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Betanodavirus Infection in Marine Fish Aquaculture in Malaysia

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Abstract

Betanodavirus is known to cause mass mortality in many marine aquaculture fish species. In this study, we detected the virus in four different marine aquaculture fish species in Malaysia. These included humpback grouper (Cromileptisaltivelis), brown marbled grouper (Epinephelusfuscoguttatus), Asian seabass (Latescalcarifer) and golden pompano (Trachinoltusblochii). Out of 246 fish specimens analyzed using RT-PCR, 60.98% detected infected by the virus. Histological pathological analysis showed extensive cell vacuolationin the brain and retina tissues of severely infected specimens. However, some of the fish specimens detected positive using RT-PCR did not exhibit cell vacoulation which indicate the carrier state of those specimens. The RT-PCR amplification method developed in this study was shown useful as biosecurity tool in monitoringBetanodavirus infection in aquaculture. Although the origin of Betanodavirus in Malaysia is difficult to ascertained, evidence showed that some infections may have been contributed by the importation of fish fingerlings form neighboring countries. Currently, effective treatment of the viral disease is still impossible hence strict biosecurity measures need to be carried out in order to control the spread of the virus in fish stocks. These can include enforcement of biosecurity check and quarantine of every batch of imported fish, the use of virus-free broodstocks in hatchery, and proper disposal of infected fish stocks. In addition, good aquaculture practices must be carried in aquaculture farms or fish nursery all the time.

Keywords: Betanodavirus, RT-PCR, marine aquaculture, Malaysia.

Introduction

Marine aquaculture in Malaysiais currently expanding with the introduction of new fish species. Among the most cultured marine fish species include Asian seabass (*Latescalcarifer*), brown-marbled grouper (*Epinephelusfuscoguttatus*), humpback grouper (*Cromileptisaltivelis*), malabar grouper (*Epinephelusmalabaricus*), orange-spotted grouper (*Epinepheluscoioides*) and sixbar grouper (*Epinephelussexfasciatus*)[1]. Recently, golden pompano (*Trachinoltusblochii*) was introduced as a new aquaculture candidate in Malaysia. These marine fishes have high demand both in local and international live fish markets. Most of the aquaculture produces in Malaysia especially groupers are exported to China, Hong Kong and Singapore[1-2]. However, the major constraint for expansion of marine fish aquaculture in Malaysia is shortage of fish fingerling. Nevertheless, this has been satisfied through importation of fish fingerlings from neighboring countries such as Indonesia, Thailand, Philippines, Taiwan and Korea[1]. The common practice of fish importation for aquaculture was in the form of fingerling (2-3 inches in total length). However, in recent years, eggs, fish larvae (less than 1 inch) and even brood fish are also imported. Such practices contribute to the spread of fish pathogen especially viruses[3-4]. One of the most detrimental viruses in marine aquaculture is the *Betanodavirus*.

Betanodavirus infection can result in mass mortalities to fish especially at larval and juvenile stages. It is a RNA virus that causes viral nervous necrosis or viral encephalopathy and retinopathy to many marine fish species[5]. It was first discovered from Japanese parrotfish (*Oplegnathusfasciatus*) by Yoshikoshi and Inoue[6]. Since, it has spread and causes severe loss to aquaculture of various marine fish species throughout the world[7-9]. In Malaysia, the virus reported to cause disease to Asian seabass[10], golden pompano[11] and brown-marbled grouper[4]. The aim of this study was to investigate whether or not other cultured marine fish species in Malaysia are also affected by the virus. To achieve the objective, we developed RT-PCR methods which enabled us to determine the presence of the virus at low copy numbers.

Material and Methods

Fish specimens: Two hundred and forty six (246) fish specimens represent the four widely cultured marine fish species in Malaysia collected over a period of approximately 3 years (February 2007 to December 2009) were examined in this study. In addition, we also examined fish specimens which were originated from Bali, Indonesia. Detail on the fish specimens is given in table-1.

