Biodiversity of viruses in fish and shellfishes of Taiwan


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- History of the investigation of viral diseases in Taiwan
  - Bacterial diseases started in 1976: Dr. G. H. Kou
  - Viral disease was initiated in 1982: Drs. G. H. Kou, S.N.Chen, John Fryer and Ron Hedrick and James Winton
  - In 1984, Dr. Y.L. Hsu came back from US and taught virology, since then a lot of young virologists produced.
### Some Properties of virus used in taxonomy

**Virion Properties**

- **Morphology**
- **Virion Size**
- **Virion Shape**
- Presence or absence and nature of peplomers
- Presence or absence of an envelope
- Capsid symmetry and structure

**Physiochemical and Physical properties**

- Virion molecular mass (Mr)
- Virion buoyant density (in CsCl, sucrose, etc.)
- PH Stability
- Thermal Stability
- Cation Stability (Mg2+, Mn2+)
- Solvent Stability
- Detergent Stability
- Irradiation Stability

**Genome**

- Type of nucleic acid (DNA or RNA)
- Size of genome in kb/kbp
- Strandedness: single-stranded or double stranded
- Linear or circular
- Sense (positive-sense, negative-sense, ambisense)
- Number and size of segments
- Nucleotide sequence
- Presence of repetitive sequence elements
- Presence of isomerisation
- G&C content ratio
- Presence or absence and type of 5' terminal cap
- Presence or absence and type of 5' terminal covalently linked protein

**Proteins**

- Number, size, and functional activities of structural proteins
- Number, size, and functional activities of nonstructural proteins
- Details of special functional activities of proteins, especially transcriptase, reverse transcriptase, hemagglutinin, neuraminidase, and fusion activities
- Amino acid sequence or partial sequence
- Glycosylation, phosphorylation, myristylation property of proteins
- Epitope mapping

**Lipids**

- Content, character, etc.

**Carbohydrates**

- Content, character, etc.

**Genome organisation and replication**

- Genome organisation
- Strategy of replication
- Number and position of open reading frames
- Transcriptional characteristics
- Translational characteristics
- Site of accumulation of virion proteins
- Site of virion assembly
- Site and nature of virion maturation and release

**Antigenic properties**

- Serologic relationships, especially as obtained in reference centers

**Biologic properties**

- Natural host range
- Mode of transmission in nature
- Vector relationships
- Geographic distribution
- Pathogenicity, association with disease
- Tissue tropisms, pathology, histopathology
Class 3: dsRNA
Family: Birnaviridae
Genus: Aquabirnavirus

• Characteristics
  - Infectious pancreatic necrosis virus (IPNV) was the first fish virus isolated by cell culture technique. It has a very broad host range in finfish and shellfish and distributed worldwide. Infected fishes exhibit the necrosis in pancreas, kidney, and with exophthalmia, abdominal distention, hemorrhage at ventral areas and bases of fins. Virulent strain of IPNV can cause 90-100% mortality, but only about 10% in avirulent strains. However, in the presence of stressors, IPNV-T42G can cause mortalities of 90-100% in grouper fry. Birnavirus has two segmented, double-stranded RNA genomes and five viral proteins.
  - Electrophoretypes of IPNV RNA and polypeptide pattern in Taiwan:
    - Major: AB
    - Minor: VR-299
    - Four rainbow trout isolates from Dan Sway (1984)
    - RNA pattern: similar to VR-299
    - early viral polypeptide pattern: Similar to AB
    - In Japan, AB type was wide distributed.
    - These VR-299 isolates of rainbow trout was originally imported from Japan. After Silver salmon cultures had been exported to Japan the VR-299 type appeared in Japan trout.
• Animal pathogens
  - trout, eel, milkfish, tilapia, grouper, perch, clam, ayu, golden arowana, Moorish idol, loach
Fig. 1. Comparison of RNA patterns of IPNV isolates from eel (E) and trout (T) with reference IPNV serotypes VR-299, AB, SP, EVE and Roo-3 separated by in 10%, SDS-PAGE and stained with silver nitrate.

