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Genetic and antigenic analysis of betanodaviruses isolated from aquatic organisms in Taiwan

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ABSTRACT: Viral nervous necrosis (VNN) is a worldwide disease among marine fishes. In Taiwan, NNN disease was first identified in 2 species of hatchery-reared grouper, *Epinephelus fuscogutatus* and *E. akaaya* in 1994. Since then, increasing mortalities have occurred among groupers *Epinephelus* spp., and also among European eels *Anguilla anguilla* L., yellow-wax pompano *Trachinotus falcatus*, firespot snapper *Lutaanus erythropterus* B., barramundi *Lates calcarifer*, cobias *Rachycentron canadum*, humpback groupers *Cromileptes altivelis* and Chinese catfish *Parasilurus asotus*. In the present study, samples were collected from affected fishes and processed for reverse transcriptase (RT) PCR amplification and virus isolation in cell culture. Infected cells (GF-1 cell line) exhibited cytopathic-effect characteristics of grouper nervous necrosis virus (GNNV). A RT-PCR product of approximately 830 bp was amplified from the brain homogenate of tested samples and sequenced. The nucleotide and deduced amino acid sequences of the amplified RT-PCR products from all isolates were strongly homologous (>97%) with the corresponding region of the published sequence of red-spotted grouper nervous necrosis virus (RGNVV). Therefore, all Taiwan NNV (nervous necrosis virus) isolates studied in this report belong to the RGNNV genotype. We used 5 neutralizing monoclonal antibodies (MAbs) against GNNV to analyze the antigenic relationship of Taiwan NNV isolates and striped jack nervous necrosis virus (SJNNV). The results of neutralization tests revealed that all Taiwan NNV isolates were closely related, but antigenically different from SJNNV in 3 neutralizing epitopes. To our knowledge, this is the first description of NNV infection in European eels, yellow-wax pompano, firespot snapper, cobia and Chinese catfish, and the first reported instance of natural NNV infection in freshwater fishes causing high mortality.

KEY WORDS: Fish nodavirus · Betanodavirus · Nervous necrosis virus · NNV · Monoclonal antibody · Neutralizing epitope

INTRODUCTION

Nervous necrosis viruses (NNV) are classified as genus betanodavirus in the family Nodaviridae, and are the pathogens of VNN. They have caused mass mortality among larvae and juveniles in 32 species of fishes (Munday et al. 2002). Of the 32 host fish species, 6 were isolated in Taiwan (Chi et al. 1997, 2001a, Lai et al. 2001). Affected fishes show abnormal swimming behavior. The most common histopathological changes observed are necrosis and vacuolation of the brain and retina (Mori et al. 1992, Comps et al. 1994, Chi et al. 1997).

The genome of betanodaviruses contains 2 segments of single-stranded, positive-sense RNA. RNA1 encodes RNA-dependent RNA polymerase, and RNA2 encodes capsid protein (Mori et al. 1992). Based on the partial sequences of RNA2 from 25 isolates of betanodavirus, they are classified into 4 genotypes: barfin flounder nervous necrosis virus (BFNNV), tiger puffer nervous necrosis virus (TPNNV), striped jack nervous necrosis virus (SJNNV) and red-spotted grouper nervous necrosis virus (RGNNV) (Nishizawa et al. 1997). The optimal growth temperatures of the 4 genotypes in the E-11 cell line are 15 to 20°C for BFNNV, 20°C for TPNNV, 20 to 25°C for SJNNV and 25 to 30°C for RGNNV (Iwamoto et al. 2000). The genetic diversity of betanodaviruses reflects phenotypic differences which may

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constitute adaptations to enable the infection of different hosts and replication at different temperatures (Totland et al. 1999).

The aquaculture of marine fishes is a very important industry in Taiwan. However, VNN has repeatedly occurred among hatchery-reared larvae and juveniles of groupers *Epinephelus* spp. since 1994 (Chi et al. 1997). A betanodavirus was isolated from moribund grouper larvae by the GF-1 cell line and was designated as grouper nervous necrosis virus (GNNV) (Chi et al. 1999a). The optimal temperature range for GNNV proliferation in the GF-1 cell line is 28 to 32°C (Chi et al. 1999b). In recent years, VNN has occurred not only in groupers but also in other economically important species of fishes (Chi et al. 2001a). This report investigates the host range of the betanodavirus in Taiwan and analyzes the genetic as well as antigenic relationships of isolates from different species of fishes.

