**Journal of Fish Diseases** 1997, **20,** 185–193

# Mass mortalities associated with viral nervous necrosis (VNN) disease in two species of hatchery-reared grouper, *Epinephelus fuscogutatus* and *Epinephelus akaara* (Temminck & Schlegel)

**S C Chi[1], C F Lo[1], G H Kou[1], P S Chang[2], S E Peng[1] & S N Chen[1]**

1 Department of Zoology, National Taiwan University, Taipei, Taiwan, ROC
2 National Kao-Hsiung Institute of Marine Technology, Kao Hsiung, Taiwan, ROC

## Abstract

Mass mortalities of hatchery-reared juvenile groupers have occurred in southern Taiwan. The diseased fish swam in a darting, corkscrew fashion. Light microscopy revealed vacuolation in the brain tissue. Electron microscopy showed numerous non-enveloped, cytoplasmic viral particles (20–25 nm in diameter) in the brain cells, and many virions were enclosed in the membrane-bound organelles of the cells. Two structural proteins of the purified grouper virus, with molecular weights of 44 and 43 kDa, were revealed by SDS-PAGE. Moreover, the results of RT-PCR and nested PCR diagnosis using primers specific to the T2 and T4 target segments of striped jack nervous necrosis virus (SJNNV) RNA2 genes suggest that this virus is a fish nodavirus, and is designated as GNNV 9410 strain (grouper nervous necrosis virus strain 9410). This is the first case report of viral nervous necrosis among marine fish in Taiwan.

## Introduction

With the rapid growth of marine aquaculture in Taiwan, new kinds of infectious viral diseases are emerging and are causing serious losses. Grouper has a high economic value in Taiwan, but during recent years, many grouper hatcheries have experienced outbreaks of an unknown disease resulting in mass mortalities. The diseased grouper juveniles showed unusual swimming behaviour, such as whirling near the surface of the water, abrupt swimming in a corkscrew fashion, sinking to the bottom and then floating to the water surface.

A new viral disease named viral nervous necrosis (VNN) has been reported among hatchery-reared Japanese parrotfish, *Oplegnathus fasciatus* (Temminck & Schlegel), (Yoshikoshi & Inoue 1990), barramundi, *Lates calcarifer* Bloch, (Glazebrook, Haesman & de Beer 1990; Renault, Haffner, Baudin Laurencin, Breuil & Bonami 1991; Munday, Langdon, Hyatt & Humphrey 1992), sea bass, *Dicentrarchus labrax* (L.), (Breuil, Bonami, Pepin & Pichot 1991), turbot, *Scophthalmus maximus* (L.), (Bloch, Gravningen & Larsen 1991), redspotted grouper, *Epinephelus akaara* (Temminck & Schlegel), (Mori, Nakai, Nagahara, Muroga, Mckuchi & Kanno 1991), and striped jack, *Pseudocaranx dentex* Bloch & Schneider, (Mori, Nakai, Muroga, Arimoto, Mushiakc & Furusawa 1992) from many parts of the world. Viral nervous necrosis disease occurs during larval and juvenile stages of marine fish, and is characterized by high mortality and accompanying vacuolation of the nerve tissues. Unenveloped spherical virus particles (25–34 nm) were found in affected fish, but so far, no cell lines are available for isolating these viruses (Yoshikoshi & Inoue 1990; Breuil *et al.* 1991; Mori *et al.* 1991; Munday *et al.* 1992).

The causative agent of VNN from diseased larvae of striped jack was successfully purified and identified as a member of the family Nodaviridae according to its nucleic acid and protein properties,

**Correspondence:** *Dr Shau-Chi Chi, Department of Zoology, National Taiwan University, Taipei, Taiwan, ROC*

and the virus was designated SJNNV, an acronym for striped jack nervous necrosis virus (Mori *et al.* 1992). Recently, a polymerase chain reaction (PCR) method was developed to amplify a portion of the coat protein gene (RNA2) of SJNNV (Nishizawa, Mori, Nakai, Furusawa & Muroga 1994).

In the present paper, the virion causing high mortalities of banded grouper juveniles was studied and identified using both light and electron microscopy, the PCR amplification method described by Nishizawa *et al.* (1994), and SDS-PAGE of purified virions. The results indicated that GNNV strain 9410, found in moribund banded grouper juveniles, is an example of fish nervous necrosis virus, also known as fish encephalomyelitis virus, and is a member of the Nodaviridae. This is the first report of VNN in marine fish in Taiwan.

