

Diagnostic Practices for Marine Fish Viral Diseases in Thailand

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ABSTRACT

The Department of Fisheries, Thailand has three institutions that are capable of virus isolation using fish cell culture system: the Aquatic Animal Health Research Institute (AAHRI), the National Institute of Coastal Aquaculture (NICA) and the Marine Shrimp Research and Development Center (MSRDC). The AAHRI is located in Bangkok while the others are in Songkhla province, south of Bangkok. Fish cell culture system was initiated in AAHRI and NICA in 1992-1993. Both institutions spent 6-12 months to develop and practice cell culture. Since then, fish cell lines have been utilized for virus isolation. Various rhabdoviruses, iridoviruses and reoviruses were isolated from diseased freshwater fishes as well as iridoviruses from cultured frog. In addition, iridoviruses and nodavirus were also isolated from diseased marine finfish. The AAHRI maintains 8 fish cell lines and 2 reptile cell lines while NICA maintains 3 fish cell lines. The MSRDC has 5 marine finfish cell lines. In the three institutions, Leibovitz -15 is the general culture medium used in both tissue culture flask and tissue culture plate systems. This medium is capable of maintaining the pH in close and open culture systems without CO₂ incubation.

Diagnostic practices for marine viral diseases in Thailand include virus isolation, histology and polymerase chain reaction (PCR) amplification technique. As diagnosis in virology is costly, only suspected virus-infected specimens submitted to the Aquatic Animal Disease Clinics are examined for viruses. An active surveillance program of marine viral diseases, with support from the Government of Japan-Trust Fund, has begun this year. The diagnostic procedures for marine viral diseases in the three institutions are similar to the techniques described in the Office International des Epizooties (OIE) Diagnostic Manual and Blue Book.

INTRODUCTION

Knowledge of viral diseases of aquatic animals is very important in the aquaculture industry. Diagnosis of viral diseases is one of the most complicated and time consuming work in aquatic animal disease laboratories. Diagnosis of viral diseases in finfish is more advanced in cold-water fish aquaculture such as the salmonids than the warm-water fishes because of the availability of expertise and funding. However, there is limited information on viral diseases in most countries in Asia.

Viruses usually cause high mortality in fry or juvenile fish. Very low mortalities due to viral infection are reported in larger or adult fish. However, adult fish may readily become infected that will possibly cause high losses. For viral infections, pathogenesis is directly related to the condition of the fish immune system. In tropical regions, lowering of environmental temperature usually reduces the ability of fish to resist viral infection.

In Thailand, initial work on viral diseases in finfish started in early 1990s at the Aquatic Animal Health Research Institute (AAHRI) and the National Institute of Coastal Aquaculture (NICA) and in mid-1990s at the Marine Shrimp Research and Development Center (MSRDC). The AAHRI was developed from the Fish Disease Group of the National Inland Fisheries Institute (NIFI). During the 15 years within NIFI, facilities and expertise in fish health research improved with support from various international agencies including FAO, UNDP, USAID, CIDA and IDRC. In 1990, the Overseas Development Administration (ODA) of the United Kingdom, now the Department for International Development (DFID), recognized the potential of the Fish Disease Group and developed it as a center of expertise in fish disease research for the Southeast Asia region. Subsequently, the Department of Fisheries of Thailand, with support from the ODA through the South East Asia Aquatic Disease Control Project, established AAHRI, which moved into its present premises in 1992. The staff and facilities were upgraded, and its function was expanded to include research on diseases of all aquatic animals.

CELL CULTURE COLLECTION

The AAHRI maintains eight fish cell lines and two reptile cell lines while NICA maintains three fish cell lines (Table 1). The MSRDC has five marine finfish cell lines.

Table 1. Cell line collection of Department of Fisheries, Thailand

Institution	Cell line code	Cell line, full name
AAHRI	EPC	Epithelioma papulosum cyprini
	BB	Brown bullhead
	BF-2	Bull gill fry
	FHM	Fathead minnow
	SSN-1	Striped snakehead fish
	SGP	Snakeskin gourami caudal peduncle
	DFT	Discus fish tail
	HCT	Hybrid catfish tail
	SCE	Siamese crocodile embryo
	STE	Soft-shelled turtle embryo
NICA	EPC, SSN-1, FHM	
MSRDC	SK	Sea bass kidney
	GF	Grouper fin
	GMF	Gray mullet fin
	GGF	Giant grouper fry
	HGF	Humpback grouper fin

The culture medium used for fish and reptile cell lines is Leibovitz-15 supplemented with 10% serum and 2 mM L-glutamine. All cell lines are maintained in an incubator without CO₂ at 25-28°C.

