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Virus Detection in Diseased and Healthy Fish and Oysters from an Indonesian Aquaculture Site: Double Infection of Viral Nervous Necrosis Virus (VNNV) and Marine Birnavirus (MABV)

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Abstract

In the south Sumatra region of Indonesia, tiger grouper, *Epinephelus fuscoguttatus*, and humpbuck grouper, *Cromileptes altivelis*, are commercially important fish species. Recently, however, mass mortalities of the two species have occurred in the region and the cause of these mortalities has not been clarified. We surveyed the distribution of fish viruses including viral nervous necrosis virus (VNNV), marine birnavirus (MABV) and red seabream iridovirus (RSIV) in the two grouper species and MABV in silver the lip oyster, *Pinctada maxima*, by PCR. VNNV was detected from both healthy and diseased groupers whereas MABV was only detected in diseased groupers, suggesting that the mass mortalities of the two grouper species were caused by co-infection of VNNV and MABV. PCR product of RSIV was not observed in any fish samples. In addition, MABV was not detected in silver lip oysters from both healthy and those exhibiting poor growth.

Key words: Tiger grouper, humpbuck grouper, lip oyster, VNNV, MABV, Indonesia

Introduction

Marine aquaculture industries including fish and shellfish species have been developed in Indonesia. In the south Sumatra region of Indonesia, tiger grouper, *Epinephelus fuscoguttatus,* and humpbuck grouper, *Cromileptes altivelis*, are commercially important fish species, however disease outbreaks due to unknown causes have been encountered in these fish species, resulting in a serious economic loss to the aquaculture industry. In shellfish species, the silver lip oyster, *Pinctada maxima*, is one of the most important cultured shellfish species to produce pearls in the region, but ventures in shellfish aquaculture have also faced problems such as poor growth of the oyster due to unknown reasons.

From the viewpoint of infectious disease, viral diseases have often caused mass mortality of cultured fish and shellfish species. For example, viral nervous necrosis virus (VNNV) belonging to genus *Betanodavirus* in family *Nodaviridae* (Muroga, 2001), red seabream iridovirus (RSIV) belonging to the genus *Megalocytivirus* in family *Iridoviridae* (Inouye et al. 1992) and marine birnavirus (MABV) belonging to genus *Aquabirnavirus* in family *Birnaviridae* (Sorimachi & Hara, 1985) were detected from many diseased fish species in Asian countries. To control viral diseases, it is necessary to understand distributions of the fish viruses in cultured fish and shellfish species as a basic research, but it has not been fully investigated in Indonesia so far. Therefore, the distribution of above three viruses in the tiger grouper and humpback grouper, and silver lip oyster cultured in south Sumatra region of Indonesia was surveyed including examine the cause of mortalities in two grouper fish species in this study.

Materials and Methods

Fish and shellfish

Healthy and diseased tiger grouper and humpbuck grouper were sampled from an aquaculture facility in Hurun Cove in south Sumatra of Indonesia in July 2003 (Fig. 1, Station 5). Diseased fish showed sluggish behavior near the water surface and resting on the bottom, and exhibited a mortality rate of >30%. Healthy and poor-growing silver lip oysters were collected from an oyster farm managed by a local private company (Fig. 1, Station 27).

Detection of fish viruses by PCR

Spleen and brain of fish were pooled and used for extraction of nucleic acids. In the case of shellfish, hepatopancreas and hemocyte of the oyster were separately collected to detect MABV because the virus could potentially infect both tissues in the Japanese pearl oyster *Pinctada fucata* (Kitamura et al. 2000). Total nucleic acids were extracted from the collected samples