## Materials and methods

### Fish samples

Diseased grouper juveniles (2–3 cm in total length) were taken from the Mito hatchery farm in Taiwan in October 1994. At this particular farm, the eggs of red spotted grouper and the black spotted grouper were mixed together, and hatched in the same tank. Juveniles of the two grouper species were also reared together in the same pond. The fish were cultured at a density of 10 000 fish $m^3$ at 26–30°C. Mortalities were nearly 95%. Live samples were fixed for observation by light and electron microscopy, and the rest were frozen at –20°C.

It is very difficult to distinguish between the black spotted grouper and the red spotted grouper at 2–3 cm body length; therefore, the specific identity of the fish from each sample examined in this study was not determined.

### Histopathological examination

Fish were fixed in 10% buffered formalin and embedded in paraffin wax. Sections of 5 μm were then stained with haematoxylin and eosin (H&E). For electron microscopy, fish were fixed in 2·5% glutaraldehyde in 0·1 M phosphate buffer at pH 7·4 and post-fixed in 1% osmium tetroxide. Ultrathin sections were stained with uranyl acetate-lead citrate and examined under a Hitachi H-600A electron microscope.

### Extraction of total nucleic acids from diseased fish

For rapid tissue preparation, the deproteinized viral RNA was prepared according to the method described by Strauss (1994). Briefly, each fish sample was frozen in liquid nitrogen and homogenized. The homogenized tissue (100 mg) was then added to 2·4 ml of digestion buffer (100 mM NaCl, 10 mM Tris-HCl at pH 8, 25 mM EDTA at pH 8, 0·5% sodium dodecyl sulphate and 0·1 mg $ml^{-1}$ proteinase K) and incubated at 65°C for 3 h. The digestion mixture was then extracted using the phenol/chloroform/isoamyl alcohol method, precipitated with ethanol, and resuspended in diethyl pyrocarbonate water. The quality and the quantity of the purified nucleic acids were determined by using a Hitachi spectrophotometer U-200.

### Primers for PCR amplification

Three primers for carrying out PCR amplification of the extracted nucleic acids were synthesized by the DNAFax Company, the primer sequences of these primers being based on those of Nishizawa *et al.* (1994). The sequence of each primer is as follows: (forward primer 1, F1) 5′-GGA-TTTGGACGTGCGACCAA-3′; (forward primer 2, F2) 5′-CGTGTCAGTCATGTGTCGCT-3′; and (reverse primer 3, R3) 3′-AGAAGTGGG-CACAACTGAGC-5′. The target region expected to be amplified from F1 to R3 is T2 (875 bp) and the target region for F2 to R3 is T4 (426 bp).

### Reverse transcription and PCR amplification

For reverse transcription, the nucleic acids extracted by the above procedure were pre-heated at 95°C for 5 min, and then incubated at 42°C for 1 h in 30 μl of reverse transcription buffer mixture consisting of 2 μg of nucleic acids, 3·3 μM of random primer, 0·5 mM of each dNTP, 50 mM Tris-HCl at pH 8·3, 75 mM KCl, 3 mM $MgCl_2$, 0·01 M DTT, 1·3 μM RNAsin and 400 U M-MLV reverse transcriptase (BRL). Following cDNA synthesis, cDNA was added to the PCR mixture to a final volume of 100 μl containing 0·5 μg forward primer, 0·5 μg reverse primer, 0·2 mM of each dNTP, 50 mM KCl, 10 mM Tris-HCl at pH 9·0, 0·1% Triton X-100, 2 mM $MgCl_2$ and 2·5 U Taq DNA polymerase (Promega)

to a final volume of 50 μl. The mixture was then incubated in an automatic thermal cycler (PTC-100 thermal cycler; MJ Research Inc., USA). The primer set used in this amplification was F1-R3. The expected target region was T2. The amplification was performed for one cycle at 94°C for 3 min; 40 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 5 min; and one cycle at 72°C for 15 min. After amplification, the PCR products were analysed on 1·5% agarose gel.

