A Virological Survey of Diseased Groupers in Thailand Using Virus Isolation and Polymerase Chain Reaction (PCR) Techniques

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ABSTRACT

A virological survey was conducted on brown-spotted grouper, Epinephelus malabaricus, cultured in cages, earthen ponds and pens during April 2001 to January 2002 in the eastern and southern coasts of Thailand. The adult grouper samples exhibited a wide range of clinical signs. In the beginning there was a darkening of the body with focal distension of the skin; this progressed to red boils in the skin and later to red spots and ulcers on the body and head. The diseased fry to juvenile stages of the groupers showed darkening of the body and swirling swimming movements. Twenty-six virus isolates were discovered using freshwater fish cell lines, SSN-1 (striped snakehead) and EPC (Epithelioma papulosom cyprini). These isolates could be grouped into two different groups of fish viruses. One viral group was identified as a betanodavirus belonging to the genotype of red spotted grouper nervous necrosis virus (RGNNV). Reverse transcriptase-polymerase chain reaction (RT-PCR) tests in this betanodavirus using specific primers to genotype striped jack nervous necrosis virus (SJNNV) revealed negative results, while specific primers to RGNNV showed a positive RT-PCR product. The second viral group could be identified as an iridovirus. This grouper iridovirus had some level of similarities to red sea bream iridovirus (RSIV). One set of specific primers for RSIV gave a positive PCR product while the second set of specific primers gave negative PCR results. The grouper iridovirus was found to be different from ranaviruses. The betanodavirus could infect both adult and fry to juvenile stages of the grouper and showed more severe in grouper fry. The grouper iridovirus seemed to cause disease in the adult fish size with a low level of mortality. Findings indicated that two different viruses were isolated and identified, a betanodavirus and a grouper iridovirus. This new isolate of grouper iridovirus had some level of variations in nucleotide sequences when compared with RSIV gene. More studies on molecular biology and pathogenesis of this new isolate of grouper iridovirus are required.

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INTRODUCTION

Groupers are one of the major economically important cultured fishes in Southeast Asia. Indonesia, the Philippines, Malaysia, Thailand, Vietnam and Australia are the principal producers of farmed groupers while Hong Kong China, Chinese Taipei, China PRC and Singapore are the major importing countries. Sources of grouper for the market are both wild caught and cultured. With the recent occurrence of an increasing awareness of over-fishing, pollution and illegal fishing, the development of grouper culture has been receiving more attention from many countries in the region. Grouper farming has existed for over 20 years along the southern and eastern coasts of Thailand. Available records indicated that the grouper aquaculture production contributed 1,443 mt in 2001 (Anonymous, 2003). Recently, with the failure of shrimp culture in some areas, some shrimp farmers have changed to culture of groupers in earthen ponds. This transition has a potential to increase production in Thailand. Unfortunately, grouper culture during the nursery and grow-out stages still has the problem of diseases. Some diseases have been reported from cultured grouper in the south of Thailand. Viruses are the major cause of serious diseases associated with high losses for which no effective treatment is available. Two different viruses, iridovirus (Danayadol *et al*., 1994; Kasornchandra and Khongpradit, 1997) and betanodavirus (Danayadol *et al*., 1995), have been identified as pathogens capable of causing high mortality in Thailand. These two kinds of viruses have also been reported in other marine cultured fishes from many countries in this region and other parts of the world. A recent review of betanodavirus infections in finfish indicated that the virus caused problems to finfish aquaculture in most parts of the world except the African continent (Munday *et al*., 2002). However, very little information is available about the occurrence of viral diseases in the groupers cultured in Thailand. Recently, scientists found that striped snakehead (SSN-1) (Frerichs *et al*., 1996; Iwamoto *et al*., 2000) and grouper fin cell lines (GF-1) (Chi *et al*., 1999) fish cell lines gave good support to betanodavirus isolation and the Epithelioma papulosum cyprini (EPC) fish cell line was generally suitable for an iridovirus isolation. Therefore, a specific virological survey among diseased groupers was conducted in this study using virus isolation in SSN-1 and EPC cell lines. The survey also used polymerase chain reaction (PCR) technique for virus detection and for virus identification.

