A Thesis
For the Degree of Doctor of Veterinary Medicine

Epidemiological study of viral nervous Necrosis in Korea

Graduate School, Cheju National University,

Department of Veterinary Medicine

Dongjoo Lim

2006. 8

Epidemiological study of viral nervous necrosis in Korea

Dongjoo Lim
(Supervised by Professor Jun Hong Park)

A thesis submitted in partial fulfillment of the requirement for the degree of
Doctor of Veterinary Medicine

2006. 6

This thesis has been examined and approved.

Thesis director, Ilsuk Yang,
Prof. of Seoul National University

Jong-Hee Bae, Prof. of Dept. of Veterinary Medicine

Se Chang Park, Prof. of Seoul National University

Tae-Sung Jung, Prof. of Gyeongsang National University

Jun Hong Park, Prof. of Dept. of Veterinary Medicine

2006. 8

Department of Veterinary Medicine
GRADUATE SCHOOL
CHEJU NATIONAL UNIVERSITY

CONTENTS

CHAPTER 3

GENERAL INTRODUCTION

For the past two decades, betanodavirus infections that caused viral nervous necrosis (VNN) have emerged as major constraints on the culture and sea ranching of marine fish in almost all parts of the world (Munday et al., 2002; OIE 2003) (Fig.1). More than 35 species (18 Families) of marine fish have been devastatingly affected during their seedling and culture process including several marine fish species in Japanese hatcheries (Table 1). VNN was first reported in larvae and juveniles of hatchery-reared Japanese parrotfish *Oplegnathus fasciatus* (Yoshikoshi and Inoue, 1990). The same viral disease, otherwise called viral encephalopathy and retinopathy (VER), has also been observed in barramundi *Lates calcarifer* (Glazebrook et al., 1990) and later in other marine fishes such as redspotted grouper *Epinephelus akaara* (Mori et al., 1991), striped jack *Pseudocaranx dentex* (Mori et al., 1992), Japanese flounder *Paralichthys olivaceus* (Nguyen et al., 1994) and others

(Munday and Nakai, 1997; Muroga 2001; Munday et al., 2002).

Fish affected with VNN showed a variety of abnormal swimming behavior. Histological examinations revealed vacuolation in the spinal cord, brain and retina, coupled by the presence of unenveloped icosahedral virus particles measuring 25-30 nm in diameter packed in the cytoplasm of nerve cells. The virus contains two segments of positive-sense single-stranded RNA. The RNA1 (3.1 kb) and RNA2 (1.4 kb) of SJNNV encode 100 kDa (presumably RNA-dependent RNA polymerase) and a major coat protein of 42 kda, respectively (Mori et., 1992) (Fig.2). The causal agent was purified from diseased striped jack and identified as a new member of the family Nodaviridae, named as striped jack nervous necrosis virus (SJNNV) (Mori et al., 1992). SJNNV belongs to a genus Betanodavirus (piscine nodaviruses) different from Alphanodavirus (insect nodaviruses) (Ball et al., 2000).

Based on partial nucleotide sequence (T4 region: 381 bases) of coat protein gene, betanodaviruses are divided into four genotypes:

SJNNV-type, tiger puffer nervous necrosis virus (TPNNV)-type, barfin flounder nervous necrosis virus (BFNNV)-type, and redspotted grouper nervous necrosis (RGNNV)-type (Nishizawa et al., 1997) (Fig. 3).

The spreading of VNN might be attributable to either vertical or horizontal transmission. A vertical transmission was demonstrated in VNN of striped jack where SJNNV was detected in the ovaries and fertilized eggs of broodfish (Arimoto et al., 1992; Mushiake et al., 1994). A similar transmission mode was also indicated in BFNNV or RGNNV infections of some other fish species such as barfin flounder *Verasper moseri*, European sea bass *Dicentrarchus labrax*, and sevenband grouper *Epinephelus septemfasciatus* (Grotmol and Totland 2000; Watanabe et al., 2000; Breuil et al., 2002; Tsuchihashi et al., 2002). However, the virus transmission mode has not yet been identified in the other VNNs, losses due to VNN has persistently occurred in fish species like groupers where vertical transmission was not confirmed to be the major transmission pathway (Nakai et al., 2001). In horizontal transmission, Le Breton et al. (1997) described

VNN in adult sea bass *D. labrax* reared in floating cages. Castric et al. (2001) also reported the importance of net pen-reared subclinically infected cultured sea bream *Sparus aurata* as potential source of nodavirus to the susceptible cultured European sea bass. In addition, VNN often occurred within a few weeks after a virus-free fish reared in tanks were transported to net-pens in the open sea (K. Mori, personal communication). So far, there is only one report in which a betanodavirus was isolated from wild winter flounder *Pleuronectes americanus*, but the isolation rate was very low at 0.23% (Barker et al., 2002).

Sensitive and specific detection-techniques for nodaviruses greatly help to prevent or control diseases in a wide range of marine fish species. A number of methods including enzyme-linked immunosorbent assay (ELISA), fluorescent antibody technique (FAT), reverse transcription-polymerase chain reaction (RT-PCR) and *in situ* hybridization (Arimoto et al., 1992; Nguyen et al., 1994; Nishizawa et al., 1994; Comps et al., 1996) have been used to detect betanodavirus from diseased fish. These methods proved to be of

great aid in detecting the virus in the gonadal samples of broodstock from striped jack and kelp grouper *E. moara* (Arimoto et al., 1992; Nakai et al., 1994). Because virus-carrying broodstock was found be an important inoculum source of VNN, control of VNN in larval striped jack at the production facilities of Fisheries Research Agency (formerly Japan Sea-Farming Association) via elimination of virus-carrying broodstock by RT-PCR and disinfection of eggs by ozonation has been successfully carried out (Mushiake et al., 1994; Mori et al., 1998). An isolation method using E-11 cells which was cloned from original SSN-1 cell line (Frerich et al., 1996) proved useful in studying the replication of nodavirus *in vitro* and *in vivo* experiments (Iwamoto et al., 1999, 2000).

RT-PCR has become a diagnostic method for fish nodaviruses and was found to be more sensitive method for detection of SJNNV by amplification of portion of the RNA2 gene (Nishizawa et al.1994), particularly as it can detect tiny quantities of viral RNA in any tissue. These assays for VNN may be conveniently used not only to eliminate virus-positive broodfish but also to check the fish status

during on-growing, especially in floating or submersible cages where contacts between wild and cultured fish are most likely to occur (Dalla Valle et al. 2000). Moreover, diagnosis of nodavirus infection by RT-PCR can be expedited by the use of combine cell culture and RT-PCR (Iwamoto et al., 2001). In spite of great benefit from PCR-based methods for pathogen detection, these methods still retain potential problems such as mismatches between primer and template sequences which failed to detect VNN in the striped jack (Nishizawa et al., 1996).

In this study, in **chapter 1**, PCR detection of betanodaviruses from apparently healthy cultured and wild marine and freshwater fish were done. In **chapter 2**, PCR detection of betanodaviruses from marine and freshwater ornamental fish imported from different countries were done. In **chapter 3**, PCR detection of betanodaviruses from several marine invertebrates were also done.

Table 1. Occurrence of clinical viral nervous necrosis in different fish species

Species	Country	Reference
Order Anguilliformes		
Family Anguillidae		
European eel *Anguilla anguilla*	Taiwan	Chi et al., 2001
Order Gadiformes		
Family Gadidae		
Atlantic cod *Gadus morhua*	United kingdom, Canada	Starkey et al., 2001; Johnson et al., 2001 Johnson et al., 2002
Order Pleuronectiformes		
Family Pleuronectidae		
Barfin flounder *Verasper moseri*	Japan	Muroga, 1995
Halibut *Hippoglossus hippoglossus*	Norway, Scotland	Grotmol et al., 1995; Starkey et al., 2000
Family Bothidae		
Japanese flounder *Paralichthys olivaceus*	Japan	Nguyen et al., 1994
Turbot *Scopthalmus maximus*	Norway	Bloch et al., 1991, Johansen et al., 2004
Family Soleidae		
Dover sole *Solea solea*	United Kingdom	Starkey et al., 2001
Order Tetraodontiformes		
Family Triodontidae		
Tiger puffer *Takifugu rubripes*	Japan	Nakai et al., 1994
Order Atheriniformes		
Family Poecilidae		
Guppy *Poecilia reticulate*	Singapore	Hedge et al., 2003
Order Perciformes		
Family Serranidae		
Red spotted grouper *Epinephelus akaara*	Japan, Taiwan	Mori et al., 1991; Chi et al., 1997
Yellow grouper *E. awoara*	Taiwan	Lai et al., 2001
Black spotted grouper *E. fuscogutatus*	Taiwan	Lai et al., 2001
Brown spotted grouper *E. malabaricus*	Thailand	Danayadol et al., 1995 Le Breton et al., 1997
Dusky grouper *E. marginatus*	Mediterranean	Nakai et al., 1994
Kelp grouper *E. moara*	Japan	Fukuda et al., 1996;
Sevenband grouper *E. septemfasciatus*	Japan, Korea	Sohn and Park, 1998 Bondad et al., 2000;
Greasy grouper *E. tauvina*	Malaysia, Philippines, Singapore	Chua et al., 1995; Hedge et al., 2002 Lin et al., 2001;
Orange spotted grouper *E. coioides*	China, Philippines	Maeno et al., 2002 Ucko et al., 2004
White grouper *E. aeneus*	Israel	Zafran et al., 2000
Humpback grouper *Cromileptes altivelis*	Indonesia	

Table 1. (continued)

Species	Country	Reference
Family Carangidae		
Striped jack *Pseudocaranx dentex*	Japan	Mori et al., 1992
Purplish amberjack *Seriola dumerili*	Japan	Muroga et al., 1995
Pompano *Trachinotus blochii*		
Family Sparidae	Taiwan	Chi et al., 2001
Gilthead sea bream *Sparus aurata*		
Family Sciaenidae	Italy	Dalla et al., 2000
Red drum *Sciaenops ocellutus*		
	Korea, Israel	Oh et al., 2001, 2002;
Shi drum *Umbrina cirrosa*		Ucko et al., 2004
	France, Italy	Comps et al., 1996;
White seabass *Atractoscion nobilis*		Bovo et al., 1999
Family Oplegnathidae	USA	Curtis et al., 2001
Japanese parrotfish *Oplegnathus fasciatus*		
Rock porgy *O. punctatus*	Japan	Yoshikoshi and Inoue, 1990
Family Rachycentridae		
Cobia *Rechycentron canadum*	Japan	Mori et al., 1992
Family Percichthydae		
Euro. Sea bass *Dicentrarchus labrax*	Taiwan	Chi et al., 2001
	Caribbean, France, Italy, Malta, Portugal, Greece, Israel	Bellance and Gallet, 1998; Breuil et al., 1991; Bovo et al., 1999; Skliris et al., 2001; Ucko et al., 2004
Family Centropomatidae		
Japanese sea bass *Lateolabrax japonicus*		Athanassopoulou et al 2003
Asian sea bass *Lates calcarifer*	Japan	Jung et al., 1996
	Australia, Tahiti, Thailand, Malaysia, Indonesia, Singapore, Israel	Glazebrook and Campbell, 1987; Renault et al., 1991; Glazebrook et al., 1990; Zafran et al., 1998; Chang et al., 1997; Ucko et al., 2004
Family Lutjanidae		
Firespot snapper *Lutjanus erythropterus*		
Family Mugilidae		
Flathead mullet *Mugil cephalus*	Taiwan	Chi et al., 2003
Order Siluriformes		
Family Siluridae		
Chinese catfish *Parasilurus asotus*	Israel	Ucko et al., 2004
Order Acipenseridae		
Family Acipenseridae		
Russian sturgeon *Acipenser gueldestaedi*	Taiwan	Chi et al., 2003
	Greece	Athanassopoulou et al., 2004