### Nested PCR amplification

Nested PCR amplification was performed immediately using the RT-PCR products as template. An F2–R3 primer set was used in this amplification. The target region was T4 (426 bp). The amplification conditions were as for RT-PCR. The amplified sequences were analysed on a 2% agarose gel.

### Purification of the virus

The virus was isolated and purified according to the method described by Mori *et al.* (1992). The purified virus was negatively stained by 1% phosphotungstic acid and observed under TEM.

### Electrophoresis of the purified virus

Purified virus was analysed by 12% SDS-PAGE and stained by Coomassie blue. The gel was then screened by a laser-based imaging densitometer (Molecular Dynamics, PDSI-PC, USA) and the molecular weights of viral proteins were determined by the image fragment analysis program of the PDSI-PC.

## Results

### Clinical signs and gross pathology

The first signs of an outbreak of this disease were behavioural; the fish lost their equilibrium and swam in a corkscrew fashion. Affected fish were generally lethargic, and some floated on their sides on the water surface, but responded to alarm or physical stimulation with a brief burst of erratic swimming. Sometimes the fish sank to the bottom and then floated to the surface again.

A few diseased fish were darker than normal. The livers of diseased fish were pale, the digestive tracts were devoid of food, the intestines filled with a greenish to brownish fluid and the spleens were red-spotted. Affected fish showed no signs of bacterial infection or parasitism.

### Histopathology

Under the light microscope, the brain of affected fish showed conspicuous vacuolation (Fig. 1). The extent of the lesions varied considerably from fish to fish. Ultrathin sections showed that numerous virions completely or partially filled the membrane-bound organelles in the cytoplasm of virus-infected brain cells (Fig. 2). These virions were non-enveloped, homogeneous, spherical to icosahedral particles with diameter of 20–25 nm, and were usually arranged in paracrystalline arrays, as shown in Fig. 3. Some membrane-bound organelles had a single layer of viral particles on the inner surface of the membrane (Fig. 4). In some virus-containing organelles, the membrane structures degenerated and four to five layers of viral particles were assembled at the site of pre-existing membranes (Fig. 5). Large vacuoles were always seen in the heavily virus-infected cells (Fig. 6).

### PCR amplification

Deproteinized nucleic acids extracted from three different diseased fish were analysed by RT-PCR and nested PCR amplification using primers specific to SJNNV RNA2 gene. The results of RT-PCR in the presence of the F1–R3 primer set are shown in Fig. 7. The three separate nucleic acid preparations consistently produced a major band with a size similar to that of the T2 region (875 bp) of SJNNV RNA2. Figure 8 shows the results of the nested PCR amplification using an internal primer set (F2–R3) for the T4 region. The 400 bp size of the nested PCR product is similar to that of the T4 region of the SJNNV RNA2 gene. Taken together, these results indicate that the viral agent in the juvenile groupers is closely related to the SJNNV, which has been identified as belonging to the Nodaviridae family (Mori *et al.* 1992).

### Virus purification

The purified virus appeared icosahedral in morphology and the average diameter of 30 viral particles was 25 nm (Fig. 9).

