

Progress and Current Status of Diagnostic Techniques for Marine Fish Viral Diseases at the SEAFDEC Aquaculture Department

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ABSTRACT

The incidence of unexplained mortalities among marine finfish in the Philippines has been increasingly observed. Considering that outbreaks of viral infections affecting similarly cultured marine fishes such as grouper and seabass were reported in many countries, a comprehensive diagnostic program to meet this challenge was initiated at the Aquaculture Department, Southeast Asian Fisheries Development Center (SEAFDEC-AQD) with funding from the Japanese Trust Fund Fish Disease Project. This activity was further boosted by the Japan International Research Center for Agricultural Sciences (JIRCAS). Overall, the program involved the staff of the marine finfish hatchery and of the Fish Health Section. Cases of unexplained mortalities observed in the hatchery were referred to the Fish Health Section. Detailed information on the culture histories of each case were provided by the hatchery staff. Diagnostic tests were performed on each case and those with potential indication of viral etiology were processed for virus detection. Presumptive diagnosis of viral infections was based on typical signs, cell culture isolation, histopathology and *in-vivo* pathogenicity tests. Confirmatory tests to identify specific viruses include RT-PCR, FAT and electron microscopy. The highlights of outbreaks of viral nervous necrosis and other virus-associated infections among marine finfish at SEAFDEC-AQD are presented.

INTRODUCTION

Grouper (*Epinephelus* spp.), snapper (*Lutjanus argentimaculatus*), sea bass (*Lates calcarifer*), rabbitfish (*Siganus gutatus*) and milkfish (*Chanos chanos*) are high valued marine fishes with promising potentials in aquaculture. However, their successful culture had been hampered with outbreaks of viral infections (Yoshikoshi and Inoue, 1990; Mori *et al.*, 1992; Munday *et al.*, 1992; Anderson *et al.*, 1993; Dayanadol *et al.* 1995; Chua *et al.*, 1995; Boonyaratpalin *et al.*, 1996; Fukuda *et al.*, 1996; Le Breton *et al.*, 1997; Chi *et al.*, 1997; Chou *et al.*, 1998; Curtis *et al.*, 2001; Lin *et al.*, 2001). In Asia, intensive research on these viral pathogens has been pursued in Japan, Taiwan, Singapore, China, Indonesia and Thailand. In the Philippines, investigations on

marine fish viral pathogens were revived recently through the support of the Fish Disease Project of the Japanese Trust Fund.

To date, there are three viral pathogens significant to the aquaculture of warmwater marine finfish namely: Viral nervous necrosis (VNN) virus, iridovirus and birnavirus. Hence, these are the viral pathogens that are the focus of the fish viral surveillance programme at SEAFDEC-AQD. The possible occurrence of new viral pathogens is, likewise, not overlooked. This report details the fish viral diagnostic procedures used and summarizes findings.

VIRAL DIAGNOSTIC SERVICES AT THE FISH HEALTH SECTION OF SEAFDEC-AQD

The diagnostic services of the Fish Health Section at SEAFDEC-AQD started in 1981. Initially, tests were limited to detection and identification of parasitic, bacterial and fungal etiologic agents. For fish viral etiology, diagnosis relied mainly on histopathology. In 1993, Lavilla detected typical vacuolations in the eyes of a diagnostic case of sea bass, *Lates calcarifer*, larvae at SEAFDEC-AQD (Lio-Po, 2001). It was subsequently confirmed as a classic case of VNN by B. Munday (Lavilla, pers. comm.). Eventually, cell cultures were established and maintained (Lio-Po *et al.*, 1999; R. Fernandez, pers. comm.). As a result, the rhabdovirus associated with the epizootic ulcerative syndrome (EUS) was the first fish virus pathogen isolated in cell culture in the Philippines (Lio-Po *et al.*, 2000). Due to budgetary and personnel constraints, virus isolation in cell cultures was not sustained.