AQUATIC ANIMAL HEALTH CLINIC

All three institutions operate an Aquatic Animal Health Clinic that provides diagnostic facility for farmers. The AAHRI and NICA issue health certificates for live aquatic animal shipments for export. The staff members often visit fish farms at the farmer's request to provide further advice on disease prevention and health management.

DIAGNOSTIC PRACTICES FOR FISH DISEASES IN THAILAND

Generally, occurrence of disease is related to a number of factors such as fish host condition, environment and pathogens. Diagnostic practices conducted at Aquatic Animal Health Clinic are as follows:

1. Obtaining information from fish farmers: all the necessary information on aquaculture activities in the farm are recorded and evaluated.
2. Measurement of water quality parameters: basic parameters measured are pH, alkalinity, hardness, color and transparency.
3. Pathogen examination: diseased specimens are examined for pathogen according to their symptoms as different techniques apply to different pathogens. The details of general diagnostic procedures for finfish diseases in Thailand are documented in Tonguthai, et al. (1999).

Diagnostic Practices for Marine Fish Viral Diseases in Thailand

1. Tissue and fluid sampling

The selection of tissues for viral assays varies according to the size and maturity of the specimen. The following tissues are sampled (Ganzhorn and LaPatra, 1994):

Size/maturity of fish	Tissue assayed
< 4 cm	Entire fish (exclude yolk sac)
4-6 cm	Entire viscera (include kidney)
> 6 cm	Kidney, spleen and gills
Sexually mature	Ovarian fluid, kidney, spleen and gills

For large fish, portions of the kidney, spleen, pancreas and gills are combined as one sample. The tissue sample should be at least 1 g. The retina layer of the eye ball and the brain are taken for fishes exhibiting clinical signs of VNN and combined as one sample.

Tissues from a maximum of 10 fry/fingerlings are pooled and treated as one sample. When larger fish are sampled, tissues from less than five fish are pooled. Pooled samples are of equal volume or weight.

2. Preparation of Tissue Extract

Diseased fish with minor clinical lesions are collected. Fish are sacrificed and wiped clean with tissue paper. Approximately 1 g of fish tissue is needed. The tissue sample can be pooled from muscle and internal organs. For muscle samples, tissue debris and surface fungus on the ulcerated lesions are removed using a clean razor blade. Pieces of muscle tissue are taken from beneath the lesions. For internal organs, the abdomen is carefully opened using clean scissors and small pieces of tissue from kidney, spleen and pancreas are taken and pooled. These tissue samples can be stored up to 48 h in transport medium or Hank's balanced salt solution (HBSS) with 2% fetal calf serum, 500 units/ml of penicillin and 500 µg/ml of streptomycin. The samples can also be immediately processed as follows:

- a. Samples are homogenized using a sterile, pre-cooled pestle and mortar until a smooth paste is obtained. Sterile fine sand is added to facilitate homogenization.
- b. Samples are diluted 1:10 by the addition of 9 ml HBSS with 2% fetal calf serum. After mixing well, the samples are transferred to sterile centrifuge tubes and spun at 1000x g at 4°C for 15 min to separate cell debris, sand and possibly some contaminants from the fluid extract.
- c. A further 1:5 dilution (or 1:50 from the original tissue sample) is carried out by filling 5 ml sterile disposable syringes with 4 ml HBSS with 2% fetal calf serum and then drawing up 1 ml supernatant.
- d. The 1:50 final dilution is mixed well, then filter-sterilized through 0.45 µm disposable filter units.
- e. The filtrates or tissue extracts are kept in 5 ml sterile bottle at 4°C and are then ready to be inoculated onto fish cell lines.