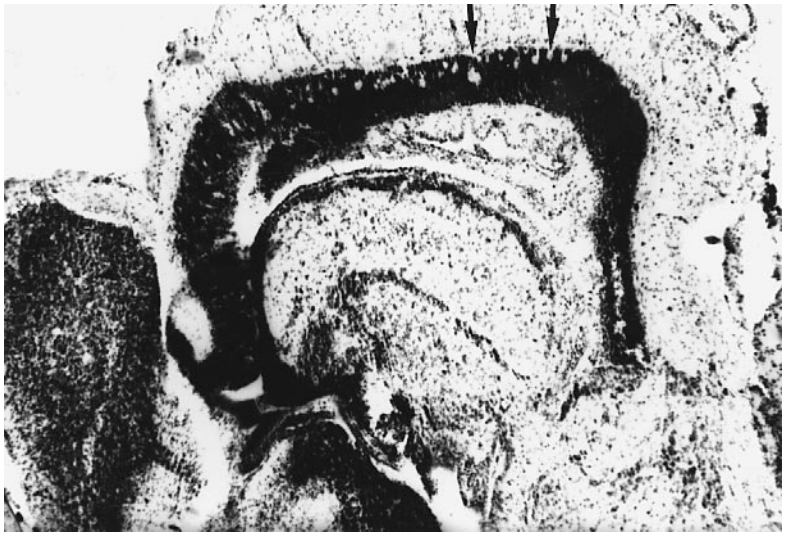

**Figure 1** Light micrograph demonstrating vacuolation (arrows) in the mid-brain of diseased juvenile grouper (H&E).

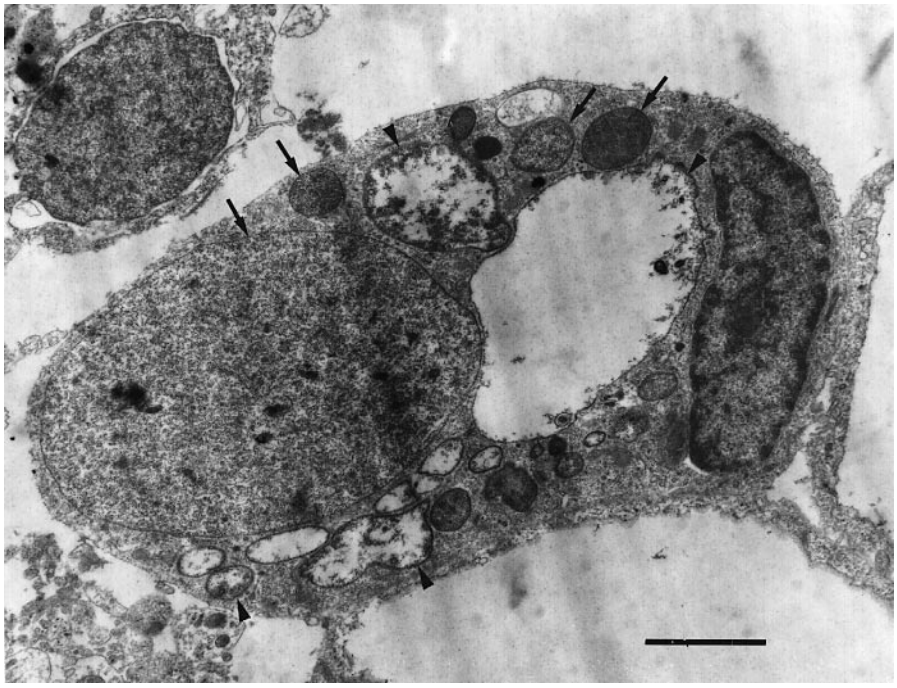

**Figure 2** Electron micrograph showing the GNNV-infected cells with different sizes of membrane-bound organelles completely filled (arrows) or partially filled (arrowheads) with viral particles (bar = 2 μm).

### Electrophoresis of viral proteins

Purified virus had structural proteins with molecular weights of 44 and 43 kDa (Fig. 10). The molecular weight was calculated by laser densitometry. The molecular weights of the viral structural proteins of grouper virus are a little higher than those of SJNNV which are 42 and 40 kDa (Mori *et al.* 1992).

## Discussion

Viral nervous necrosis (VNN) in marine fish, also known as fish encephalomyelitis, has been reported