Modified from Munday et al.（2002）

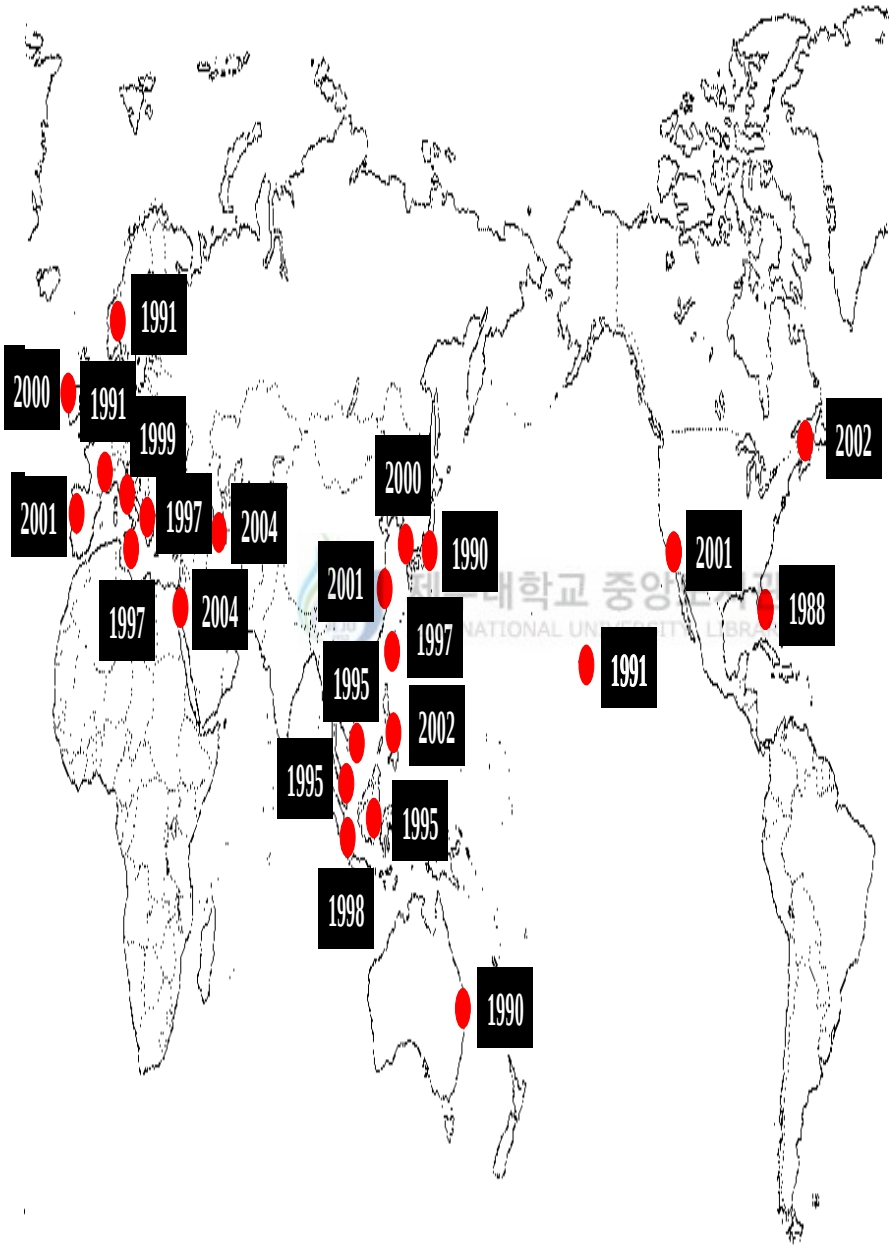


Fig. 1. Global distribution of viral nervous necrosis (VNN).

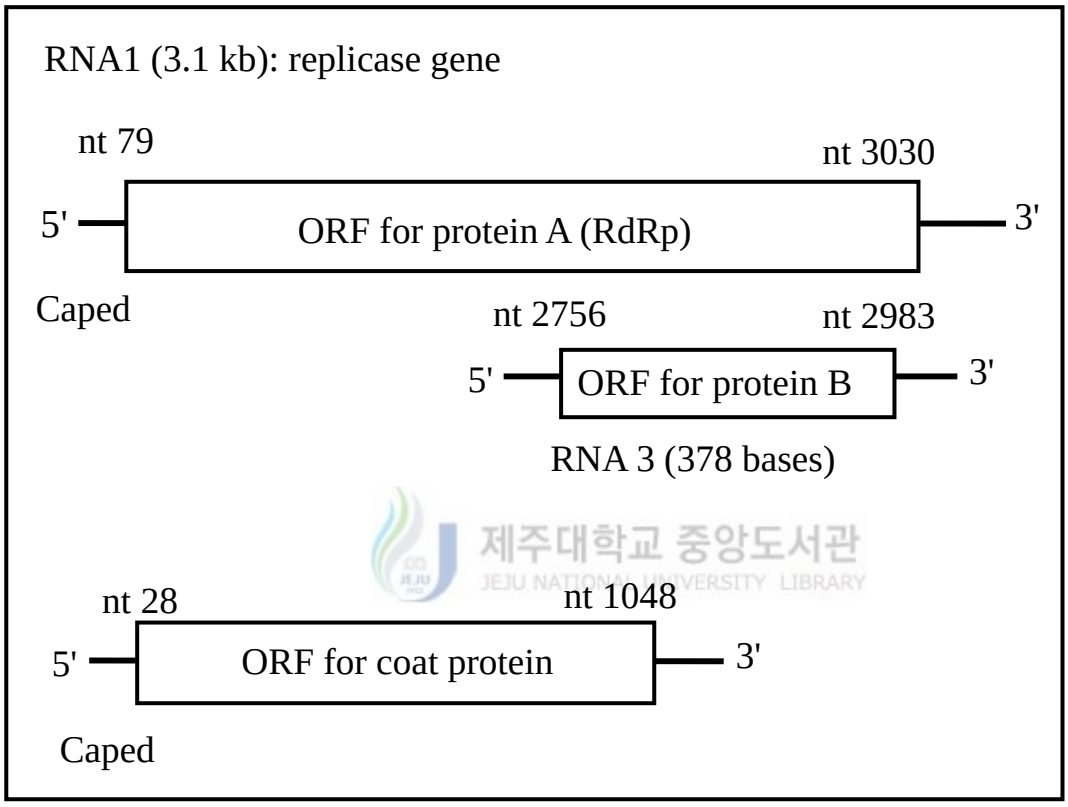
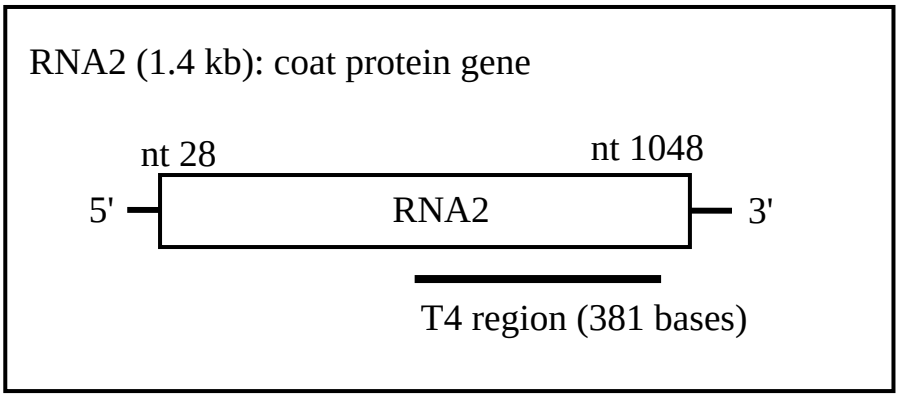


Fig. 2. Schematic illustration of striped jack nervous necrosis virus (SJNNV) genome construction.



Genotype	Representative Virus	Host
SJ	SJNNV	Striped jack
TP	TPNNV	Tiger Puffer
BF	BFNNV	Halibut, flounder and other cold water fish
RG	RGNNV	Grouper, seabass and other warm water fish

Fig.3. Genotypic classification of fish nodaviruses and their corresponding representative host fish based on the nucleotide sequence of the T4 region of the coat protein gene.

CHAPTER 1

Detection of Betanodaviruses from Cultured and Wild Marine and Freshwater Fish with No Clinical Signs

ABSTRACT

Apparently healthy 63 cultured fish (1 species) were collected from flounder culture facility of Jeju Island. And also 386 apparently healthy wild marine fish consisting of 57 species were collected near the culture facilities in three different areas [Donghae (East), Hwanghae (West), Namhae (South)] of Korea. A total of 36 (5 species) apparently healthy freshwater fish were also collected in the river near the culture facilities in Hwanghae (West) area. The brains of fish were examined by reverse transcriptase polymerase chain reaction (RT-PCR) and nested PCR assays. In the flounder culture

facility at Jeju Island, all samples were negative for nodavirus in both RT-PCR and nested PCR tests. In Donghae (East) areas, 14 wild marine fish were negative for nodavirus in both RT-PCR and nested PCR tests. In Hwanghae (West) areas, 80 wild marine and 36 wild freshwater fish were all negative for nodavirus in both RT-PCR and nested PCR tests. While in Namhae (South) area, 10 of 292 wild marine fish were positive only for nodavirus in nested PCR test. These results indicates that nodavirus is widespread among the large populations of wild marine fish in southern part of Korea. The test also identified that 7 species of these marine fish were subclinically infected suggesting an importance of such fish as carriers or reservoir of betanodaviruses.

INTRODUCTION

The spread of VNN might be attributable to either vertical or horizontal transmission. A vertical transmission in a broad sense was demonstrated in VNN of striped jack, *Pseudocaranx dentex*, where SJNNV was detected in the ovaries and fertilized eggs of broodfish (Arimoto et al., 1992; Mushiake et al., 1994) and a similar transmission mode was indicated in BFNNV or RGNNV infections of other fish species such as barfin flounder, *Verasper moseri*, European sea bass, *Dicentrarchus labrax*, and sevenband grouper, *Epinephelus septemfasciatus* (Grotmol & Totland 2000; Watanabe et al., 2000; Breuil et al., 2002; Tsuchihashi et al., 2002). However, the virus transmission mode has not been identified in the other VNNs, even though the importance of horizontal transmission of nodavirus from subclinically infected fish has been discussed (Le Breton et al., 1997; Castric et al., 2001; Barker et al., 2002).

In the present study, to investigate subclinical infection of

betanodaviruses in cultured and wild marine and freshwater fish populations, apparently healthy fish around marine aquaculture areas were examined for the presence of betanodavirus by polymerase chain reaction (PCR)-based methods.

MATERIALS AND METHODS

Fish samples

Sixty three cultured fish (1 species) were collected in the flounder culture facility of Jeju Island, Korea in October, 2005 (Fig.1). Apparently healthy wild marine fish were collected near the culture facilities in three different areas of Korea (Fig. 1). A total of 14 wild fish (1 species) were collected in Donghae (East) area in September, 2005; 80 wild fish (12 species) in Hwanghae (West) area in October, 2005 and also 292 wild fish (47 species) in Namhae (South) area from October to November, 2005. A total of 36 (5 species) apparently healthy freshwater fish were also collected in the river near the culture facilities in Hwanghae (West) area in October, 2005. The brains were aseptically collected from fish and stored at -80^{o}C until used.

RNA extraction

Total RNA was extracted from the brain tissues by using RNA extraction kit, TRIzol Reagent (Invitrogen, USA) according to manufacturer's instruction. Briefly, the brain tissues homogenized with TRIzol reagent were shaken with chloroform and centrifuged at 12,000 *g* for 15 min. RNA in the aqueous phase was precipitated with isopropanol and was dissolved in diethylpyrocarbonate-treated water (DEPC).

