Journal of Fish Diseases 1999, **22,** 315–318

Short communication

Nodavirus isolated from experimentally infected tilapia, *Oreochromis mossambicus* (Peters)

G P Skliris and R H Richards

Institute of Aquaculture, University of Stirling, Stirling, UK

Viral encephalopathy and retinopathy is considered to be one the most serious viral diseases affecting not only larvae but also juvenile marine fish. Mediterranean sea bass, *Dicentrarchus labrax* (L.), Australian sea bass, *Lates calcarifer* (Bloch), turbot, *Scophthalmus maximus* (L.), halibut, *Hippoglossus hippoglossus* L., grouper, *Epinephelus malabaricus* (Bloch and Schneider), and several marine fish from Japan, have been reported to be susceptible to nodaviruses (Munday & Nakai 1997).

The causative agents of the disease have been purified from larvae of striped jack, *Pseudocaranx dentex* (Bloch and Schneider), (Mori, Nakai, Muroga, Arimoto, Mushiake & Furusawa 1992), *D. labrax* and *L. calcarifer* (Comps, Pepin & Bonami 1994), and were designated as striped jack nervous necrosis virus (SJNNV), *D. labrax* encephalitis virus (DLEV) and *L. calcarifer* encephalitis virus (LCEV), respectively. The latter two viruses share similar biochemical properties and are closely related to SJNNV, and thus, were identified as members of the Nodaviridae family. After unsuccessful attempts to culture the virus, it was isolated for the first time from the brains of diseased juvenile *D. labrax* in a cell line derived from striped snakehead, *Channa striatus* (Bloch), (SSN-1) by Frerichs, Rodger & Peric (1996).

Tilapia, *Oreochromis mossambicus* (Peters), is an important species for freshwater and marine aquaculture, particularly in developing countries. There is limited information in the literature concerning viral infections of tilapia. However, a birnavirus was reported and isolated from *Tilapia mossambica* in Taiwan in the early 1980s (Hedrick, Fryer, Chen & Kou 1983), while a Bohle iridovirus epizootic was reported from *O. mossambicus* by Ariel & Owens (1997). The survival of nodavirus under extreme environmental conditions (e.g. pH 3–9 and at a wide range of temperatures) strongly suggests that the virus may be able to infect a range of economically important species in a variety of habitats. Therefore, it was decided to investigate the susceptibility of tilapia to nodavirus, using an isolate recovered from naturally infected *D. labrax*.

Thirty tilapia, ≈ 10 cm long and 5–8 g in weight, were maintained in sea water (35‰, pH 8.1) in five 60-L glass tanks, each holding six fish. The temperature of the tanks was maintained at 27 ± 1 °C by immersion heaters. Fish were fed twice daily with pelleted feed and tanks were siphoned at the end of the day to remove waste material. A virulent nodavirus isolate originating from Greece was titrated in a 96-well microtitre plate containing SSN-1 cells and exhibited a titre of 10^8 $TCID_{50}$ mL^{-1}. The fish were intraperitoneally injected with 0.1 mL of the isolate at 1×10^7, 1×10^5, 1×10^3 and 1×10^1 $TCID_{50}$ mL^{-1}. The fifth control group received 0.1 mL of Hanks' balanced salt solution (HBSS).

Mortalities were recorded for 12 days, after which time the experiment was terminated and all the fish were sacrificed. Samples from the brain, eyes, spinal cord, gills, kidney, liver, spleen and muscle were fixed, processed and cut for histology using standard procedures. One set of the sections

Correspondence *George P Skliris, Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, UK.*
E-mail: george.skliris@stir.ac.uk

was stained with haematoxylin and eosin (H&E) for histopathological analysis and another was screened using immunohistochemistry (IHC) with the aid of a rabbit polyclonal antiserum raised against a Maltese nodavirus isolate (G.N. Frerichs, unpublished data).

Only the brains were collected for virological analysis, and two samples from each challenged group were pooled and homogenized in HBSS (Sigma–Aldrich Co., Irvine, UK) supplemented with 2% heat-inactivated foetal bovine serum (Gibco BRL, UK) to provide a 1:50 dilution and filtered through a 0.45-μm sterile filter (Minisart, Sartorius, AG, Gottingen, Germany). The filtered tissue extracts were simultaneously inoculated into SSN-1 cells and *O. mossambicus* caudal peduncle cells (OMCP-10) in 24-well plates and 25-mL flasks (Nunc, Roskilde, Denmark) and incubated at 25 °C. Cultures were checked daily for the presence of a cytopathic effect (CPE). When these were assessed as positive, subsequent passages were performed. Negative cultures were also passaged.

Virus titrations were performed by inoculating the samples in 96-well microtitre plates (Nunc). Briefly, HBSS was added to all the wells except those of the first column, where 100 μL of the virus suspension was added and titrated ten-fold across the plate. Then, 100 μL of SSN-1 cell suspension was added to every well of the plate, which was sealed with nescofilm (Nescofilm, Kobe, Japan) and incubated at 25 °C. Fifty per cent end-points ($TCID_{50}$) were calculated according to Karber (1931).