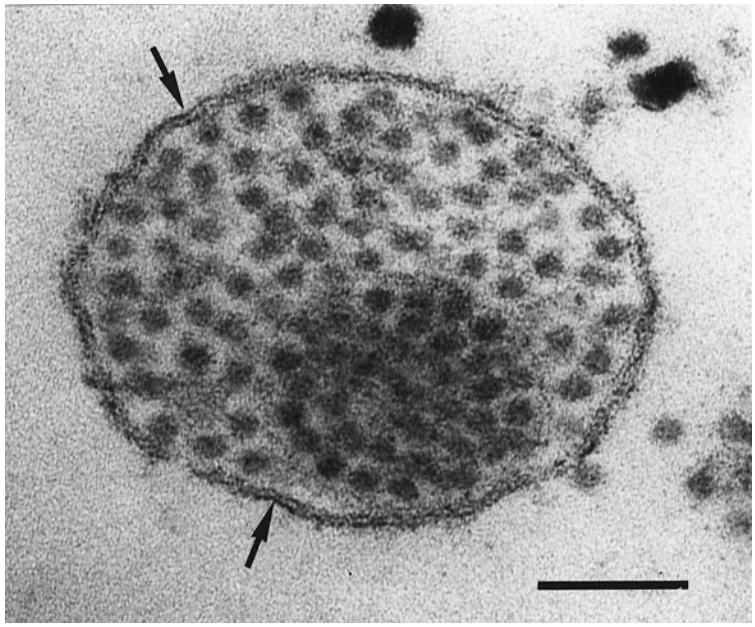

**Figure 3** Electron micrograph showing non-enveloped, spherical viral particles packed in paracrystalline arrays in membrane-bound organelles. Arrows indicate the lipid bilayer structure of the membrane (bar = 100 nm).

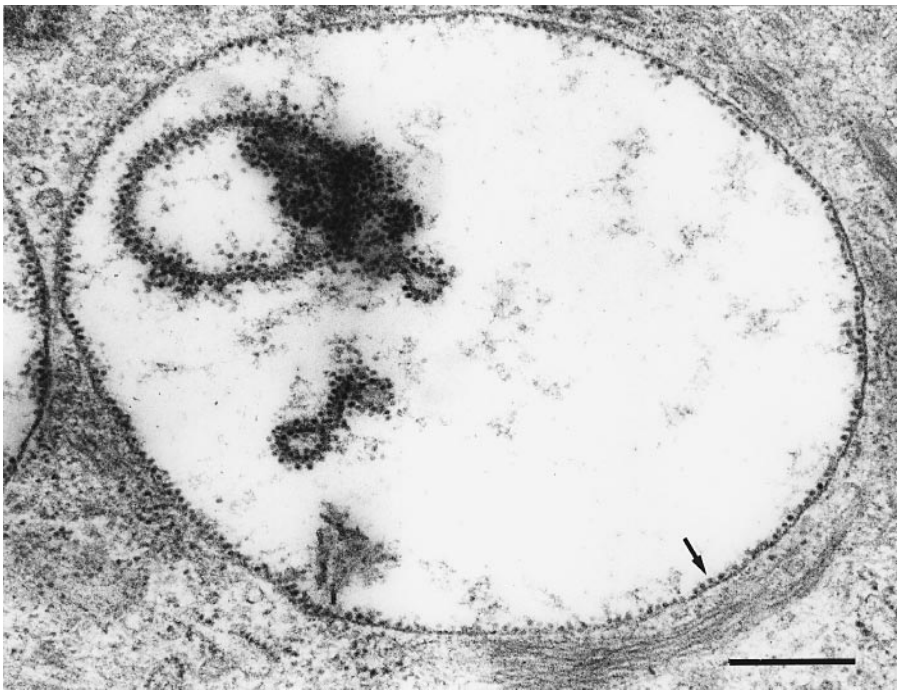

**Figure 4** Electron micrograph showing a single layer of virions (arrow) lining the inner surface of a membrane-bound organelle in an infected brain cell (bar = 400 nm).

in a number of countries: Malaysia (Awang 1987), Japan (Yoshikoshi & Inoue 1990; Mori *et al.* 1991), North Australia (Glazebrook *et al.* 1990; Munday *et al.* 1992), France (Breuil *et al.* 1991; Renault *et al.* 1991) and Norway (Bloch *et al.* 1991). In this paper, we reported the existence of a viral strain,

