Short communication

Susceptibility of the fish cell line SAF-1 to betanodavirus

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Betanodavirus infections of fish, generally known as viral nervous necrosis or viral encephalopathy and retinopathy, are a serious threat to marine fish farming throughout the world (Munday, Kwang & Moody 2002). The culture of betanodavirus has proved complex because most commonly available fish cell lines do not allow the growth of these viruses, as reviewed by Munday & Nakai (1997). The difficulties in finding cell lines that supported betanodavirus replication delayed isolation of the virus (Frerichs, Rodger & Peric 1996) and limited knowledge concerning viral infectious mechanisms. The first successful isolation of a nodavirus was made from sea bass, Dicentrarchus labrax (L.), using the SSN-1 cell line derived from striped snakehead, Ophicephalus striatus (Bloch) (Frerichs et al. 1996). It has been demonstrated that this cell line shows a high permissivity for other fish nodaviruses although some isolates do not produce cytopathic effect (CPE) (Iwamoto, Mori, Arimoto & Nakai 1999). To overcome these and other problems shown by SSN-1 cells, Iwamoto, Nakai, Mori, Arimoto & Furusawa (2000) cloned this cell line and reported that the clones obtained were more useful than the SSN-1 cells for qualitative and quantitative analysis of betanodaviruses. However, these cloned cell lines, like SSN-1, are persistently infected by a C-type retrovirus. Other authors have reported that another fish cell line (GF-1), derived from grouper, Epinephelus coioides (Hamilton), was useful for the isolation and replication of a nodavirus obtained from the same fish species, but did not show susceptibility of this cell line to other nodaviruses (Chi, Hu & Lo 1999).

Recently, a new fish cell line, SAF-1 (ECACC no. 00122301), derived from gilthead seabream, Sparus auratus (Bejar, Borrego & Alvarez 1997), has proven to be suitable to detect and quantify lymphocystis viruses that do not replicate in standard fish cell lines, as well as other fish viruses (Perez-Prieto, Rodriguez-Saint Jean, García-Rosado, Castro, Alvarez & Borrego 1999). In the present study, we report the suitability of SAF-1 cells to support replication and quantification assays of nodavirus strains and compare the results obtained using this cell line with those obtained using SSN-1 cells. The SAF-1 cell line is not infected with retrovirus (J. J. Borrego, unpublished data).

Eleven samples (Table 1) obtained from three fish species farmed in the Iberian Peninsula were used in this study. These samples had previously been demonstrated to be positive for nodavirus by reverse transcriptase-polymerase chain reaction (RT-PCR) in a routine analysis performed in the Instituto de Acuicultura facilities. Briefly, the samples (whole individuals when using larvae, the head in the case of small fish < 1 g, and a pool of eyes and brain from larger fish) were mixed (1:10) with Earle’s balanced salt solution (Gibco® Invitrogen, Carlsbad, CA, USA) supplemented with antibiotics.
(1000 IU mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin, 500 µg mL⁻¹ gentamycin and 10 µg mL⁻¹ amphotericin B) and homogenized. After centrifugation of the homogenates at 2000 g for 15 min, the supernatants were transferred to new tubes and incubated for 4 h at 15/25°C and inoculated immediately onto cell monolayers. In addition, the nodavirus strains 411/96/ERV (kindly provided by Dr G. Bovo, Italy), SJ93Nag (kindly provided by Dr T. Nishizawa, Japan) and AHNV692/9/98 (kindly provided by Dr B. H. Dannevig, Norway) were used for comparative purposes.

Both SSN-1 and SAF-1 cells were grown at 25°C using Leibovitz L-15 medium supplemented with 10% foetal bovine serum. For the infection assay, SSN-1 cells were cultured in 25 cm² flasks whereas the SAF-1 cells were grown on 24-well plates. Both cells were inoculated in duplicate with 0.1 mL (SAF-1) and 0.5 mL (SSN-1) of viral homogenates. Virus samples were adsorbed at room temperature for 1 h, then the inoculum was removed, and fresh medium was added to the cells. Infected monolayers were incubated at 20 and 25°C and examined daily for the presence of CPE. After 15 days, one of the duplicate wells inoculated with either positive or negative samples (cultures showing CPE or no CPE) was subjected to a second or blind passage, whereas the other well was maintained until complete destruction of the cells was observed, or up to a maximum of 30 days. The second passage was finished when complete destruction was observed within 30 days.

To determine the virus yield produced from the SAF-1 cell line after first and second passage, resultant viral suspensions were diluted 10-fold with Earle’s balanced salt solution. Diluted virus (100 µL) was inoculated onto SAF-1 monolayers in 48-well plates in triplicate and incubated at 20 and 25°C. Infected cultures were examined every day for CPE. The virus titre was determined from the CPE by using the procedure of Reed & Muench (1938) at day 15 post-inoculation.

