Vol. 43: 81–89, 2000 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published November 14

Cloning of the fish cell line SSN-1 for piscine nodaviruses

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ABSTRACT: Six cell clones were derived from the SSN-1 cell line, which is composed of a mixed cell population and persistently infected with a C-type retrovirus (SnRV). These clones were susceptible to 4 piscine nodavirus strains belonging to different genotypes (SJNNV, RGNNV, TPNNV and BFNNV [striped jack, redspotted grouper, tiger puffer and barfin flounder nervous necrosis viruses]). Three clones, designated A-6, E-9, and E-11, were highly permissive to nodavirus infection and production. The virus-induced cytopathic effects appeared as cytoplasmic vacuoles and intensive disintegration at 3 to 5 d post-incubation. These observations were highly reproducible and formed the basis for a successful virus titration system. Quantitative analysis using the cloned E-11 cell line clearly revealed differences in the optimal growth temperatures among the 4 genotypic variants: 25 to 30°C for strain SGWak97 (RGNNV), 20 to 25°C for strain SJNag93 (SJNNV), 20°C for strain TPKag93 (TPNNV), and 15 to 20°C for strain JFIwa98 (BFNNV). Electron microscopy demonstrated SnRV retrovirus particles only in A-6 and E-9 cells, but PCR amplification for the *pol* gene and LTR region of the proviral DNA indicated the presence of the retrovirus in the other clones, including E-11. The cell clones obtained in the present study will be more useful for qualitative and quantitative analyses of piscine nodaviruses than the SSN-1 cell line.

KEY WORDS: Nodavirus · Viral nervous necrosis, VNN · SSN-1 cell line · Cell cloning · C-type retrovirus · Snakehead retrovirus

INTRODUCTION

Viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER) has appeared in a wide variety of larval and juvenile marine fish in various countries during this decade (Munday & Nakai 1997, Office International des Epizooties 1997). Early studies on isolation of the causative piscine nodaviruses, in which some established fish cell lines such as RTG-2, CHSE-214, FHM, EPC, and BF-2 were used, reported unsuccessful results (Breuil et al. 1991, Mori et al. 1991, Munday et al. 1992, Nguyen et al. 1994, Grotmol et al. 1995). This led to the rapid development of alternative methods to detect virus including enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody technique (FAT), reverse transcription-polymerase chain reaction (RT-PCR), and *in situ* hybridization (Arimoto et al. 1992, Nguyen et al. 1994, Nishizawa et al. 1994, Comps et al. 1996). However, these methods were not easily used as tools to investigate the pathogenesis of piscine nodaviruses or epidemiology of the disease. The first successful isolation of a piscine nodavirus was made from diseased sea bass *Dicentrarchus labrax* using the SSN-1 cell line, which had been established from whole fry tissue of striped snakehead *Ophicephalus striatus* (Frerichs et al. 1996). Subsequently, it was reported by Delsert et al. (1997) that a simian cell line (Cos1) and 3 fish cell lines (SBL, RTG-2, BF-2) were semipermissive for a piscine nodavirus (DlEV, *Dicentrarchus labrax* encephalitis virus) isolated from sea bass, and Chi et al. (1999) reported

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that a new cell line (GF-1) derived from grouper *Epinephelus coioides* was useful for the isolation and proliferation of a piscine nodavirus (GNNV, grouper nervous necrosis virus).