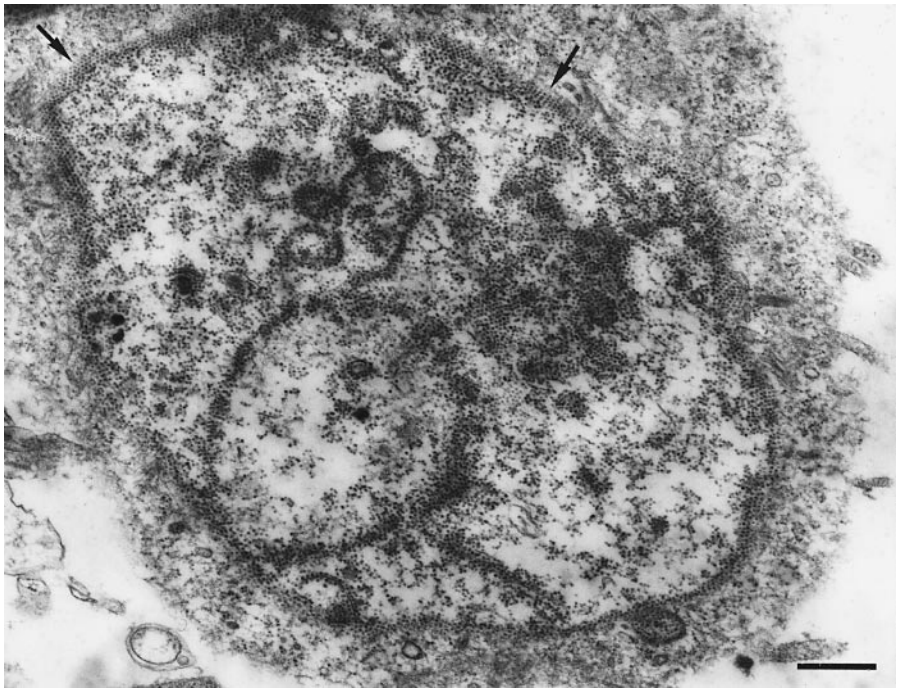

**Figure 5** Electron micrograph showing degeneration of membrane structures of cellular organelles. Arrows indicate four to five layers of viral particles (bar = 500 nm).

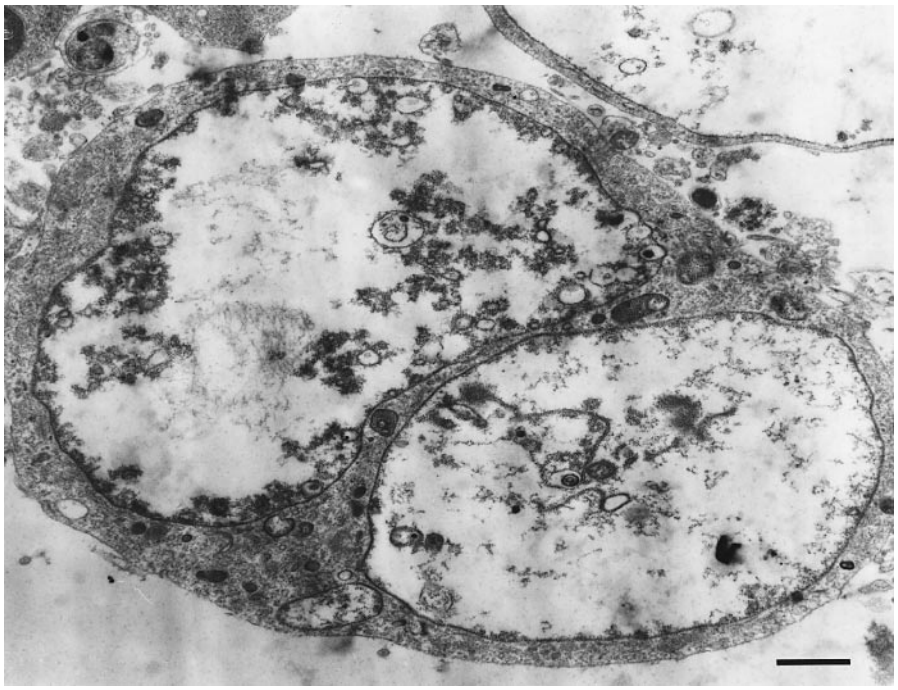

**Figure 6** Electron micrograph showing heavily virus-infected brain cell with large vacuoles in the cytoplasm (bar = 1 μm).