In 2000, the Japanese Trust Fund Fish Disease Project was implemented at SEAFDEC-AQD. With this development and the availability of more cell lines and advanced molecular techniques for the detection of fish viral pathogens, research on fish virus was revived including fish virus detection in the diagnostic services of the Fish Health Section. Thus, the current Fish Health Section's diagnostic service for microbial infections is more comprehensive (Fig. 1). Fish samples from cases of unexplained mortalities were then processed for the isolation and detection of viral pathogens. In addition, parasitic, bacterial and fungal etiologies were ruled out for each case. The program involved the staff of the marine fish hatchery and of the Fish Health Section. Unexplained mortality cases and details on their culture histories were provided by the fish hatchery staff. Diagnostic tests were performed on each case and those with potential indication of viral etiology were processed for virus detection.

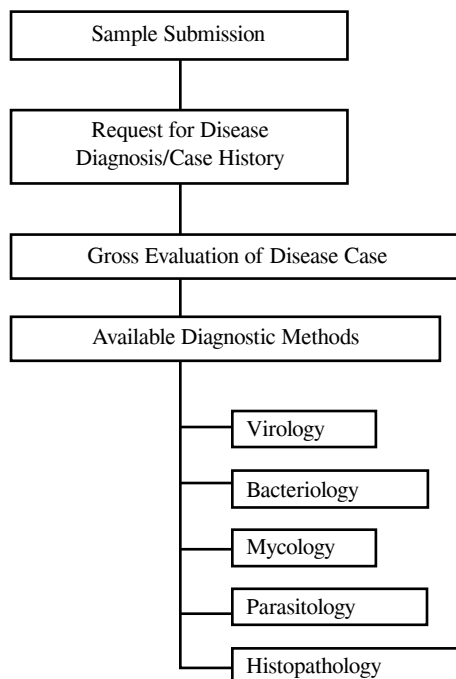


Figure 1. Flow chart of diagnostic sample processing

For viral diagnosis, samples were subjected to presumptive tests and then confirmatory tests to confirm viral etiology. Presumptive tests consisted of typical disease signs, cell culture isolation, histopathology and pathogenicity assay. The confirmatory diagnostic tests used were electron microscopy (EM), reverse transcriptase-polymerase chain reaction (RT-PCR) for detection of VNN and fluorescent antibody test (FAT) using monoclonal antibodies against iridovirus.

Cell Cultures

Established fish cell lines derived from fresh and marine fish species were maintained (Table 1). The cells were cultured in minimum essential medium (MEM) or Leibovitz medium (L15) with 10% fetal bovine serum (FBS). The pH of all media used was adjusted to 7.2 - 7.4 with 7.5% sodium bicarbonate and supplemented with 100 i.u. penicillin G sodium, 100 μg streptomycin sulfate, 25 μg amphotericin B per ml medium.

Table 1. Fish cell lines at SEAFDEC Aquaculture Department

| Cell lines | | Source |
|------------|--|-----------------------------------|
| BF-2 | (Bluegill fry) | N. Ohseko/Y. Maeno |
| CFS | (Catfish spleen) | G. Lio-Po/R. Hedrick |
| EPC | (<i>Epithelioma papulosum cyprini</i>) | K. Nakajima/N. Ohseko/Y. Maeno |
| FHM | (Fathead minnow) | K. Nakajima |
| GF | (Grouper fin) | M. Yoshimizu/J. Kasornchandra |
| SBK-2 | (Sea bass kidney) | M. Yoshimizu |
| SHS | (Snakehead spleen) | G. Lio-Po/R. Hedrick |
| SSN-1 | (Striped snakehead whole fry) | S. Kanchankhan/N. Ohseko/Y. Maeno |
| WSSk | (White sturgeon skin) | R. Hedrick |
| WSS2C1 | (White sturgeon spleen clone 1) | R. Hedrick |