Fig. 2. Comparison of RNA patterns of mixed samples of IPNV isolates from eel and trout with reference IPNV serotypes and mvirus in a silver nitrate-stained 10%, SDS-PAGE gel.

Fig. 3. Autoradiogram of viral polypeptides of IPNV isolates from eel with those of reference strains labelled in early infected CHSE-214 cells. Infected cells were u.v.-irradiated at 3 h post-infection (C + u.v.), and labelled for 1 h at 5 post-infection. α, β1, γ1 and γ2 are virus-induced polypeptides. H denotes a host cell polypeptide.

Fig. 4. Autoradiogram of viral polypeptides of IPNV isolates from trout with those of reference strains labelled in early infected CHSE-214 cells. Method as described in legend for Fig. 3.
Class 3: dsRNA
Family: Reoviridae
Genus: Aquareovirus

Characteristics:
This typical double-layered capsid of icosahedral virion with a 78 nm diameter was appeared in EM, and a buoyant density in CsCl is 1.365 g/ml, both are typical characteristics of Reoviridae. However, LSV shows 11 segmented dsRNA genomes and 5 major virion proteins, and has aquatic animal hosts with an optimal temperature of 20 ℃, all which are characteristics of aquareovirus, and are different from viruses of the defined 6 genera of reoviridae. In 1988, another aquareovirus isolated from rainbow trout (RTV) and hard clam (HCV) were compared with LSV, all of them expressed the same RNA and virion protein patterns, but different from CSV, GSV, CRV and 13P2. Only the cultured hard clam pond had 47.4% mortality.

Animal pathogens
Landlocked salmon virus (LSV), Rainbow trout virus (RTV), Hard clam virus (HCV)
Landlocked Salmon virus

A Reo-like Virus from Landlocked Salmon

Table 1. The multiplication of landlocked salmon virus (LSV) in selected fish cell lines at 18°C

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Abbreviation</th>
<th>TCID₅₀/ml</th>
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<tbody>
<tr>
<td>Atlantic salmon</td>
<td>AS</td>
<td>2.2 x 10⁶</td>
</tr>
<tr>
<td>Bluegill fry</td>
<td>BF-2</td>
<td>7.0 x 10⁵</td>
</tr>
<tr>
<td>Brown bullhead</td>
<td>BB</td>
<td>5.0 x 10⁴</td>
</tr>
<tr>
<td>Chum salmon ovary</td>
<td>COO</td>
<td>1.6 x 10⁴</td>
</tr>
<tr>
<td>Chinook salmon embryo</td>
<td>CHSE-214</td>
<td>1.0 x 10⁴</td>
</tr>
<tr>
<td>Epitheloma papulosus cystic</td>
<td>EPC</td>
<td>3.2 x 10³</td>
</tr>
<tr>
<td>Fathead minnow</td>
<td>FHM</td>
<td>5.0 x 10³</td>
</tr>
<tr>
<td>Grouper kidney</td>
<td>GK</td>
<td>1.6 x 10³</td>
</tr>
<tr>
<td>Milkfish heart</td>
<td>MHR</td>
<td>6.8 x 10²</td>
</tr>
<tr>
<td>Perch heart</td>
<td>PH</td>
<td>6.8 x 10²</td>
</tr>
<tr>
<td>Perch liver</td>
<td>PL</td>
<td>4.7 x 10²</td>
</tr>
<tr>
<td>Rainbow trout gonad</td>
<td>RTG-2</td>
<td>6.8 x 10²</td>
</tr>
<tr>
<td>Tilapia ovaries</td>
<td>TO-2</td>
<td>3.2 x 10³</td>
</tr>
</tbody>
</table>

Fig. 3. Electron micrography of negatively stained LSV.

Fig. 4. Electrophoretypes of RNA genomes of LSV, CSV, Reo-3, GSV and CRV.