The *alpha* serum neutralization procedure was carried out (Rovozzo & Burke 1973). The Maltese nodavirus antiserum, diluted in HBSS, was added to three replicate rows of serial viral dilutions in a 96-well microtitre plate. Hanks' balanced salt solution was added to a further three rows of viral dilutions and the mixtures incubated at room temperature for 60 min. The samples were assayed for infectivity by adding confluent SSN-1 and OMCP-10 monolayers, and were incubated at 25 °C. Cultures were checked daily for between 3 and 11 days for the presence of a cytopathic effect (CPE). Neutralization indices (NIs) for the recovered viruses and the isolate used for the inoculation of tilapia were calculated as the ratio of the virus titre without antiserum to the virus titre in the presence of antiserum. Neutralization indices less than 10 (<1 log) were considered insignificant, those between 10 and 50 (1.0–1.7 logs) were questionable and those over 50 (>1.7 logs) were considered significant (Rovozzo & Burke 1973).

No mortalities occurred during the experimental period and the fish appeared healthy. Histologically, minor changes of multifocal vacuolation, focal necrosis in neurons, microgliosis and congestion were observed in all the brains of the fish receiving the high doses (1×10^7 and 1×10^5 $TCID_{50}$ mL^{-1}, respectively). Focal vacuolation was observed in the ganglion cell layer of the retina and in the spinal cords of the fish receiving the high doses. No significant histopathological changes were observed at the two lower doses or in the controls. A positive reaction was evident in the central nervous system (i.e. the brain and spinal cord) and the retinas at the two higher doses with immunohistochemistry.

When the brain extracts of the fish injected with the two highest doses were inoculated onto the SSN-1 cell line, a well-defined CPE occurred. Vacuolation started 4 days post-inoculation, progressed and became evident during the next 5 days with degeneration and cell destruction in the monolayer. Passages of the original cultures were positive 3 days post-inoculation, and the cytopathic effect was extended, progressed and led to the complete destruction of the monolayer 7 days post-inoculation, when the extracts were harvested and kept for future analysis (Fig. 1a,b). A further passage was also considered positive 3 days post-inoculation. Filtered tissue extracts inoculated onto OMCP-10 cells and control samples inoculated onto SSN-1 and OMCP-10 cells did not induce any CPE. Subsequent passages failed to induce any changes.

Samples from positive SSN-1 cultures were removed and used in an *alpha* neutralization test. According to serological tests previously performed (unpublished data), the Greek and Maltese nodavirus isolates were classified in the same serogroup (unpublished data). The Greek isolate used for the inoculation of the fish and the viruses recovered from the brains of the highly dosed tilapia exhibited the same $TCID_{50}$ when titrated in SSN-1 cells. The NI for both viruses was 10 000 (4 $\log_{10}$). The difference between the NIs of the two viruses was less than 20 (<1.3 $\log_{10}$) when tested against the Maltese nodavirus antiserum, and therefore, the isolates were considered identical.

Evidence of virus infection, associated with minimal pathology, was detected in the central nervous tissue and in the ganglion cell layer of the

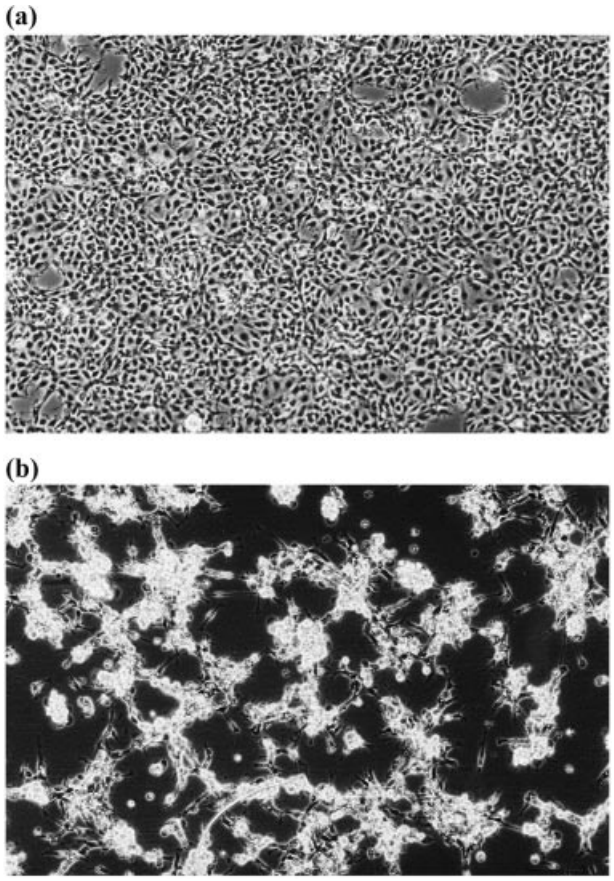

Figure 1(a) Normal monolayer of striped snakehead cell line (SSN-1); and (b) the characteristic CPE of nodavirus (vacuolation) induced in SSN-1 cells 7 days post-inoculation with nodavirus re-isolated from juvenile tilapia (bar = 100 μm).