Piscine nodaviruses can be divided into 4 genotypic groups based on partial sequences of the coat protein gene (Nishizawa et al. 1997): SJNNV (striped jack nervous necrosis virus), RGNNV (redspotted grouper nervous necrosis virus), TPNNV (tiger puffer nervous necrosis virus), and BFNNV (barfin flounder nervous necrosis virus). In a previous study, we demonstrated that the SSN-1 cell line was useful for propagating and differentiating 17 isolates of piscine nodavirus collected from 13 host fish species in 5 countries (Iwamoto et al. 1999). However, one problem with the practical use of the SSN-1 cell line was that this cell line was composed of a mixed population of cells, causing inconsistencies in the cytopathic effects (CPE) observed during virus infection. For this reason, FAT was used to titrate the virus instead of CPE as described in our previous study (Iwamoto et al. 1999). FAT was laborious and costly due to the requirement of special test chambers, and it has proved to be inadequate for the quantitative analysis of a large number of samples. In addition, the fact that the SSN-1 cell line is spontaneously infected by a C-type retrovirus designated as SnRV (Frerichs et al. 1991, Hart et al. 1996) may be another disadvantage in using this cell line. In order to overcome these problems, we cloned the SSN-1 cells, and each cell clone was examined for permissiveness to piscine nodaviruses infection and the presence of SnRV.

MATERIALS AND METHODS

Virus strains. Four fish species naturally infected with piscine nodaviruses, strains SJNag93, SGWak97, TPKag93, and JFIwa98, which belong to SJNNV, RGNNV, TPNNV, and BFNNV genotypes respectively, were the sources of virus used in this study (Table 1). The virus inocula were prepared as previously described (Iwamoto et al. 1999). Briefly, infected larvae or juveniles were homogenized with 9 volumes of Hanks' balanced salt solution (HBSS), centrifuged at 3000 × *g* for 10 min, and the supernatants were filtered through 0.45 µm membrane filter. The virus filtrates were stored at –80°C until used.

Cell culture and cloning. The SSN-1 cells were cultured in a 25 cm^2 tissue culture flask (Sumitomo Bakelite Co. Ltd) at 25°C using Leibovitz L-15 medium (Gibco) supplemented with 10% FBS (fetal bovine serum). Cell cloning was conducted at 25°C throughout the process. A monolayer culture of SSN-1 cell line was treated with 0.1% trypsin solution and the individual cells were cloned by limiting dilution using L-15 medium (10% FBS) in 96-well tissue culture plates. When the individually cloned cells became confluent in the wells, cells from each well were transferred to a separate well of a 24-well plate and cultured until confluency. Subculture was continued in this manner until complete monolayers were obtained in 25 cm^2 flasks.

Table 1. Four genotype strains of piscine nodavirus used in this study

Diseased fish species	Virus strain	Isolation year	Genotype[a]
Striped jack *Pseudocaranx dentex*	SJNag93	1993	SJNNV
Sevenband grouper *Epinephelus septemfasciatus*	SGWak97	1997	RGNNV
Tiger puffer *Takifugu rubripes*	TPKag93	1993	TPNNV
Japanese flounder *Paralichthys olivaceus*	JFIWa98	1998	BFNNV

[a]Determined by restriction fragment length polymorphism (RFLP) analysis of coat protein gene T2 regin (870 bp) (Iwamoto et al. 1999)

Test for sensitivity to virus infection. The monolayer covering 70 to 80% of the 25 cm^2 flask was washed twice with HBSS and then 500 µl of the virus filtrate was inoculated in the cell culture. After incubation at 25°C for 1 h, the flask was washed with HBSS, supplemented with L-15 medium (2% FBS), and incubated at 25 or 20°C. CPE were observed daily for 10 d. In the FAT test for piscine nodavirus antigens, 100 µl of the virus filtrate was inoculated into a 0.8 cm^2 chamber of Lab-Tek chamber slide (Nunc) which contained semi-confluent layers of cells. After incubation at 25 or 20°C for 3 d, cells were immuno-stained according to a method described previously (Iwamoto et al. 1999). Briefly, cells in the chamber were incubated with a rabbit anti-SJNNV serum and then reacted with FITC (fluorescein isothiocyanate)-conjugated swine Ig to rabbit Ig (Dako). The intensity of the fluorescence signals was given as a roughly estimated number of positive cells: +, less than 100; ++, 100 to 1000; and +++, more than 1000 in a chamber (0.8 cm^2).