Fig. 5. Electrophoretypes of major virion proteins of CSV, CRV, GSV, 13P2, and LSV.
Class 1: dsDNA
Family: Iridoviridae
Genus: unnamed

- **Characteristics**
  - Since 1992, red sea bream culture in Taiwan’s Pon Fu Island has been plagued by outbreaks of a new disease. Based on EM observation, an iridovirus-like infection was appeared (Chou et al., 1994), later grouper and sea bass were also appeared the similar infection. In 1995, a new epizootic viral disease causing up to 60% mortality has threatened cultured grouper (Epinephelus sp.) in Taiwan, an icosahedral particles with a diameter of 230±10 nm were observed in the spleen of moribund fish. This grouper iridovirus of Taiwan (TGIV) can induce cytopathic effect (CPE) in KRE cell line, and the susceptibility of the virus to chloroform and ether indicates that virus may be enveloped. That acridine orange staining and IUDR treatment suggest viral genome is double-stranded DNA. While healthy juvenile groupers were experimentally challenged with TGIV by intraperitoneal injection, cumulative mortality reached 100% within 11 days, and no grouper died in control groups, and similar virus can be re-isolated.

- **Animal pathogens**
  - Groupers, sea bass, red sea bream.
Grouper Iridovirus of Taiwan (TGIV)

Table 2. General properties of TGIV

<table>
<thead>
<tr>
<th>Characterization</th>
<th>Yield of TGIV (Log TCID₅₀/ml)</th>
<th>Reference virus (IPNV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>to Ether</td>
<td>2.92</td>
<td>N/A</td>
</tr>
<tr>
<td>Chloroform</td>
<td>2.72</td>
<td>N/A</td>
</tr>
<tr>
<td>Control</td>
<td>4.63</td>
<td>N/A</td>
</tr>
<tr>
<td>Stability</td>
<td></td>
<td></td>
</tr>
<tr>
<td>at pH 3</td>
<td>2.70</td>
<td>N/A</td>
</tr>
<tr>
<td>pH 7.2</td>
<td>5.40</td>
<td>N/A</td>
</tr>
<tr>
<td>pH 11</td>
<td>3.40</td>
<td>N/A</td>
</tr>
<tr>
<td>Susceptibility</td>
<td></td>
<td></td>
</tr>
<tr>
<td>to IUDR</td>
<td>3.50</td>
<td>8.0</td>
</tr>
<tr>
<td>Control</td>
<td>6.00</td>
<td>8.0</td>
</tr>
</tbody>
</table>

N/A: not available

Fig. 1. External appearance of spontaneously diseased grouper which were sampled from Chihs-Ting area of Kao-Hsing. Eviscerated fish are off their food and lethargic.

Fig. 2. Giant cells (arrows) in the spleen of spontaneously diseased grouper. Thick section, toluidine blue stain, x 1200.

Fig. 3. Transmission electron micrograph of the spleen from spontaneously diseased grouper. (A) Viral particles in the cytoplasm of a giant cell. Nuc: nucleaus. (B) High magnification of viral particles. Scale bars = 5 μm in A, 500 nm in B.

Fig. 4. Cytopathic effect (CPE) in KRE cells 3 days after inoculating filtrate from diseased groupers. (A) Uninfected KRE cells, (B) infected KRE cells.

Fig. 5. Mortality (%) of groupers (Epinephelus sp.) experimentally infected with intraperitoneal injection with TGIV (10⁶ TCID₅₀/0.1 ml/fish). Groupers injected with medium only served as control. Data shown are the mean of 2 replicates.

Fig. 6. Cumulative mortality (%) of groupers (Epinephelus sp.) experimentally infected with TGIV (10⁶ TCID₅₀/0.1 ml/fish). Groupers injected with medium only served as control. Data shown are the mean of 2 replicates.

Fig. 7. Transmission electron micrograph of spleen from a grouper experimentally infected with TGIV. (A) Aggregation of viral particles in the cytoplasmic membrane. (B) High magnification of viral particles. Scale bars = 5 μm in A, 1 μm in B.
Class 1: dsDNA
Family: Herpesviridae
subfamily: Alloviridae
Genus: unnamed

