Cell culture isolation of piscine neuropathy nodavirus from juvenile sea bass, *Dicentrarchus labrax*

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A virus causing a vacuolating encephalopathy and retinopathy in juvenile sea bass, *Dicentrarchus labrax*, was isolated from brain tissue in a fish cell line (SSN-1) derived from striped snakehead, *Channa striatus*. The isometric, non-enveloped, 30 nm diameter virus particles were resistant to pH 2-9 and heating at 56 °C for 30 min. Infectious particles had a buoyant density of approximately 1.31 g/cm³ in CsCl. Two structural polypeptides of molecular mass 40 and 42 kDa were identified and the ssRNA consisted of two fragments of molecular mass 1.10 and 0.51 x 10⁶ Da. From these characteristics the virus was identified as a nodavirus. Due to the broad range of susceptible fish hosts and the consistent neuropathology of the disease condition, the generic term piscine neuropathy nodavirus (PNN) is proposed for this infectious agent.

During the past 10 years, a new infectious neuro-pathological condition of hatchery-reared fish has been encountered with increasing frequency in the marine aquaculture industry. The disease is characterized by the development of a vacuolating encephalopathy and retinopathy with high mortality in larval and juvenile stages of affected fish. The central nervous system lesions are consistently associated with aggregates of unenveloped, roughly spherical virus particles 25–30 nm in diameter, hitherto suggestive of a picornavirus infection.

First accounts of the disease relate to outbreaks causing heavy losses at the early larval stage in sea bass (*Dicentrarchus labrax*) hatcheries in Martinique and the French Mediterranean (Breuil et al., 1991) and barramundi (*Lates calcarifer*) in Queensland, Australia (Glazebrook et al., 1990). Comparable clinical conditions with central nervous system lesions, picornavirus-like infections and high mortalities have now also been recorded for larval and juvenile forms of barramundi in Tahiti (Renault et al., 1991), turbot (*Scophthalmus maximus*) in Norway (Bloch et al., 1991), the Japanese parrotfish (*Oplegnathus fasciatus*) (Yoshikoshi & Inoue, 1990), red-spotted grouper (*Epinephalus akean*) (Mori et al., 1991), striped jack (*Pseudocaranx dentex*) (Mori et al., 1992) and a number of other marine fish species in Japan (Nishizawa et al., 1995). The very recent report of the disease in hatchery-reared halibut (*Hippoglossus hippoglossus*) in Norway (Grotmol et al., 1995) underlines the increasing significance of this infection in terms of susceptible fish species, geographical distribution and the future development of marine aquaculture.

Although the infectious agent could not be recovered in cell culture, the transmissibility of the disease has been experimentally established in red-spotted grouper (Mori et al., 1991) and striped jack (Arimoto et al., 1993) by injection or bath challenge with homogenized tissue filtrates from affected fish. Other reported attempts at virus isolation in fish cell lines were also unsuccessful but the agent was nevertheless identified as a nodavirus by biochemical characterization of the nucleic acid and structural protein of purified virion material obtained directly from diseased striped jack larvae (Mori et al., 1992). The same identification has been similarly established for viral particles recovered from the brains of affected sea bass and barramundi (Comps et al., 1994) and an analysis of coat protein gene sequences has since confirmed a close relationship between the striped jack nodavirus and the viruses from four other Japanese fish species with the same disease condition (Nishizawa et al., 1995). We now report the successful isolation in cell culture of the virus from characteristically diseased sea bass juveniles and confirm the identification of this infectious agent as a nodavirus.

Within 3 days of transfer of juvenile sea bass of approximately 2 g weight to an ongrowing Mediterranean mariculture site, the fish started to exhibit abnormal swimming behaviour. Affected specimens were seen near the water surface swimming on their sides or spiralling. Initial low level mortalities gradually increased during the ensuing 6 month period when the total stock loss reached just over 10%. Sibling stocks on other farms were reported to have shown the same abnormal behaviour but mortalities were even more severe, varying from 30–60% total stock loss depending upon site.

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Tissue samples from diseased fish were fixed in 10% buffered formalin, processed, wax embedded, sectioned (5 μm) and stained with haematoxylin and eosin (H&E). On histological examination, pathology was restricted to the brain, spinal cord and nervous tissue layer in the retina. There was a diffuse reaction in all areas of the brain with neuronal cytoplasmic vacuolation, neuronophagia, microgliosis and congestion of blood vessels with some perivascular haemorrhages. Oval or round intracytoplasmic eosinophilic inclusion bodies (2.5–4 μm) were also observed. In the cerebellum the Purkinje cells appeared atrophied and eosinophilic and seemed to be degenerating. Vacuolation, neuronophagia and microgliosis were also observed in the spinal cord. In the nuclear layer of the inner nervous layer in the retina, cytoplasmic...
vacuolation was a common finding. All other organs sampled, including gills, stomach, intestine, liver, kidney, spleen, muscle and skin showed no significant pathology.