Titration of virus. Virus samples used here were prepared by culturing the original virus filtrates (SJNag93, SGWak97, TPKag93, JFIwa98) in cloned E-11 cells. The virus-containing culture supernatant was diluted serially in 10-fold increments to 10^{-11} with HBSS, and 50 µl from each dilution was inoculated onto E-11 monolayers in 96-well plates. Four wells were used for each diluted sample. Plates were incubated at 15, 20, 25, and 30°C respectively and observed daily for CPE. After 10 d, the 50% tissue culture infectious dose ($TCID_{50}$ ml^{-1}) was calculated by the method of Reed & Muench (1938).

Enumeration of virus growth. The same virus samples used above were inoculated at an MOI (multiplicity of infection) of 1.0 onto the E-11 monolayer culture in a 24-well plate. Plates were incubated at 15, 20, 25, or 30°C for 96 h. Culture supernatants collected by centrifugation (3000 × *g*, 10 min) were serially diluted in 10-fold increments with HBSS. The virus titer was determined at 25°C for SJNag93 and SGWak97 or at 20°C for TPKag93 and JFIwa98. In a separate experiment, strain SJNag93 was inoculated at an MOI of 1.0 onto monolayer cultures of SSN-1, E-11 or RTG-2 in 24-well plates. During incubation at 25°C for 168 h, 10 µl each of culture supernatants was taken from the wells at 12 or 24 h intervals and diluted serially in 10-fold increments with HBSS; the virus titer was determined for each sample at 25°C.

PCR for C-type retrovirus detection. Genomic DNA was extracted from SSN-1 and from cloned cells using an ISOGEN extraction kit (Nippon gene Co. Ltd) according to the manufacturer's directions. BF-2 cells were used as a negative control. Proviral DNA was detected by PCR amplification with the primers described by Hart et al. (1996): ML1/GPOL2 primers for the proviral *pol* gene encoding reverse transcriptase (ML1: 5′-TGGTACCCATGGATACAGGTACCTCA-3′; GPOL2: 5′-TGTCAGACATGGCCTGTACTTTAGCAGC-3′) and LTR10/LTR20 primers for the LTR (long terminal repeats) region (LTR10: 5′-TGACTCATATCCTGCTTAGTAGAC-3′; LTR20: 5′-GAAAGTACGACTCAGGCTCAAGAC-3′). PCR amplification was performed with an initial denaturation of 2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 61°C for *pol* or 58°C for LTR, and 1 min at 72°C, with a final extension of 5 min at 72°C. PCR amplicons were examined by 2% agarose (NuSieve 3:1 agarose, FMC) gel electrophoresis.

Electron microscopy. Cloned cells, either uninfected or infected with piscine nodaviruses, were cultured at 25°C for 3 d and fixed in a 2.5% glutaraldehyde-2% paraformaldehyde mixture (pH 7.4), post-fixed with 1% osmium tetroxide and embedded in Quetol-812 (Oken). Thin sections were stained with 1% uranyl acetate and 1% lead citrate, then examined with an electron microscope (Hitachi H-600A) at 80 kV accelerating voltage.

RESULTS

Cloning of SSN-1 cells

A total of 6 clonal lines were established from the SSN-1 cells (Table 2). Five clones designated as A-6, B-7, E-2, E-9 and E-11 grew well at 25 and 20°C but clone C-3 failed to grow at 20°C. Two clones, B-7 and E-11, were round-shaped and the others were spindle-shaped (Fig. 1). These cells formed a monolayer within 36 h at 25°C or within 72 h at 20°C in the well of a 24-well plate, when they were seeded at 10^5 cells well^{-1}, or within 2 d at 25°C or 5 d at 20°C in a 25 cm^2 flask. They were also maintained without any significant changes for 2 wk at 25°C or 4 wk at 20°C in a 25 cm^2 flask.