MATERIALS AND METHODS

Specimen collection

Groupers were sampled monthly for 10 months from farms on the southern and eastern coasts of Thailand. Fish collected during April to November 2001 were young adult to adult stages which had low mortality rate or no mortalities in the farms. Fish collected in December 2001 were adult groupers with medium to high mortality rates in the culture farms. Samples collected in January 2002 were fry to juvenile groupers (1-3 inches), and had a severe mortality rate. Specimens were sacrificed, separated into four parts, and treated differently for virus isolation, total RNA extraction, DNA extraction and histology processing. The specimens collected from the South were processed at the National Institute of Coastal Aquaculture (NICA) in Songkhla province while the samples collected from the East were investigated at the Aquatic Animal Health Research Institute (AAHRI) in Bangkok.

Virus isolation. Approximately 1 g of pooled tissue from the internal organs, eyes and brain was needed for virus isolation. Tissues were homogenized and their extracts were collected, diluted at 1:50 and filter-sterilized according to Thoesen (1994). The extracts were inoculated onto selected cell lines, EPC (Fijian *et al*., 1983), SSN-1 (Frerichs *et al*., 1991), maintained in L-15 medium plus 2% fetal calf serum and incubated at 25°C. Cytopathic effects (CPE) were observed daily for 7-10 days and one blind passage of supernatant fluid was performed using fresh cell lines. Viruses obtained were propagated in larger tissue culture flasks and kept at -20°C for further study.

Total RNA and DNA extraction for PCR / RT-PCR

Approximately 200-400 mg tissue of eyes, brain and internal organs were collected from the fish and treated immediately with Tri-reagent (Sigma). Total RNA and DNA extractions were conducted according to suggested protocols supplied with the reagent. A standard phenol-chloroform method was applied for DNA extraction. Some samples were also extracted using QIAmp Viral RNA Mini kit (QIAGEN, UK). The total RNAs were used directly for reverse transcriptase-polymerase chain reaction (RT-PCR) detection using a OneStep RT-PCR kit from QIAGEN.

PCR and RT-PCR

DNA and RNA samples from the south coast were sent to AAHRI for PCR and RT-PCR. Two sets of primers for betanodavirus detection were used as suggested by Nishizawa et al. (1994). Primer set RGNNV770 was designed to amplify the coat protein gene at nucleotide sequence position 53 - 823 from the RGNNV nucleotide sequence from GenBank accession #D38636 (Nishizawa *et al*., 1995). A known betanodavirus genotype RGNNV obtained from NICA was used for positive control in RT-PCR amplification. PCR primers for red sea bream iridovirus and ranavirus detection were used according to the published literatures (Oshima *et al*., 1998; Mao *et al*., 1999) with some modifications (Table 1.). Sea bass iridovirus

Table 1. List of primers used in the PCR and RT-PCR detections from fish tissue.

	Primer set code	Forward primer 5'-3'	Reverse primer 5'-3'	Exp. product (bp)	References
Betanod avirus	SJNNV-426	CGT-GTC-AGT-CAT-GTG-TCG-CT	CGA-GTC-AAC-ACG-GGT-GAA-GA	426	Nishizawa *et al*., 1994
	SJNNV-1147	GGA-TTT-GGA-CGT-GCG-ACC-AA	GAC-AAG-ACT-GGT-GAA-GCT-GG	1147	Nishizawa *et al*., 1994
	RGNNV-770	GGG-ACA-GGA-ACA-GAC-GGA-TA	AAC-AGG-CAG-CAG-GAT-TTG-AC	770	Designed from RGNNV genotype, GenBank #D38636
Iridovirus	Ranavirus-300	GTT-TCA-TCG-ACT-TGG-CCA-CT	ATG-TTG-TGC-ATG-GGG-TTC-TT	300	Designed from *Ranavirus* FV3 (Mao *et al*., 1999)
	RSIV-613	CAC-GTG-TTG-GCT-TTC-TTC-GC	GAG-CAT-CAA-GCA-GGC-GAT-CT	613	Oshima *et al*., 1998
	RSIV-187	GCA-TGT-ATG-CTG-TTT-AGA-GA	GAG-CAT-CAA-GCA-GGC-GAT-CT	187	Oshima *et al*., 1998