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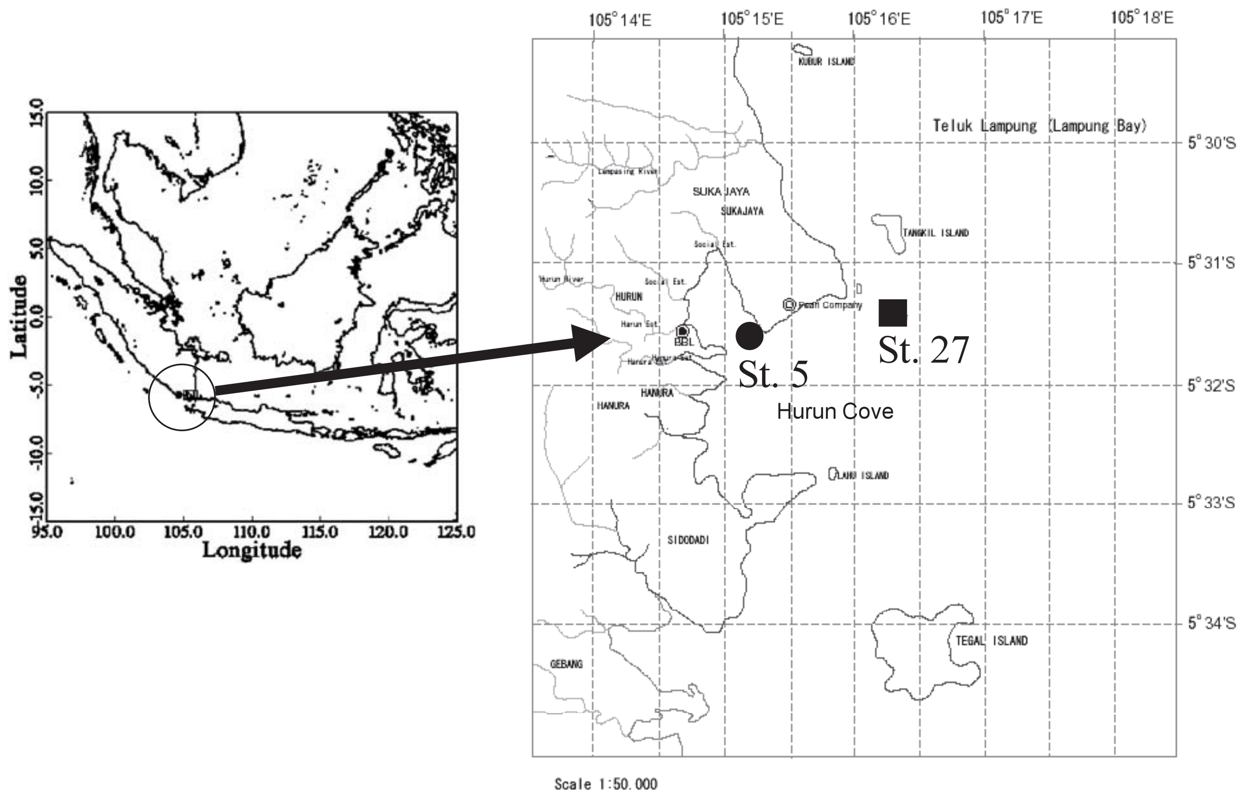

Fig.1. Map of sampling stations. ●; sampling station of tiger grouper and humpbuck grouper, ■; sampling station of silver lip oyster.

using the method described by Suzuki et al. (1997). Briefly, the organ was homogenized in 4-fold volume of TE (0.2 M Tris HCl, pH 8.0, 0.1 M EDTA), and was centrifuged at 3,000 x g for 5 min. The 45 μl of supernatant was taken and 5 μl of proteinase K (1mg/ml; TaKaRa Co., Shiga, Japan) was added. The mixture was incubated at 55 ℃ for 2 h. Nucleic acids were extracted using phenol-chloroform. A portion of the nucleic acids were subjected to reverse transcription (RT) for VNNV and MABV detection, described by Nishizawa et al. (1994) and Suzuki et al. (1997), respectively; PCR was then performed. In order to detect RSIV, the remaining nucleic acids were directly used for PCR by the method of Kurita et al. (1998). PCR amplification was performed with a DNA thermal cycler (TaKaRa Co., Shiga, Japan). Amplified products were analyzed by agarose gel electrophoresis, and were visualized with ethidium bromide and UV light irradiation.

Results and Discussion

In fish samples collected from the south Sumatra region of Indonesia, VNNV was detected from both healthy and diseased tiger and humpbuck grouper. The detection rate of VNNV was 100% from diseased individuals of both species, whereas it was <66.6% in what were designated as healthy fish (Table 1). Since Zafran et al. (2000) detected the same virus from diseased juvenile humpbuck

Table 1. PCR detection of three fish viruses from tiger grouper, humpbuck grouper and oyster

Species	Condition	Fish no.	mean B.W. (g)	PCR positive rate (%)		
				VNNV	MABV	RSIV
Tiger grouper	Healthy	3	41.4	33.3	0	0
Tiger grouper	Diseased	3	52.2	100	100	0
Humpbuck grouper	Healthy	3	28.9	66.6	0	0
Humpbuck grouper	Diseased	3	36.7	100	66.6	0
Silver lip (Hepatopancreas)	Healthy	10	49.9	NT*	0	NT
Silver lip (Hemocyte)	Healthy	10	−	NT	0	NT
Silver lip (Hepatopancreas)	Poor growing	10	26.9	NT	0	NT
Silver lip (Hemocyte)	Poor growing	10	−	NT	0	NT

*Not tested

grouper in the Bali region of Indonesia, the virus is thought to be widely distributed in the country. In the present study, symptoms of a diseased fish, sluggish behavior and resting on the bottom, was similar to that observed for fish affected with VNNV, suggesting that the main cause of mortality in the two grouper species is VNNV infection. RSIV was not detected from any of the grouper samples. Since the virus has caused mass mortality in many fish species, including grouper, many Southeast Asian countries require sustained surveillance of the virus (Miyata et al. 1997; Sudthongkong et al. 2002; Gibson-Kueh et al. 2004).