Permissivity of cell clones for nodaviruses

Only 1 clone, B-7, had a low permissivity against all 4 nodavirus strains both at 25 and 20°C (Table 2). When the incubation was performed at 25°C, the other 5 clones were highly permissive for SJNag93 and SGWak97, but were less or non-permissive for TPKag93 and JFIwa98. The CPE observed in these cells were characterized by vacuole formation in the cytoplasm and almost complete cell disintegration within 3 to 5 d (Fig. 2). High permissivity against all 4 genotype strains was also observed in the 20°C incubation, particularly in 3 clones (A-6, E-9, and E-11). These results were reproduced in 3 different trials. Small spherical

Table 2. Nodavirus-susceptibility of cell clones derived from SSN-1 cell line and detection of spontaneously infected C-type retrovirus (SnRV). SS: spindle-shaped; RS: round-shaped; ND: not done

Cell clone		Growth at:		Susceptibility[a] at 25°C/20°C against nodavirus strain (genotype)				Detection of SnRV		
Name	Morphology	25°C	20°C	SJNag93 (SJNNV)	SGWak97 (RGNNV)	TPKag93 (TPNNV)	JFIWa98 (BFNNV)	TEM	PCR *pol* gene	PCR LTR
A-6	SS	+	+	+++/+++	+++/++	+/++	–/+++	+	+	+
B-7	RS	+	+	+/+	+/+	–/+	–/+	–	+	–
C-3	SS	+	–	+++/ND	+++/ND	–/ND	–/ND	–	+	+
E-2	SS	+	+	+++/++	+++/++	–/+	–/++	–	+	+
E-9	SS	+	+	+++/+++	+++/++	+/++	–/+++	+	+	+
E-11	RS	+	+	+++/+++	+++/++	+/++	–/++	–	+	+
SSN-1	Mixed	+	+	+++/+++	+++/++	+/++	–/+++	+	+	+

[a]Intensity of susceptibility was given as number of FAT-positive cells less than 100 (+), 100 to 1000 (++), more than 1000 (+++) in 0.8 cm^2