• Characteristics
  • A herpesvirus isolated from Taiwan cultured European eels Anguilla anguilla was first characterized in 2002 (Chang et al., 2002), the syncytia formation was detected both in EP-1 (eel epidermis) cell and EK (eel kidney) cells, under EM examination, numerous nucleocapsids of about 100 nm in diameter were found within the nucleus of infected cells, whereas enveloped particles were observed within the cytoplasm. The mature viral particle, about 235 nm in diameter had an electron-dense core with a hexagonal nucleocapsid surrounded by a coarse capsule. A 402 bp fragment was amplified by PCR and cloned from genomic DNA of EEHV (European eel herpesvirus). The nucleotide homology was 99% (298 of 300) with DNA polymerase of eel herpesvirus. The in situ hybridization (ISH) of EEHV was also established and detected in skin, liver, spleen and kidney, later, Shih et al. (2003) also detect Herpesvirus anguillae (HVA) DNA using in situ hybridization or dot blot.
  • In 2003, high mortality of abalone occurred among land-based and ocean-based ponds in north eastern Taiwan that resulted in losses of US$11.5 million to the domestic abalone industry, a herpes-like virus is demonstrated for the first time to be associated with high mortality rates in this maricultured abalone Haliotis diversicolor supertexta (Chang et al., 2005). The nerve system was the primary target tissue, and the tissue necrosis accompanied with infiltration of haemocytes. EM demonstrated viral particles within the degenerated cerebral ganglion cells, the virus was hexagonal, approximately 100 nm in diameter and had a single coat. The minced visceral organs and muscle of moribund abalone induced 100% mortality through both intramuscular injection and bath treatments.
  • In 2002, mass mortality occurred in 2-year-old koi (fancy carp) Cyprinus carpio in Taiwan, no external signs except swollen gills appeared, only the histological changes were observed in gills: hyperplasia of epithelial cells, infiltration of eosinophilic granular cells and fusion of the secondary lamellae (Tu et al., 2004). Negatively stained nucleocapsids were icosahedral and 112+1 nm in diameter. Koi herpesvirus (KHV) was detected in the diseased fish fry by PCR using specific primers for KHV, and sequence showed a 99% identity with the published data.
European eel HerpesVirus (EEHV)

Fig. 2. Anguilla anguilla. Transmission electron micrographs of EP-1 cell infected with European eel herpesvirus. (A) Viral particles replicated in the nucleus (arrow) and released into the cytoplasm (arrow); scale bar = 1 μm. (B) Enveloped virions in the cytoplasm (arrow) and nucleocapsids in the nucleus (inset); scale bar = 450 nm.

Fig. 3. Anguilla anguilla. Histopathology of natural infected European eel. (A) Diseased fish showing epidermal hyperplasia with melanomacrophage inclusions (arrow) in the skin; scale bar = 100 μm. (B) Diseased fish with melanomacrophage aggregates in the kidney; scale bar = 25 μm.

Fig. 4. Anguilla anguilla. Agarose gel electrophoretic analysis of the cloned fragment (approximately 400 bp) from PCR product of EEHV obtained with Primers F1 and F2. Lane M: 100 bp DNA ladder; Lane S: sample.

Fig. 5. Anguilla anguilla. In situ hybridization of European eels with natural infection. (A) Strong signals detected throughout the melanomacrophage aggregates in head kidney observed with fluorescent microscope (FR-TR). (B) Melanomacrophage aggregates corresponding with location of (A) are black-brown when observed under light microscope (FR-TR); (A) and (B) = 200 x.
Class 5: ssRNA (-)
Family: Rhabdoviridae
Genus: Norirhabdovirus

Characteristics
The first isolate of infectious hematopoietic necrosis virus (IHNV) from rainbow trout was found in 1983 in Taiwan (Chen et al., 1985), the virion appeared bullet shape 90 x 180 nm under electron microscopy, and hematopoietic tissue necrosis, serum neutralization and fluorescent antibody tests all showed the positive IHNV infection. IHN disease was not reported after that until another disease outbreak occurred in rainbow trout ponds in the central part of Taiwan during Nov. 1994 to February 1995 (Wang et al., 1996). The infected fry, about 2 to 3 cm in length, were lethargic and anorexic. Most of them lay on the bottom of ponds for several days before dying. The morbidity and mortality of the outbreak were 90% (22000/25000) and 95% (21000/22000), respectively. Three isolates were made from these outbreaks, and polymerase chain reaction (PCR), several IHNV monoclonal and rabbit polyclonal antibodies were used to confirm the IHNV PCR assay.