PCR amplification

Five oligonucleotide primers were used in this study; BNV-RT.BNV-UR1 and BNV-UF1 for RT-PCR, and BNV-UR2 and BNV-UF2for nested PCR. Target regions to be amplified by these primers were 570 bp for RT-PCR and 420 bp for nested PCR. After reverse transcription with Reverse Transcriptase SuperScript II (Invitrogen, USA) at 45^0C for 60 min, PCR was performed using Ex *Taq*

polymerase (Takara, Japan) with 30 cycles of denaturation at 94^0C for 30 s, annealing at 57^0C for 20 s, and extension at 72^0C for 60 s. Nested PCR was done with the same protocol as above. The PCR products were analyzed by 2% agarose gel electrophoresis. RNAs from uninfected striped jack larvae were uses as a negative control for RT-PCR and nested PCR.

RESULTS

PCR amplification

In 63 cultured marine fish samples (1 species) collected at the flounder culture facility at Jeju Island, all samples were negative for nodavirus in both RT-PCR and nested PCR tests (Table 1). Of the 14 wild marine fish (1 species) collected at Donghae (East) areas were also negative for nodavirus in both RT-PCR and nested PCR tests (Table 1). Of the 80 wild marine fish (12 species) collected at Hwanghae (West) areas were also negative for nodavirus in both RT-PCR and nested PCR tests (Table 1). Of the 292 wild marine fish (47 species) collected in Namhae (South) area, 10 wild fish were positive only for nodavirus in nested PCR test (Table 1). Of the 36 (5 species) wild freshwater fish collected in the river near the culture facilities at Hwanghae (West) areas were also negative for nodavirus in both RT-PCR and nested PCR tests (Table 1.). Positive nested PCR results were obtained in the brains of the apparently healthy

wild marine fish species, *Apogon lineatus* Indian perch, *Doederleinia berycoides* Black throat sea perch, *Muraenesox cinereus* Daggertooth pike conger, *Pennahia argentata* White croaker, *Scomber japonicus* Chub mackerel, *Takifugu niphobles* Grass puffer, *Trachurus japonicus* Japanese jack mackerel. The detection rate in nested PCR (10/485 = 2.1%) (Table 1) and the representative amplicons by nested PCR are shown in Fig.2.

DISCUSSION

The data of positive nested PCR results indicates that nodavirus is present and were obtained from the brains of at least 7 different marine fish species, *Apogon lineatus* Indian perch, *Doederleinia berycoides* Black throat sea perch, *Muraenesox cinereus* Daggertooth pike conger, *Pennahia argentata* White croaker, *Scomber japonicus* Chub mackerel, *Takifugu niphobles* Grass puffer, *Trachurus japonicus* Japanese jack mackerel, in southern part of Korea. It seems likely to assume that nodavirus is widespread in the Namhae area, while the results from eastern (Donghae) and western (Hwanghae) indicates negative for nodavirus among the cultured and wild marine and freshwater fish species tested. The test also identified that 7 species of these marine fish from southern part of Korea were carriers of nodavirus. n every case, the brains were usually negative in RT-PCR but were confirmed to be positive in nested PCR and the detection rate in nested PCR (10/485 = 2.1%),

suggesting that most of these marine fish were latently infected with betanodaviruses. The lack of any clinical symptoms and obvious pathology is typical of viral infections reported from wild fishes. Most infections are presumably latent and pathology does not manifest itself until the fishes are stressed. Fishes will quickly die when suffering from acute infections; thus it is difficult to find such infections among a wild population. Almost all of the infected fish identified showed no gross pathology consistent with that reported for wild winter flounder with VNN (Barker et al., 2002) which suggests that virus may have been present at low concentrations in brain tissues of fish that can be classes as asymptomatic carriers.

What does the detection of betanodaviruses from apparently healthy wild fish near the mariculture areas mean? There is one report in which a betanodavirus was isolated from wild fish (winter flounder *Pleuronectes americanus*) in Canada but the isolation rate was very low (only 1 out of 440 flounder: 0.23%) (Barker et al., 2002). Castric et al. (2001) suggested that net pen-reared subclinically infected sea

bream *Sparus aurata* is a potential carrier of betanodavirus for viral encephalopathy and retinopathy (VER) in European sea bass. Also about 67% of the apparently healthy cultured and wild marine fish were also detected by nested PCR near the mariculture areas in Japan (Gomez et al., 2004). So far, four hatcheries located at Yochon and Yosu in the south coast of Korea have experienced outbreaks of VNN at least once, and particularly as some VNN-affected *Sciaenops ocellatus* red drum larvae were found in the cultured tanks during 1999-2000. This marine fish species is considered as one of the target species of aquaculture in Korea. The cultured or wild marine fish populations have possibly been infected with betanodavirues originating from diseased fish. Furthermore, these subclinically infected wild fish may be a persistent potential source of the virus for susceptible fish species cultured at the facilities.

We have suspected for a long time that the infection was caused by horizontal transmission and not via vertical transmission. The present results support this possibility. In addition, other marine fish

species positive in PCR assay have not previously been reported as a host species for nodavirus, but the isolation of betanodaviruses from apparently uninfected pelagic and highly migratory fish such as Japanese jack mackerel may be an effective vector to transmit betanodaviruses to geographically remote areas, as speculated previously (Curtis et al., 2001; Chi et al., 2003, Gomez et al., 2004) and have been described as carriers (Gomez et al., 2004).

The general conclusions appear to be that nodavirus occurs considerably more often in marine fish than in freshwater. This raises several important questions: (a) are the present virus isolates from apparently healthy wild fish pathogenic to wild or cultured fish? (b) are the marine isolates antigenetically and/or genetically distinguishable from freshwater isolates? (c) how should the marine isolates be regarded in terms of veterinary regulations for prevention and control of VNN disease? In order to answer these several important questions, however, further intensive molecular epidemiological, pathological, and virological analyses will be required.

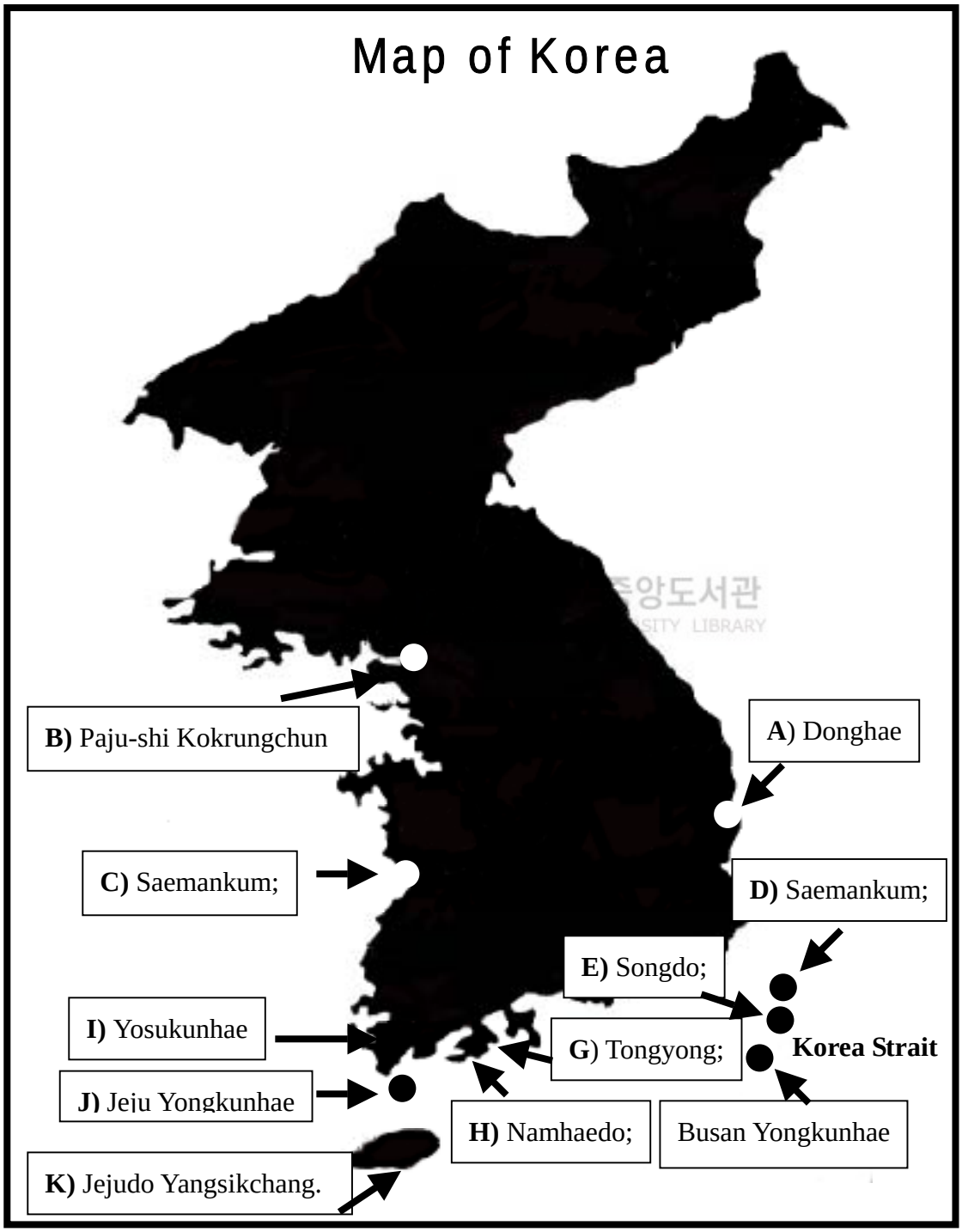


Fig. 1. Location of the sampling sites.

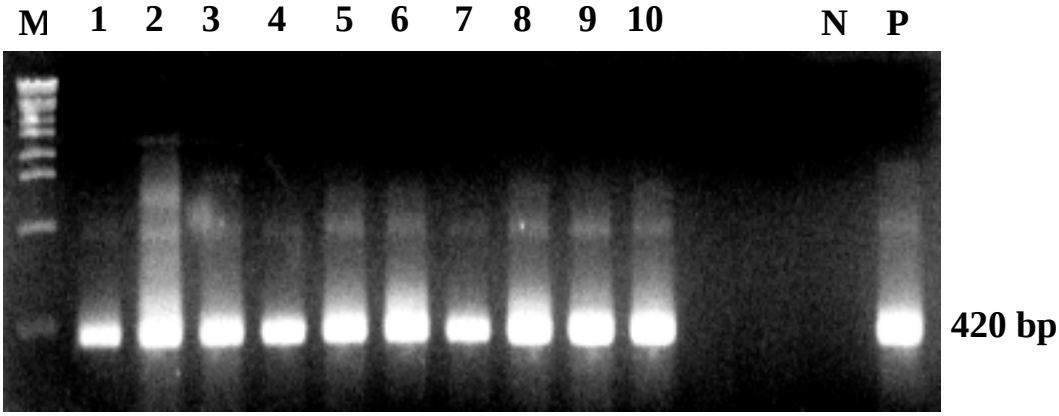


Fig. 2. Representative nested polymerase chain reaction (PCR) profiles. RNA samples were extracted from the brains of cultured and wild marine and freshwater fish and were used for nested PCR. *Apogon lineatus* (Lane 1), *Doederleinia berycoides* (Lane 2), *Muraenesox cinereus* (Lane 3), *Pennahia argentata* (Lane 4), *Scomber japonicus* (Lane 5-8), *Takifugu niphobles* (Lane 9), *Trachurus japonicus* (Lane 10), negative control (N), positive control (P) and molecular size (100 bp ladder) (M).