(SIV) DNA was selected as a positive PCR control for marine fish iridovirus (Khongpradit and Kasornchandra, 1996), while tiger frog iridovirus (TFIV) was used as a positive PCR control for ranavirus (Kanchanakhan *et al*., 1999).

RESULTS

Grouper samples collected from cage culture, pen culture or earthen pond farms during April - December 2001 were young adult or adult fish stages, 5-14 in total length. Numbers of sampled fish, the code of the samples and the sampling times from the east and south coasts are shown in Table 2. Most samples collected during the April (code GP1) - July (code GP4) period and October (code GP7) exhibited normal appearance and gave negative results for virus isolation and PCR/RT-PCR detections. Fish sample codes 5, 6, 8, 9 and 10 showed some positive results for virus isolations and PCR/RT-PCR tests, which are described in detail in this report.

Table 2. Grouper samples collected for virus isolation, PCR and RT-PCR diagnosis during the survey of the east and south coasts of Thailand (April 2001 - January 2002). Sample codes GP1-GP4 and GP7 were found negative for all tests.

Sample code	Date	Number of samples	
		East Coast	South Coast
GP1	April	8	5
GP2	May	10	5
GP3	June	10	5
GP4	July	10	5
GP5	August	9	5
GP6	September	10	5
GP7	October	11	5
GP8	November	9	5
GP9	December	27	5
GP10	January	15	5

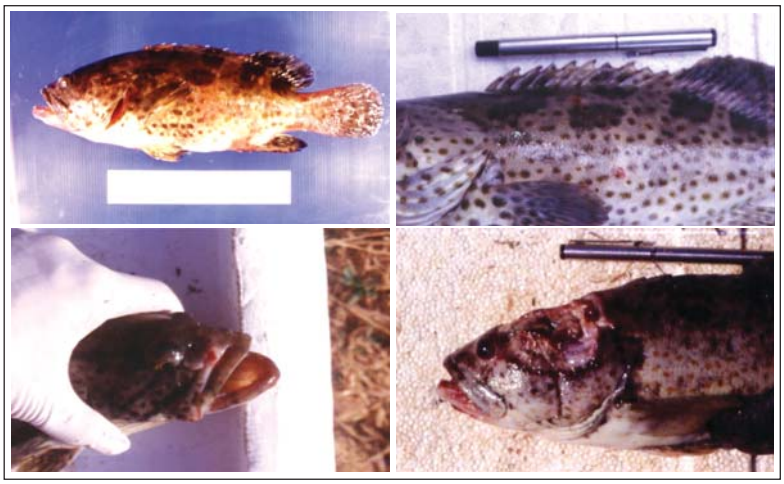

Figure 1. Clinical signs of diseased groupers sampled during the survey. The diseased fish exhibited hemorrhagic lesions on the body and mouth. Some fish had focal hemorrhages, red spots or red boils on the body while some fish showed deep ulceration in the muscle tissue.