retina of highly dosed fish. Histochemically, the virus was also demonstrated in the same tissues. Virological analysis in these fish revealed that the re-isolated viruses from brain tissue caused a significant cytopathic effect. The recovered viruses were confirmed to be nodaviruses since these were neutralized in the *alpha* neutralization test with nodavirus antiserum. Extracts from tilapia challenged with the lower doses were negative for the presence of the virus. Blind passages were also considered negative.

Even though no mortalities occurred during the short time course of the experiments reported in the present study, these results indicate that nodavirus might be transmitted to seawater-reared tilapia. A possible reason why the fish did not succumb to the virus in the present experiments could be that, as previously reported in other species, juveniles are more resistant (Breuil, Bonami, Pepin & Pichot 1991; Arimoto, Mori, Nakai, Muroga & Furusawa 1993; Thiery, Peducasse, Castric, Le Ven, Jeffroy & Baudin Laurencin 1997).

Hedrick *et al.* (1983) reported that the birnaviruses from *T. mossambica* in Taiwan were widespread among fish populations and may have exerted secondary effects on the host, such as enhanced susceptibility to other infectious agents. In the present study, the virus recovered from the highly dosed tilapia and the original isolate used for the inoculation of the fish exhibited identical $TCID_{50}$ when titrated in SSN-1 cells. This observation suggests that the virus was viable and did not lose infectivity in tilapia during the experimental period, and indicates the possibility of the development of a carrier state in juvenile tilapia in sea water.

Acknowledgments

We thank Dr G.N. Frerichs for providing the

nodavirus antiserum, Dr Kollanoor Riji John for his kind suggestions concerning neutralization tests and Mr S. Millar for making tilapia available for our use. Fiona Muir, Alison Tweedie, William Hamilton and Keith Ranson are acknowledged for their assistance. G. Skliris was funded by a scholarship from the State Scholarships Foundation (I.K.Y), Greece, and the last year of his studies was supported by the A. G. Leventis Foundation (France).

References

Ariel E. & Owens L. (1997) Epizootic mortalities in tilapia *Oreochromis mossambicus*. *Diseases of Aquatic Organisms* **29,** 1–6.

Arimoto M., Mori K., Nakai T., Muroga K. & Furusawa I. (1993) Pathogenicity of the causative agent of viral nervous necrosis disease in striped jack, *Pseudocaranx dentex*. *Journal of Fish Diseases* **16,** 461–469.

Breuil G., Bonami J.R., Pepin J.F. & Pichot Y. (1991) Viral infection (picorna-like virus) associated with mass mortalities in hatchery-reared sea bass (*Dicentrarchus labrax*) larvae and juveniles. *Aquaculture* **97,** 109–116.

Comps M., Pepin J.F. & Bonami J.R. (1994) Purification and characterisation of two fish encephalitis viruses (FEV) infecting *Lates calcarifer* and *Dicentrarchus labrax*. *Aquaculture* **123,** 1–10.

Frerichs G.N., Rodger H.D. & Peric Z. (1996) Cell culture isolation of piscine neuropathy nodavirus (PNN) from juvenile sea bass, *Dicentrarchus labrax*. *Journal of General Virology* **77,** 2067–2071.

Hedrick R.P., Fryer J.L., Chen S.N. & Kou G.H. (1983) Characteristics of four birnaviruses isolated from fish in Taiwan. *Fish Pathology* **18,** 91–97.

Karber G. (1931) Beitragzur kollektiven behandlung pharmakologischer reihenversuche. *Archiv für Experimentelle Pathologische und Pharmakologie* **162,** 480–483.

Mori K., Nakai T., Muroga K., Arimoto M., Mushiake K. & Furusawa I. (1992) Properties of a new virus belonging to Nodaviridae found in larval striped jack (*Pseudocaranx dentex*) with nervous necrosis. *Virology* **187,** 368–371.

Munday B.L. & Nakai T. (1997) Special topic review: nodaviruses as pathogens in larval and juvenile marine finfish. *World Journal of Microbiology and Biotechnology* **13,** 375–381.

Rovozzo G.C. & Burke C.N. (1973) *A Manual of Basic Virological Techniques*. Prentice Hall, Englewood Cliffs, NJ.

Thiery R., Peducasse J., Castric J., Le Ven A., Jeffroy J. & Baudin Laurencin F. (1997) Experimental transmission of viral encephalopathy to juvenile seabass (*Dicentrarchus labrax*). *Bulletin of the European Association of Fish Pathologists* **17,** 118–122.