MATERIALS AND METHODS

Sources of NNV isolates. Table 1 summarizes the aquatic organisms collected for NNV examination. All organisms were collected from hatchery farms in southern Taiwan except European eels (EE98PH), which were collected from Pong-Hu, an island off Taiwan. Brine shrimp *Artemia* sp. nauplii, the copepod *Tigriopus japonicus* and the shrimp *Acetesinte medius* were also collected from grouper hatchery farms where VNN had occurred, and were used for RT-PCR and semi-nested PCR examination.

Mass mortality had been observed among groupers *Epinephelus* spp. (G9410YA, G9508KS), European eels *Anguilla anguilla* (EE98PH), yellow-wax pompano *Trachinotus falcatus* (YP99PD), baramundi *Lates calcarifer* (BOOGD), cobia *Rachycentron canadum* (Co00CC1, Co00CC2), and Chinese catfish *Parasilurus asotus* (CC01YL), and samples collected during outbreaks of mass mortality were also examined for bacteria and parasites.

Virus isolation by GF-1 cell line. The GF-1 cell line (Chi et al. 1999a) was used for virus isolation. Homogenates of brain and retina were filtered through 0.2 µm Millipore membranes, and inoculated into GF-1cells. After 6 d incubation, a cytopathic effect (CPE) was evident and the supernatants were collected for further study.

Extraction of viral RNA and PCR amplification. Total RNA was isolated from fish brain tissue or (for live food organisms) from the whole organism following the single-step acid guanidinium thiocyanate-phenol-chlorofrom extraction method described by Chomczynski & Sacchi (1987). For reverse-transcription, extracted viral RNA was incubated at 42°C for 30 min in 40 µl 2.5 × PCR buffer (25 mM Tris-HCl, pH 8.8, 3.75 mM $MgCl_2$, 125 mM KCl, 0.25% Triton X-100) that contained 2 U Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega), 0.4 U RNasin (Promega), 0.25 mM dNTP, and 0.5 µM reverse primer R3 (5′-CGAGTCAACACGGGT-GAAGA-3′). Following cDNA synthesis, 40 µl of the cDNA mixture was diluted 2.5-fold with diethyl pyrocarbonate (DEPC)-treated H_2O that contained 0.025 U *Tag* DNA polymerase (Boehringer Mannheim Biochemicals), 0.1 mM dNTP and 0.5 µM forward primer F1 (5′-GGATTTGGACGTGCGAC-CAA-3′). This mixture was then incubated in an automatic thermal cycler (TouchDown™ thermal cycler, Hybaid). Cycling conditions were 94°C for 3 min followed by 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s, with a final extension of 5 min at 72°C. Nucleic acids extracted from GNNV-affected grouper larvae were used as positive controls, and DEPC-treated water was used as a negative control.

Table 1. Sources of NNV isolates used in this study

Virus isolate	Source of isolate	Stage	Year of isolation
G9410YA	Black-spotted grouper *Epinephelus fuscoguttatus*	Juvenile	1994
G9508KS	Grouper *Epinephelus coioides* H.	Larvae	1995
EE98PH	European eel *Anguilla anguilla* L.	Juvenile	1998
EE00TN	European eel *Anguilla anguilla* L.	Juvenile	2000
YP99PD	Yellow-wax pompano *Trachinotus falcatus*	Juvenile	1999
YP00FL	Yellow-wax pompano *Trachinotus falcatus*	Juvenile	2000
FS99PD	Firespot snapper *Lutaanus erythropterus* B.	Larvae	1999
B99GD	Barramundi *Lates calcarifer*	Larvae	1999
B00GD	Barramundi *Lates calcarifer*	Larvae	2000
Co00GD	Cobia *Rachycentron canadum*	Juvenile	2000
Co00CC1	Cobia *Rachycentron canadum*	Juvenile	2000
Co00CC2	Cobia *Rachycentron canadum*	Juvenile	2000
HG00GD	Humpback grouper *Cromileptes altivelis*	Juvenile	2000
CC01YL1	Chinese catfish *Parasilurus asotus*	Juvenile	2001

Primers F2 (5′-CGAGTCAACACGGGTGAAGA-3′) and R3 were used for the semi-nested PCR. Target regions T2 (870 bp) and T4 (421 bp) were amplified by RT-PCR and semi-nested PCR respectively. Primer sequences were based on published nucleotide sequence data (Nishizawa et al. 1994). Reaction products were analyzed using 1.5 % agarose gel.