The RT-PCR assay was performed by using the SuperScript™ One-Step RT-PCR kit (Invitrogen) and the primers R3 and F2 designed for the T4 region (430 bp) of SJNNV coat protein gene by Nishizawa, Mori, Nakai, Furusawa & Muroga (1994). Each reaction tube containing viral RNA and both specific primers (0.5 µl each) was denatured (98°C for 5 min) and reannealed (48°C for 5 min). Then, RT/Platinum® Taq Mix (Invitrogen) and the reaction mix buffer were added and the mixture was subjected to incubation at 48°C for 35 min and 5 min at 94°C, followed by 35 cycles of PCR (denaturation for 30 s at 94°C, annealing for 30 s at 60°C, and amplification at 68°C for 45 s) in a Mastercycler Personal Thermal Cycler (Eppendorf, Hamburg, Hamburg, Germany).
Germany). The polymerisation was concluded by an extension period of 15 min at 68 °C. The RT-PCR products were visualized by electrophoresis in 1.5% agarose gels.

The experiments performed to study nodavirus infectivity in both SAF-1 and SSN-1 cells revealed that all field samples (Table 1) replicated and developed CPE in the SAF-1 cell line at 25 °C, but only three (27%) did so in the SSN-1 cells at a first passage. Two more samples produced CPE after a blind passage and the remaining samples (six) failed to cause CPE in SSN-1 cells after the fourth blind passage. In addition, the positive samples produced CPE earlier in SAF-1 than in SSN-1 cells (Table 1). The replication of nodavirus isolates in SAF-1 cells resulted in a CPE characterized by rounded cells with large cytoplasmic vacuoles (Fig. 1) within 4–7 days (only two samples took up to 11 days) in the first passage, and about 4 days in the second passage. Once CPE appeared it took 5–13 days for complete destruction of the cell monolayer. Similar results were obtained at 20 °C, although the appearance of CPE was delayed for 3–4 days compared with cells incubated at 25 °C. The identity of all viral isolates producing CPE in both cell lines was confirmed as nodavirus by RT-PCR.

Viral titres obtained in the SAF-1 cell line were measured as the end-point dilution showing CPE, as total monolayer destruction was observed only at low dilutions. Viral titres of different isolates at 20 and 25 °C are shown in Table 2. Levels of viral production in the first passage in SAF-1 cells were quite high at 25 °C (average TCID$_{50}$ 10$^{6.5}$ mL$^{-1}$) and as expected increased after the second passage up to an average of 10$^{8}$ TCID$_{50}$ mL$^{-1}$. At 20 °C, titre values were slightly lower. Only strain 411/96/ERV showed identical titre values at both the temperatures.

The difficulties in finding cell lines that supported betanodavirus replication delayed isolation of the virus and limited full understanding of the viral infectious mechanisms and the epidemiology of the disease. After the first isolation of a fish nodavirus using SSN-1 cells (Frerichs et al. 1996), this cell line has been commonly used to isolate and propagate nodavirus (Iwamoto et al. 1999; Dannevig, Nielsen, Modahal, Jankowska, Taksdal & Press 2000; Barker, MacKinnon, Boston, Burt, Cone, Speare, Griffiths, Cook, Ritchie & Olivier 2002). However, Johansen, Sommerset, Tørud, Korsnes, Hjortaas, Nilsen, Nerland & Dannevig (2004) reported that some nodaviruses cannot be isolated using this cell line. In addition, SSN-1 cells have caused problems in the quantification of the virus, making the use of FAT (fluorescent antibody technique) necessary to titrate the virus instead of

![Figure 1](a) Uninfected SAF-1 monolayer. (b) Cytotoxic effect caused by betanodavirus isolate 160.03 at day 7 post-infection at 25 °C.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>20 °C</th>
<th>25 °C</th>
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<tbody>
<tr>
<td></td>
<td>first passage</td>
<td>second passage</td>
</tr>
<tr>
<td>004.03</td>
<td>5.5</td>
<td>6.75</td>
</tr>
<tr>
<td>160.03</td>
<td>4.5</td>
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</tr>
<tr>
<td>74.05</td>
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</tr>
<tr>
<td>76.05</td>
<td>5.5</td>
<td>7.25</td>
</tr>
<tr>
<td>411/96/ERV</td>
<td>8.5</td>
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Virus titres were measured by end-point cytopathic effect and are expressed as log TCID$_{50}$ mL$^{-1}$.
CPE (Iwamoto et al. 1999, 2000). In the present study, we have demonstrated the suitability of the cell line SAF-1, derived from gilthead seabream, for isolation and quantification based on the CPE and without using FAT, of a number of nodavirus field samples obtained from five fish species. The development of a cell line suitable for quantification of betanodavirus has also been reported by Iwamoto et al. (2000), using a cloned cell line obtained from SSN-1, designated E-11, which yielded higher viral titres than SAF-1. However, E-11 cells maintain one of the disadvantages of the SSN-1 cells, i.e. the infection by a C-type retrovirus (SnRV). Iwamoto et al. (2000) suggest that the SSN-1 retrovirus plays an important role in the replication of nodavirus in the cells and is involved in the induction of a specific receptor for viral adhesion. The results obtained in the present study indicate that nodavirus can infect and develop a clear and characteristic CPE in cells not infected by retrovirus.

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