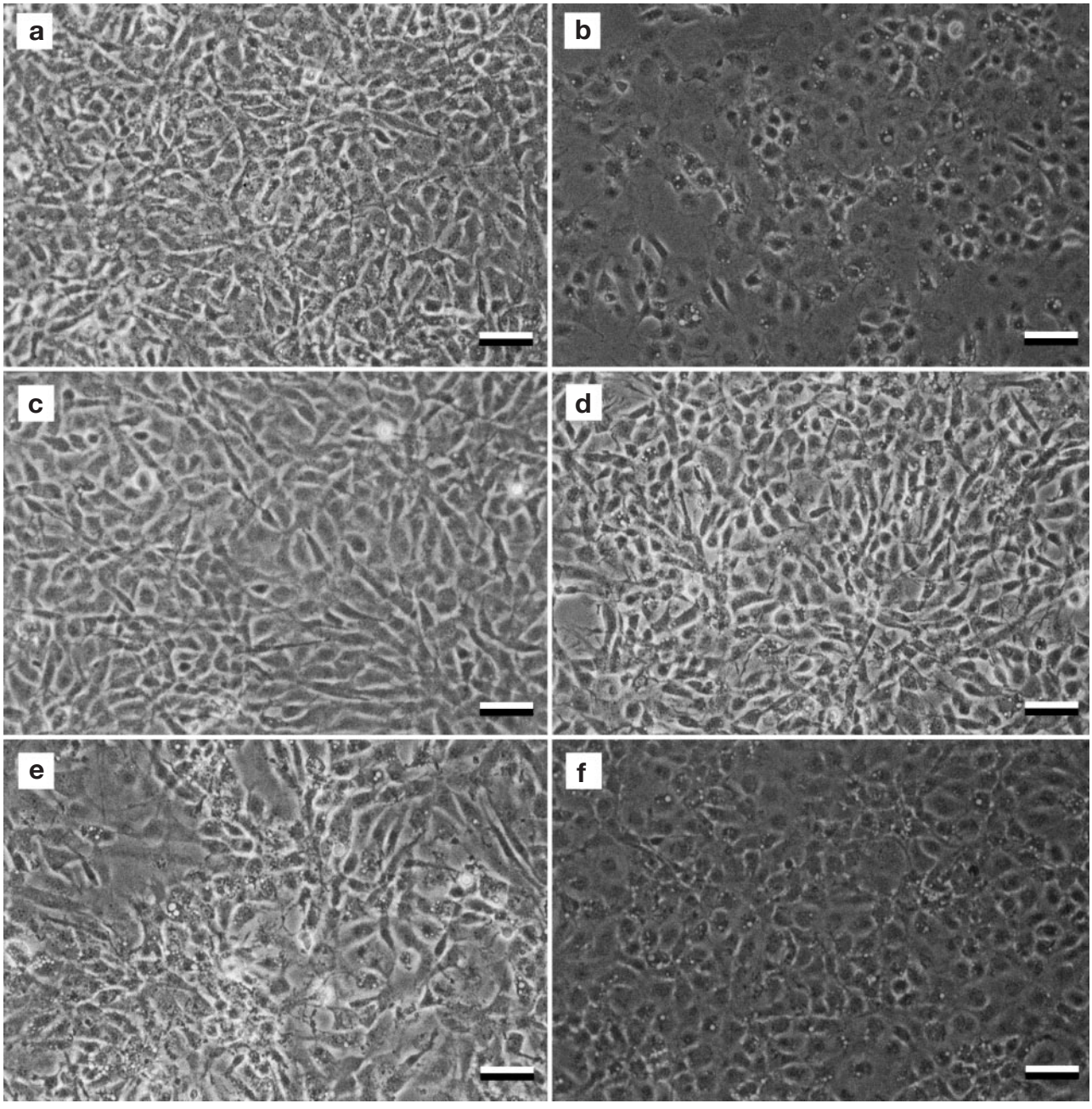

Fig. 1. Six cell clones derived from the SSN-1 cell line. (a) A-6, (b) B-7, (c) C-3, (d) E-2, (e) E-9, and (f) E-11; 3 d culture in L-15 medium (10 % FBS) at 25°C. Scale bar = 50 µm

virus particles (ca 25 nm in diameter) arranged in paracrystalline arrays or in membrane-bounded vesicles were abundantly observed in the cytoplasm of the affected cells by electron microscopy (Fig. 3).

Optimal temperatures for titration and growth of virus

Virus titrations conducted on the E-11 clone gave the highest titers, ranging from $10^{9.0}$ to $10^{10.0}$ $TCID_{50}$ ml^{-1}, at 25°C for SGWak97 and SJNag93 or at 20°C for TPKag93 and JFIwa98 (Fig. 4a). Titers higher than $10^{7.8}$ $TCID_{50}$ ml^{-1} were also obtained at 20 and 30°C for SGWak97, at 20°C for SJNag93, or at 15°C for JFIwa98, while they were not detectable (less than $10^{3.0}$ $TCID_{50}$ ml^{-1}) at 30°C for TPKag93 or at 25 and 30°C for JFIwa98.

The virus titres at 96 h of culture in E-11 cells at the different temperatures are shown in Fig. 4b. Virus particles at titers higher than $10^{8.6}$ $TCID_{50}$ ml^{-1} were ob-