Deduced amino acid sequence alignment and phylogenetic tree construction. PCR products were extracted from agarose gel, purified, ligated into pGEM-T easy vector (Promega), transformed into *Escherichia coli* XL1-Blue (BRL, Stratagene), and then sequenced. Automatic sequencing was performed commercially (Mission Biotech). Multiple alignments of determined nucleotide and deduced amino acid sequences were constructed with the Lasergene program (DNASTAR).

In the phylogenic analysis we used 16 nucleotide sequences of Nucleotides 159 to 988 of the capsid protein gene. The analysis was performed using the neighbor-joining (NJ) (Saitou & Nei 1987) and maximum parsimony (MP) (Swofford 1993) methods with the Phylogenetic Analysis Using Parsimony (PAUP) 4.0b1 program. Bootstrap analysis was performed using 1000 data re-samplings.

The GenBank accession numbers of the new sequences reported in this paper are Accession No. AY140793 (barramundi, B00GD), AY140794 (Chinese catfish, CC01YL1), AY140795 (cobia, Co00CC1), AY140796 (European eel EE98PH), AY140797 (Firespot snapper, FS99PD), AY140798 (grouper, G9508KS), AY140799 (humpback grouper, HG00GD), AY140800 (yellow-wax pompano, YP99PD) and AY140801 (black-spotted grouper, G9410YA). The sequences of 4 NNV genotypes (Nishizawa et al. 1997) and 2 Taiwan NNV isolates (Lin et al. 2001) were retrieved from the GenBank: D30814 (striped jack, SJNNV), D38637 (tiger puffer, TPNNV), D38635 (barfin flounder, BFNNV), D38636 (red-spotted grouper, RGNNV), AF245003 (manabaricus grouper, MGNNV) and AF245004 (dragon grouper, DGNNV). The nucleotide sequence of another Taiwan NNV isolate (yellow grouper, YGNNV) was taken from the study of Lai et al. (2001).

Neutralization test. We established 5 neutralizing monoclonal antibodies (2E, 9B, 9D, 10G and 12E) using GNNV as an immunogen which was propagated and characterized in the GF-1 cell line (Chi et al. 2001c). The 50% neutralization doses (ND_{50}) of these 5 monoclonal antibodies (MAbs) against 100 $TCID_{50}$ GNNV are 2^{6-12}. MAb 9D and 10G can react with denatured GNNV capsid polypeptide in the Western immunoblot assay, but MAbs 2E, 9B and 12E cannot. In an enzyme-linked immunosorbant assay (ELISA), MAbs 9D and 10G showed a positive reaction with purified GNNV proliferated in GF-1, but a negative reaction with the recombinant GNNV capsid protein expressed in *E. coli* (Chi et al. 2001c).

In the present study, the alpha neutralization method (constant antibody plus virus dilution) was applied for the antigenic analysis of betanodaviruses. The culture medium from each monoclonal hybridoma clone was initially diluted 5-fold, and then used as the stock MAb solution; 1 ml of this solution was mixed with an equal volume of serial 10-fold diluted viral solution and incubated at room temperature for 1 h with gentle shaking. The mixture was then inoculated into GF-1 cells preseeded in a 96-well plate. The culture plates were incubated at 28°C for 6 d. The neutralization index (NI) was determined as the difference between the titer of the control and that of the MAb-treated viral samples. The neutralization effect is expressed as $\log_{10}$NI, and is considered to be significant when $\log_{10}$ NI exceeds 1.7 (Mahy & Kangro, 1996).

RESULTS

Clinical signs and gross pathology

Some fish samples collected during the outbreak of mortality, including groupers (G9410YA, G9508KS), European eels (EE98PH), yellow-wax pompano (YP-99PD), barramundi (B99GD, B00GD), cobia (Co00CC1, Co00CC2) and Chinese catfish (CC01GY), displayed abnormal swimming behavior, loss of appetite, and mass mortality (>80%). Diseased fishes had no bacterial or parasitic infections, and examination of their internal organs revealed no special morphological changes in most cases. The mortalities of EE00TN (European eel), YP00FL (yellow-wax pompano), FS99PD (firespot snapper), B99GD (barramundi), Co-00GD (cobia) and HG00GD (humpback grouper) were much lower (<30%).