3. Viral Isolation

Simultaneous cell culture and sample inoculation are carried out using at least 2 different fish cell lines. For diseased marine fish, the extract is inoculated onto SSN-1 and EPC or SCE cell lines while SSN-1 or BF-2 and EPC are used for isolation of viruses from fresh water fish. Viral isolation is done in 24-well plates if there are many samples. The following are the general practices at AAHRI:

- a. Each plate is first seeded with a single cell suspension of the fish cell line in maintenance medium (L-15 medium containing 2% fetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin).
- b. Each well receives 1.3-1.4 ml of cell suspension. Cells with complete monolayer in 25 cm² tissue culture flask is sufficient to produce an 80-90% confluent monolayer in 1 day after seeding in one 24-well tissue culture plate.
- c. One tissue extract (1:50 dilution) is immediately inoculated into two wells. The first well receives 200 µl inoculum while the second well receives 50 µl inoculum. The same number of replicate wells is used as negative control for each plate. However, a 1:10 dilution of the tissue extract is prepared if low viral infection is suspected.
- d. The tissue extract-inoculated cells are incubated at 23-25°C and observed daily for CPE for at least 14 days. Observation time is longer for marine viruses.
- e. A first blind passage of culture fluid is performed on days 7-10 for freshwater fish viruses. For marine fish viruses, the first blind passage is performed on days 10-14. Viral passage or subculture is done by transferring 200 µl of supernatant from each well to fresh 24-well culture plate. Observations for CPE is continued further for 5-7 days in the old plates. A second and third blind passages are also carried out.
- f. Samples showing CPE in which the cell monolayer changed, disintegrated, sloughed off from the surface of the tissue-culture wells or ended with cell lysis, will be passaged further to provide larger quantities of the suspected virus.
- g. A 500 µl of supernatant from a single well exhibiting CPE is inoculated into 25 cm² flasks containing 80-90% confluent cell monolayer. The suspected virus is allowed to be adsorbed for 1 h.
- h. The cells are washed once with 5 ml PBS then 7-8 ml of maintenance medium is added.
- i. Flasks are incubated at 23-25°C together with un-inoculated control flasks for comparison.
- j. When the cells show complete CPE, they are centrifuged at 1000x g at 4°C for 15 min.
- k. The supernatants are collected, aliquoted in tubes with 1 ml quantities and stored at 4°C and 20°C or -80°C, for further characterization.

VIRAL CHARACTERIZATION AND IDENTIFICATION

Once viruses are isolated in cell culture, efforts are needed to maintain them. Viruses are checked after storage to ensure viability. The basic properties of viruses, such as type of nucleic acid, envelop testing and particle morphology, are examined for viral Family classification.

Since the establishment of cell culture system in Thailand in the early 1990s, various fish rhabdoviruses, iridoviruses and reoviruses have been isolated from diseased freshwater fishes as well as iridoviruses from cultured frog. Iridoviruses and nodavirus have also been isolated from disease marine finfish.

For viral identification, serology and molecular biology are basically used. Polyclonal antibodies are produced from rabbits and neutralization and immuno-blot techniques are conducted for serological tests. A classic neutralization test is normally used to identify viruses and identify the sero-group. With the accessibility of different sequences of viral genes in database such as the Gene Bank, it is easy to obtain sequences that can be used for gene probes and primers for PCR.

With these advanced molecular tools, viruses are readily identified within a short time. An active surveillance program on viral diseases of grouper in Thailand using viral isolation and PCR screening has begun this year, with support from the Government of Japan-Trust Fund.

CONCLUSION

It does take time to get familiar with cell culture and learn how to prevent bacterial contamination, especially in laboratories where the culture medium is bought in powder form and pipettes and bottles are re-used. Most of the procedures for cell culture and virus isolation performed at the three institutes of the Department of Fisheries generally follow the standard procedures published elsewhere such as the OIE Diagnostic Manual (OIE, 2000) and the Blue Book (Thoesen, 1994)). Surprisingly, after the establishment of cell culture and virus isolation, many viruses have been isolated from diseased fishes in Thailand. Most of these viruses isolated were found to be new strains. It is most important to encourage scientists in the region to conduct researches in aquatic animal viruses, as viral diseases in many countries have not been explored.

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