that newly developing cells are sensitive to infection of the virus. We also found that the CPE were increase at the onset of infection. The number of CPE were stable or decrease along the time. In this study we did not find many CPE at 6 dpi or up. Study of lympocystis virus (LV) infection in grass carp cell line showed that CPE were produced at 1 dpi and extended up to 75% at 3 dpi but no extension after 4 dpi. They concluded that LV infection initiates rapid cell death and thus prevents further infection and virus propagation (Zang *et al.*, 2003).

Propagating GSDIV in GF cell line was not removed the pathogenecity to the host fish. GSDIV derived from GF-infected cells caused mortality to humpback grouper about 10%-100% depend on viral concentration. The abundance of CPE seems to be involved in the rate of mortality. This result indicated that GSDIV propagated in GF cells was viable and conserved their pathogenic character. As described above, ultrastructural similarity of *Megalocityvirus* infection *in vivo* and *in vitro*, might be encourage this founding.

In summary, this study has provided report of GSDIV propagation on GF cell line. The development of new sub-cultured GF cells contributed to GF sensitivity to GSDIV. Propagated GSDIV in GF cells line conserve their capability to infect humpback grouper. GF cells sensitivity is potential to increased, thus elaborate study still needed. This data will be useful in understanding and developing viral propagation in cell line.

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REFERENCES

Anonymous. 1961. Fish cell lines. Proc Soc Exp Biol Med; 108: 762; *Ann Ny Acad Sci.*, 1965;126;343

Ariel, E., Skall, H.F., & Olesen, N.J. 2009. Susceptibility testing of fish cell lines for virus isolation. *Aquaculture*, 298: 125-130.

Chang, S.F., Ngoh, G.H., Kueh, L.F.S., Qin, Q.W., Chen, C.L., Lam, T.J., & Sin, Y.M. 2001. Development of a tropical marine fish cell line from Asian seabass (*Lates calcarifer*) for virus isolation. *Aquaculture*, 192: 133-145.

Chew-Lim, M., Ngoh, G.H., Ng, M.K., Lee, J.M., Chew, P., Li, J., Chan, Y.C., & Howe, J.L.C. 1994. Grouper cell line for propagating grouper viruses. *Sing. J. Pri. Ind.*, 22: 113-116.

Chi, S.C., Hu, W.W., & Lo, B.J. 1999. Establishment and characterization of a continuous cell line (GF-1) derived from grouper, *Epinephelus coioides* (Hamilton): a cell line susceptible to grouper nervous necrosis virus (GNNV). *Journal of Fish Disease*, 22: 173-182.

Chinchar, G., Essbauer, S., He, J.G., Hyatt, A., Miyazaki, T., Seligy, V., & Williams, T. 2005. Family *Iridoviridae*. *In.* Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., & Ball, L.A. (Eds.) Virus Taxonomy. Classification and Nomenclature of Viruses. *Eighth Report of the International Committee on the Taxonomy of Viruses.* Academic Press, San Diego, p. 145-162.

Chou, H.Y., Hsu, C.C.W., & Peng, T.Y. 1998. Isolation and characterization of a pathogenic iridovirus from cultured grouper (*Epinephelus* sp.) in Taiwan. *Fish Pathology*, 33: 201-206.

Fryer, J.L. & Lannan, C.N. 1994. Three decades of fish cell culture: a current listing of cell lines derived from fish. *J. Tissue Cult. Methods*,16: 87-94.

Go, J. & Whittington, R. 2006. Experimental transmission and virulence of a megalocytivirus (Family *Iridoviridae*) of dwarf gourami (*Colisa lalia*) from Asia in Murray cod (*Maccullochella peelii peelii*) in Australia. *Aquaculture*, 258: 140–149.

Kurita, J., Nakajima, K., Hirono, I., & Aoki, T. 1998. Polymerase chain reaction (PCR) amplification of DNA of red sea bream iridovirus (RSIV). *Fish Pathology*, 33: 17-23.