Virus isolation by GF-1 cell line

Complete CPE was observed in GF-1 cells separately inoculated with the brain homogenates of groupers (G9410YA, G9508KS), European eels (EE98PH), yellow-wax pompano (YP99PD), barramundi (B00GD), cobia (Co00CC1, Co00CC1, Co00CC2) and Chinese catfish (CC01YL). CPE developed initially as areas of rounded, granular cells, and finally the whole cell-sheet degenerated and detached from the flask surface. The characteristics of CPE observed here are identical to those of the CPE of GNNV-infected GF-1 cells described by Chi et al. (1999a).

Table 2. Mortality and diagnosis results of PCR and virus isolation in fishes with VNN. Mortality = ++: cumulative mortality >70%; +: cumulative mortality <30%; –: no mortality; cytopathic effect (CPE) = ++: whole cell-sheet detached and degenerated; +: some infected cells swollen and detached

Fish	Mortality	PCR		CPE of GF-1
		RT (T2)	Semi-nested (T4)	
Grouper (G9410YA)	++	+	+	++
Grouper (G9508KS)	++	+	+	++
European eel (EE98PH)	++	+	+	++
European eel (EE00TN)	+	–	+	+
Yellow-wax pompano (YP00FL)	+	–	+	+
Yellow-wax pompano (YP99PD)	++	+	+	++
Firespot snapper (FS99PD)	–	–	+	+
Barramundi (B99GD)	+	–	+	+
Barramundi (B00GD)	++	+	+	++
Cobia (Co00GD)	+	–	+	+
Cobia (Co00CC1)	++	+	+	++
Cobia (Co00CC2)	++	+	+	++
Humpback grouper (HG00GD)	–	–	+	+
Chinese catfish (CC01YL1)	++	+	+	++

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          54         64         74         84         94         104        114        124        134        144
RGNNV     DVHLSGMSRI SQAVLPAGTG TDGYVVVDAT IVPDLLPRLG HAARIFQRYA VETLEFEIQP MCPANTGGGY VAGFLPDPTD NDHTFDALQA TRGAVVAKWW
G9410YA   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
G9508KS   .......... .......... .......... .......... .T........ .......... .......... .......... .......... ..........
EE98PH    .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
YP99PD    .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
FS99PD    .......... .......... .......... .......... .......... ....G..... .......... .......... ....L..... ..........
BOOGD     .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Co00CC1   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
HG00GD    .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
CC01YL1   .......... .......... .......... .......... .......... .......... .......... A......... .......... ..........
DGNNV     .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
MGNNV     .......... .......... .......... .......... .......... ..A....... .......... .......... .......... ..........
YGNNV     .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
SJNNV     ...I...... A...V..... ...KI...S. ...E...... .......... .......... .......... .......... .......... ..........
TPNNV     .......... A....T.... A......NEI ...E...... .........I ......D... .......... .......... .......I.. ..........
BFNNV     ....T..... A...E..... ....I...E. ...E...... F......... .......... .......... S......I.. ..........
          154        164        174        184        194        204        214        224        234        244
RGNNV     ESRTVRPQYT RTLLWTSSGK EQRLTSPGRL ILLCVGNNTD VVNVSVLCRW SVRLSVPSLE TPEETTAPIM TQGSLYNDSL ST--NDFKSI LLGSTPLDIA
G9410YA   .......... .......... .......... .......... .A........ .......... .......... .......... ..--...... ..........
G9508KS   .......... .......... .......... .......... .......... .......... .......... .......... ..--...... ..........
EE98PH    .......... .......... .......... .......... .......... .A........ .......... .......... ..--...... ..........
YP99PD    .......... .......... .......... .......... .......... .......... .......... .......... ..--T..... ..........
FS99PD    .......... .......... .......... .......... .......... .......... .......... .......... ..--...... ..........
B00GD     .......... .......... .......... .......... .......... .......... .......... .......... ..--...... ..........
Co00CC1   .......... .......... ....M..... .......... .......... .......... .......... .......... ..--...... ..........
HG00GD    .......... .......... .......... .......... .......... .......... .......... .........P ..--...... ..........
CC01YL1   .......... .......... .......... .......... .......... .......... .......... .......... ..--...... ..........
DGNNV     .......... .......... .......... .......... .......... .......... .......... .......... ..--...... ..........
MGNNV     .......... .......T.. .......... .......... .......... .......... .......... .......... ..--...... ..........
YGNNV     .......... .......... .......... .......... .......... .......... .......... .......... ..--...... ..........
SJNNV     .......... ......T.. .......... V.....S... ......M... .......... ...D.....T ..AP.H...I NNGYTG.R.. .....Q..L.
TPNNV     .........A .......T.. .......... ......S... .......... .....F...T S..P.....I T.ATSG.R.. ....GQ....
BFNNV     ....I....A .A.....V.. .......... .......... .......... ...D.F...L .L.P...... AA--...... .....Q....
          254        264        274        284        294        304        314        324
RGNNV     PDGAVFQLDR PLSIDYSLGT GDVDRAVYWH IKKFAGNAGT PAGWFRWGIW DNFNKTFTDG VAYYSDEQPR QILLPVGT
G9410YA   .......... .......... .......... L......... .......... .......... .......... ........
G9508KS   .......... .......... .......... L......... .......... .......... .......... ........
EE98PH    .......... .......... .......... L......... .......... .......... .......... ........
YP99PD    .......... .......... .......... L...G..... .......... .......... .......... ........
FS99PD    ......R... .P.......I .......... L......... .......... .D........ .......... ........
BOOGD     .......... .......... .......... L......... .......... .......... .......... ........
Co00CC1   .......... .......... .......... L......... .......... .......... .......... ........
HG00GD    .......... .......... .......... L......... .......... .......... .......... ........
CC01YL1   .......... .......... .......... L......... .......... .......... .......... ........
DGNNV     .......... .......... .......... L......... .......... .......... .......... ........
MGNNV     .......... .......... .......... L......... .......... .......... .......... ........
YGNNV     .......... .......... .......... L......... .......... .......... .......... ........
SJNNV     .AN...VT.K ..P...N..V .......... LQ.K..DTQV ...Y.D..L. .D......V. AP....Q... .....A..
TPNNV     .P.T.YSI.. ......N..V .......... LL.KK.DPNN ...FLD..L. .D...VLH.C ......Q... ........
BFNNV     .E...YS... .......... .......... V..V...V.A .....H..L. .........Q. ......A... ........
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Fig. 1. Multiple alignment of deduced amino acids encoded by T2 region of RNA2 of NNV isolates (RG: red-spotted grouper; DG: dragon grouper; MG: manabaricus grouper; SJ: striped jack, TP: tiger puffer; BF: barfin flounder; other isolate abbreviations as in Table 1). Dots: amino acid residue identical to that at same position; dashes: gap inserted; AN: neutralization epitope of SJNNV

