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Detection of betanodaviruses in apparently healthy aquarium fishes and invertebrates

Dennis Kaw Gomez[1], Dong Joo Lim[2], Gun Wook Baeck[3], Hee Jeong Youn[1,4], Nam Shik Shin[1,4], Hwa Young Youn[1,4], Cheol Yong Hwang[1,4], Jun Hong Park[2], Se Chang Park[1,4,*]

[1]*KRF Zoonotic Disease Priority Research Institute, and* [4]*College of Veterinary Medicine, Seoul National University, Seoul 151-742, Korea*
[2]*Department of Veterinary Medicine, College of Applied Life Sciences, Cheju National University, Jeju 690-756, Korea*
[3]*Faculty of Marine Technology, Chonnam National University, Yeosu 550-749, Korea*

Betanodaviruses are the causative agents of viral nervous necrosis (VNN) in cultured marine fish. A total of 237 apparently healthy aquarium fish, marine (65 species) and freshwater (12 species) fishes and marine invertebrates (4 species), which were stocked in a commercial aquarium in Seoul, South Korea, were collected from November 2005 to February 2006. The brains of the fish and other tissues of the invertebrates were examined by reverse transcriptase-polymerase chain reaction (RT-PCR) and nested PCR to detect betanodavirus. Positive nested PCR results were obtained from the brains of 8 marine fish species (shrimp fish *Aeoliscus strigatus*, milkfish *Chanos chanos*, three spot damsel *Dascyllus trimaculatus*, Japanese anchovy *Engraulis japonicus*, pinecone fish *Monocentris japonica*, blue ribbon eel *Rhinomuraena quaesita*, look down fish *Selene vomer*, yellow tang *Zebrasoma flavesenes*), 1 marine invertebrate species (spiny lobster *Pamulirus versicolor*), and 2 freshwater fish species (South American leaf fish *Monocirrhus polyacanthus* and red piranha *Pygocentrus nattereri*). The detection rate in nested PCR was 11/237 (4.64%). These subclinically infected aquarium fish and invertebrates may constitute an inoculum source of betanodaviruses for cultured fishes in the Korean Peninsula.

Key words: aquarium fish, betanodavirus, invertebrate, PCR detection, subclinical infection, viral nervous necrosis

Introduction

Betanodaviruses (family Nodaviridae) are the causative agents of viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER) in a variety of cultured marine fishes worldwide. This disease particularly occurs during the seedling period and the culturing process [17,19,21]. Necrosis and the vacuolation of central nervous tissues (brain and spinal cord) and eye retina are the most characteristic lesions of VNN, and affected fish shows abnormal swimming behavior. The spread of VNN among populations of cultured marine fish may be attributable to either vertical [2,4,26] or horizontal [3,5,10] transmission. The first description of VNN in Korea was reported apparently in cultured groupers (*Epinephelus septemfasciatus*), although in this case, the causative agent was not identified [23]. Recently, Oh *et al.* [22] reported mass mortality in hatchery-reared red drum (*Sciaenops ocellatus*), which was associated with a betanodavirus. VNN in the guppy (*Poicelia reticulata*), a freshwater ornamental fish, was described previously [11], though the viral etiology was not fulfilled.

Korea imports a variety of aquarium (marine or freshwater) fishes from Southeast Asian countries; the number of such imported species increases annually [13]. However, appropriate quarantine practices are often neglected or overlooked in the importation of aquarium fishes and invertebrates in many countries. As a result, imported fishes and invertebrates sometimes die of infections soon after arrival, or during transport. In order to know, we evaluated apparently healthy fish and invertebrate samples collected from a commercial aquarium in Korea for the presence of betanodavirus, by polymerase chain reaction (PCR)-based techniques, to determine whether these imported aquarium fishes and invertebrates might be one of the sources of VNN.