Table-1
Fish specimens examined in this study

Sampling No.	Fish species	Place	Age (dph)	Collection Date	Number of positive specimen detected by RT-PCR	Number of positive specimens detected by histology	Lab Reference
1	*Latescalcarifer*	Kota Kinabalu, Malaysia	32	February 2007	12/12	Not done	SB0207
2	*Latescalcarifer*	Kota Kinabalu, Malaysia	37	June 2007	14/14	Not done	SB0607
3	*Cromileptisaltivelis*	Bali, Indonesia	45	July 2008	17/20	Not done	MG0708
4	*Epinephelusfuscogutattus*	Bali, Indonesia	42	July 2008	20/20	Not done	TG0708
5	*Latescalcarifer*	Kota Kinabalu, Malaysia	35	August 2008	12/12	Not done	SB0808
6	*Trachinotusblochii*	Langkawi Island, Malaysia	52	November 2008	6/6	Not done	GP1108
7	*Latescalcarifer*	Kota Kinabalu, Malaysia	20	November 2008	16/16	2/2	SB1108
8	*Epinephelusfuscogutattus*	Tuaran, Malaysia	30	December 2008	4/5	2/2	TG1208
9	*Epinephelusfuscogutattus*	Langkawi Island, Malaysia	50	January 2009	6/10	Not done	TG0109
10	*Cromileptisaltivelis*	Bali, Indonesia	34	April 2009	0/15	Not done	MG0409
11	*Epinephelusfuscogutattus*	Bali, Indonesia	30	April 2009	0/15	Not done	TG0409
12	*Latescalcarifer*	Kota Kinabalu, Malaysia	28	July 2009	9/13	1/2	SB0709
13	*Latescalcarifer*	Tuaran, Malaysia	28	August 2009	0/5	Not done	SB0809
14	*Epinephelusfuscogutattus*	Tuaran, Malaysia	22	August 2009	24/38	0/2	TG0809
15	*Latescalcarifer*	Sandakan, Malaysia	27	September 2009	10/10	2/2	SB0909
16	*Latescalcarifer*	Kota Kinabalu, Malaysia	23	October 2009	0/6	0/2	SB1009
17	*Latescalcarifer*	Tuaran, Malaysia	15	November 2009	0/14	0/2	SB1109
18	*Epinephelusfuscogutattus*	Tuaran, Malaysia	34	November 2009	0/3	0/2	TG1109
19	*Latescalcarifer*	Tuaran, Malaysia	26	December 2009	0/8	0/2	SB1209
20	*Epinephelusfuscogutattus*	Tuaran, Malaysia	40	December 2009	0/4	0/2	TG1209

Note: dph=day post-hatching.

Table-2
Primer combinations used to amplify the RdRp and Cp genes in golden pompano nervous necrosis virus (GPNNV)

Target gene	PCR	Primer combination	Expected PCR product	Reference
RdRp	PCR 1a	JRNV1F1 + JRNV1R1	1122bp	Ransangan and Manin[12]
RdRp	PCR1b	JRNV1F2 + JRNV1R2	1032bp	Ransangan and Manin[12]
RdRp	PCR1c	JRNVF3 + JRNV1R3	1099bp	Ransangan and Manin[12]
Cp	PCR 2a	JRNV2F1 + JRNV2R1	1363bp	Ransangan and Manin[12]
Cp	PCR 2b	*JRNV2F2 + JRNV2R2	1026bp	This study

*JRNV2F2: 5'ATGGTACGCAARGGTGAKAAG3'; JRNV2R2: 5'ATGACCCGGTTAGTTTYCCG3'

RNA extraction: RNA was extracted from brain and eye tissue or whole fish specimen (50mg – 150mg) by using TRIzol® reagent following manufacturer's instruction. First, tissue from individual fish specimen was grounded using sterile pestle and mortar in the presence of TRIzol® reagent. Second, the tissue homogenate was transferred into a 1.5 ml tube. As for viral culture, golden pompano nervous necrosis virus (GPNNV) infected SSN-1 cells were harvested at 13,000 rpm. Thereafter, tissues and infected SSN-1 cells were homogenized with 1.0ml TRIzol® reagent and let stand at room temperature for 5 min. RNA was precipitated using isopropanol and washed with 70% (v/v) ethanol. The air-dried RNA pellet was dissolved in 200µl diethyl pyrocarbonate (DEPC) treated water and kept at – 20°C. The concentration and the purity of RNA were determined using RNA/DNA calculator (Gene Quant *pro*, England).