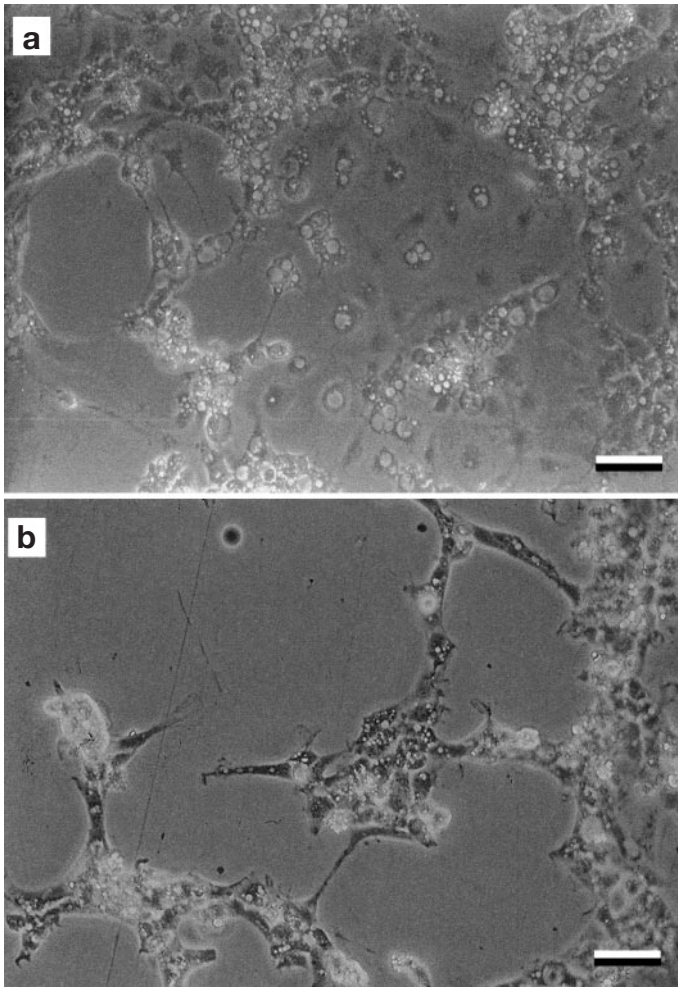

Fig. 2. Cytopathic effects (CPE) found in the E-11 cells infected with strain SJNag93. (a) 2 d culture at 25°C, (b) 4 d culture at 25°C. Scale bar = 50 µm

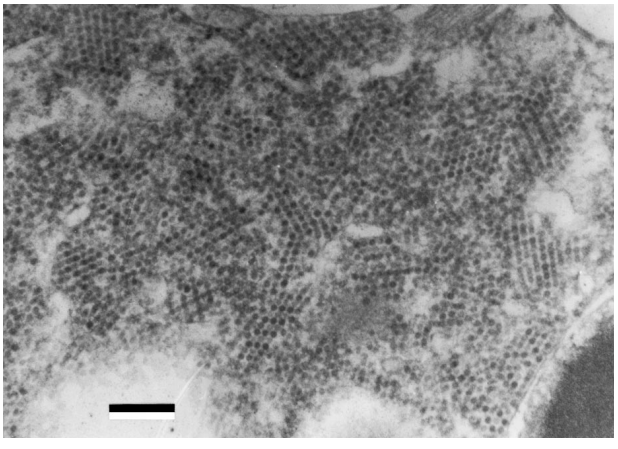

Fig. 3. Nodavirus particles found in E-11 cells. After being inoculated with strain SJNag93, cells were incubated at 25°C for 3 d. Scale bar = 200 nm

tained from the cultures at 20 to 30°C for SGWak97, at 20 and 25°C for SJNag93, at 20°C for TPKag93, and at 15 and 20°C for JFIwa98. The highest virus yields at 30 and 15°C were obtained in SGWak97 and JFIwa98, respectively. Growth kinetics of SJNag93 in SSN-1 and E-11 cells at 25°C is shown in Fig. 5. Virus titers in the both culture supernatants increased to 10^4–10^5 $TCID_{50}$ ml^{-1} at 24 h post-incubation and reached $10^{9.3}$ $TCID_{50}$ ml^{-1} in SSN-1 cell and $10^{9.6}$ $TCID_{50}$ ml^{-1} in E-11 cell at 96 h post-incubation. Virus was not detected in the tissue culture media from RTG-2 infected cells at a minimum detection level of $10^{3.0}$ $TCID_{50}$ ml^{-1} throughout the entire course of the experiment.