Materials and Methods

Fish samples

From November 2005 to February 2006, 237 samples of marine (65 species) and freshwater (12 species) fishes and marine invertebrates (4 species) were collected from a commercial aquarium in Seoul, Korea. From these aquarium

*Corresponding author
Tel: +82-2-880-1282; Fax: +82-2-880-1213
E-mail: parksec@snu.ac.kr

fish samples, 20 samples (8 species) were obtained from Korea. Other aquarium fishes were imported from different sources, such as Japan with 58 samples (25 species), Singapore with 58 samples (17 species), the Amazon River with 45 samples (11 species), Indonesia with 22 samples (5 species), the Philippines with 13 samples (5 species), Australia with 3 samples (2 species), the United States of America with 9 samples (1 species), Canada with 1 sample (1 species), the Caribbean Sea with 1 sample (1 species) and the Pacific Ocean with 1 sample (1 species). With regard to the invertebrate samples, 2 samples (2 species) were from Japan, 2 samples (1 species) were from Hawaii, and 2 samples (1 species) were from the Pacific Ocean. The brains or other pooled organs were collected aseptically from the samples, then maintained at a temperature of –80°C until use.

RNA extraction

Total RNA was extracted from the brains or pooled organ tissues using an RNA extraction kit with TRIzol Reagent (Invitrogen, USA), in accordance with the manufacturer's instructions. In brief, the tissues were homogenized with TRIzol reagent and shaken with chloroform, then centrifuged for 15 min at 12,000 × *g*. RNA in the aqueous phase was precipitated with isopropanol, then dissolved in diethyl-pyrocarbonate-treated water (Biosesang, Korea).

PCR amplification

PCR amplification was conducted using primers (BNV-RT, BNV-UR1, BNV-UF1) for RT-PCR and (BNV-UR2, BNV-UF2) for nested PCR designed for the target regions (570 bp and 420 bp) of the SGNNV RNA2, as previously described [10]. After reverse transcription using Reverse Transcriptase SuperScript II (Invitrogen, USA) at 45°C for 60 min, PCR was conducted using Ex *Taq* polymerase (Takara, Japan) with 30 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 20 s, and a final extension step at 72°C for 60 s. Nested PCR was conducted using the protocol described above. The PCR products were then analyzed by 2% agarose gel electrophoresis. The RNAs from the uninfected redspotted grouper (*E. akaara*) larvae were used as negative controls for both RT-PCR and nested PCR.

Results

The results of betanodavirus detection by RT-PCR and nested PCR are summarized in Table 1, and the detection rate in nested PCR was 11/237 (4.64%). Marine aquarium fish with no clinical signs positive for nodavirus by nested PCR were as follows: 3 of the 58 samples from Japan, 3 of 58 from Singapore, 1 of 13 from Indonesia and 1 of 9 from the United States of America. 2 of 45 freshwater aquarium fish from the Amazon River also tested positive for nodavirus by nested PCR. 1 of 2 marine invertebrates obtained from Japan also tested positive for nodavirus by nested PCR. Other marine aquarium fish from Korea (20), the Philippines (13), Australia (3), Canada (1), the Pacific Ocean (1), the Caribbean Sea (1) as well as freshwater aquarium fish from Indonesia [9]; and marine invertebrates from Hawaii (2) and the Pacific Ocean (2) tested negative for nodavirus by both RT-PCR and nested PCR tests.

Positive nested PCR results were obtained from the brain samples of 8 apparently healthy marine aquarium fish species: shrimp fish (*Aeoliscus strigatus*), milkfish (*Chanos chanos*), three spot damsel (*Dascyllus trimaculatus*), Japanese anchovy (*Engraulis japonicus*), pinecone fish (*Monocentris japonica*), blue ribbon eel (*Rhinomuraena quaesita*), look down fish (*Selene vomer*) and the yellow tang (*Zebrasoma flavesenes*). Other positive nested PCR results were obtained from the brains of 2 apparently healthy freshwater fish species, South American leaf fish (*Monocirrhus polyacanthus*), red piranha (*Pygocentrus nattereri*) and the marine invertebrate, the spiny lobster (*Pamulirus versicolor*). Representative amplicons of the fish and invertebrate samples tested positive on nested PCR are shown in Fig. 1.