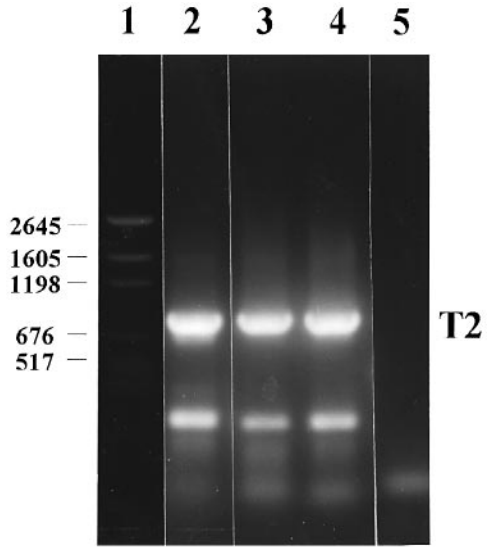


**Figure 7** Agarose gel electrophoresis of the products by RT-PCR amplification using primers specific to the T2 target region of SJNNV RNA2 and the nucleic acids extracted from three diseased juvenile groupers as templates. An F1–F3 primer set specific for the T2 target region was used in RT-PCR amplification. The expected size of the T2 region is 875 bp. Lane 1 is pGEM marker; lanes 2–4 show the PCR product from nucleic acids extracted from the three different fish; and lane 5 shows a template-free control reaction.

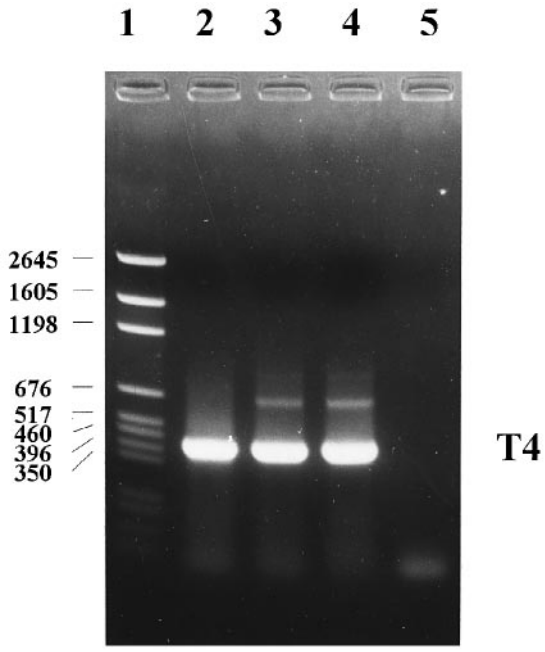


**Figure 8** The products in the nested PCR amplification using the F2–R3 primer set specific to the T4 target region of SJNNV RNA2 and the product of RT-PCR as DNA templates. Lane 1 shows pGEM marker; lanes 2–4 show PCR products of three different diseased fish; lane 5 shows a template-free control reaction.

named GNNV strain 9410, found in the CNS of juvenile grouper reared at the Mito hatchery in southern Taiwan, and determined its classification using RT-PCR and nested PCR amplification techniques with SJNNV RNA-2 specific primer sets, and the structural protein profile analysis of purified virus by electrophoresis. This is the first record of viral nervous necrosis among hatchery-reared grouper in Taiwan.

Under light microscopy, many vacuoles were found in the brain tissue of the diseased fish, especially in the mesencephalon. With the electron microscope, viral particles were found only in the cytoplasm of the brain cells in close association with intracytoplasmic cell membranes. Mature viral particles were packed inside the endoplasmic reticulum cisternae, resulting in membrane-bound organelles filled with virus particles, and then released into the cytoplasm by destruction of the organelle membrane. High concentrations of cytoplasmic viruses gradually displace the cellular organelles. These observations suggest that the viral agent in the juvenile grouper is an RNA virus and that intracellular membranes support its replication.

It is believed that GNNV is the main cause of mortality among juvenile grouper because of the corkscrew swimming behaviour displayed by affected fish, the fact that neither parasites nor pathogenic bacteria were detected in moribund fish, and the similarity of histo- and cytopathological signs to the characteristics of VNN disease among marine fish previously described (Yoshikoshi & Inoue 1990; Renault *et al.* 1991; Mori *et al.* 1992).

Nervous necrosis virus was originally tentatively identified as a Picornavirus based on its ability to replicate in the cytoplasm, its association with membrane structures, its size (25–30 nm) and its RNA content (Glazebrook *et al.* 1990). Later, Mori *et al.* (1992) clearly identified the VNN virus isolated from the striped jack as a member of the family Nodaviridae because SJNNV has two single-stranded, positive-sense RNAs with molecular weights of $1{\cdot}0\times10^6$ Da (RNA1) and $0{\cdot}49\times10^6$ Da (RNA2), and the RNAs of SJNNV do not have a poly(A) tail. Also, RNA1 directs the synthesis of 1a protein (100 kDa), and RNA2 directs the synthesis of 2a protein (42 kDa), which is the coat protein of the virus (Mori *et al.* 1992).

