NEW MEGALOCYTIVIRUS INFECTED TO THE CULTURED FRESH WATER GIANT GOURAMI, *Osphronemus gouramy* Lac. IN INDONESIA

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ABSTRACT

Giant gourami (*Osphronemus gouramy* Lac.) is one of freshwater fish having high economic value and very popular in Indonesian food restaurant. This fish have been cultured throughout Indonesia. Since two years ago, cultured fish in ponds infected by unknown pathogenic agent and may cause diseased fish and mass mortality. Mass mortality of pond cultured giant gourami occurred in West Java, Central Java, and Bali-Indonesia. The symptoms of diseased fish are the body color becomes darker, resting on the water surface, and internal organs of fish characterized by enlargement of spleen. In the present study diseased fish samples were diagnose by Polymerase Chain Reaction (PCR) techniques using specific primers of red sea bream iridovirus (RSIV) and histopathologically analysis to determine the causative agent of mortality. Based on PCR analysis, show that all fish sampled were positive infected by megalocytivirus and infected fish mostly died. This is a new and first time reported virus infection in cultured giant gourami in Indonesia and it is proposed to be named as giant gourami iridovirus (GGIV).

KEYWORDS: megalocityvirus, giant gourami, PCR detection, histopathology

INTRODUCTION

Giant gourami, *Osphronemus gouramy* Lac. Is one most important fish among fresh water fish in Indonesia and others Asian countries? The demand of marketable size fish is increase year by year, and this increasing demand triggered the farmer to produce more fish by adopting semi intensive culture. Previously there was no report on serious diseases problem in giant gourami culture for decade. The common bacterial disease in giant gourami are *Streptococcus iniae, Streptococcus agalactiae, Nocardia sp., Aromonas hydrophila, Aeromonas carviae, Staphylococcus saprophyticus, Flavobacterium sp.*, and *Mycobacterium fortuitum* (Lusiastuti *et al.*, 2008; Minaka *et al.*, 2012; Kitao *et al.*, 2011; Purwaningsih & Taukhid, 2010). Those bacteria have been succeeded to control. Since 2011 mass mortality has been occurred in pond culture giant gourami at Cianjur, Parung, Depok-West Java, Purwokerto-Central Java, and at Karangasem-Bali. Clinical signs of diseased fish are darker in body surfaces, resting on the water surfaces, and enlargement of spleen. Symptoms of diseases fish are mass mortality. Similar clinical signs and symptoms have been reported by several scientists for marine fish such as histopathology study by Mahardika *et al.* (2004); Sudthongkong *et al.* (2002b); Go & Whittington (2006); Go *et al.* (2006); OIE (2009). All of them reported that diseases cause by megalocytivirus. Megalocytivirus infection is a serious constrain on grouper, sea bass culture, and imported ornamental fish dwarf gourami (*Colisa lalia*) in Indonesia

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(Koesharyani *et al.*, 2001; Mahardika *et al.*, 2003; Mahardika *et al.*, 2004; Koesharyani *et al.*, 2009). This virus also attack to several fin fishes culture in other countries, like to brown spotted grouper *Epinephelus tauvina* Forskall as a sleepy grouper disease (SGD) in Singapore (Chua *et al.*, 1994); *E. malabaricus* in Thailand (Danayadol *et al.*, 1997); Pagrus major (RSIV) in Japan (Kurita *et al.*, 1998); *Epinephelus* sp. (TGIV) in Taiwan (Chou *et al.*, 1998; Chao *et al.*, 2002).

At present, megalocytivirus not only infected to marine fish but also to freshwater fish, such as ornamental fish *Colisa lalia*, dwarf gouramy known by the name Dwarf Gouramy Iridovirus (DGIV) and African lampeye *Aplocheilichthys normani* as a African Lampeye Iridovirus or ALIV (Sudthongkong *et al.*, 2002). The newest finding marine ornamental fish *Bangai cardinal* was infected by iridovirus (unpublished data).

Present studies try to determine pathogenic agent or causative agent of diseased of giant gourami (*Osphronemus gouramy* Lac.) that caused mass mortality by applying PCR-DNA molecular technique and histopathological examination.

MATERIALS AND METHODS

Fish samples: giant gourami sample were collected from different location fish ponds: i.e., Bogor, Depok, Cianjur, Purwokerto, Pemalang, and Bali Indonesia on periods of 2011 until 2013. Internal organ and tissues of fresh sample from ponds cultured fish were transported by keeping on ice to the laboratory and buffered formalin and 70% ethanol for identification purposes.