Discussion

The positive data from the nested PCR results show that betanodavirus is present in both marine and freshwater fish, as well as marine invertebrates, displayed in one commercial aquarium in Korea. Korea imports a variety of aquarium fish from different countries, and the majority of these fish or invertebrates are imported without appropriate quarantine protocol [12]. In the present system, there were no specific rules regarding quarantine practices on the importation of aquarium fishes and invertebrates in Korea, and there was also an absence of proper documentation from the exporting countries with regard to whether the fishes or invertebrates were pathogen-free or not prior to export. Positive samples were obtained from the brains of 8 different marine fish species imported from 4 different countries: Indonesia [28,29], Japan [10,16,18,27], Singapore [6,15] and the USA [9], in which VNN has previously occurred, and have also been reported in different marine fish species [17,19]. Nodavirus positive samples were also obtained from the brains of 2 freshwater fish species imported from the Amazon River. It is interesting to note that, until now, there have been no cases of VNN reported on the Latin American continent. Other nodavirus-positive sample was obtained from the brain of the spiny lobster, a marine invertebrate, imported from Japan. In Taiwan, Chi *et al.* [7,8] reported the detection of VNN in other live food organisms, such as crustaceans including the brine shrimp (*Artemia* sp.) nauplii, the copepod (*Tigriopus japonicus*), and the shrimp (*Acetesinte medius*). It has also been reported in Taiwan, China, and the French West Indies [25], that freshwater shrimp (*Macrobrachium rosenbergii*) with white tail disease (WTD) have also been infected with a virus, and the causative pathogen has been

Table 1. Betanodavirus detection by nested PCR in apparently healthy aquarium fishes and invertebrates

Species	Source	No. of positive sample No. of fish examined
Marine Fish		
Indo pacific sergeant (*Abudetdut vaigiensis*)	J	0/3
Longnose unicorn (*Acanthurus unicornis*)	PO	0/1
Shrimp fish (*Aeoliscus strigatus*)	S	1/3
African pompano (*Alectis ciliaris*)	J	0/1
Golden damselfish (*Amblyglyphidodon aureus*)	S	0/1
Orange-striped goby (*Amblygobius decussates*)	Ph	0/1
Orange clownfish (*Amphiprion percula*)	Ph	0/2
Autralian cardinal fish (*Apogon opercularis*)	Au	0/2
Smooth lumpsucker (*Aptocyclus ventricosus*)	K	0/10
Dog faced puffer (*Arothron nigropunctatus*)	J	0/1
Velvet fish (*Caracanthus maculatus*)	J	0/1
Oceanic white tip shark (*Carcharinus longimanus*)	J	0/1
Coral beauty angelfish (*Centropyge bispinosus*)	J	0/1
Tassle filefish (*Chaetodermis penicilligerus*)	S	0/2
Butterfly fish (*Chaetodon semilarvatus*)	J	0/7
Tear's drop butterfly (*Chaetodon unimaculatus*)	J	0/1
Milkfish (*Chanos chanos*)	J	1/2
Napoleon fish (*Cheilinus undulates*)	J	0/2
Copper band butterflyfish (*Chelmon rostratus*)	I	0/1
Brown banded bamboo shark (*Chiloscyllium punctatum*)	I	0/3
Rabbit fish (*Chimaera monstrosa*)	I	0/1
Scanbreast turkfish (*Choerodon azurio*)	J	0/1
Blue green chromis (*Chromis viridis*)	J	0/11
Sapphire devilfish (*Chrysiptera cyanea*)	S	0/16
Two spot coralfish (*Coradion melanopus*)	Ph	0/2
Three spot damsel (*Dascyllus trimaculatus*)	S	1/1
Red stingray (*Dasyatis akajei*)	K	0/1
Longspine porcupinefish (*Diodon holocanthus*)	J	0/1
Banded pipe fish (*Doryrhamphus dactyliophorus*)	S	0/2
Japanese anchovy (*Engraulis japonicus*)	J	1/4
Redspotted grouper (*Ephinephelus akaara*)	J	0/1
Blue face angelfish (*Euxiphipops xanthometopon*)	J	0/1
Longnose butterflyfish (*Forcipiger flavissimus*)	Ph	0/6
Golden trevally (*Gnathanodon speciosus*)	S	0/2
Soldier fish (*Holocentrus rufus*)	CS	0/1
Rat fish (*Hydrolagus colliei*)	C	0/1
Slender shad (*Ilisha elongata*)	K	0/1
Red wrasse (*Labrus mixtus*)	S	0/1
Thornback cowfish (*Lactoria fornasini*)	J	0/2
Blue striped snapper (*Lutjanus kasmira*)	J	0/1
Stripey foot baller (*Microcanthus strigatus*)	K	0/1
Pinecone fish (*Monocentris japonica*)	J	1/1
Common eagle ray (*Myliobatis aquila*)	J	0/1
Fire goby (*Nemateleotris decora*)	Ph	0/2
Whitespotted box fish (*Ostracion meleagris*)	J	0/1
Butterfish (*Pampus argenteus*)	K	0/1
Arc eye hawkfish (*Paracirrhites arcatus*)	S	0/1
Blue damselfish (*Pomacentrus coelestis*)	S	0/4