Table 1. Detection of betanodaviruses from apparently healthy cultured and wild marine and freshwater fish by PCR-based methods

Fish Species	Sources[a]	No of Fish examined	No of positive fish	
			RT-PCR	Nested PCR
Paralichthys olivaceus (Olive flounder)	CM	63	0	0
Leiognathus nuchalis (Silver ponyfish)	WM (D)	14	0	0
Amblychaeturichthys hexamena (Pink gray goby)	WM (H)	6	0	0
Chaeturichthys stigmatias (Fine spot goby)	WM (H)	5	0	0
Cynoglossus joyneri (Red tongue sole)	WM (H)	5	0	0
Johnius grypotus (Belenger's jewfish)	WM (H)	6	0	0
Konosirus punctatus (Dotted gizzard shad)	WM (H)	25	0	0
Pampus argenteus (Silver pomfret)	WM (H)	5	0	0
Paralichthys olivaceus (Olive flounder)	WM(H)	1	0	0
Repomucenus koreanus (Korean Dragonet fish)	WM (H)	7	0	0
Setipinna tenuifilis (Common hairfin anchovy)	WM (H)	5	0	0
Thryssa kammalensis (Kammal thryssa)	WM (H)	5	0	0
Trichurius lepturus (Largehead hairtail)	WM (H)	4	0	0
Zebrias fasciatus (Many banded sole)	WM (H)	6	0	0
Acanthopagrus schlegeli (Black porgy)	WM (N)	14	0	0
Acropoma japonicum (Glowbelly)	WM (N)	11	0	0
Amblychaeturichthys sciistius (Spottysail goby)	WM (N)	3	0	0
Apogon lineatus (Indian perch)	WM (N)	1	0	1
Argyrosomus argentatus (White croaker)	WM (N)	5	0	0
Caelorinchus multispinulosus (Spearnose grenadier)	WM (N)	17	0	0
Cheilidonichthys spinosus (Blue fin sea robin)	WM (N)	28	0	0
Chromis notata (White saddled reef fish)	WM (N)	1	0	0
Clupea pallasi (California herring)	WM (N)	10	0	0
Coilia nasus (Japanese taper tail anchovy)	WM (N)	7	0	0
Conger myriaster (Common Japanese conger)	WM (N)	12	0	0
Cygnoglossus joyneri (Red tongue sole)	WM (N)	7	0	0
Cynoglossus robustus (Robust tonguefish)	WM (N)	1	0	0
Ditrema temminckii (Japanese sea perch)	WM (N)	1	0	0
Doederleinia berycoides (Black throat sea perch)	WM (N)	10	0	1
Eopsetta grigorjeur (Roundnose flounder)	WM (N)	1	0	0
Halichoeres poecilopterus (Multicolor rainbow fish)	WM (N)	5	0	0
Helicolenus hilgendorfi (Hilgendorf saucord)	WM (N)	3	0	0
Hypodytes rubripinnis (Red fin velvet fish)	WM (N)	5	0	0
Kareius bicoloratus (Stone flounder)	WM (N)	1	0	0
Konosirus punctatus (Dotted gizzard shad)	WM (N)	5	0	0
Leiognathus nuchalis (Spotnape pony fish)	WM (N)	5	0	0
Lepidotrigla guentheri (Red-banded sea robin)	WM (N)	1	0	0
Limanda yokohamae (Marbled sole)	WM (N)	1	0	0
Liparis tanakai (Tanaka's snail fish)	WM (N)	8	0	0
Lophius litulon (Yellow goosefish)	WM (N)	6	0	0
Microcanthus strigatus (Stripey fish)	WM (N)	12	0	0
Muraenesox cinereus (Daggertooth pike conger)	WM (N)	3	0	1
Myctophum mitidulum (Metallic lantern fish)	WM (N)	4	0	0
Okamejei kenojei (Skate ray fish)	WM (N)	6	0	0
Pennahia argentata (White croaker)	WM (N)	5	0	1
Pleuronectes yokohamae (Marbled sole)	WM (N)	11	0	0
Psenopsis anomala (Butter fish)	WM (N)	2	0	0
Pseudolabrus sieboldi (Bamboo leaf wrasse)	WM (N)	1	0	0
Pseudorhombus pentophthalmus (Five spots flounder)	WM (N)	13	0	0
Rudarius ercodes (Network filefish)	WM (N)	1	0	0
Saurida undosquamus (Lizardfish)	WM (N)	2	0	0
Sebastes schlegeli (Jacopever)	WM (N)	5	0	0

[a] CM, Cultured marine fish from Jeju Island; WM (D) wild marine fish from Donghae area; WM (H) wild marine fish from Hwang-hae area; WM (N) wild marine fish from Nam-hae area.

Table 1. (continued)

Fish Species	Sources[b]	No of Fish examined	No of positive fish RT-PCR	Nested PCR
Sebastes thompsoni (Goldeneye rockfish)	WM (N)	5	0	0
Seriola quinqueradiata (Yellowtail)	WM (N)	11	0	0
Scomber japonicus (Chub mackerel)	WM (N)	10	0	4
Stephanolepis cirrhifer (Filefish)	WM (N)	4	0	0
Takifugu niphobles (Grass puffer)	WM (N)	4	0	1
Thunnus thynnus (Bluefin tuna)	WM (N)	1	0	0
Trachurus japonicus (Japanese jack mackerel)	WM (N)	18	0	1
Trichurus lepturus (Pacific cutlass fish)	WM (N)	7	0	0
Xenocephalus elongates (Bluespotted stargazer)	WM (N)	1	0	0
Abbottina rivularis (Chinese false gudgeon)	WF (H)	8	0	0
Carassius auratus (Crussian carp)	WF (H)	6	0	0
Hemibarbus labeo (Steel barbell)	WF (H)	2	0	0
Pseudorasbora parva (False dace)	WF (H)	6	0	0
Synechogobius hasta (Javelin goby)	WF (H)	6	0	0
	WF (H)	8	0	0
Total		485	0	10
Rate (%)			0	2.1

[b] WM (N) wild marine fish from Nam-hae area; WF (H) wild freshwater fish from Hwang-hae area.

CHAPTER 2

Detection of Betanodaviruses from Marine and Freshwater Ornamental Fish with No Clinical Signs

ABSTRACT

Sixty seven samples (24 species) of marine and freshwater ornamental fishes were collected from COEX Aquarium in Seoul from November to December, 2005. Of the 9 marine ornamental fish samples from Japan, 1 sample was positive for nodavirus by nested PCR test. Of the 23 marine ornamental fish from Singapore, also 1 sample was positive for nodavirus by nested PCR tests. Of the 12 marine ornamental fish samples (3 species) from Korea, all samples were negative for nodavirus in both RT-PCR and nested PCR tests. Of the 4 marine ornamental fish samples, each fish samples imported

from Indonesia, Canada, USA and Pacific Ocean, all samples were negative for nodavirus in both RT-PCR and nested PCR tests. Of the 19 freshwater ornamental fish collected in the Amazon River were also negative for nodavirus in both RT-PCR and nested PCR tests. Positive nested PCR results were obtained in the brains of the apparently healthy marine ornamental fish species, *Monocentris japonica* Pinecone fish and *Zebrasoma* flavesenes Yellow tang. The detection rate in nested PCR (2/67 = 3%) (Table 1) and the representative amplicons by nested PCR are shown in Fig.2. These results indicates that nodavirus is present from imported marine ornamental fish in the commercial aquarium. These subclinically infected marine ornamental fish can be one the sources of betanodavirus infection among the fishes in Korea.

INTRODUCTION

The ornamental fish industry worldwide is estimated to be worth up to 900 million US dollars, and occupies a significant part of international trade (Evans and Lester, 2001). Recently, ornamental fishes have been cultured mainly in Southeast Asia and Asian countries on a large scale, and then exported worldwide. However, appropriate quarantine practices are neglected for imported ornamental fishes in many countries, and as a result, imported fishes sometimes die of infections soon after arrival or during transportation (Kim et al., 2002a). Korea also imports various kinds of ornamental aquarium fishes from Southeast Asian countries; the scale and number of imported species are increasing (Kim et al., 2002b). In the present study, to investigate subclinical infection of betanodaviruses from imported marine ornamental fish populations, apparently healthy, moribund or dead fish were collected from the commercial aquarium and were examined for the presence of betanodavirus by polymerase chain reaction (PCR)-based methods.

MATERIALS AND METHODS

Fish samples

Sixty seven samples (24 species) of marine and freshwater ornamental fishes were collected from COEX aquarium in Seoul from November to December, 2005. Of the 67 aquarium fish samples, 12 samples (3 species) were collected from Korea. Other aquarium fishes were imported from different sources such as Japan with 9 samples (9 species), Singapore with 23 samples (5 species), Indonesia with 1 sample (1 species), Canada with 1 sample (1 species), United States of America with 1 sample (1 species), Pacific Ocean with 1 sample (1 species) and Amazon River with 19 samples (3 species). The brains were aseptically collected from fish and stored at -80^{o}C until used.

RNA extraction

Total RNA was extracted from the brain tissues by using RNA extraction kit, TRIzol Reagent (Invitrogen, USA) according to manufacturer's instruction. Briefly, the brain tissues homogenized with TRIzol reagent were shaken with chloroform and centrifuged at 12,000 *g* for 15 min. RNA in the aqueous phase was precipitated with isopropanol and was dissolved in diethylpyrocarbonate-treated water (DEPC).

PCR amplification

Five oligonucleotide primers were used in this study; BNV-RT.BNV-UR1 and BNV-UF1 for RT-PCR, and BNV-UR2 and BNV-UF2for nested PCR. Target regions to be amplified by these primers were 570 bp for RT-PCR and 420 bp for nested PCR. After reverse transcription with Reverse Transcriptase SuperScript II (Invitrogen, USA) at 45^0C for 60 min, PCR was performed using Ex *Taq*

polymerase (Takara, Japan) with 30 cycles of denaturation at 94^0C for 30 s, annealing at 57^0C for 20 s, and extension at 72^0C for 60 s. Nested PCR was done with the same protocol as above. The PCR products were analyzed by 2% agarose gel electrophoresis. RNAs from uninfected striped jack larvae were uses as a negative control for RT-PCR and nested PCR.

RESULTS

PCR amplification

In 9 marine ornamental fish samples (9 species) imported from Japan, 1 sample was positive for nodavirus by nested PCR test (Table 1). Of the 23 marine ornamental fish (5 species) imported from Singapore, also 1 sample was positive for nodavirus by nested PCR tests (Table 1). Of the 12 marine ornamental fish samples (3 species) from Korea, all samples were negative for nodavirus in both RT-PCR and nested PCR tests (Table 1). Of the 4 marine ornamental fish samples (4 species), each fish samples imported from Indonesia, Canada, USA and Pacific Ocean, all samples were negative for nodavirus in both RT-PCR and nested PCR tests (Table 1). Of the 19 (3 species) of freshwater ornamental fish collected in the Amazon River were also negative for nodavirus in both RT-PCR and nested PCR tests (Table 1.). Positive nested PCR results were obtained in the brains of the apparently healthy marine ornamental

fish species, *Monocentris japonica* Pinecone fish and *Zebrasoma* flavesenes Yellow tang. The detection rate in nested PCR (2/67 = 3%) (Table 1) and the representative amplicons by nested PCR are shown in Fig.2.

DISCUSSION

The data of positive nested PCR results indicates that betanodaviruses is not only positive in wild marine fish of the southern coastal areas of Korea but also present in the marine ornamental fishes displayed in the commercial aquarium. The positive samples were obtained from the brains of at least 2 different marine fish species imported from different countries, *Monocentris japonica* Pinecone fish from Japan and *Zebrasoma flavesenes* Yellow tang from Singapore. In every case, the brains were usually negative in RT-PCR but were confirmed to be positive in nested PCR and the detection rate in nested PCR (2/67 = 3%), suggesting that these marine ornamental fish were latently infected with betanodaviruses. This type of latent infection status of VNN in fishes showing no clinical signs of nervous necrosis disease has also been recorded in some other fish species such as tilapia and brown meager. Recently, Skliris and Richards (1999) have demonstrated the presence of NNV

in healthy tilapia undergoing experimental infection. Dalla Valle et al. (2000) also observed NNV infection in the brown meager *Sciaena umbra,* showing no signs of viral nervous necrosis disease following their RT-PCR analysis.