Reverse transcription: Ten microlitre (10µl) of RNA (0.2µg/µl) mixed with 1.0µl (0.5µg/µl) of random primer (Promega), 1.0µl (40U/µl) of RNAsin (Promega) and 28µl of nuclease free water in a 1.5ml tube. Thereafter, the RNA solution was incubated at 70°C for 10 min. Meanwhile, the M-MLV reverse transcriptase solution was prepared by mixing 20µl of 5X M-MLV buffer (Promega) with 5.0µl (10mM) of dNTPs (Promega), 1.0µl (200U/µl) of M-MLV reverse transcriptase enzyme (Promega) and 34µl of nuclease free water in a 0.2ml PCR tube. The two solutions were mixed and incubated at 37°C for 90 min in a PCR machine (GeneAmp® PCR System 9700, Applied Biosystem). Subsequently, the reaction was stopped at 95°C for 5 min. Finally, complementary DNA (cDNA) was kept at – 20°C until further use.

PCR amplification: PCR primers for amplification of RNA dependent RNA polymerase (RdRp) and capsid protein genes in GPNNV were followed primers designed by Ransangan and Manin[12] except for one primer pair (JRNV2F2 and JRNV2R2). Detail information about the PCR primers is given in Table-2. The specificityof PCR primers was tested against DNA from five viruses which included GPNNV, Infectious hematopoietic necrosis virus (IHNV), Iridovirus, Koi herpes virus (KHV) and Lymphocystis virus. Meanwhile, the sensitivity of PCR primers were performed as described by Thiéryet al.[13] using plasmid containing DNA fragments of GPNNV[11]. Based on the results of specificity and sensitivity tests, the PCR 1a and PCR 2b methods were finally selected for the viral detection.

The PCR reaction was prepared by mixing 2.0µl cDNA, 5.0µl 10X PCR buffer (Fermentas), 1.0µl (10mM) dNTPs (Promega), 3.4µl (25mM) $MgCl_2$, 2.0µl (10µM) of each forward and reverse primers, 1.0µl (5.0U/µl) Taq DNA polymerase (Fermentas) and 33.6µl of nuclease free water. Then, the PCR reaction was amplified using a thermal cycler (GeneAmp® PCR System 9700, Applied Biosystem) at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 30s, annealing at 58°C for 30s and extension at 72°C for 30s. The amplification was terminated by a single extension step at 72°C for 5 min. Finally, PCR products were analyzed on 1.5% agarose gel electrophoresis. Gel images were captured using Gel Documentation System (Alpha Innotech Corporation).

Histological analysis: Histological analysis was only conducted on fresh fish specimens. The whole fish larvae (10dph to 40dph) or only the head portion for larger fish specimens were used for histological sectioning according to the method described by Ransangan and Manin[10].

Results and Discussion

In this study, a total of 246 fish specimens were examined using RT-PCR amplification method and 20 specimens were subjected to histopathological examination. The fish specimens were collected from several hatcheries and aquaculture farms in Malaysia (Langkawi Island, Kota Kinabalu, Tuaran and Sandakan) and Indonesia (Bali). Large number of the fish specimens analyzed in the present study comprised of Asian seabass and brown-marbled grouper. This is because the two species are the widely cultured marine fish species in Malaysia[1,10]. Although the culture of humpback grouper is preferred because of its market advantage, its relatively slow growth[14-15] and inconsistent seed supply due to viral infection[16] limits the aquaculture of this species. The golden pompano is a newly introduced culture fish species in Malaysia[11]. With exception to Asian seabass, the seeds for groupers and golden pompano are often imported from Indonesia[1] and Taiwan[11], respectively.