Table 1. Continued

Species	Source	No. of positive sample No. of fish examined
Damselfish (*Pomancentrus sp.*)	S	0/11
Anthias fish (*Pseudoanthias sp.*)	S	0/1
Purple anthias (*Pseudoanthias pascalus*)	S	0/1
Sea goldie fish (*Pseudoanthias squamipinnis*)	S	0/2
Red lion fish (*Pterois volitans*)	K	0/4
Blue ribbon eel (*Rhinomuraena quaesita*)	I	1/8
Rockfish (*Sebastes schlegeli*)	K	0/1
Look down fish (*Selene vomer*)	USA	1/9
Bulgyhead wrasse (*Semicossyphus reticulatus*)	K	0/1
Scorpion fish (*Scorpaenopsis oxycephala*)	J	0/2
Fox faced fish (*Siganus virgatus*)	J	0/1
Bicolor damselfish (*Stegastes partitus*)	S	0/2
Blue spotted ribbontail ray (*Taeniura lymma*)	J	0/7
Fiddler ray (*Trygonorrhina fasciata*)	Au	0/1
Moorish idol (*Zanclus cornutus*)	J	0/3
Yellow tang (*Zebrasoma flavesenes*)	S	1/7
Brown tang (*Zebrasoma scopes*)	S	0/1
Freshwater Fish		
Oscar (*Astornotus ocellatus*)	A	0/1
Amber cichlid (*Cichlasoma citrinellus*)	A	0/1
Sucker mousefish (*Corydoras sp.*)	A	0/2
Electric eel (*Electrophorus electricus*)	A	0/1
South American leaf fish (*Monocirrhus polyacanthus*)	A	1/17
Cichlid (*Neolamprologous cylindricus*)	A	0/2
Silver arowana (*Osteoglossum bicirrhosum*)	A	0/3
Neon tetra (*Paracheirodon innesi*)	A	0/10
Golden angelfish (*Pterophyllum scalare*)	A	0/2
Red piranha (*Pygocentrus nattereri*)	A	1/4
Chocolate gourami (*Sphaerichthys osphromenoides*)	I	0/9
Red discus (*Symphysodon discus*)	A	0/2
Marine Invertebrates		
Chambered nautilus (*Nautilus pompilius suluensis*)	PO	0/2
Spiny lobster *(Pamulirus versicolor*)	J	1/1
King crab (*Parlithodes camtschaticus*)	J	0/1
Hawaiian slipper lobster (*Scyllarides haauii*)	H	0/2
Total		11/237
Rate (%)		4.64

A, Amazon; Au, Australia; C, Canada; CS, Caribbean Sea; H, Hawaii; I, Indonesia; J, Japan; K, Korea; PO, Pacific Ocean; Ph, Philippines; S, Singapore; USA, United States of America.

identified as the *M. rosenbergii* nodavirus (*Mr*NV) [1]. However, thus far, there have been no reports of betanodavirus infections in aquarium fish and invertebrates in Korea. To our knowledge, this study is the first report of betanodavirus in subclinically infected aquarium fish and invertebrates in a Korean commercial aquarium.