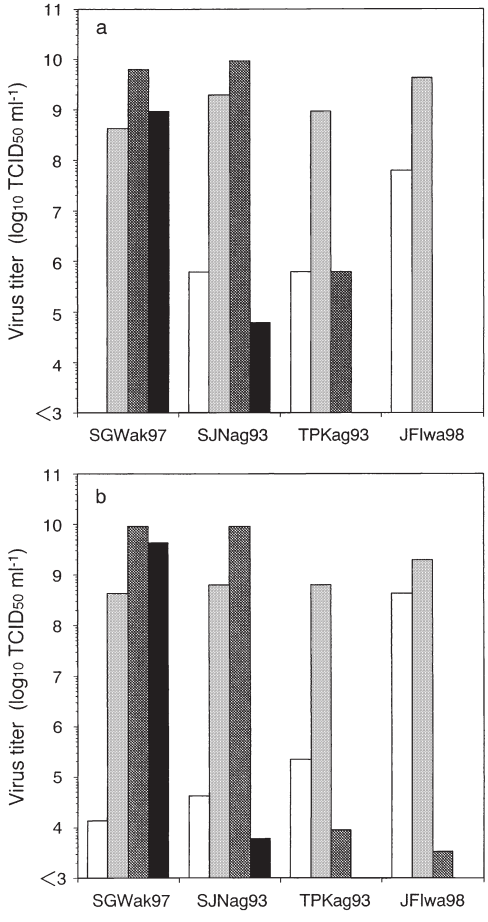

Fig. 4. Influence of incubation temperature on titration and growth of virus. (a) Serially 10-fold-diluted virus samples of 4 different genotypic strains (SGWak97, SJNag93, TPKag93, and JFIwa98) were inoculated into E-11 cells and incubated at different temperatures for 10 d before the titers ($TCID_{50}$ ml^{-1}) were determined based on the CPE. (b) The same virus samples as above were inoculated into E-11 cells at a multiplicity of infection (MOI) of 1.0 and incubated at different temperatures for 96 h. Virus yields in the culture spernatants were determined by the above-mentioned titration procedure at 25°C for SGWak97 and SJNag93 or at 20°C for TPKag93 and JFIwa98. (□) 15°C, (▒) 20°C, (▩) 25°C, (■) 30°C

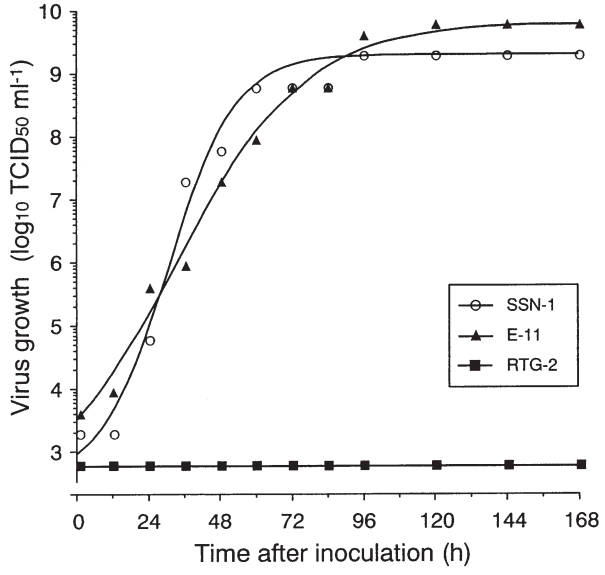

Fig. 5. Growth kinetics of strain SJNag93 in cultured cells. The SJNag93 was inoculated at MOI 1.0 into (○) SSN-1, (▲) E-11, or (■) RTG-2 cells and incubated at 25°C. Released virus was quantified at 12 or 24 h intervals by the titration procedure (25°C) using E-11 cells