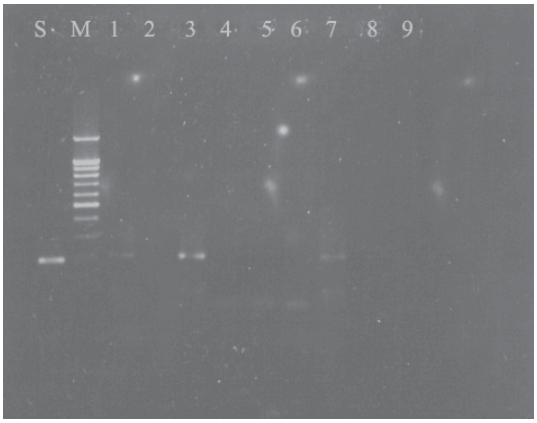

Figure 2. PCR amplification of grouper code GP5 using primers specific to RSIV showed a product at ~187 bp of sample number 1, 3 and 7. (M = 100 bp Ladder marker; S = sea bass iridovirus positive control)

Sample Code GP5

Grouper specimens collected from cage culture farms in August on the east coast gave 4/9 positive virus isolations on SSN-1 cells and 2/9 positive isolations on EPC cells as shown in Table 3. All positive virus isolations obtained from diseased groupers exhibited focal hemorrhagic lesions on the body and mouth (Fig. 1). The hemorrhages were also found deep in the muscle tissue. PCR tests using RSIV primers showed a band at ~187 bp on fish samples 1, 3 and 7 (Fig. 2), while the other 5 sets of primers gave negative PCR or RT-PCR results. Fish samples from the South showed normal appearance and were negative in all tests.

Table 3. Sample code GP5 collected in August 2001 showing virus isolations, PCR and RT-PCR results.

Code GP5	Sample number													
	East Coast									South Coast				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Virus isolation														
SSN-1	-	-	+	-	+	+	+	-	-	-	-	-	-	-
EPC	-	-	+	-	-	-	+	-	-	-	-	-	-	-
PCR														
RSIV-187	+	-	+	-	-	-	+	-	-	-	-	-	-	-
RSIV-613	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ranavirus-*300*														
RT-PCR	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SJNNV-426	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SJNNV-1147	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RGNNV-770														

Table 4. Sample code GP6 collected in September 2001 showing virus isolations, PCR and RT-PCR results.

Code GP6	Sample number														
	East Coast										South Coast				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Virus isolation															
SSN-1	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
EPC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PCR															
RSIV-187	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
RSIV-613	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ranavirus-*300*															
RT-PCR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SJNNV-426	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SJNNV-1147	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RGNNV-770															

Sample Code GP6

Sample code GP6 were collected from cage culture farms in September in east and south coast. Fish did not exhibit severe clinical signs, however, few fish per surveyed farm died every day during the past 30 days. Only one sample gave positive virus isolation results on SSN-1 cells as shown in Table 4. Only DNA extracted from the virus-positive fish gave a positive PCR test by using primers RSIV-187, while the other 5 sets of primers gave negative PCR and RT-PCR results, respectively.

Sample Code GP8

Fourteen samples were collected in the November survey. No virus could be isolated from the eastern grouper samples and their PCR and RT-PCR tests were also negative. However, one virus isolate could be obtained from SSN-1 cells from one diseased adult grouper from the South (Table 5). The RT-PCR test for betanodavirus found only one positive specimen when using primer set RGNNV-770. The other tested primers showed negative PCR or RT-PCR results in all samples.

Table 5. Sample code GP8 collected in November 2001 showing virus isolations, PCR and RT-PCR results.

Code GP8	Sample number													
	East Coast									South Coast				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Virus isolation														
SSN-1	-	-	-	-	-	-	-	-	-	+	-	-	-	-
EPC	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PCR														
RSIV-187	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RSIV-613	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ranavirus-*300*														
RT-PCR	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SJNNV-426														
	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SJNNV-1147														
	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RGNNV-770														

Table 6. Sample code GP9 collected in December 2001 showing virus isolations, PCR and RT-PCR results.