What does the detection of betanodaviruses from apparently healthy marine ornamental fish mean? So far, there is only one report of nodavirus infection from a freshwater ornamental fish, *Poicelia reticulate* guppy in freshwater farms in Singapore (Hedge et al., 2003). But several isolates of fish nodaviruses from diseased marine finfishes were also obtained in Singapore (Lim et al., 1997). Chua et al. (1995) also reported a vacuolating encephalopathy and retinopathy (VER) in juvenile greasy grouper *Epinephelus tauvina* caused by a nodavirus-like agent in Singapore. While in Japan, several isolates of fish nodaviruses from both the diseased and subclinically infected marine fishes were also reported (Muroga 2001; Munday et al., 2002; OIE 2003; Gomez et al., 2004). In this present study, it's not unusual for these marine ornamental fishes to be subclinically infected and become carriers or reservoir of betanodavirues because they were

imported from these two countries that have experienced nodavirus outbreaks. However, as of now there is no report of a nodavirus infection in marine ornamental fish from Korea. And this present study is so far the first reported cases not only in Korea but also anywhere around the world of marine ornamental fish to be subclinically infected with betanodaviruses.

When trading live animals or animal products, quarantine practices should be implemented in order to prevent the introduction of transmissible pathogens which can cause serious outbreaks of disease and consequent economic losses. Aquatic animals are no exception. The Office International des Epizooties (OIE) has detailed provisions for the import and export of aquatic animals and aquaculture products aimed at avoiding the risk of spreading aquatic animal diseases (OIE 1997). However, ornamental fishes are not included in these provisions and, in fact, in many countries; the tropical ornamental fish trade operates without appropriate quarantine practices and consequently, the fishes infected with undetected pathogens can be distributed to retailers and sold to consumers (Kim

et al., 2002a). These fish may cause problems in the importing country, since they can die of infections soon after their arrival, or during transportation due to the combination of stress and pathogen infection, resulting in economic losses. From an environmental perspective, infected fish can cause problems to indigenous fish species if they escape into the natural environment of the importing countries. We cannot predict what the consequences are likely to be when the exotic pathogens are introduced in the importing countries, due to the limited information on the factors relevant to the establishment of exotic pathogens to the importing countries. Hence, to avoid the introduction of fish pathogens it is necessary to introduce quarantine practices to the ornamental fish trade, as well as to the trade in fish for human consumption. Until such laws have been introduced, ornamental fishes should be examined, either on an ad hoc or routine basis, to confirm that they are pathogen-free; if they are infected, appropriate treatments should be administered before clearance. Importing countries should also examine imported fishes and treat them before domestic distribution.

Fig. 1. Location of the sampling site in COEX Aquarium, Seoul, South Korea

Fig. 2. Representative nested polymerase chain reaction (PCR) profiles. RNA samples were extracted from the brains of marine ornamental fish and were used for nested PCR. *Monocentris japonica* (Lane 1), *Zebrasoma flavesenes* (Lane 2), negative control (N), positive control (P) and molecular size (100 bp ladder) (M).

Table 1. Detection of betanodaviruses from apparently healthy wild marine ornamental fish by PCR-based methods

Fish Species	Sources[a]	No of fish Examined	No of positive fish RT-PCR	Nested PCR
Abudetdut vaigiensis (Indo pacific sergeant)	J	1	0	0
Acanthurus unicornis (Longnose unicorn)	PO	1	0	0
Alectis ciliaris (African pompano)	J	1	0	0
Aptocyclus ventricossus (Smooth lumpsucker)	K	10	0	0
Centropyge bispinosus (Coral beauty angel)	J	1	0	0
Chaetodon semilarvatus (Butterfly fish)	J	1	0	0
Chimaera monstrosa (Rabbit fish)	I	1	0	0
Choerodon azurio (Scanbreast turkfish)	J	1	0	0
Chrysiptera cyanea (Damsel fish)	S	15	0	0
Gnathanodon speciosus (Golden trevally)	S	2	0	0
Hydrolagus colliei (Rat fish)	C	1	0	0
Labrus mixtus (Red wrasse)	S	1	0	0
Lutjanus kasmira (Blue striped snapper)	J	1	0	0
Microcanthus strigatus (Stripey foot baller)	K	1	0	0
Monocentris japonica (Pinecone fish)	J	1	0	1
Monocirrhus polyacanthus (South American Leaf fish)	A	7	0	0
Osteoglossum bicirrhosum (Silver arowana)	A	2	0	0
Paracheirodon innesi (Neon tetra)	A	10	0	0
Pseudoanthias sp. (Anthias)	S	1	0	0
Sebastes schlegeli (Rockfish)	K	1	0	0
Selene vomer (Look down fish)	USA	1	0	0
Scorpaenopsis oxycephata (Scorpion fish)	J	1	0	0
Taeniura lymma (Blue spot ray)	J	1	0	0
Zebrasoma flavesenes (Yellow tang)	S	4	0	1
Total		67	0	2
Rate (%)			0	3

[a] J, Japan; PO, Pacific Ocean; K, Korea; I, Indonesia; S, Singapore; C, Canada; A, Amazon; USA, United States of America.

CHAPTER 3

Detection of Betanodaviruses from Several Invertebrates

ABSTRACT

One hundred samples (20 species) of wild marine invertebrates were collected from western and southern part of Korea. In 40 wild marine invertebrate samples (5 species) collected at Hwanghae (West) areas, all samples were negative for nodavirus in both RT-PCR and nested PCR tests. Of the 60 wild marine invertebrates (12 species) collected at Namhae (South) area, 4 invertebrate samples were positive only for nodavirus in nested PCR test. Positive nested PCR results were obtained in the brain of crab species, *Charybdis*

bimaculata Charybdid crab; hepatopancreas of shrimp species, *Pandalus hypsinotus* Southern humpback shrimp and pooled organs of mussel species, *Mytilus galloprovincialis* Mediterranean mussel. The detection rate in nested PCR is 4/100 = 4%. These results indicates that nodavirus is present from wild marine invertebrates in the southern areas of Korea. These subclinically infected marine invertebrates can be one the sources of betanodavirus infection among the fishes in Korea.

INTRODUCTION

Marine invertebrates such as crustaceans and shellfishes are also very important aquaculture commodities in Korean aquaculture economy. Shrimp farming has rapidly increased for the past two decades in Korea. However, due to more frequently occurring outbreaks of infectious diseases constitute the main barrier to the development and continuation of crustacean aquaculture. And then, we also speculated that marine invertebrates are also one of the sources of viruses such as nodavirus, the causative agent of viral nervous necrosis (VNN), that cause high mortalities to marine fishes in aquaculture. In the present study, to investigate subclinical infection of betanodaviruses from marine invertebrates, different samples were collected and examined for the presence of betanodavirus by polymerase chain reaction (PCR)-based methods.

MATERIALS AND METHODS

Invertebrate samples

Wild marine invertebrates were collected near the culture facilities in two different areas of Korea(Fig. 1). A total of 40 wild marine invertebrates (5 species) were collected in Hwanghae (West) area in October, 2005 and 60 wild marine invertebrates (16 species) in Namhae (South) area from October to November, 2005 and The organs were aseptically collected from invertebrates and stored at -80^{o}C until used.

RNA extraction

Total RNA was extracted from the brain tissues by using RNA extraction kit, TRIzol Reagent (Invitrogen, USA) according to manufacturer's instruction. Briefly, the brain tissues homogenized with TRIzol reagent were shaken with chloroform and centrifuged at

12,000 *g* for 15 min. RNA in the aqueous phase was precipitated with isopropanol and was dissolved in diethylpyrocarbonate-treated water (DEPC).

PCR amplification

Five oligonucleotide primers were used in this study; BNV-RT.BNV-UR1 and BNV-UF1 for RT-PCR, and BNV-UR2 and BNV-UF2for nested PCR. Target regions to be amplified by these primers were 570 bp for RT-PCR and 420 bp for nested PCR. After reverse transcription with Reverse Transcriptase SuperScript II (Invitrogen, USA) at 45^{0}C for 60 min, PCR was performed using Ex *Taq* polymerase (Takara, Japan) with 30 cycles of denaturation at 94^{0}C for 30 s, annealing at 57^{0}C for 20 s, and extension at 72^{0}C for 60 s. Nested PCR was done with the same protocol as above. The PCR products were analyzed by 2% agarose gel electrophoresis. RNAs from uninfected striped jack larvae were uses as a negative control for RT-PCR and nested PCR.

RESULTS

PCR amplification

In 40 wild marine invertebrate samples (5 species) collected at Hwanghae (West) areas, all samples were negative for nodavirus in both RT-PCR and nested PCR tests (Table 1). Of the 60 wild marine invertebrates (12 species) collected at Namhae (South) area, 4 invertebrate samples were positive only for nodavirus in nested PCR test (Table 1). Positive nested PCR results were obtained in the brain of crab species, *Charybdis bimaculata* Charybdid crab; hepatopancreas of shrimp species, *Pandalus hypsinotus* Southern humpback shrimp and pooled organs of mussel species, *Mytilus galloprovincialis* Mediterranean mussel. The detection rate in nested PCR (4/100 = 4%) (Table 1) and the representative amplicons by nested PCR are shown in Fig.2.

DISCUSSION

Nodaviruses are already known to infect insects, freshwater and marine fishes. The data of positive nested PCR results indicates that betanodaviruses is also present in marine invertebrates in the southern coastal areas of Korea. The positive samples were obtained from three different species of marine invertebrates (shrimp, crab and mussel). In every case, the samples were usually negative in RT-PCR but were confirmed to be positive in nested PCR. The detection rate in nested PCR is 4/100 = 4%, suggesting that these marine invertebrates were also latently infected with betanodaviruses. It seems likely to assume that nodavirus is not only widespread in wild marine fish but also to the marine invertebrates. So far this is the first reported case of wild marine invertebrates to be subclinically infected with betanodavirus in Korean Peninsula.

In the present study, crustacean samples such *Pandalus hypsinotus* Southern humpback shrimp and *Charybdis bimaculata* Charybdid

crab were subclinically infected with nodavirus in the hepatopancreas and in the brain of the samples, respectively. The presence of virus in crustacean diseases has previously been reported (Bonami 1980, Mari 1987). It has been suggested that the occurrence of viral synergic actions might be due to the lack of an interferon-like reaction in invertebrates (Tanada 1956, Odier 1974). In Taiwan, Chi et al. (2000, 2003) also reported VNN in other live food organisms such as crustaceans including *Artemia* sp. nauplii, the copepod *Tigriopus japonicus*, and the shrimp *Acetesinte medius*. It was also reported from Taiwan, China and French West Indies (Sri Widada et al., 2003), that freshwater shrimp *Macrobrachium rosenbergii* with white tail disease (WTD) was also infected with virus and the causative pathogen has been identified as *M. rosenbergii* nodavirus (*Mr*NV) (Arcier et al., 1999). This *Mr*NV has al ready been placed in the family Nodaviridae based on these characteristics (Garzon and Charpentier, 1992; van Regenmortel et al., 2000; Romestand and Bonami, 2003). Sahul Hameed et al. (2004) found *Mr*NV in all the organs except eyestalks and the hepatopancreas of adult

Macrobrachium rosenbergii. This was consistent with the results of Sri Widada et al. (2003), but not with those of Arcier et al. (1999), who also observed positive tests for MrNV in the heptopancreas. In the present study, the sample tested was consistent with the report of Arcier et al (1999), that nodavirus was positive in the hepatopancreas of the shrimp. For *Mytilus galloprovincialis* Mediterranean mussel, nodavirus was positive in the pooled organs of the sample. Recently, it was also observed in Japan that marine invertebrates such as mollusks, Japanese common squid *Todarodes pacificus* used as feeds for aquaculture was also suclinically infected in the brain by betanodaviruses (D.K. Gomez, personal communication). So far, this is the first report of nodavirus in the affected mollusk species in Japan.