Virus Isolation

External lesions and visceral organs (pool of spleen, liver, kidney, brain, eyes and gills) were aseptically excised and suspended in L15 medium containing 200 i.u. penicillin per ml and 200 μ g streptomycin per ml. Tissue homogenates were prepared, diluted to 10% with Earle's balanced salt solution (EBSS) containing antibiotics and 50 μ g amphotericin B per ml (antibiotic-antimycotic mixture A) (Lio-Po *et al.*, 2000). The homogenates were then centrifuged at 3000 x g for 15 min at 4°C. The supernatant was filtered through a 0.45 μ m pore size membrane filter (Millipore) and stored at -70°C until virus assay.

Primary viral isolations were conducted in EPC, SHS, FHM, SSN-1 cells in 24-well plates (Falcon) with MEM or L15 medium containing 2% FBS (L15-4) buffered with 1M N-2-hydroxyethylpiperazine-N'-2ethanesulfonic acid (Hepes). The plates were then incubated at 25°C for 7 days. Blind passages were carried out on negative samples at least three times. Only isolates manifesting consistent cytopathic effects (CPE) in all subsequent passages were considered positive. Viral titers were determined following the method applied by Lio-Po *et al.* (2000).

Pathogenicity Bioassay

In-vivo viral effects were determined by pathogenicity assays following the method of Lio-Po *et al.* (2001). Aliquots of either tissue filtrates or supernatants of cell culture assays positive for CPE were inoculated into healthy, susceptible fish by either intraperitoneal or intramuscular injection. The manifestation of disease signs in the test fish similar to that observed

in natural infection was interpreted as pathognomonic of viral infection. In addition, further viral assays of tissue filtrates derived from the experimental fish should show the presence of the inoculated virus in cell assays on susceptible cell lines.

Histopathology

For detection of typical histopathological lesions, sections of fish organs (kidney, brain, eyes, spleen) were fixed in 10% buffered formalin or Bouin's solution for at least 24 h then replaced with 70% ethanol. Fixed tissues were processed and sectioned following standard histological techniques (Luna, 1968). Histological sections were stained with Hematoxylin and Eosin stains then analyzed by light microscopy.

Electron Microscopy

In the absence of a transmission electron microscope at SEAFDEC-AQD, electron microscopic visualization of viral isolates was tentatively conducted in Japan in collaboration with JIRCAS scientists. Where appropriate, electron microscope facilities at the University of the Philippines or the St. Luke's Hospital in Manila, may also be tapped. Briefly, virus were inoculated onto susceptible cells in L15 medium and virus purified from cell-free supernatants by gradient ultracentrifugation. Negative staining were applied on purified virus. Infected cell cultures were fixed in glutaraldehyde and post-fixed with osmium tetroxide. Processed cells were embedded, sectioned and stained with uranyl acetate and lead citrate for transmission electron microscopy viewing.

RT-PCR for Detection of VNN

Two methods of tissue sampling were used: the destructive method and non-destructive method. Tissue samples collected for non-destructive sampling include eggs, milt, gills and blood. For destructive sampling, the whole fish was processed for larvae up to 15 days old. In larvae greater than 15 days old only the head portion was processed. For destructive sampling in adult fish, the brain and eyes were processed.

Tissue samples weighing 50-100 mg were dissected aseptically and homogenized in 1 ml of TRIzol reagent (Gibco) and centrifuged at 10,000 x g for 10 min. The supernatant was mixed with 200 μ l chloroform, centrifuged at 10,000 x g for 15 min. The aqueous phase was separated, added with 500 μ l isopropyl alcohol and incubated at room temperature for 10 min. After centrifugation at 10,000 x g for 10 min, the RNA was pelleted by adding 1 ml of 75% ethanol. RNA pellets were dried at room temperature, dissolved in DEPC-treated distilled water and incubated at 58C for 10 min. RNAs were stored at -80C until use.