Code GP9	Sample number December																
	East Coast																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Virus isolation																	
SSN-1	-	-	-	-	-	+	-	+	+	-	-	+	+	-	-	-	-
EPC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PCR / RT-PCR																	
RSIV-187	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ranavirus-*300*																	
	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
RGNNV-770																	

Code GP9	Sample number December															
	East Coast										South Coast					
	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	
Virus isolation																
SSN-1	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	
EPC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
PCR / RT-PCR																
RSIV-187	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Ranavirus-*300*																
	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	
RGNNV-770																

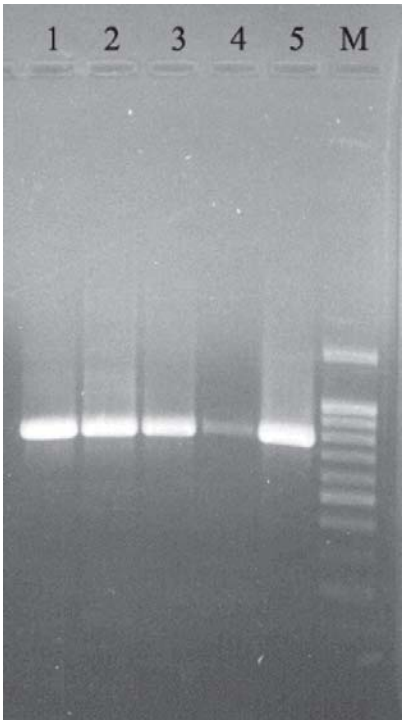

Figure 3. RT-PCR amplification of diseased groupers code GP8, GP9 and GP10 from the south coast showed positive results with 770 bp products. (Lane1 = RGNNV positive control; Lane 2 = GP9/#28; Lane3 = GP9/#29; Lane4 = GP8/#10; Lane5 = GP10/#16; M = 100 bp Ladder.)

Sample Code GP9

Thirty-two samples were collected from seven grouper farms during the December survey. The fish exhibited a wide range of clinical signs beginning with darkening of body color with focal distension of the skin, then red boils in the skin and then red spots and ulcers on the body and head. Viruses could be isolated from 8/32 diseased groupers using SSN-1 cells (Table 6). RT-PCR using specific primer set RGNNV-770 to betanodavirus gave 4/32 positive results (Fig. 3). No PCR products were obtained from any samples when using primer RSIV-187 and primer ranavirus-300.

Table 7. Sample code GP10 collected in January 2002 showing virus isolations, PCR and RT-PCR results.

Code GP10	**Sample number**																			
	East Coast															**South Coast**				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Virus isolation																				
SSN-1	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	-	-
EPC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PCR / RT-PCR																				
RSIV-187	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ranavirus-300	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RGNNV-770	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	-	-

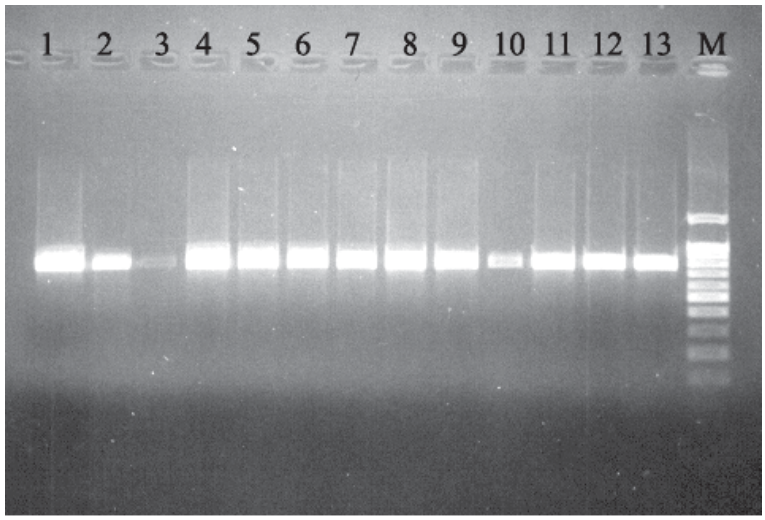

Figure 4. RT-PCR amplification of diseased groupers code GP8 and GP10 from the east coast showed positive results with 770 bp products. (Lane1 = RGNNV positive control; Lane2 = GP8/#8; Lane3 = GP8/#9; Lane4-13 = GP10/#1-#10, respectively; M = 100 bp Ladder)