The reason for this discrepancy in the presence of nodavirus in different organs of invertebrates is still unknown. Knowing pathogen distribution especially nodavirus in tissues and organs of invertebrates can help us to understand issues related to disease susceptibility and transmission and to choose optimal samples for

pathogen isolation and detection, especially for potential carriers like that may require monitoring for control measures (Sahul Hameed et al., 2004). The virus-host interactions and the modes of transmission are still unknown. But we have already suspected for as long time that the infection was caused by horizontal transmission and not via vertical transmission. This present results also imply the spread of betanodavirus from apparently healthy wild marine fish to wild marine invertebrates or vice versa. In order to demonstrate these speculations, however, further intensive molecular epidemiological, pathological, and virological analyses will be required.

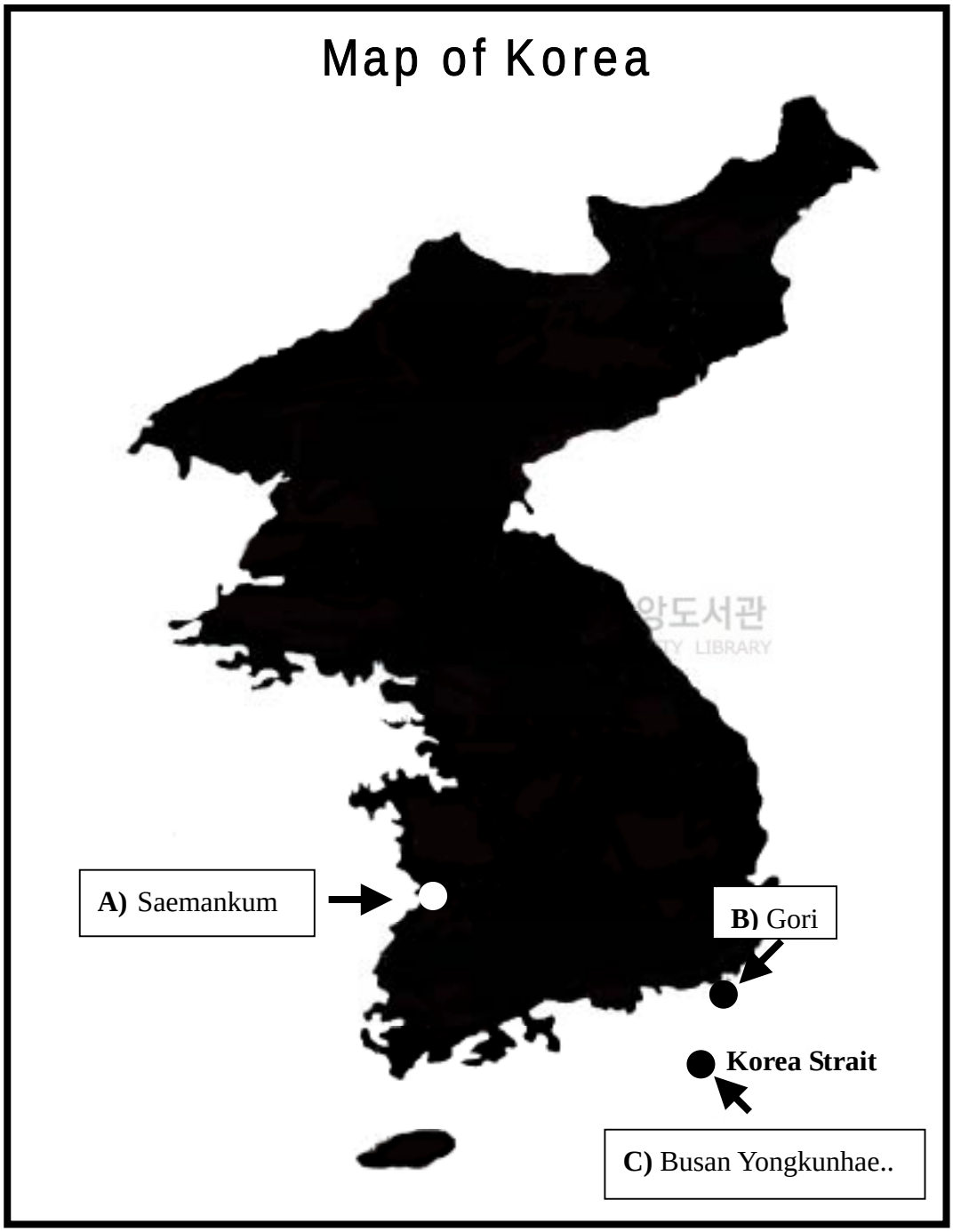


Fig. 1. Location of the sampling sites.

Fig. 2. Representative nested polymerase chain reaction (PCR) profiles. RNA samples were extracted from different organs of invertebrates and were used for nested PCR. *Charybdis bimaculata* (Lane 1), *Mytilus galloprovincialis* (Lane 2-3), *Pandalus hypsinotus* (Lane 4), negative control (N), positive control (P) and molecular size (100 bp ladder) (M).

Table 1. Detection of betanodaviruses from apparently healthy wild marine invertebrates by PCR-based methods

Invertebrates Species	Sources[a]	No of invertebrates examined	No of positive invertebrates RT-PCR	Nested PCR
Charybdis japonica (Swimming crab)	WMI (H)	5	0	0
Ibacus ciliatus (Spiny lobster)	WMI (H)	3	0	0
Loligo beak (Beka squid)	WMI (H)	7	0	0
Ovalipes punctatus (Swimming crab)	WMI (H)	20	0	0
Portunus trituberculatus (Swimming crab)	WMI (H)	5	0	0
Acila divaricata (Divaricated nut clam)	WMI (N)	5	0	0
Alpheus japonicus (Snapping shrimp)	WMI (N)	2	0	0
Alpheus rapax (Goby shrimp)	WMI (N)	1	0	0
Carcinoplax longimana (Monkey crab)	WMI (N)	7	0	0
Charybdis bimaculata (Charybdid crab)	WMI (N)	6	0	1
Crangon hakodatei (Sand shrimp)	WMI (N)	4	0	0
Latreutes planirostris (Flatnose shrimp)	WMI (N)	1	0	0
Mytilus galloprovincialis (Mediterranean mussel)	WMI (N)	2	0	2
Pandalus hypsinotus (Southern humpback shrimp)	WMI (N)	8	0	1
Parapenaeopsis tenellus (Smooth shell shrimp)	WMI (N)	1	0	0
Portunus sanguinolentus (Three spots swimming crab)	WMI (N)	1	0	0
Portunus trituberculatus (Swimming crab)	WMI (N)	1	0	0
Sepia officinalis (Cuttlefish)	WMI (N)	5	0	0
Siphonalia cassidariaeformis (Bonnet whelk)	WMI (N)	11	0	0
Solenocera melantho (Big head shrimp)	WMI (N)	1	0	0
Squilla oratoria (Mantis shrimp)	WMI (N)	4	0	0
Total		100	0	4
Rate (%)			0	4

[a] WM (H) wild marine invertebrates from Hwang-hae area; WM (N) wild marine invertebrates from Nam-hae area.

SUMMARY

Viral nervous necrosis (VNN) caused by piscine nodavirus (*Nodaviridae,* Betanodavirus) has emerged as major constraints on aquaculture of marine fish worldwide. The spreading of VNN might be attributable to either vertical or horizontal transmission of the causal agent. There was strong evidence for vertical transmission of infection in VNNs of some fish species like striped jack *Pseudocaranx dentex*. However, the mode of horizontal transmission has not yet been verified thoroughly, although the importance of subclinically infected fish as a source of nodavirus was suggested. And also other biological organisms such invertebrates might be one of the potential sources of nodavirus that can cause VNN in both cultured and wild marine fishes.

The present study has demonstrated that large populations of wild marine fish and invertebrates near the mariculture areas in the southern part of Korea are apparently infected with betanodavirus.

These wild fish and invertebrates could be a natural host or reservoir of betanodavirus and pose a serious risk for the spread of VNN to the cultured fish in Korea.

Chapter 1. Detection of betanodaviruses from cultured and wild marine and freshwater fish with no clinical signs

Apparently healthy 63 cultured fish (1 species) were collected from flounder culture facility of Jeju Island. And also 386 apparently healthy wild marine fish consisting of 57 species were collected near the culture facilities in three different areas [Donghae (East), Hwanghae (West), Namhae (South)] of Korea. A total of 36 (5 species) apparently healthy freshwater fish were also collected in the river near the culture facilities in Hwanghae (West) area. The brains of fish were examined by reverse transcriptase polymerase chain reaction (RT-PCR) and nested PCR assays. In the flounder culture facility at Jeju Island, all samples were negative for nodavirus in both RT-PCR and nested PCR tests. In Donghae (East) areas, 14

wild marine fish were negative for nodavirus in both RT-PCR and nested PCR tests. In Hwanghae (West) areas, 80 wild marine and 36 wild freshwater fish were all negative for nodavirus in both RT-PCR and nested PCR tests. While in Namhae (South) area, 10 of 292 wild marine fish were positive only for nodavirus in nested PCR test. These results indicates that nodavirus is widespread among the large populations of wild marine fish in southern part of Korea. The test also identified that 7 species of these marine fish were subclinically infected suggesting an importance of such fish as carriers or reservoir of betanodaviruses.

Chapter 2. Detection of betanodaviruses from marine and freshwater ornamental fish with no clinical signs

Sixty seven samples (24 species) of marine and freshwater ornamental fishes were collected from COEX Aquarium in Seoul from November to December, 2005. Of the 9 marine ornamental fish samples from Japan, 1 sample was positive for nodavirus by

nested PCR test. Of the 23 marine ornamental fish from Singapore, also 1 sample was positive for nodavirus by nested PCR tests. Of the 12 marine ornamental fish samples (3 species) from Korea, all samples were negative for nodavirus in both RT-PCR and nested PCR tests. Of the 4 marine ornamental fish samples, each fish samples imported from Indonesia, Canada, USA and Pacific Ocean, all samples were negative for nodavirus in both RT-PCR and nested PCR tests. Of the 19 freshwater ornamental fish collected in the Amazon River were also negative for nodavirus in both RT-PCR and nested PCR tests. Positive nested PCR results were obtained in the brains of the apparently healthy marine ornamental fish species, *Monocentris japonica* Pinecone fish and *Zebrasoma* flavesenes Yellow tang. The detection rate in nested PCR (2/67 = 3%) (Table 1) and the representative amplicons by nested PCR are shown in Fig.2. These results indicates that nodavirus is present from imported marine ornamental fish in the commercial aquarium. These subclinically infected marine ornamental fish can be one the sources of betanodavirus infection among the fishes in Korea.

Chapter 3. Detection of betanodaviruses from several invertebrates

One hundred samples (20 species) of wild marine invertebrates were collected from western and southern part of Korea. In 40 wild marine invertebrate samples (5 species) collected at Hwanghae (West) areas, all samples were negative for nodavirus in both RT-PCR and nested PCR tests. Of the 60 wild marine invertebrates (12 species) collected at Namhae (South) area, 4 invertebrate samples were positive only for nodavirus in nested PCR test. Positive nested PCR results were obtained in the brain of crab species, *Charybdis bimaculata* Charybdid crab; hepatopancreas of shrimp species, *Pandalus hypsinotus* Southern humpback shrimp and pooled organs of mussel species, *Mytilus galloprovincialis* Mediterranean mussel. The detection rate in nested PCR is 4/100 = 4%. These results indicates that nodavirus is present from wild marine invertebrates in the southern areas of Korea. These subclinically infected marine invertebrates can be one the sources of betanodavirus infection among the fishes in Korea.

REFERENCES

Arcier J. M., F. Herman, D. V. Lightner, R. Redman, J. Mari and J. R. Bonami (1999): A viral disease associated with mortalities in hatchery-reared postlarvae of the giant freshwater prawn *Macrobrachium rosenbergii*. *Dis Aquat Org* **38**, 177-181.

Arimoto M., K. Mushiake, Y. Mizuta, T. Nakai, K. Muroga and I. Furusawa (1992): Detection of striped jack nervous necrosis virus (SJNNV) by enzyme-linked immunosorbent assay (ELISA). *Fish Pathol.* **27** (4), 191-195.