RT-PCR and semi-nested PCR examination

Table 2 summarizes the results of PCR amplification of fish brain homogenates. The T2 target fragment of the NNV capsid gene was easily detected by RT-PCR in those fish samples suffering mass mortality, while only the T4 target fragment of NNV was detected in semi-nested PCR in fish samples suffering minor or no mortality. The T4 target fragment of NNV was also detected in *Artemia* sp. nauplii, the copepod *Tigriopus japonicus* and the shrimp *Acetesinte medius* by semi-nested PCR amplification.

Comparisons of nucleotide and deduced amino acid sequences

The identities of the sequences of nucleotide 159-988 of the NNV capsid protein gene between all Taiwan isolates and RGNNV were >98.7%; however, the identities among all Taiwan isolates and the other 3 NNV genotypes varied from 75.1 to 83.1%. Furthermore, the nucleotides at Positions 564, 619 and 844 were highly conserved as C in all Taiwan isolates compared to T (564, 619) and A (844) in RGNNV.

The identities of the deduced amino acid (aa) sequence of the T2 region (aa 54 to 331) between Taiwan isolates and RGNNV were >97.5%, while the identities among the Taiwan isolates and the other 3 NNV genotypes ranged from 80.4 to 89%. The alignment of deduced amino acid sequences for the T2 region revealed a highly conserved region at aa 88 to 216 among 4 NNV genotypes and the Taiwan isolates (Fig. 1). Cysteine residues at Positions 115, 187 and 201 were fully conserved in all isolates analyzed; 2 cysteine side groups can form a covalent disulphide bond S. Disulphide bonds between cycteines are widespread in proteins and are important in determining 3-dimensional conformation and in holding together multisubunit proteins. Therefore, it is suggested that the conservation of these 3 cysteines in all the NNV isolates is important for the structural stability of the NNV coat protein. All Taiwan isolates and the RGNNV lack 2 amino acids at the positions corresponding to aa 236 and 237 of the SJNNV. Among the deduced amino acids in the T2 region of the RGNNV isolate from Japan and the corresponding residues in all Taiwan isolates, substitutions at 17 positions are observed (Fig. 1). The leucine (L) residue at Position 284 is highly conserved in all Taiwan isolates, whereas the isoleucine (I) residue is in RGNNV.