PCR Analysis

Total DNA extraction was done by Kit DNAzol, 25-30 mg spleen excised from naturally infected fish in 1.5 mL clean micro tube, homogenize in 1 mL Diazole. Homogenate tissue was centrifuged at 12 000 *x g* for 10 min. at room temperature; transfer the resulting supernatant to fresh tube, this step removes insoluble tissue fragment, RNA and excess polysaccharides from the homogenate. DNA was precipitated from the homogenate with 0.5 mL of 100% ethanol per 1 mL Diazole reagent used for the isolation. Samples were mixed by inversion and store at room temperature (27°C to 30°C) for 1 to 3 min. DNA should quickly become visible as a cloudy precipitate. DNA precipitate was centrifuged at 8 000 x *g* for 3 min. at room temperature; carefully decant the supernatant leaving the DNA pellet. Wash the DNA precipitate twice with 0.5 mL of 95% ethanol. Each of wash suspend the DNA in ethanol was done by inverting the tubes 2-6 times. Store the tubes vertically for 0.5 to 1 min. to allow the DNA to settle to the bottom of the tubes and remove ethanol by pipetting or decanting. Air dries the DNA by storing in an open tube for 5 to15 second after removing the ethanol (If the DNA is exposed to air for more than a few seconds, it will be much more difficult to dissolve). DNA were dissolved in 100 to 200 µL Tris EDTA (TE) or Water, Dnase, RNase free (Deionized water treated with 0.001 DEPC filtered through 0.2 micron filter and sterilized by autoclave), and as template DNA for PCR amplification.

PCR amplification was done in Go Tag Green Master Mix (2x), contained: Go Tag DNA Polymerase Reaction Buffer (pH. 8.5), dNTPs (400 µM dATP, 400 µM dGTP, 400 µM dCTP; 400 µM dTTP), $MgCl_2$ (3 mM) and stabilizer and additionally contains two inert dyes (yellow and blue; each 1 µL Reverse and Forward primers 10 pMol and Nuclease-Free Water. Primers were designed from the nucleotide sequence of the 959-bp *Pst* I restriction fragment-DDBJ accession number AB006954 of RSIV genome DNA: 1F: 5'- CTCAAACACTCTggCTCATC-'3; 1R: '5 - gCACCAACACATCTCCTATC- '3. The cycle condition program was for 30 cycles with condition of denaturation for 30 sec. at 94°C, annealing for 1 min. at 58°C and elongation for 1 min. at 72°C, followed by an extension period for 5 min. at 72°C after the last cycle. The expected PCR product size was 570 bp. (Kurita *et al.*, 1998). Reaction PCR mixes was amplified in an automatic thermal cycler (Biometra Tpersonal Serial No. 19104331). The PCR mix prepared in 25 µL with final concentration 1x for Go Tag Green Master Mix, 0.4 µM of each primer, DNA template < 100 ng and nuclease-free water adjust to 25 µL volume.

Analysis of PCR amplified product, PCR products were analyzed or separated by electrophoresis in 1.5% agarose gel-1xTAE buffer (40 mM Tris, 20 mM acetic acid, 1mM EDTA) and visualized by UV-transilluminator and staining with ethidium bromide at concentration 0.05%. (1µg mL^{-1}). PCR product was compared against molecular size marker

100 bp (Fermentas). For positive control of PCR, genomic DNA was isolated from spleen of megalocytivirus grouper infected and for negative control were used nuclease free water.

Histopathology Analysis

The fixed tissue (spleen, liver and gill) in 10% formalin were then prepared according to standard technique for histological examination. Tissue section were stained with Hematoxylin and eosin (H & E).

RESULT AND DISCUSSION

In field observation shown that the diseased fish were mass mortality, lethargy swimming, and resting on the surface of water, loss appetite, darker/black or pale of body color, sunken eyes, and internal organ such as spleen become enlarge. (Figure 1). This kind of sign and symptoms usually can be use as indicator of virus infection.

The results of amplification DNA product have shown that all samples are positive infected by virus (Figure 2 and Table 1).

This is the first study to apply PCR technique in identification of giant gourami diseased. Based on the gross sign and PCR product there is a high probability that the fish were infected by megalocytivirus. In the PCR test for diseased fish, specific primer of red sea bream (RSIV) was used, and shows DNA product of 570 bp which was exactly same with iridovirus infection in red sea bream (Kurita *et al.*, 1998). This support that giant gourami in Indonesia was infected by megalocytivirus.