Interestingly, the high detection rate (>66%) of MABV was observed in only diseased groupers, indicating that co-infection by VNNV and MABV occurred in the fish. Regarding the co-infection by birnavirus and the other fish virus, from experiemental infection of Japanese flounder (*Paralichthys olivaceus*). Pakingking et al. (2003) reported that primary aquabirnavirus (ABV) infection suppressed the secondary viral hemorrhagic septicemia virus (VHS) infection. However, our observations suggest that MABV enhanced the pathogenicity of VNNV because MABV was detected from only diseased individuals of grouper, despite VNNV having been detected from both healthy and diseased fish. To clarify the relationship between mortality and co-infection of these viruses in grouper, the experimental co-infection in consideration of the contagious turn of VNNV and MABV is necessary.

Alternatively, in the single-infection of birnavirus in grouper (*Epinephelus* sp.) fry, the pathogenicity of the virus was low in experimental infections, but it increased when fish were exposed to a stressor such as heavy metals and changes in salinity (Chou et al. 1999). This evidence suggests that the virus is an opportunistic pathogen in grouper and its pathogenicity could be enhanced by environmental pollution and physiological factors. Recently, high population densities of cultured fish coupled with environmental pollution have created ideal conditions for infectious diseases, making it a serious problem in Asian countries. Therefore, monitoring of cultured fish species in Indonesia for opportunistic pathogens such as MABV is also highly warranted.

In shellfish samples of silver lipped oysters, MABV was not detected from any individuals (i.e. poor or regular growth), and therefore the cause of poor growth remains unclear. Further examination is needed to clarify the potential pathogenic and environmental causes of poor growth in this commercially important species.

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References

Chou HY, Peng TY, Chang SJ, Hsu YL, Wu JL (1999) Effect of heavy metal stressors and salinity shock on the susceptibility of grouper (*Epinephelus* sp.) to infectious pancreatic necrosis virus. Virus Res 63: 121－129

Gibson-Kueh S, Ngoh-Lim GH, Netto P, Kurita J, Nakajima K, Ng ML (2004) A systemic iridoviral disease in mullet, *Mugil cephalus* L., and tiger grouper, *Epinephelus* fuscoguttatus Forsskal: a first report and study. J Fish Dis 27: 693－699

Inouye K, Yamano K, Maeno Y, Nakajima K, Matsuoka M, Wada Y, Sorimachi M (1992) Iridovirus infection of cultured red sea bream, *Pagrus major*. Fish Pathol 27: 19－27.

Kitamura SI, Jung SJ, Suzuki S (2000) Seasonal change of infective state of marine birnavirus in Japanese pearl oyster *Pinctada fucata*. Arch Virol 145: 2003－2014

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

Miyata M, Matusno K, Jung SJ, Danayadol Y, Miyazaki T (1997) Genetic similarity of iridoviruses from Japan and Thailand. J Fish Dis 20: 127－134

Muroga K (2001) Viral and bacterial diseases of marine fish and shellfish in Japanese hatcheries. Aquaculture 202: 23－44

Nishizawa T, Mori K, Nakai T, Furusawa I, Muroga K (1994) Polymerase chain reaction (PCR) amplification of RNA of striped jack nervous necrosis virus (SJNNV). Dis Aquat Org 18: 103－107

Pakingking Jr R, Takano R, Nishizawa T, Mori K, lida Y, Arimoto M, Muroga K (2003) Experimental coinfection with aquabirnavirus and viral hemorrhageic septicemia virus (VHSV), *Edwardsiella tarda or Streptococcus iniae* in Japanese Flounder Paralichthys olivaceus. Fish Pathol 38: 15－21

Sorimachi M, Hara T (1985) Characteristics and pathogenicity of virus isolated from yellowtail fingerlings showing ascites. Fish Pathol 19: 231－238

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: 2089－2109

Suzuki S, Hosono N, Kusuda R (1997) Detection of aquatic birnavirus gene from marine fish using a combination of reverse transcription- and nested PCR. J Mar Biotechnol 5: 205－209

Zafran I, Koesharyani FJ, Yuasa K, Harada T, Hatai K (2000) Viral nervous necrosis in humpback grouper *Cromileptes altivelis* larvae and juveniles in Indonesia. Fish Pathol 35: 95－96