Detection of the virus by RT-PCR amplification was carried out according to the procedure described by Nishizawa et al. (1994) selecting the T4 (430 base pairs) region as the target sequence for PCR amplification. Briefly, complementary DNA was synthesized from extracted RNAs using MMLV reverse transcriptase (Gibco) and reverse primer (R3:5'-CGA GTC AAC ACG GGT GAA GA-3') at 42C for 30 min and at 99C for 10 min. After addition of the forward primer (F2:5'-CGT

GTC AGT CAT GTG TCG CT-3') and Taq DNA polymerase (Gibco) to the mixture, each cycle of amplification was repeated 30 times at 95C (40 sec), 55C (40 sec) and 72C (40 sec) using a thermal cycler (Mastercycler gradient, Eppendorf). Amplified DNA was analyzed by agarose gel electrophoresis using 2% agarose (Agarose 1000, Gibco).

Fluorescent Antibody Technique (FAT) for Iridovirus Detection

This diagnostic technique was based on the method of Nakajima *et al.* (1995). The spleen of a fish was obtained, dissected and used to dab onto a clean slide. The impression smear was air-dried then fixed in pre-chilled acetone (-20C) for 10 min. After drying, the slide was stored at -20C until further use.

The fixed imprints of the spleen were covered with monoclonal antibodies (M10) and incubated at 37C for 30 min in a humid chamber. The slide was then rinsed three times for 1 min with phosphate buffered saline (PBS) by dipping 10 times in each beaker. The slide was overlain with the secondary antibody (anti-mouse IgG, Cappel) and incubated at 37C for 30 min in a humid chamber. After the slide was washed three times with PBS, two drops of glycerol was added as mounting fluid. The slide was examined under a fluorescent microscope to identify immunofluorescence positive cells.

CASES PROCESSED FOR VIRAL PATHOGENS IN YEARS 2000~2001

Under the Japanese Trust Fund Project, processing of fish diagnostic samples for fish viral pathogens were conducted following the above-mentioned procedures since October 2000 to date. In 2000, a total of 15 fish cases suspected of viral etiology were processed for cell culture inoculation in EPC, FHM, GF, SBK-2 and SNN-1 cells. Of these specimens, filtrates from 4 rabbitfish cases, 5 grouper fingerlings cases and one snapper case were positive for virus.

The following year, samples of 33 cases of grouper eggs, fry/larvae, juvenile and broodstock; 6 cases of milkfish eggs, larvae and juveniles; 1 case of seabass larvae; 3 cases of rabbitfish juveniles and 6 cases of snapper eggs and juveniles were assayed for virus in CFS, EPC, FHM, GF, SBK-2, SHS, SSN-1 and WSS2C1 cells. Only samples from grouper eggs and larvae were positive for virus.

The use of RT-PCR test for VNN at SEAFDEC-AQD were initiated in 2001. This test detected the presence of VNN in 20 cases (43%) of grouper and in a case of snapper larvae. Histopathologic analyses of grouper larvae and fingerlings likewise showed the presence of typical vacuolations in the brain samples of 8 cases (17%) (Fig. 2). Furthermore, Maeno *et al.* (2002) experimentally reproduced VNN in healthy fish and demonstrated the virus by TEM (Fig. 3), thereby confirming the occurrence of VNN in grouper, *E. coioides* in the Philippines.