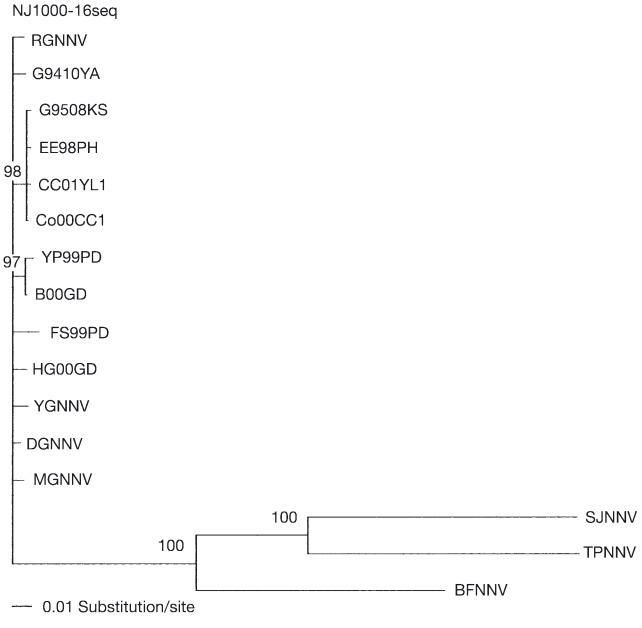

Fig. 2. Neighour-joining phylogenetic tree deduced from analysis of T2 region of nucleotide sequence of coat protein gene from 16 NNV isolates. Numbers on branch nodes indicate percent bootstrap support for that node with 1000 replications. Virus isolates abbreviated as in Table 1 (see also Nishizawa et al. 1997, Lai et al. 2001 and Lu et al. 2001)

Phylogenetic analysis

Nucleotide sequences of the T2 region of the NNV capsid protein gene from 16 isolates were used to construct a phylogenetic tree to examine the relationship between the NNV isolates identified in this study and previously identified isolates. Since both the NJ and the maximum parsimony tree generated similar results, only the NJ tree is shown here (Fig. 2). Although the host range of Taiwan NNV isolates includes 11 species of fishes, the phylogenetic analysis revealed that all Taiwan isolates belong to the RGNNV genotype.

Neutralization test

A neutralization test analyzed the antigenic relationships among viral isolates from different species of fishes, and compared neutralization epitopes among different genotypes (Table 3). All the tested Taiwan isolates could be significantly neutralized by all 5 MAbs antibodies, except for the catfish isolate (CC01YL1), which was poorly neutralized by MAb 2E (log NI = 1.6). Moreover, the striped jack isolate SJNNV could be neutralized only by MAbs 2E and 10G, and not by MAbs 9B, 9D or 12E. Therefore, the result of antigenic analysis between Taiwan isolates and the SJNNV isolate is in good agreement with the results of the phylogenetic analysis.

Table 3. Neutralization indexes of monoclonal antibodies (MAbs) reacted with NNV isolates. Values are $\log_{10}$ NI (NI = viral titer before MAb treatment/viral titer after MAb treatment). Isolates labelled as in Tables 1 & 2

Virus isolate	Neutralizing monoclonal antibodies				
	2E	9B	9D	10G	12E
G9508KS	3.3	6.3	6.4	5.0	6.3
EE98PH	3.9	7.3	5.5	4.5	7.3
YP99GD	3.0	5.7	5.3	3.6	5.9
B00GD	3.8	5.5	5.8	4.2	6.2
Co00CC1	3.9	6.8	6.5	4.0	6.2
HG00GD	3.9	7.4	7.0	4.7	7.5
CC01YL1	1.6	5.9	6.5	4.4	5.0
SJNNV	3.0	0.0	0.5	2.8	0.0

DISCUSSION

In this study, the host range of NNV in Taiwan was screened, and the genetic and antigenic relationships of Taiwan isolates were analyzed. Prior to this study, only isolates from groupers had been identified in Taiwan; the present study brings the number of NNV-infected fishes up to at least 11 species belonging to 7 families and 3 orders. Among these, European eel, yellow-wax pompano, firespot snapper, cobia and Chinese catfish isolates are characterized for the first time.