Sample Code GP10

Twenty samples of diseased groupers were collected from four grouper farms during the January survey. Samples 11-15 were diseased adult groupers showing hemorrhagic ulcers on the bodies. The others were fry to juvenile stages of grouper (1-3 in in total length) exhibiting swirling swimming movements, with some fish floating near the water surface in the cages and earthen ponds. Viruses could be isolated from 12/20 diseased groupers using SSN-1 cells (Table 7). No virus isolate was obtained using EPC cells. RT-PCR using a specific primer set RGNNV-770 to betanodavirus gave 12/20 positive results (Fig. 4). No PCR product was obtained from any samples when using primer RSIV-187 and ranavirus-300.

DISCUSSION

At least two different marine fish viruses have been isolated from diseased groupers using fresh- water fish cell lines, SSN-1 and EPC, during the 10-month survey (April 2001 - January 2002) in the eastern and southern coast of Thailand. One virus isolate has been identified as a strain of betanodavirus as the primers specific to genotype RGNNV could give a product band with RT-PCR. The specific primer targeting 426 bp of SJNNV RNA2 used in the RT-PCR test system failed to amplify the new isolate of grouper nodavirus. This failure also indicated a different genotype of the betanodavirus as the sensitivity of the RT-PCR test varies depending the strains of the virus (Munday *et al*., 2002). A preliminary nucleotide sequence of this RT-PCR product at 770 bp showed over 98% homology to RGNNV. Results indicate that this isolated betanodavirus can be identified as a virus member in genotype RGNNV. The second virus isolate could be identified as an iridovirus as a preliminary nucleotide sequencing of the 187 bp PCR product showed 88% homology with the RSIV gene. There are some differences in the gene of this grouper iridovirus and red sea bream iridovirus as the specific primer set RSIV-613 could not give any products. Results suggest that this new isolate of grouper iridovirus has some level of variations in nucleotide sequences when compared with RSIV gene. Further molecular study on this new isolate of grouper iridovirus is required. The recent cell line susceptibility tests showed that the grouper iridovirus also caused CPE in sea bass kidney or SK cell line.

Betanodavirus infection in groupers seems to be more severe to the fry stage of the grouper with a high mortality. The virus can infect both adult and fry to the juvenile stage of the grouper. It has been recognized that betanodavirus or piscine nodavirus could infect and cause high losses in older fish or adult stage of marine fish such as European sea bass (Le Breton *et al*., 1997) and grouper (Fukuda *et al*., 1996). Grouper iridovirus seems to cause disease in adult size fish, with a low level of mortality. The virus seems to infect the fish predominantly in August and September or during rainy season in Thailand.

The major capsid protein (MCP) gene of iridovirus has been found to be a very well conserved nucleotide sequence among freshwater fish/frog iridoviruses (Mao *et al*., 1999). However specific primers designed from MCP gene of ranaviruses could not give any PCR products from all of the grouper samples. Results suggest that freshwater fish/frog iridoviruses and marine fish iridoviruses should be grouped as different genera in the family Iridoviridae.

Twenty-six viral isolates were isolated from 161 tissue extracts during this survey. There were 6 grouper specimens that gave positive virus isolation in fish cell culture, but the PCR and RT-PCR detection from the fish tissue revealed negative results. This might have been caused by a low degree of virus infection in the fish or a low amount of viral particles in the fish tissue. All virus isolates in fish cell cultures needed to be checked again with PCR or RT-PCR. If new viruses are found, there is a need to do further characterization and identification.

Viral disease is one of the major problems in grouper culture in Thailand as many viruses have been isolated during this short survey. The diseases have already spread to the main coastal aquaculture areas in the south and east coasts. It is important to understand the epizootiology of marine fish viral diseases in order to minimize their impact and for control and prevention.

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