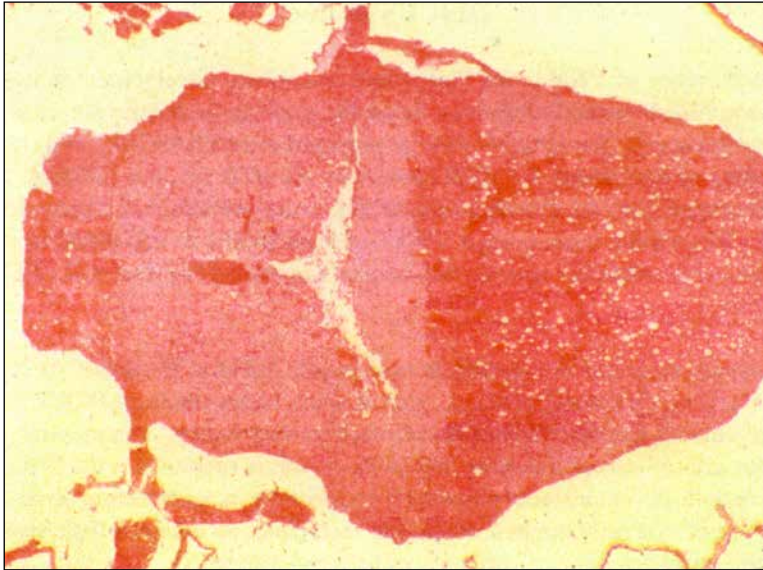


Figure 2. Histological section of the brain of grouper larva showing vacuolations (Hematoxylin and Eosin, 40x)

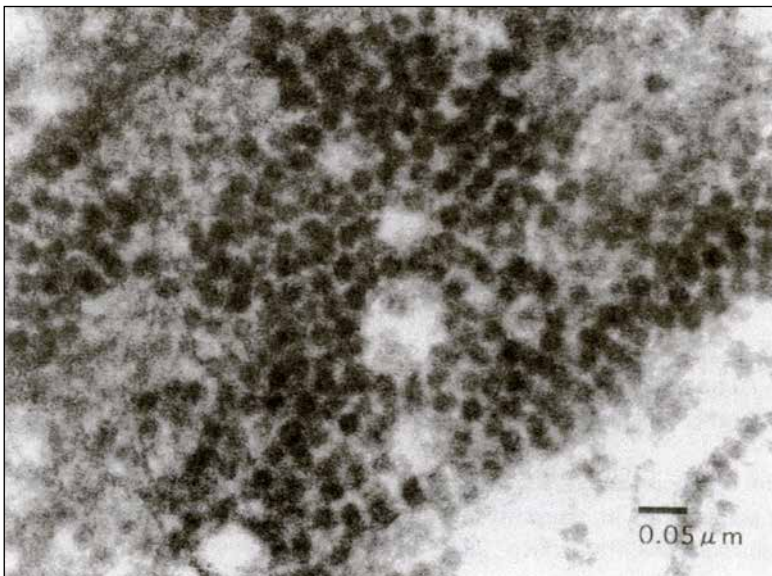


Figure 3. Transmission electron photomicrograph of the piscine nodavirus isolated in SSN-1 cells (bar= 0.05 μm)

DISCUSSION

The occurrence of VNN in the Philippines is a critical development in the successful culture of marine finfish in the Philippines. Since there are no treatments for viral infections, preventive measures are the best options and were proposed at the SEAFDEC-AQD fish hatchery. For one, screening of broodstock for VNN carriers by RT-PCR test was initiated. In addition, washing fertilized eggs with ozone-treated seawater following Arimoto *et al.*'s procedure (1996) is being planned. Vaccination trials using a VNNV vaccine similar to that tested in groupers, *E. septemfasciatus* and *Cromileptes altivelis* will be also be pursued (Tanaka *et al.*, 2001; Yuasa *et al.*, 2002).

In summary, diagnosis of viral infections in marine finfish at SEAFDEC-AQD was based on presumptive and confirmatory tests, *e.g.*, typical signs, histopathology, PCR/RT-PCR, virus isolation in cell culture, pathogenicity tests and electron microscopy. The presence of VNN in grouper, snapper and sea bass as well as an unidentified virus in rabbitfish in the Philippines were confirmed. Meantime, development of more rapid diagnostic tests for detection of viral pathogens and improvement of currently applied diagnostic techniques to enhance their sensitivity and specificity for the early detection of viral pathogens is recommended.

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