European eels (EE98PH) with body lengths of ~30 cm were cage-reared in the sea nearby Pong-Hu island from larvae transported from southern Taiwan a few months before the outbreak of mortality. Whether the eels (EE98PH) were infected by NNV before or after transportation is uncertain. However, at the end of April 1998, the water temperature increased significantly, and the eels began dying at the beginning of May. Diseased eels showed clinical symptoms of VNN and cumulative mortality eventually reached 100%. Increases in water temperature are considered to be an important stress factor causing mass mortality in NNV-infected eels.

As NNV was also detected in freshwater-reared European eels (EE00TN: 30% mortality) and in freshwater-reared Chinese catfish (CC01YL1: 100% mortality), salinity is obviously not a limiting factor in NNV transmission. In Taiwan, individual hatchery farms usually rear several species of fishes in separate tanks or ponds. NNV can easily be transmitted from one pond to another via the closed-circulation water system or via tools. Barramundi larvae are usually reared in seawater; the young juveniles are then gradually acclimatized to freshwater to promote growth, and are subsequently sold to other freshwater farms for the next stage of culture. NNV has been detected in many tissues of the barramundi juvenile survivors after the outbreak of VNN in the larval stage (data not shown). Consequently, it is possible for NNV to be transmitted from marine fish-culture areas to freshwater fish-culture areas via barramundi carriers.

In addition to fishes, NNV was also detected by semi-nested PCR in the live food organisms including *Artemia* sp. nauplii, the copepod *Tigriopus japonicus*, and the shrimp *Acetesinte medius*. The sequences of the amplified T4 region of the capsid protein gene were determined, and identified as the RGNNV genotype (data not shown). Whether the live food organisms were contaminated by NNV on their body surface only, or/and in their digestive tracts, or whether they are natural hosts for NNV replication needs further study. However, NNV-contaminated live food organisms may also be a pathway for horizontal infection.

The phylogenetic analysis clustered all Taiwan NNV isolates as the RGNNV genotype. However, cytosine (C) is highly conserved in the T2 region of the caspsid protein gene at the positions 564, 619 and 844 in all Taiwan isolates, while the nucleic acids at these 3 corresponding positions in RGNNV isolate are thymine, thymine and adenine separately (data not shown). Moreover, the deduced amino acid residue at position 284 of the T2 region is leucine (L) in all the Taiwan isolates, but is isoleucine (I) in the RGNNV isolate. Therefore, it is suggested that all Taiwan NNV isolates had the same origin. Because the first case of VNN in Taiwan occurred in hatchery-reared groupers (G9410YA) in 1994, it is suggested that an NNV-infected grouper was the original source of the NNV epizootic infection. Furthermore, a molecular evolution rate of 4.03×10^{-3} nucleotide replacement/site/year was calculated based on the base substitution mutation rate of 12 Taiwan isolates.

The phenotypic properties of RGNNV and SJNNV genotypes are very different. Isolates of the SJNNV genotype have been found only in Japan, while the RGNNV genotype comprises isolates from at least 8 countries across European and Asia (Nishizawa et al. 1997; Skliris et al. 2001). In Taiwan, the host range of betanodaviruses of RGNNV genotype covers 11 species of fishes belonging to 7 families and 3 orders (Table 4), while the SJNNV genotype has been found only in striped jack and red sea bream (Nishizawa et al. 1997, Skliris et al. 2001). The low host-specificity of RGNNV will increase the spread of NNV and the economic loss of the fish industry through its inter-specific transmission among fishes reared in the same aquatic area or through international fish transportation.

It is interesting that over the past 8 yr (1994 to 2001) only the RGNNV genotype has been found in Taiwan. Warm water temperatures possibly explain this phenomenon. The water temperature range (24 to 30°C) in southern Taiwan is optimal for RGNNV (25 to 30°C), suboptimal for SJNNV (20 to 25°C), and unsuitable for TPNNV (20°C) or BFNNV (15 to 20°C). To date, all the

fish species reported as being infected with the RGNNV genotype are warm-water fishes (Nishizawa et al. 1997, Iwamoto et al. 1999, Curtis et al. 2001, Skliris et al. 2001, Munday et al. 2002). Totland et al. (1999) reported that while a Japanese strain (SJ93Nag) of the SJNNV genotype was highly virulent to larvae of striped jack at 20°C; no replication was detected in larvae of Atlantic halibut at 6°C. On the other hand, a Norwegian nodavirus strain (AH95NorA) was highly virulent to Atlantic halibut larvae reared at 6°C, but did not replicate in striped jack larvae reared at 20°C. Our previous study (Chi et al. 1999b) indicated that cumulative mortality of GNNV bath-challenged grouper larvae 1 d post-infection was 80% for larvae cultured at 28°C, but only 10% for those cultured at 24°C. Genetic variation in adaptations to water temperature may be related to the activity of RNA-dependent RNA polymerase, and temperature adaptation seems to be a more important phenotypic property for the RGNNV genotype than other properties such as host range and geographical distribution.