Based on the symptoms of the diseased fish especially of enlargement the spleen and the results of PCR detection test, the virus infected in giant gourami was most probably belong to Family *Iridoviridae*. *Iridoviridae* consists of large double stranded DNA viruses approximately 120-350 nm in size which posse's icosahedral symmetry, a 140-303 kb genome and replicate in both the nuclease and cytoplasm. Five genera are recognized by the international committee on taxonomy of viruses: *Iridovirus, Ranavirus, Chloridovirus, Ranavirus, Lymphocystivirus*, and *Megalocytivirus* (Chincar *et al.*, 2005 *in* Go *et al.*, 2006). Data blast sequences using fragment gen *Pst* I, gourami iridovirus from Indonesia was belong to genus Megalocytivirus and showed relatively high homology up to100% (98%) to Red Sea bream Iridovirus (RSIV); infectious spleen and kidney necrosis virus (ISKNV); Grouper disease Iridovirus (GSDIV); and other fish. Based on the similarity of irido-like from Indonesia gourami, it was proposed to be named as Giant Gourami Iridovirus (GGIV). Viruses belong to megalocy-

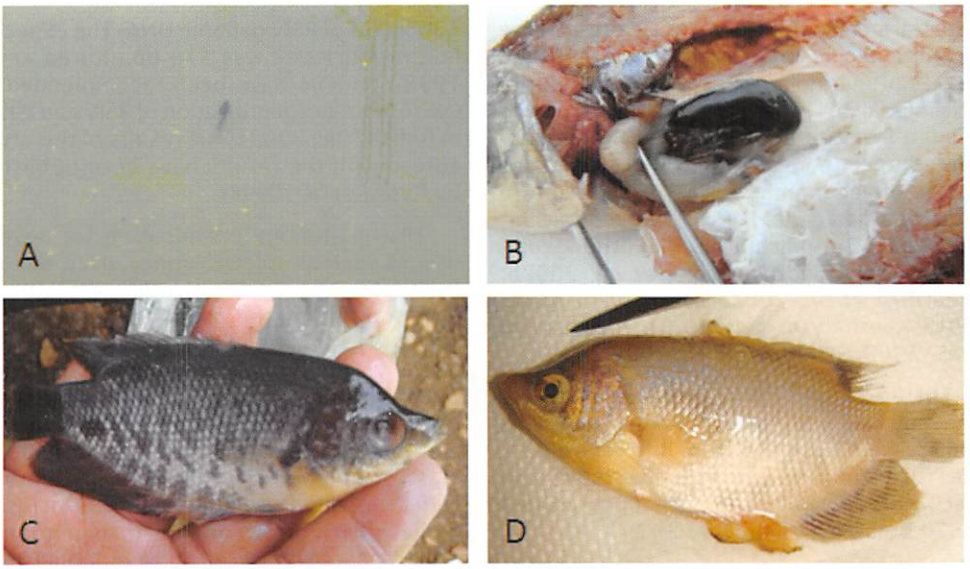

Figure 1. Gross signs giant groupers infected, A: resting on the surfaces, B: enlargement of spleen organ, C and D: sunken eyes and dark or pale body coloration

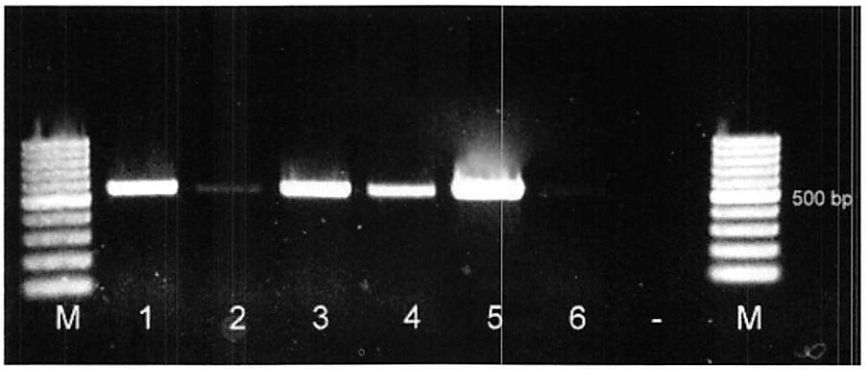

Figure 2. Amplification of PCR product (570 bp) of giant gouramy iridovirus from different location gourami grow out culture, 1. Cianjur; 2. Purwokerto; 3. Pemalang; 4. Ciseeng-Bogor; 5. Karangasem-Bali; 6. Depok; N = Negative control; M = Marker 100 bp

Table 1. Megalocytivirus giant gourami iridovirus detection from different farm giant gourami culture using PCR technique

Time		Size (cm)	Location	PCR Tested
April	2011	± 10	Nagrak, Cianjur	Positive
August	2011	± 10	Beji, Purwokerto	Positive
November	2011	± 10	Kebondalam, Pemalang	Positive
May	2012	± 10	Cise'eng, Bogor	Positive
October	2012	± 15	Karangasem, Bali	Positive
July	2013	± 8	Depok	Positive

tivirus are causative agent of severe disease accompanied by high mortality in multiple species of marine and freshwater fish (Kurita & Nakajima, 2012). Megalocytivirus have been isolated from different fishes and were considered as pathogenic agent that may cause significant economic losses in aquaculture industry (Chou *et al.*, 1998; Chua *et al.*, 1994; Nakajima & Sorimachi, 1995; Sudthongkong *et al.*, 2002a; Sudthongkong *et al.*, 2002b; Mahardika *et al.*, 2004. Koesharyani *et al.*, 2001a; Koesharyani *et al.*, 2001b; Koesharyani *et al.*, 2009; Shinmoto *et al.*, 2009).