Fresh brain material from 30 fish was pooled, homogenized in Eagle's minimal essential medium (EMEM) with antibiotic supplement, filtered (0.2 μm) and the fluid extract diluted in 30 ml of EMEM. The greater portion of this material was clarified by high speed centrifugation at 10,000 g for 15 min and the supernatant then ultracentrifuged at 150,000 g for 90 min in an SW41 swinging bucket rotor (Beckman). Pelleted material was resuspended in a drop of PBS, placed on a formvar–carbon film grid, negatively stained with methylamine tungstate (Bio-Rad) and examined in a Philips 301 transmission electron microscope. Small, roughly spherical ‘full’ and ‘empty’ virus particles 30 nm in diameter were visualized. The lesser portion of brain extract was used to simultaneously inoculate and seed EPC (carp), FHM (fathead minnow) and a third cell line, SSN-1, derived from a striped snakehead fish (Frerichs et al., 1991). Inoculated cultures were grown as monolayers in EMEM or Leibovitz L-15 medium supplemented with 5% fetal bovine serum and antibiotics in 25 cm² tissue culture flasks at 20 °C. No cytopathic changes developed in EPC or FHM cultures over a 17 day observation period but evidence of viral infection was noted in SSN-1 cells within 3 days of inoculation. A specific cytopathic effect (CPE) developed initially as localized areas of rounded, granular, refractile cells which spread through the cell sheet over a further 3–4 days to form a meshwork of degenerating cells before complete disintegration of the monolayer within about 10 days. The CPE was readily transmitted by inoculation of clarified tissue culture fluid to fresh suspensions or preformed monolayers of SSN-1 cells.

Electron microscope examination of ultrathin sections of infected SSN-1 cells fixed in glutaraldehyde, post-fixed in OsO₄ and stained with uranyl acetate–lead citrate showed intracytoplasmic, membrane bound, aggregates of virus particles (Fig. 1a). Particle morphology and size were never clearly delineated in fixed tissue sections, but negative stained preparations of virions pelleted from culture fluid revealed full and empty, non-enveloped, 30 nm diameter isometric particles with small surface projections (Fig. 1b), indistinguishable from the particles pelleted directly from brain tissue extract.

Fig. 2. SDS-PAGE analysis of neuropathy nodavirus structural protein. Lanes 1 and 3, molecular mass markers. Lane 2, nodavirus polypeptides.

Fig. 3. Agarose-formaldehyde gel electrophoresis analysis of viral genomic RNA. Lane 1, RNA molecular mass marker II. Lane 2, nodavirus RNA. The white bar marks indicate RNA fragments. Lane 3, brome mosaic virus RNA.
Virus replication in SSN-1 cells was not inhibited by the incorporation of 50 μg/ml ddUdR in the culture medium and acridine orange staining of 48 h infected culture monolayers revealed clearly demarcated, flame-red, intracytoplasmic inclusion bodies establishing the genome as ssRNA (Rovozzo & Burke, 1973). The non-enveloped nature of the particle was confirmed by infectivity resistance to chloroform treatment (Feldman & Wang, 1961). Isopycnic centrifugation of pelleted virus for 17 h at 150 000 g in a 20–40% (w/w) CsCl gradient and assay of recovered fractions in SSN-1 cells established a buoyant density of approximately 1.31 g/cm³ for infective particles. The virus was resistant to exposure across the range pH 2.9 for 30 min with less than 1 log₁₀ drop in infectivity titre, and likewise was resistant to heat treatment at 50 °C for 1 h (Rovozzo & Burke, 1973).

The structural protein of CsCl gradient purified virus was analysed essentially according to the method of Laemmli (1970) using the Mini-V 8.10 Vertical Gel Electrophoresis System (Gibco BRL). Purified virus was disrupted by boiling in SDS reducing buffer and the polypeptides separated by discontinuous polyacrylamide gel electrophoresis (SDS-PAGE) using a 4% stacking gel and 12% resolving gel. (1970) using the Mini-V 8.10 Vertical Gel Electrophoresis System (Gibco BRL). Purified virus was disrupted by boiling in SDS reducing buffer and the polypeptides separated by discontinuous polyacrylamide gel electrophoresis (SDS-PAGE) using a 4% stacking gel and 12% resolving gel. Low range molecular mass standards (Bio-Rad) and viral polypeptide bands were stained with 0.1% Coomassie blue R-250. By interpolation of the molecular mass standards migration curve, two closely associated virus polypeptides were identified at molecular mass 40 and 42 kDa (Fig. 2).

Viral nucleic acid was recovered from purified virions by proteinase K–SDS treatment, phenol–chloroform extraction and ethanol precipitation according to the protocol of Burleson et al. (1992). Electrophoretic analysis of denatured RNA in gel loading buffer was carried out in a 10-cm-long 1.2% agarose–formaldehyde gel containing MOPS running buffer but excluding ethidium bromide (Burleson et al., 1992). Brome mosaic virus RNA (Promega) and RNA molecular mass marker II (Boehringer Mannheim) were co-electrophoresed at 10 V for 16 h and the gel stained with 10 μg/ml ethidium bromide in running buffer followed by destaining for several hours in the dark with DEPC-treated distilled water. The gel was exposed to UV light and photographed. Two genomic fragments of 1.10 and 0.51 × 10⁶ Da were identified using the conversion 1 kb = 3.4 × 10⁵ Da (Fig. 3).

The virion morphology, buoyant density, pH stability, capsid structural protein and bipartite genomic RNA of the isolated virus conformed with the general properties of Nodavirus (Murphy et al., 1995). The same virus identified by Mori et al. (1992) has been designated striped jack nervous necrosis virus (SJNNV) and by Comps et al. (1994) as fish encephalitis virus (FEV). In view of the present cell culture isolation and confirmation of identity of the virus as a nodavirus, the apparently broad range of susceptible fish hosts and the consistent vacuolating encephalopathy and retinopathy characteristic of the clinical condition, we propose that this agent be generically termed piscine neuropathy nodavirus (PNN). The in vitro isolation of the virus from infected fish tissue should now facilitate further studies of this important pathogen.

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References


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