Nishizawa et al. (1997) indicated that the amino acid sequences at aa 254 to 256 of RGNNV, SJNNV, TPNNV, and BFNNV are PDG, PAN, PPG and PEG respectively, and are completely preserved in each NNV genotype. All Taiwan isolates belong to the RGNNV genotype, and the aa residues at positions 254 to 256 are also conserved as PDG (Fig. 1), reconfirming that 4 NNV genotypes are serologically distinguishable. Although NNV has been studied for more than 10 yr, information on the serological relationship among different virus genotypes and the neutralizing epitope of the capsid protein is still limited. Nishizawa et al. (1999) proposed a SJNNV-specific neutralization epitope PAN at aa 254 to 256.

In this study, 5 neutralizing monoclonal antibodies against GNNV were used to analyze the antigenic relationship of Taiwan isolates and the SJNNV isolate from Japan. Epitopes recognized by MAbs 10G and 9D are suggested to exist on the non-polypeptide part of the capsid protein, since these 2 MAbs react with denatured GNNV capsid protein purified from GNNV-infected GF-1 cells (eukaryotic system) but do not react with recombinant GNNV capsid proteins expressed and purified from *E. coli* (prokaryotic system) (Chi et al. 2001c). Furthermore, periodic silver staining has revealed that GNNV capsid proteins contain carboxyl groups (Chi et al. 2001b), so it is possible that these 2 epitopes are present on the carboxyl group of the capsid protein.

An alpha neutralization test was applied in this study. The neutralization effect of the antibody is expressed by the neutralizaiton index (NI). In general, titration in 10-fold dilutions vary by a factor of 10, or $\log_{10} = 1$. Therefore, neutralization indexes of <10 (log <1) are considered not significant, indexes between 10 and 50 (log 1.0 to 1.6) are questionable, and indexes >50 (log ≥1.7) are significant. The neutralization test revealed that all Taiwan isolates and SJNNV isolate can be significantly neutralized by MAb 2E, except the Chinese catfish isolate CC01YL1 (log NI = 1.6). The multiple alignment analysis of deduced amino acid sequences of the T2 region of the capsid protein indicated that valine (V) at position 124 was highly conserved in all the Taiwan isolates and the SJNNV isolate except in the Chinese catfish isolate CC01YL1. Therefore, we suggest that the epitope for MAb 2E contains V at position 124.

However, there are 3 neutralizing epitopes separately recognized by MAbs 9B, 9D and 12E for the Taiwan isolates, but are absent in SJNNV isolate (Table 3), and these 3 MAbs can be used to distinguish RGNNV and SJNNV genotypes. MAbs 9B and 12E recognize conformation-dependent epitopes of the capsid polypeptide, and the epitope recognized by MAb 9D is on the non-polypeptide part of the capsid protein (Chi et al. 2001c). Further antigenic epitope characterization and virulence comparisons among NNV isolates from different species of fishes are necessary to develop a specific diagnosis system and a vaccine, and will be the subject of future studies.

Table 4. Fish species affected with betanodaviruses in Taiwan

Species	Source
Oder Percifomes	
Family Centropomatidae	
Barramundi *Lates calcarifer*	Chi et al. (2001a)
Family Serranidae	
Black-spotted grouper *E. fuscoguttatus*	Chi et al. (1997)
Dragon grouper *Epinephelus lanceolatus*	Lin et al. (2001)
Brown-spotted grouper *Epinephelus malabaricus*	Lin et al. (2001)
Yellow grouper *Epinephelus awoara*	Lai et al. (2001)
Humpback grouper *Cromileptes altivelis*	Chi et al. (2001)
Family Lutjanidae	
Firespot snapper *Lutjarnus erythropterus*	Chi et al. (2001a)
Family Carangidae	
Yellow-wax pompano *Trachinotus falcatus*	Chi et al. (2001a)
Family Rachycentridae	
Cobia *Rachycentron canadum* L.	Chi et al. (2001a)
Order Anguiliformes	
Family Anguilidae	
European eel *Anguilla anguilla* L.	Chi et al. (2001a)
Order Siluriformes	
Family Siluridae	
Chinese catfish *Parasilurus asotus*	Present study

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