Animal pathogens
Rainbow trout (imported eggs)
Infectious Hematopoietic Necrosis Virus

Fig. 1. Amplified viral products from isolates by thermal cycle reaction were analyzed on an ethidium bromide-stained 1.5% agarose gel. Lane 1: NCH1 isolate; lane 2: NCH2 isolate; lane 3: NCH3 isolate; lane 4: IHNV positive control virus; lane 5: aquatic animal barnavirus; lane 6: uninfected CHSF-214 cells.

Table 1. Identification of isolates from rainbow trout as IHNV by immunodot assay. +: positive reaction; -: negative reaction.

<table>
<thead>
<tr>
<th>Virus</th>
<th>IHNV MAb</th>
<th>IHNV MAb polyclonal</th>
<th>IPNV MAb</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCH1</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>NCH2</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>NCH3</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>IHNV</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>IPNV</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CHSF-214</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 5. IHNV (chinook salmon isolate) budding from an FHM cell membrane. The plasma membrane is continuous with the outer membrane of virus. An irregular fringe extends beyond the outer membrane. ×105,400. From Darlington et al. (1972). Reprinted with the permission of Springer-Verlag.
Class 4: ssRNA (+)
Family: Nodaviridae
Genus: betanodavirus

• Characteristics
  A mass mortality of hatchery-reared grouper larvae and juveniles has occurred repeatedly in Taiwan, the virion was isolated and identified by RT-PCR as a fish nodavirus, designated grouper nervous necrosis virus (GNNV) (Chi et al., 1997). In order to amplify GNNV in vitro, a cell line GF-1 was developed from the fin tissue of a grouper, Epinephelus coioides (Hamilton) (Chi et al., 1999), and was used to study the biochemical and biophysical properties of GNNV. The causal pathogen of VNN disease has been characterized as a small, non-enveloped, bi-segmented, single-stranded, positive-sense RNA virus with a diameter of 25-30 nm (Chi et al., 2001; Lai et al., 2001). Genotypes: RG (red spotted grouper), SJ (striped jack), TP (tiger puffer), JF (Japanese flounder).

• Animal pathogens
  Red spotted Grouper Nervous Necrosis Virus (RGNNV), various groupers, cobia, eel, barramundi
Nervous Necrosis Virus

Figure 1: Electron micrograph showing necrotic cells in tissue culture infected with Nervous Necrosis Virus (bar = 100 nm).

Figure 2: Electron micrograph showing degenerated structures in infected tissue (bar = 100 nm).

Figure 3: The morphology of the purified virus isolated from diseased grouper (bar = 100 nm).

Figure 4: Agarose gel electrophoresis of the products by RT-PCR amplification using primers specific to the T2 region of SNNV RNA2 and the nucleic acids extracted from three diseased juvenile groupers as templates. Lane 1 is pGEM marker; lanes 2–4 show the PCR products of nucleic acids extracted from three different fish; and lane 5 shows a template-free control reaction.

Figure 5: The products in the nested PCR amplification using the R2–R3 primer set specific to the T4 region of SNNV 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.

Figure 6: The structural proteins of the purified viruses from diseased groupers analysed by 12% SDS-PAGE.
How Important of IPNV?

Virulent strain -----------80-100% mortality
Avirulent strain---------10-15% mortality
ZnCl\_2  5ppm----------
CdCl\_2  3ppm-----------5-15% mortality
CuCl\_2  1ppm----------
IPNV + heavy metal------96-100% mortality
IPNV + salinity change-----80-100% mortality
Virio carchariae---------0-40% mortality
IPNV + Vibrio----------100% mortality

Worldwide distributed in various species of fish and shellfishes, and avirulent + stressors can induce 90-100% mortality.
Five viral proteins: 3 of them are kinases and 1 phosphastase—How significant are them?
How virus manipulates host cell.

“Signaling Redirection Hypothesis”
Conclusion

- Proposed
  Emerging species: Herpesvirus
  Endangering species: IPNV, Herpesvirus, Iridovirus

Future prospects:
Model system such as IPNV for “Signaling Redirection Hypothesis”
Impacts would be on life processes, drug screen, virus prevention