The percentage of infected specimens detected using RT-PCR and histopathological methods were recorded at 60.98% and 35.0%, respectively. The finding shows a worrying degree of *Betanodavirus* infection in the aquaculture of marine fish species in Malaysia. *Betanodavirus* has also been well documented in aquaculture of humpback grouper and brown-marbled grouper in Indonesia [17-18]. Over the study period, we received humpback grouper and brown-marbled grouper specimens from Bali, Indonesia twice. Those specimens received in July 2008 were found infected with *Betanodavirus* but specimens received in April 2009 were free from the virus. However, precaution is necessary since RT-PCR has been reported unable to detect low concentration of virus. Similar observation was also supported by the findings of Nguyen et al.[19] and Barker et al.[20].

Both brown-grouper specimens collected from a hatchery in Tuaran (Malaysia) in December 2008 and August 2009 were found infected by the virus. However, the Asian seabass specimens collected in August 2009 from the same hatchery were free from the virus. The situation in the brown-marbled grouper may be explained by the vertical transmission of the virus from brood fish to offspring. The brood fish used to establish the seed production in the hatchery were caught from natural environment and kept in captivity until maturity. Interestingly, the Asian seabass specimens were free from virus infection despite being reared in the same hatchery. From our observation, the situation can be explained by the strict culture practices employed by the hatchery management. Both fish were cultured in different tanks separated from each other, different persons were assigned to handle each fish species, different water system and equipment were used.

It was reported that *Betanodavirus* can easily spread within and between hatcheries through contaminated equipments[21], contaminated water [9,12] and through physical contact with asymptomatic carrier fish[4]. This could explain the VNN outbreak in golden pompano cultured in Langkawi Island in April 2006[11] that reoccurred in November 2008. In January 2009, another VNN outbreak occurred in brown-marbled grouper cultured in the same facility. We suspected presence of carries fish may have contributed to the re-occurrence of the outbreak.

Histological examination of *Betanodavirus*infected fish specimens showed cell vacuolation (Figure-1) especially in brain and retina tissues. This is consistent with the observations by other researchers[22-24]. However, some numbers of infected fish specimens showed a minor or no cell vacuolation. One instancewas the brown-marbled grouper specimens collected from Tuaran in August 2009 which did not show any cell vacuolation but experienced high mortality. This could be explained by the high pathogenicity of the virus. Although histological method is less sensitive particularly at early stage of infection[25], it is useful in revealing the degree of cell damage due to infection.

The RT-PCR conditions (Figure-2) in this study were very useful for detection of *Betanodavirus* especially for the RGNNV genotype which is widely distributed throughout the Southeast Asian region. Although we did not use nested RT-PCR which thought to be more sensitive than the conventional RT-PCR[12,26], our RT-PCR methods have shown to amplify low viral copy number equivalent. The use of specific primers has the advantage over the use of degenerate primers on sensitivity. However, degenerate primers are useful in the amplification of different strains of the *Betanodavirus*. Nishizawaet al.[27] has reported that the detection of *Betanodavirus* can be improved by increasing the PCR cycles but in our study 30 cycles were sufficient. In fact extending the amplification to 40 cycles did not show much different in term of PCR yield. Unlike nested PCR, conventional RT-PCR is less affected by carry over contamination. However, the disadvantages will become apparent when handling large number of specimens[12].