So, what is the significance of the detection of betanodaviruses from apparently healthy marine or freshwater aquarium fish or marine invertebrates? It is possible that samples from exporting countries in which VNN infections have occurred were already infected vertically from broodstock to larvae, or horizontally from fish via water or other by biological organisms, thus becoming carriers of the nodavirus prior to transport to the importing countries. The results of the present study indicated that approximately 4.64% of the fish or invertebrate samples imported from different countries

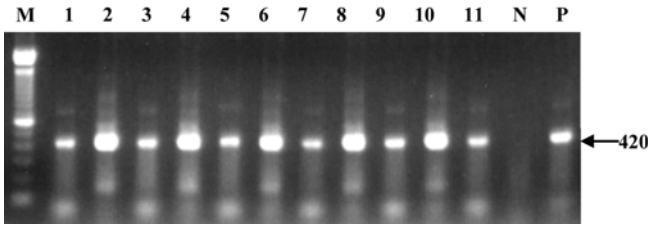

Fig. 1. Representative nested polymerase chain reaction (PCR) profiles. RNA samples were extracted from the brains of marine or freshwater aquarium fishes and marine invertebrates were used for nested PCR. M, 100 bp DNA marker; Lane 1, *Aeoliscus strigatus*; Lane 2, *Chanos chanos*; Lane 3, *Dascyllus trimaculatus*; Lane 4, *Engraulis japonicus*; Lane 5, *Monocentris japonica*; Lane 6, *Rhinomuraena quaesita*; Lane 7, *Selene vomer*; Lane 8, *Zebrasoma flavesenes*; Lane 9, *Monocirrhus polyacanthus*; Lane 10 *Pygocentrus nattereri*; Lane 11, *Pamulirus versicolor*; N, negative control; P, positive control.

were tested positive for nodavirus in the Korean commercial aquarium. In the cases in this study, the brains were usually negative on RT-PCR, but were confirmed positive on nested PCR, which suggests that the samples had been latently infected with betanodaviruses. Another possibility is that the imported samples might also be infected soon after transport via horizontal transmission, as the fishes are usually maintained together in one aquarium with other subclinically infected wild fishes collected from the Korean sea. Sohn *et al*. [23,24] previously reported the first incidence of VNN in a cage-cultured grouper (*E. septemfasciatus*) on the southern coast of Korea. Recently, incidences of nodavirus disease outbreaks have been recorded in cultured marine fish such as the red drum (*S. ocellatus*) [14,22].

These nodavirus-contaminated fish or invertebrates may cause problems, as they may die soon after arrival or during transport, due to the combination of stress and nodavirus infection which could entail economic losses. We are also unable to predict what the consequences are likely to be when betanodaviruses are introduced to different countries, due primarily to a lack of proper documentation and limited information regarding the factors relevant to betanodavirus infections in the exporting countries. The Office International des Epizooties (OIE) has detailed provisions regarding the importation and exportation of aquatic animals [20], as well as aquaculture products targeted at avoiding the risk of spreading aquatic animal diseases [21]. Furthermore, these subclinically infected samples may constitute a persistent potential source of nodavirus from exporting countries for susceptible fish species cultured in Korea.

It cannot yet be established as to what the source of the virus may be, as well as the most likely route of infection. Due to the paucity of reports regarding the detection of betanodaviruses in healthy aquarium fish and invertebrates, the results of this study indicate the possibility of a new route of the infection into cultured fish populations. The fact that this virus was detected in produce imported from foreign countries implies an urgent necessity for the establishment of a quarantine system in Korea. Until such laws have been implemented, imported aquarium fishes or invertebrates should be randomly assessed by PCR-based techniques, either on a routine basis, to verify that they are pathogen-free; in cases in which fish or invertebrate are infected, a particular batch of samples should not be accepted, or appropriate treatments should be administered prior to clearance and domestic distribution throughout Korea.

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