Barker D.E., A. M. MacKinnon, L. Boston, M.D.B.Burt, D.K. Cone, D.J. Speare, S. Griffiths, M. Cook, R. Ritchie and G. Olivier (2002): First report of piscine nodavirus infecting wild winter flounder *Pleuronectes americanus* in Passamaquoddy Bay, New Brunswick, Canada. *Dis. Aquat. Org.* **49**, 99-105.

Bonami J. R. (1980): Recherches sur les infections virales des crustaces marins: etude des maladies a otiologie simple et complexe chez les Decapodes des cotes francaises. Ph. D. thesis, Universite des Sciences et Techniques du Languedoc, Montpellier 2.

Breuil G. and B. Romestand (1999): A rapid ELISA method for detecting specific antibody level againsts nodavirus in the serum of the sea bass, *Dicentrarchus labrax* (L.): application to the screening of spawners in a sea bass hatchery. *J Fish Dis* **22**, 45-22.

Breuil G., J.F.P. Pepin, S. Boscher and R. Thiery (2002): Experimental vertical transmission of nodavirus from broodfish to eggs and larvae of the sea bass, *Dicentrarchus labrax* (L.). *J. Fish Dis.* **25**, 697-702.

Castric J., R. Thiery, J. Jeffroy, P. de Kinkelin and J. C. Raymond (2001) Sea bream *Sparus aurata*, an asymptomatic contagious fish host for nodavirus. *Dis. Aquat. Org.*, **47**, 33-38.

Chi S.C., C.F. Lo, G.H. Kou, P.S. Chang, S.E. Peng and S. N. Chen (1997): Mass mortalities associated with viral nervous necrosis (VNN) disease in two species of hatchery-reared grouper, *Epinephelus fuscogutatus* and *Epinephelus akaara* (Temminck & Schlegel). *J Fish Dis* **20**, 185-193.

Chi S.C., J.R. Shieh and S.J. Lin (2003): Genetic and antigenic analysis of betanodaviruses isolated from aquatic organisms in Taiwan. *Dis Aquat Org* **55**, 221-228.

Chua F. H. C., J. J. Loo and J. Y. Wee (1995): Mass mortality in juvenile greasy grouper, *Epinephelus tauvina*, associated

with vacuolating encephalopathy and retinopathy. In: *Diseases in Asian aquaculture II*. (ed. by M. Shariff, J. R. Arthur and R. P. Subasinghe), pp. 235-241. Fish Health Section, Asian Fisheries Society, Manila.

Comps M., M. Trindade and C. Delsert (1996): Investigation of fish encephalitis viruses (FEV) expression in marine fishes using DIG-labeled probes. *Aquaculture* **143**, 113-121.

Curtis P.A., M. Drawbridge, T. Iwamoto, T. Nakai, R.P. Hedrick and A.P. Gendron (2001): Nodavirus infection of juvenile white seabass cultured in Southern California: First record of viral nervous necrosis (VNN) in North America. *J Fish Dis* **24**, 263-271.

Dalla Valle L., L. Zanella, P. Patarnello, L. Paolucci, P. Belvedere and L. Colombo (2000): Development of a sensitive diagnostic

assay for fish nervous necrosis virus based on RT-PCR plus nested PCR. *J Fish Dis* **23**, 321-327.

Delsert C., Morin N. and M. Comps (1997) A fish encephalitis virus that differs from other nodaviruses by its capsid protein processing. *Arch Virol* **142**, 2359-2371.

Erlich H A (1989): PCR Technology, Principles and applications for DNA amplification. Stockton, New York.

Evans B. B. and R. J. G. Lester (2001): Paasites of ornamental fish imported to Australia. *Bull Eur Assoc Fish Pathol* **21**, 51-55.

Frerichs G.N., H.D. Rodger and Z. Peric (1996): Cell culture isolation of piscine neuropathy nodavirus from juvenile sea bass, *Dicentrarchus labrax*. *J Gen Virol* **77**, 2067-2071.

Gagne N., S. C. Johnson, M. Cook-Versloot, A. M. MacKinnon and G. Olivier (2004): Molecular detection and characterization of nodavirus in several marine fish species from the northeastern Atlantic. *Dis Aquat Org* **62**, 181-189.

Garzon S. and G. Charpentier (1992): Nodaviridae. In: Adams J. R. and Bonami J. R. (eds.) *Atlas of invertebrate viruses*. pp. 351-370. CRC Press, Boca Raton, Fl.

Glazerbrook J. S., M.P. Heasman and S.W. de Beer (1990): Picorna-like viral particles associated with mass mortalities in larval barramundi, *Lates calcarifer* (Bloch). *J Fish Dis* **13**, 245-249.

Gomez D. K., J. Sato, K. Mushiake, T. Isshiki, Y. Okinaka, and T. Nakai (2004): PCR-based detection of betanodaviruses from cultured and wild marine fish with no clinical signs. *J Fish Dis* **27**, 603-608.

Grotmol S. and G.K. Totland (2000): Surface disinfetion of Atlantic halibut *Hippoglossus hippoglossus* eggs with ozonated seawater inactivates nodavirus and increases survival of the larvae. *Dis Aquat Org* **39**, 89-96.

Grotmol S., A.H. Nerland, E. Biering, G.K. Totland and T. Nishizawa (2000): Characterization of the capsid protein gene from nodavirus strain affecting the Atlantic halibut, *Hippoglossus hippoglossus* and design of an optimal reverse-transcriptase polymerase chain reaction (RT-PCR) detection assay. *Dis Aquat Org* **39**, 79-88.

Hedge A., H.C. Teh, T.J. Lam and Y.M. Sin (2003): Nodavirus infection in freshwater ornamental fish, guppy, *Poicelia reticulata* – comparative characterization and pathogenicity studies. *Arch Virol* **148** (3) 575-587.

Huang B., C. Tan, S.F. Chang, B. Munday, J.A. Matthew, G.H. Ngoh and J. Kwang (2001): Detection of nodavirus in barramundi, *Lates calcarifer* (Bloch), using recombinant coat protein-based ELISA and RT-PCR. *J Fish Dis* **24**, 135-141.

Iwamoto T., K. Mori, M. Arimoto and T. Nakai (1999): High permissivity of the fish cell line SSN-1 for piscine nodaviruses. *Dis. Aquat. Org.*, **39**, 37-47.

Iwamoto T., T. Nakai, K. Mori, M. Arimoto and I. Furusawa (2000): Cloning of the fish cell line SSN-1 for piscine nodaviruses. *Dis Aquat Org* **43**, 81-89.

Iwamoto T., T. Nakai, K. Mori, M. Arimoto, K. Mise and I. Furusawa (2001): Transfection of striped jack nervous necrosis virus (SJNNV) RNA into fish cells. *J Fish Dis* **24**, 185-188.

Iwamoto T., Y. Okinaka, K. Mise, K. Mori, M. Arimoto, T. Okuno and T. Nakai (2004): Identification of Host-Specificity Determinants in Betanodaviruses by Using Reassortants between Striped Jack Nervous Necrosis Virus and Sevenband Grouper Nervous Necrosis Virus. *J Virol* **78** (3) 1256-1262.

Jung S. J. and M. J. Oh (2000): Iridovirus-like infection associated with high mortalities of striped beakperch, *Oplegnathus fasciatus* (Temminck et Schlegel), in southern coastal areas of the Korean Peninsula. *J Fish Dis* **23**, 223-226.

Kim J. H., C. J. Hayward and G. J. Heo (2002a): Nematode worm infections (*Camallanus cotti*; *Camallanidae*) in guppies (*Poecilia reticulate*) imported to Korea. *Aquaculture* **205**, 231-235.

Kim J. H., C. J. Hayward, S. J. Joh and G. J. Heo (2002b): Parasitic infections in live freshwater tropical fishes imported to Korea. *Dis Aquat Org* **52**, 169-173.

Le Breton A., L. Grisez, J. Sweetman and F. Ollevier (1997): Viral nervous necrosis (VNN) associated with mass mortalities in cage-reared seabass, *Dicentrarchus labrax* (L.). *J. Fish Dis.*, **20**, 145-151.

Lim M. G. B., S. Y. Chong and S. Kueh (1997): Some observations of grouper (*Epinephelus tauvina*) and seabass (*Lates calcarifer*) nodavirus infection in Singapore. *Singapore Vet J* **21**, 47-51.

Lin L., J. He, K. Mori, T. Nishioka, J.L. Wu, W. Shiaoping, K. Mushiake, M. Arimoto and T. Nakai (2001): Mass

mortalities associated with viral nervous necrosis in hatchery-reared groupers in the People's Republic of China. *Fish Pathol* **36** 186-188.

Mari J. (1987): Recherches sur les maladies virales du Crustace Decapode marin Carcinus mediterraneus Czerniavsky 1884. PhD thesis, Universite de Montpellier 2.

Melchers W.J.G., R. Schrift, E. Stolz, J. Lindeman and W.G.V. Quint (1989): Human papilloma virus detection in urine samples from male patients by the polymerase chain reaction. *J Clin Microbiol* **27**, 1711-1714.

Mori K., T. Nakai, M. Nagahara, K. Muroga, T. Mekuchi and T. Kanno (1991): A viral disease in hatchery-reared larvae and juveniles of redspotted grouper. *Fish Pathol* **26**, 209-210.

Mori L., T. Nakai, K. Muroga, M. Arimoto, K. Mushiake and I. Furusawa (1992): Properties of a new virus belonging to Nodaviridae found in larval striped jack (*Pseudocaranx dentex*) with nervous necrosis. *Virology* **187**, 368-371.

Mori K., K. Mushiake and M. Arimoto (1998): Control measures for viral nervous necrosis in striped jack. *Fish Pathol* **33**, 443-444.

Mori K., T. Mangyoku, T. Iwamoto, M. Arimoto, S. Tanaka and T. Nakai (2003): Serological relationships among genotypic variants of betanodavus. *Dis Aquat Org* **57**, 19-26.

Munday B.L. and T. Nakai (1997): Special topic review Nodavirus as pathogens in larval and juvenile marine finfish. *World J Microbiol Biotech* **13**, 357-381.

Munday B.L., J. Kwang and N. Moody (2002): Betanodavirus infections of teleost fish: a review. *J Fish Dis* **25**, 127-142.

Muroga K., T. Furusawa and I. Furusawa (1998): A review: viral nervous necrosis in striped jack, *Pseudocaranx dentex*. *Suisan Zoshoku* **46**, 473-480 (In Japanese)

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

Mushiake K., T. Nishizawa, T. Nakai, I. Furusawa and K. Muroga (1994): Control of VNN in striped jack: selection of spawners based on the detection of SJNNV gene by polymerase chain reaction (PCR). *Fish Pathol* **29** (3), 177-182.

Nakai T., H.D. Nguyen, T. Nishizawa, K. Muroga, M. Arimoto and K. Ootsuki (1994): Occurrence of viral nervous necrosis in kelp grouper and tiger puffer. *Fish Pathol* **29**, 211-212.

Nakai, T., T. Iwamoto, K. Mori, M. Arimoto and S. Tanaka (2001): Recent developments in identification and control of viral nervous necrosis (VNN) of grouper pp. 81-85. In: Bomdad-Reantas, J., Humprey S., Kanchankhan and S. chinabut (Eds.) *Report and proceedings of APEC FWG Project 02/2000 "Development of a Regional Research Programme on Grouper Virus Transmission and Vaccine Development", 18-20 October 2000 Bangkok Thailand*. Aisa Pacific Economic cooperation (APEC) Aquatic animal Health Research Institute (AAHRI) Fish health Section of the Asian Fisheries society (FHS/AFS) and network of Aquaculture Centres in Asia-Pacific (NACA). Bangkok, Thailand.