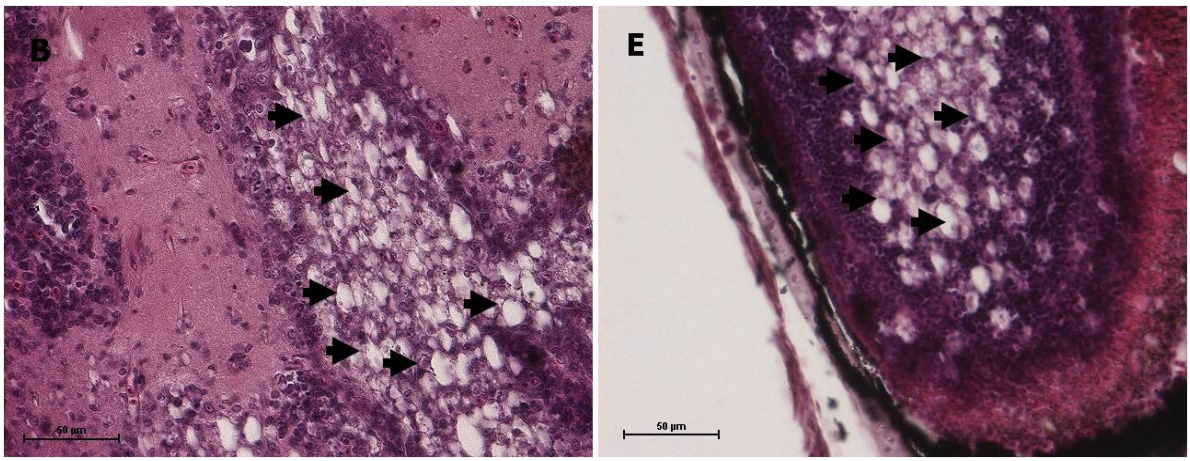

Figure-1
Histological sections of B) brain and E) eye tissues of Asian sebass (SB0909) specimens collected from hatcheries in Sandakan, Malaysia. Notice the cell vacuolations in the brain and eye tissues (black arrows) of infected fish specimen

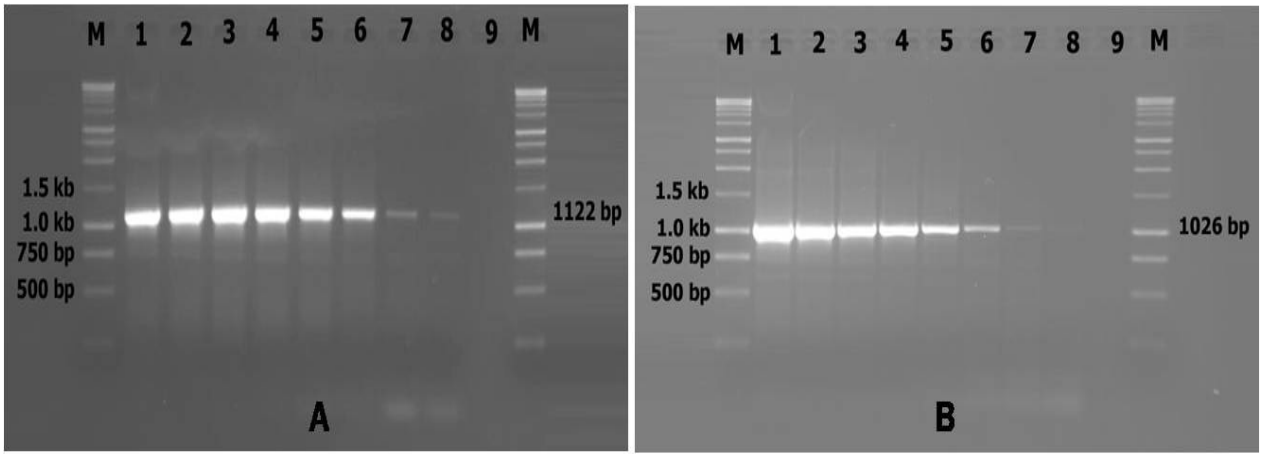

Figure-2
Agarose gel electrophoresis of A) PCR 1a and B) PCR 2b against different gene copies of GPNNV, Lane M: 1.0 kb DNA ladder (Promega); Lane 1: 4.6 X 10^7 gene copies; Lane 2: 4.6 X 10^6 gene copies; Lane 3: 4.6 X 10^5 gene copies; Lane 4: 4.6 X 10^4 gene copies; Lane 5: 4.6 X 10^3 gene copies; Lane 6: 4.6 X 10^2 gene copies; Lane 7: 4.6 X 10^1 gene copies; Lane 8: 4.6 gene copies; Lane 9: negative control (nuclease free water)

Conclusion

Betanodavirus infection in marine fish aquaculture in Malaysia is already at a worrying stage. The detection of the virus infection in the four widely cultured fish species (Asian seabass, hum back grouper, brown-marbled grouper and golden pompano) indicates that there is high possibility of other fish species could experience some degrees of infection. Such situation can cause great impact to the development and economic aspects of aquaculture in Malaysia. Hence, in order to stay viable and competitive in the industry, biosecurity policies need to be implemented in all aquaculture facilities throughout the country.

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