There are many primers have been used for detection megalocytivirus in fin fish and those primers were designed from different genes. The PCR examination assay in this study using specific product of PCR amplification, resulted in 570 bp amplicon. (Figure 2).

The primers were used here designed from the nucleotide sequence of the 959-bp *Pst* I restriction fragment-DDBJ accession number AB006954 of RSIV genome DNA. The expected PCR product size was 570 bp. (Kurita *et al.*, 1998). Primers have been recommended by OIE for routine identification of RSIV and ISKNV in finfish (OIE, 2009). The results of this study agreed with the results have been published by Kurita *et al.* (1998).

Histopathology examination for several tissues such as spleen, kidney, liver, and gill from infected megalocytivirus giant gourami have shown that, the virus was induced inclusion body or enlarged cells (black arrows) and it was visible by light microscopy. Photomicrograph spleen of fish infected by virus has shown many inclusion bodies (black arrow) and necrosis in white pulp or and lymphoblast of splenic tissue (Figure 3A). Kidney of fish infected virus, also shown inclusion bodies (black arrow) and necrosis in tubules inter-renal of kidney cell and lymphoblast cell (Figure 3B). Other organ, liver of fish infected virus, shows inclusion bodies (black arrow),

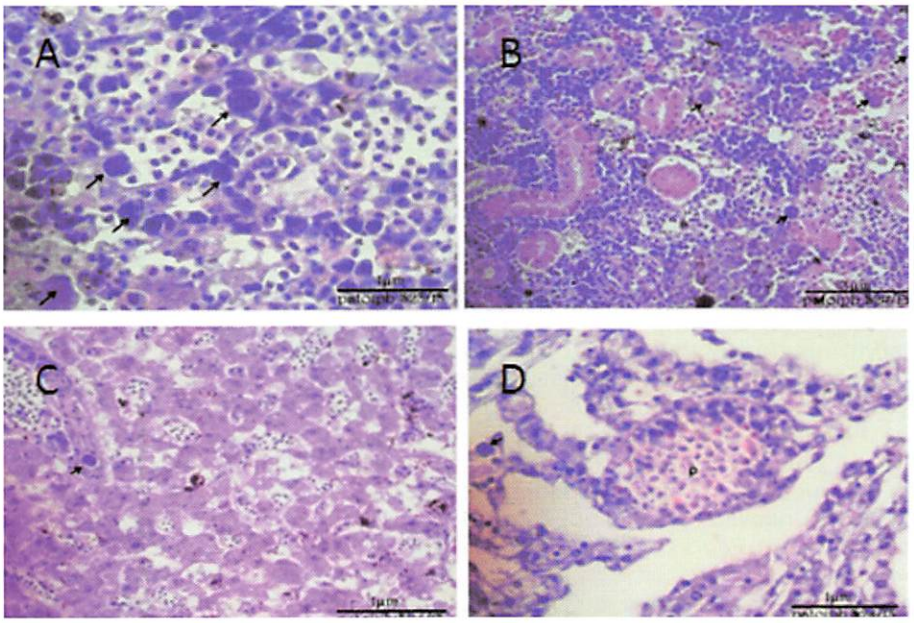

Figure 3 Photomicrograph of, spleen (A), kidney (B), liver (C), and gill (D) of gourami naturally infected with megalocytivirus, there are many inclusion bodies (black arrow) and necrosis in tissue

and congestion of Kupffer cell in liver sinusoid (Figure 3C). Photomicrography gills of fish by artificial infection using tissue supernatant virus, resulted in hyperplasia, degeneration, necrosis on the ephitel and inclusion of virus (black arrow), and there are parasite protozoa cyst (p) (Figure 3D). In megalocytivirus infection, diseased fish show numerous accumulation of virus particles in spleen and kidney (Chou *et al.*, 1998; Sudthongkong *et al.*, 2002a; Mahardika *et al.*, 2004). Spleen, Kidney, heart, and liver were the best organ for PCR examination (OIE, 2009; Kurita *et al.*, 1998).

Present study concludes that, based on the gross clinical sign symptoms, PCR product, and histological examination, it is concluded that the Indonesian diseased giant gourami is infected by Iridovirus. This result is the first report dealing with Indonesian giant gourami virus, so that it is proposed or submitted this virus named by Giant Gouramy Irido Virus (GGIV). For prevention, further research will be conducted on vaccines for giant gourami freshwater.

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