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

Nishizawa T., K. Muroga and M. Arimoto (1996): Failure of the polymerase chain reaction (PCR) method to detect striped jack nervous necrosis virus (SJNNV) in striped jack *Pseudocaranc dentex* selected as spawners. *J Aquat Animal Health* **8**, 332-334.

Nishizawa T., M. Furuhashi, T. Nagai, T. Nakai and K. Muroga (1997) Genomic classification of fish nodaviruses by molecular phylogenetic analysis of the coat protein gene. *Appl. Environ Microbiol* **63**, 1633-1636.

Nguyen H.D., T. Mekuchi, K. Imura, T. Nakai, T. Nishizawa and K. Muroga (1994): Occurrence of viral nervous necrosis (VNN) in hatchery-reared juvenile Japanese flounder *Paralichthys olivaceus*. *Fish Sci* **60**, 551-554.

Odier F. (1974): Mise en evidence et etude dun complexe de maladies a parvovirus, baculovirus et iridovirus. *Entomophaga* **22**, 397-404.

Office International des epizooties OIE (1997): International aquatic animal health code. In: *Manual of Diagnostic Tests for Aquatic Animals*, p. 267.OIE, Paris.

Office International des epizooties OIE (2003): Viral encephalopathy and retinopathy. In: *Manual of Diagnostic Tests for Aquatic Animals*, pp. 135-141.OIE, Paris.

Oh M. J. and T. J. Choi (1998): A new rhabdovirus (HRV-like) isolated in Korea from cultured Japanese flounder *Paralichthys olivaceus*. *J Fish Pathol* **11(2)**, 129-136.

Oh M. J. and S. J. Jung (1999): Viral diseases of fish in Korean mariculture. In: Shin J. A. (ed.), *Efficient Application and Preservation of Marine Biological resources. Proceedings of Fifth International Symposium, 7-8 Oct. 1999*. pp. 25-32. Yosu, Korea.

Oh M. J., S. J. Jung, S. R. Kim, K. V. Rajendran, Y. J. Kim, T. J. Choi, H.R. Kim, J. D. Kim (2002): A fish nodavirus associated with mass mortality in hatchery-reared red drum, *Sciaenops ocellatus*. *Aquaculture* **211**, 1-7.

Park M. A. and S. G. Sohn (2001): Status of diseases of seven band grouper, Epinephelus sepetemfasciatus, in Korea RO. In:

Report and proceeding of APEC FWG Project 02/2000 "Development of a Regional Research Programe on Grouper Virus Transmission and Vaccine Development", 18-20 October 2000, Bangkok, Thailand. pp. 35-37. Asia Pacific Economic Cooperation (APEC), Aquatic animal Health Research Institute (AAHRI), Fish Health Section of the Asian Fisheries Society (FHS/AFS) and the Network of Aquaculture Centres in Asia-Pacific (NACA). Bangkok, Thailand.

Persing D.H., T.F. Smith, F.C. Tenover and T.J. White (1993): Diagnostic molecular microbiology: Principles and Application. American Society Microbiology, Washington, D.C.

Qian D., Z. Shi, S. Zhang, Z. Cao, W. Liu, L. Li, Y. Xie, I. Cambournac and J. R. Bonami (2003): Extra small virus-like particles (XSV) and nodavirus associated with whitish

muscle disease in the giant freshwater prawn, *Macrobrachium rosenbergii*. *J Fish Dis 26*, 521-527.

Romestand B. and J. R. Bonami (2003): A sandwich enzyme linked immunosorbent assay (S-ELISA) for detection of MrNV in the giant freshwater prawn, *Macrobrachium rosenbergii* (de Man.). *J Fish Dis* **26**, 71-75.

Sahul Hameed A. S., K. Yoganandhan, J. Sri Widada and J. R. Bonami (2004): Experimental transmission and tissue tropism of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and its associated extra small virus (XSV). *Dis Aquat Org* **62**, 191-196.

Skliris G.P. and R.H. Richards (1999): Induction of nodavirus disease in seabass, *Dicentrarchus labrax*, using different infection models. *Virus Res* **63**, 85-93.

Sohn S. G., M. A. Park, M. J. Oh and S. K. Chun (1998): A fish nodavirus isolated from cultured sevenband grouper, *Epinephelus septemfasciatus*. *J Fish Pathol* **11**, 68-73.

Sri Widada J., S. Durand, I. Cambournac, D. Qian, Z. Shi, E. Dejonghe, V. Richard and J. R. Bonami (2003): Genome-based detection methods of *Macrobrachium rosenbergii* nodavirus, a pathogen of the giant freshwater prawn, *Macrobrachium rosenbergii*: dot-blot, in situ hybridization and RT-PCR. *J Fish Dis* **26**, 583-590.

Tan C., B. Huang, S.F. Chang, G.H. Ngoh, B. Munday, S.C. Chen and J. Kwang (2001): Determination of the complete nucleotide sequence of RNA1 and RNA2 from greasy grouper (*Epinephelus tauvina*) nervous necrosis virus, Singapore strain. *J Gen Virol* **82**, 647-653.

Tanada Y. (1956): Some factors affecting the susceptibility of the armyworms to virus infections. *J Econ Entomol* **49**, 52-57.

Thiery R., S. Peducasse, J. Castric, A. Le Ven, J. Jeffroy and F.B. Laurencin (1997): Experimental transmission of viral encephalopathy and retinopathy to juvenile sea bass (*Dicentrarchus labrax*). *Bull Eur Assoc Fish Pathol* **17** (3/4), 118.

Thiery R., J.C. Raymond and J. Castric (1999): Natural outbreak of viral encephalopathy and retinopathy in juvenile sea bass, *Dicentrarchus labrax*: study by nested reverse transcriptase-polymerase chain reaction. *Virus Res* **63**, 11-17.

Tsuchihashi Y., I. Kuriyama, Y. Kuromiya, M. Kashiwagi and M. Yoshioka (2002): Control of viral nervous necrosis (VNN) in

seedling production of sevenband grouper, *Epinephelus septemfasciatus*. *Suisanzoshoku* **50**, 355-361. (In Japanese)

van Regenmortel M. H. V., C. M. Fauquet, D. H. L. Bishop, E. B. Cartens and 7 others (2000): Virus taxonomy: classification and nomenclature of viruses. Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, CA.

Watanabe K., T. Nishizawa and M. Yoshimizu (2000): Selection of broodstock candidates of barfin flounder using an ELISA system with recombinant protein of barfin flounder nervous necrosis virus. *Dis Aquat Org* **41**, 219-223.

Yoshikoshi K and K. Inoue (1990): Viral nervous necrosis in hatchery-reared larvae and juveniles of Japanese parrotfish, *Oplegnathus fasciatus* (Temminck & Schlegel). *J Fish Dis* **13**, 69-77.

어류와 무척추동물의 바이러스성 신경괴사증

(지도교수 : 박 전 홍)

임 동 주

제주대학교 수의학과

바이러스성 신경괴사(Viral Nervous Necrosis, VNN)증이 1990 년 일본에서 패럿 피쉬(*Oplegnathus fasciatus*) 알과 치어(새끼)에서 보고 된 이후 전 세계 바다 어류 양식장에서 Betanoda 바이러스를 원인체로 한 바이러스에 18 과 35 종 이상의 양식어가 감염되어 폐사되는 큰 경제적 피해가 있었다. 국내에서는 많은 수생동물이 원인을 모르고 폐사를 하고 있어 최근 국제적으로 문제가 되고 있으며 수생동물의 많은 폐사를 야기하는 VNN 을 초점으로 해수어, 담수어, 관상어 및 갑각류에서 RT-PCR 법과 nested PCR 법으로 VNN 감염 여부를 조사하였다.

제주 양식장에서 외관상으로 건강한 넙치 63 마리, 동해와 서해 그리고 남해에서 자연산 해수어 57 종 386 마리, 서해안으로 흐르는 곡릉천(경기도 파주) 에서 건강한 담수어 5 종 36 마리를 채취하여 RT-PCR 법과 nested PCR 법으로 VNN 감염여부를 조사하였다. 제주도 넙치양식장에서 채취한 넙치, 동해 자연산 해수어 14 마리, 서해 자연산 해수어 80 마리와 자연산 담수어 36 마리는 RT-PCR 과 nested PCR 법으로 음성이다. 그러나 남해 자연산 해수어 292 마리 중 10 마리에서 nested PCR 법으로 Betanoda 바이러스가 검출되었다.

서울 소재 수족관의 도움을 받아 외국에서 수입되는 관상어 총 24 종 69 마리를 채취하여 RT-PCR 법과 nested PCR 법으로 VNN 감염여부를 조사하였다. 이중 일본에서 수입된 관상어 9 마리 중 1 마리와 싱가포르에서 수입된 23 마리의 해수어 중 1 마리는 nested PCR 법으로 Betanoda 바이러스 양성이었다. 이 바이러스 양성인 해수어는 외관상으로 임상증상이 없는 파인콤 피쉬(*Monocentris japonica*)와 옐로우 탱(*Zebrasoma flavesenes*) 이며 검출률은 3%다. 그러나 한국산 해수어 3 종 12 마리,

인도네시아, 캐나다, 미국 그리고 태평양에서 수입된 4 마리의 해수 관상어, 남미 아마존 강에서 채취한 19 마리의 담수어는 RT-PCR 과 nested PCR 법으로 Betanoda 바이러스가 검출되지 않았다. 서해와 남해에서 20 종 100 마리의 무척추동물을 채취하여 RT-PCR 법과 nested PCR 법으로 VNN 감염여부를 조사하였다. 서해에서 채집한 5 종 40 마리의 무척추동물에서는 Betanoda 바이러스가 검출 되지 않았으나 남해에서 채집한 12 종 60 마리의 무척추동물 중 4 마리가 양성이었다. 바이러스 양성인 무척추동물은 게의 일종인 채리디드 게 (*Charybdis bimaculata*)와 곱사등이 새우 (*Pandahus hypsinotus*) 그리고 지중해 홍합 (*Mytilus galloprovincialis*) 이며 Nested PCR 법으로 검출률은 4%다.

이와 같은 실험결과 한반도 남해에 서식하는 일부 어류와 무척추동물이 Betanoda 바이러스에 감염되었으며 감염된 동물은 Betanoda 바이러스를 전파하는 보균자 또는 숙주로서 위험성을 시사하고 있다. 더욱이 일부 수입되는 관상어가 Betanoda 바이러스에 감염된 것을 알 수 있어

무분별한 관상어 수입은 우리나라에 Betanoda 바이러스를 퍼트리는 요인이 될 수도 있다. 따라서 종묘생산에 있어 친어의 질병진단을 통한 건강한 치어의 생산과 생사료(꽁치, 멸치 등을 갈아 양식어류의 먹이로 쓰고 있음) 사용금지를 통한 질병의 재발방지 그리고 검역강화를 통한 외부유래의 질병을 막을 필요가 있다.

감사의 글

이번 박사학위 논문을 쓰면서 그간 이 실험을 지도해주고 격려를 아기지 않았던 제주대학교 수의학과 박전홍 교수를 위시하여 생리학교실의 대학원생들에게 감사를 드린다.
또한 이 실험의 완성을 위해 여러 가지의 일들과 자문을 해준 서울대학교 수의과대학 양일석 학장과 수의학과 수생동물질병학교실의 박세창 교수와 그리고 어류 질병학자 Gomez 박사에게도 심심한 감사를 드린다.

이뿐만이 아니라 실험 기간 중 많은 도움을 준 경상대학교 수의과대학 어패류질병학 교실 정태성 교수에게도 지면을 빌어 감사를 표한다.
이 외에도 일일이 이름을 밝히지는 않지만 물고기의 채취에 협력을 아끼지 않았던 여러 분들에게도 감사의 뜻을 표한다.
또한 일요일에도 마다 않고 나와 서류정리에 힘을 써준 차근희 양에게도 감사를 드린다.

또한, 불평 한마디 없이 뒤에서 묵묵히 지원해주었던 아내에게도 감사를 드린다.

임동주