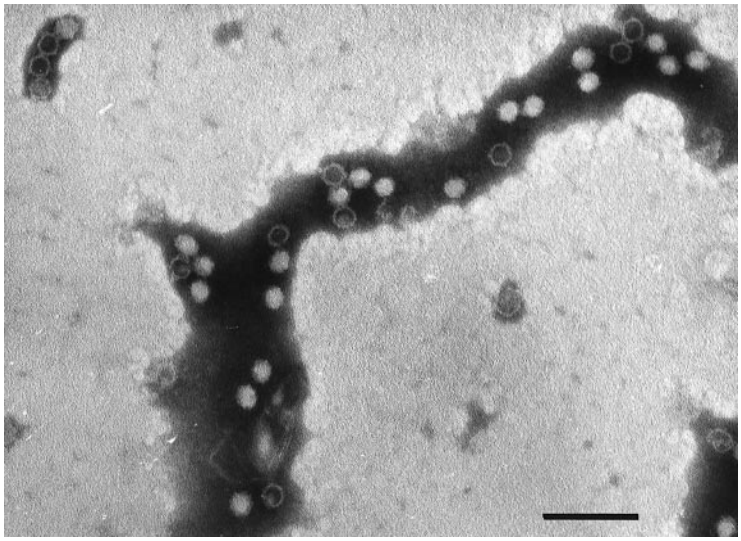

**Figure 9** The morphology of the purified virus isolated from diseased grouper juveniles (bar = 100 nm).

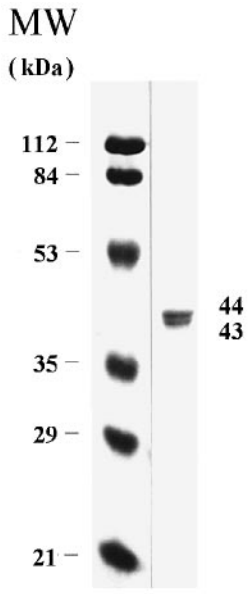


**Figure 10** The structural proteins of the purified viruses from diseased grouper analysed by 12% SDS-PAGE.

Five primer sets specific for the SJNNV RNA2 target regions T1, T2, T3, T4 and T5 were designed by Nishizawa *et al.* (1994). Among these five, the T4 region was recommended as the most suitable target region for a PCR amplification of the coat protein gene of fish nodavirus, and the T4 target region has been detected in PCR products of VNN viruses from four other kinds of marine fish (Nishizawa, Mori, Nakai, Furusawa & Muroga 1995).

In this study, we used two SJNNV RNA-2 specific primer sets, F1–R3 and F2–R3, in a PCR amplification test designed to identify suspected viral nucleic acids in infected grouper tissue. A major band similar in size to the T2 region (875 bp) in the RT-PCR, and an obvious band similar in size to the T4 region (426 bp) in the nested PCR test, were detected. These results indicate that the virus from banded groupers is closely related to SJNNV. In addition, the purified viral particles were non-enveloped, spherical to icosahedral in shape, 20–25 nm in diameter, and the molecular weights of structural proteins were 44 and 43 kDa, which were similar to SJNNV (Mori *et al.* 1992) and other insect nodaviruses (Glazon & Charpentier 1991). These results, together with the clinical signs of the diseased fish and the light and electron microscope observations, suggest that the virus isolated from the grouper juveniles, designated as GNNV 9410 strain, is an aquatic nodavirus. Comparison of the gene sequences of the GNNV 9410 strain and SJNNV will further elucidate the differences between these two NNV strains.

It will be important to carefully monitor the spread of VNN in Taiwan because this disease always results in very high mortality rates in the larval and juvenile stages of marine fish and causes severe economic losses at fish hatcheries. In this paper, we have reported the existence of viral nervous necrosis disease in cultured fish in Taiwan and

propose that screening for the VNN virus infection at all hatcheries and in different developmental stages of cultured grouper should be undertaken immediately in order to prevent the further spread of this disease.

## Acknowledgments

This work was supported by the Council of Agriculture under grant No. 85-AST-1.13-FID-06(16) and by the National Science Council under grant NSC 86-2311-B-002-048.

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