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

Isti Koesharyani and Lila Gardenia

Centre for Aquaculture Research and Development

(Received January 16th, 2013; Accepted April 14th, 2013)

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: megalocytivirus, 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., Aeromonas hydrophila, Aeromonas carviae, Staphylococcus saprophyticus, Flavobacterium sp., and Mycobacterium fortuitum (Lusiautut 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.
(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 tawina Forskall as a sleepy grouper disease (SGD) in Singapore (Chua et al., 1994); E. malabaricus in Thailand (Danayadol et al., 1997); Pagus 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 lamp-eye Aplocheilichthys normani as a African Lamp-eye 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 to 15 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 dTTP; 400 μM dCTP), MgCl₂ (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'CTCAACACTCTTggCTCATC-'3; 1R: '5' - gCACACACACATCTCCTATC-'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 mix was amplified in an automatic thermal cycler (Biorad 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–1%TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM 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 posses 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 PstI, gourami iridovirus from Indonesia was belong to genus Megalocytivirus and showed relatively high homology up to 100% (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](image1.png)

**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>
<thead>
<tr>
<th>Time</th>
<th>Size (cm)</th>
<th>Location</th>
<th>PCR Tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>April</td>
<td>2011</td>
<td>± 10</td>
<td>Nagra, Cianjur</td>
</tr>
<tr>
<td>August</td>
<td>2011</td>
<td>± 10</td>
<td>Beji, Purwokerto</td>
</tr>
<tr>
<td>November</td>
<td>2011</td>
<td>± 10</td>
<td>Kebondalam, Pemalang</td>
</tr>
<tr>
<td>May</td>
<td>2012</td>
<td>± 10</td>
<td>Cise'eng, Bogor</td>
</tr>
<tr>
<td>October</td>
<td>2012</td>
<td>± 15</td>
<td>Karangasem, Bali</td>
</tr>
<tr>
<td>July</td>
<td>2013</td>
<td>± 8</td>
<td>Depok</td>
</tr>
</tbody>
</table>

Iridovirus 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; Shinamoto 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),
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 epithel 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 Iridine Virus (GGIV). For prevention, further research will be conducted on vaccines for giant gourami freshwater.

ACKNOWLEDGMENTS

This work was supported by Indonesian government for research budget (APBN) and gratefully also to Ibu Ratih at Department clinics, Reproduction and Pathology, Faculty of Veterinary Medicine, Institute Pertanian Bogor (IPB) for supported histopathology examination and to our colleagues in Center for Aquaculture Research and Development Laboratory in Pasar Minggu and Depok for supporting this work.

REFERENCES


Danayadol, Y., Direkbusarakom, S., Boonyaratpalin, S., Miyazaki, T., & Miyata, M. 1997. Iridovirus infection in Brown-spotted grous-
Go, J., Lanaster, M., Deece, K., Dhunyel, O., & Whittington, R. 2006a. The molecular epidemiology of iridovirus in Murray cod (Maccullochella peeli peeli) and dwarf gourami (Colisa laiba) from distant biogeographical region suggests a link between trade in ornamental fish and emerging iridoviral diseases. *Molecular and Cellular Probes*, 20: 212-222.
Go, J. & Whittington, R. 2006b. Experimental transmission and virulence of a megalocytivirus (Family Iridoviridae) of dwarf gourami (Colisa laiba) from Asia in Murray cod (Maccullochella peeli peeli) in Australia. *Aquaculture*, 258: 140-149.
Purwaningsih, U. & Taikhd. 2010. Diagnosis penyakit mycobacteriosis, *Mycobacterium fortuitum* pada ikan gurami (Osphrone-
mus gouramy) dengan teknik Polymerase Chain Reaction (PCR). Prosiding Forum Inovasi Teknologi Akuakultur, p. 969-975.


INDONESIAN AQUACULTURE JOURNAL
Instruction for Authors

Scope
The Indonesian Aquaculture Journal publishes research results on aquaculture, coastal, and freshwater fisheries, and the environment with special reference to South East Asian countries.

Manuscript Submission
Manuscripts submitted to the Journal must be original with clear definition of the objective, materials used, method applied and results and should not have been published or offered for publication elsewhere. The manuscript should be written in English, in double line spacing on single-sided A4-size white paper. Two copies of the manuscript and a disk containing the manuscript file(s) produced using MS-Word must be submitted to the Managing Editor, Jl. Ragunan 20, Pasar Minggu, Jakarta Selatan 12540, Indonesia, e-mail: publikasi.p4b@gmail.com. There are no page charges for manuscripts accepted for publication, unless otherwise indicated below.

Manuscripts Preparation
◆ A title page should be provided which shows the title and the name(s) of author(s). The author(s) position and affiliation should be written as footnotes at the bottom of the title page
◆ Title should not be more than 15 words
◆ Abstract summarising the study to be no more 250 words. Keywords (3-5) must be provided which clearly indicate the subject of the work. Do not use any sub-headings.
◆ Materials and methods should clearly and concisely describe the experiment with sufficient details for independent repetition.
◆ Results are presented with optimum clarity and without unnecessary detail. Results should be presented in tables or figures but not duplicated in both formats. Tables (same font size as text, lower case superscript letters to indicate footnotes), and illustration/figures should be printed on separate sheets and given consecutive Arabic numbers.
◆ Conclusion should be short and should relate the objectives, with discussion of the results
◆ Acknowledgements, if necessary, should be kept to a minimum (less than 40 words).
◆ References should be cited in the text by the author(s)' family or last name and date in one or two forms: Sugama (2000) or (Sugama, 2000). For references with more than two authors, cite the first author plus et al. full citation in alphabetical order in required.
◆ Acronyms or uncommon abbreviations must given in full at the first mention; new abbreviations should be coined only for unwieldy names and should not be used at all unless the names occur frequently.
◆ Latin name and family of the species should be given besides its common name at the first mention in the manuscript, and the common name only for subsequent mentions.
◆ SI system should be used for all measurements.