Lai, Y.-S., John, J.A.C., Lin, C-H., Guo, I.-C., Chen, S-C., Fanf, K., Lin, C-H., & Chang, C-Y. 2003. Estabilishment of cell lines from a tropical grouper, *Epinephelus awoara* (Temminck & Schlegel), and their susceptibility to grouper irido-and nodaviruses. *J. Fish. Dis.*, 26: 31-42.

Lai, Y-S., Chiou, P.P., Chen, W.-J., Chen, Y.-C., Chen, C.-W., Chiu, I.-S., Chen, S.-D., Cheng, Y.-H., & Chang, C.-Y. 2008. Characterization of apoptosis induced by grouper iridovirus in two newly established cell lines from barramundi, *Lates calcarifer* (Bloch). *J. Fish Dis.*, 31: 825-824.

Mahardika, K., Koesharyani, I., Sugama, K., Priyono, A., & Yuasa, K. 2001. Studi histopatologi iridovirus pada *Epinephelus coioides* dan *Epinephelus bleekeri*. *Kumpulan Makalah Teknologi Budidaya Laut dan Pengembangan Sea Farming di Indonesia*. ISBN 979-8186-82-6. Departemen Kelautan dan Perikanan bekerjasama dengan JICA. (Indonesian with English abstract). p. 334-341.

Mahardika, K., Zafran, Yamamoto, A., & Miyazaki, T. 2004. Susceptibility of juvenile humpback grouper (*Cromileptes altivelis*) to grouper sleepy disease iridovirus (GSDIV). *Dis. Aquat. Org.*, 59: 1-9.

Mahardika, K., Haryanti, Muzaki, A., & Miyazaki, T. 2008. Histopathological and ultrastructural features of enlarged cells of humpback grouper *Cromileptes altivelis* challenged with *Megalocytivirus* (Family *Iridoviridae*) after vaccination. *Dis. Aquat. Org.*, 79: 163-168.

Mahardika, K. & Miyazaki, T. 2009. Electron microscopic features of cultured grunt fin cells infected with *Megalocytivirus*. *Aquaculture Sci.*, 57(1): 9-18.

Nakajima, K. & Sorimachi, M. 1994. Biological and physico-chemical properties of the iridovirus isolated from cultured red sea bream, *Pagrus major*. *Fish Pathol.*, 29: 29-33.

Oh, M.J., Kitamura, S.-I., Kim, W.S., Park, M.K., Jung, S.J., Miyadai, T., & Ohtani, M. 2006. Susceptibility of marine fish species to a *Megalocytivirus*, turbot iridovirus, isolated from turbot, *Psetta maximus* (L.). *J. Fish Dis.*, 29: 415-421.

Qin, Q.W., Wu, T.H., Jia, T.L., Hegde, A., & Zhang, R.Q. 2006. Development and characterization of a new tropical marine fish cell line from grouper, *Epinephelus coioides* susceptible to iridovirus and nodavirus. *Journal of Virological methods*, 131: 58-64.

Sudthongkong C., Miyata, M., & Miyazaki, T. 2002. Viral DNA sequences of genes encoding the ATPase and the major capsid protein of tropical iridovirus isolates which are pathogenic to fishes in Japan, South China Sea, and Southeast Asian countries. *Arch. Virol.*, 147: 2,089–2,109.

Tsai, C.T., Ting, J.W., Wu, M.H., Wu, M.F., Guo, I.C., & Chang, C.Y. 2005. Complete genome sequence of the grouper iridovirus and comparison of genomic organization with those of other iridoviruses. *Journal of Virology*, 79: 2,010–2,023.

Zhang, Q.Y., Ruan, H.M., Li, Z.Q., Yuan, X.P., & Gui, J.F. 2003. Infection and propagation of lymphocystis virus isolated from the cultured flounder *Paralichthy solivaceus* in grass carp cell lines. *Diseases of Aquatic Organisms*, 57: 27-34