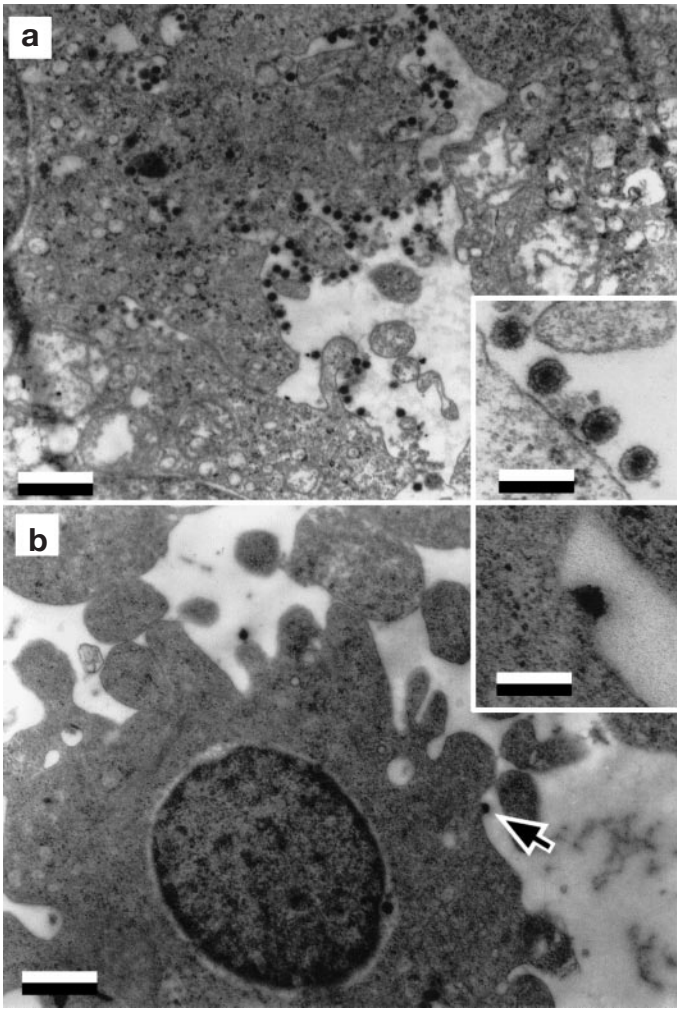

Fig. 6. C-type retrovirus SnRV particles found in cell clones derived from the SSN-1 cell line. (a) A-6 cell, (b) E-9 cell. Arrow in (b) shows a particle in the budding process. Scale bar = 1 µm (0.2 µm for high magnification)

Detection of retrovirus

SnRV-like particles, about 90 nm in diameter, were found to be abundant in the extracellular spaces of A-6 and E-9 cell lines. Particles in the budding process were also observed (Fig. 6). However, such virus particles were not observed in the other 4 clones, including E-11. A clear band at ca 730 bp, showing the proviral *pol* gene of SnRV in genomic DNA, was detected in PCR amplifications of the genomic DNAs extracted from all of the cloned cell lines as well as in the uncloned SSN-1 cells (Fig. 7a). In addition, the amplicon of ca 500 bp, showing the proviral LTR region, was detected in 5 cloned lines (A-6, C-3, E-2, E-9, and E-11) and in the uncloned SSN-1 cells (Fig. 7b). No PCR amplicons were detected in the reactions obtained from BF-2 cells.

DISCUSSION

The purpose of this study was to obtain cloned cell lines from SSN-1 suitable for isolation and propagation of piscine nodavirus. The cloned cell lines obtained, A-6, E-9, and E-11, were highly permissive for all the genotypic variants of piscine nodaviruses; additional advantages over the parental SSN-1 cell line are their steady, faster growth to confluency and longer stability in monolayer cultures for 2 wk at 25°C or 4 wk at 20°C and their reproducible, clear CPE characterized by cytoplasmic vacuole formation followed by intensive disintegration. These cell clones will be useful for both qualitative and quantitative analyses of piscine nodaviruses.

Lack of reliable and simple quantitative measurement of virus titre was a problem in previously reported investigations on piscine nodaviruses or the disease VNN. Virus titration and growth experiments at different incubation temperatures using the present E-11 cells (Fig. 4) clearly revealed differences in the optimal culture temperature among 4 genotypic variants of piscine nodavirus: 25 to 30°C for strain SGWak97 (RGNNV genotype), 20 to 25°C for strain SJNag93 (SJNNV), 20°C for strain TPKag93 (TPNNV), and 15 to 20°C for strain JFIwa98 (BFNNV). This quantitative

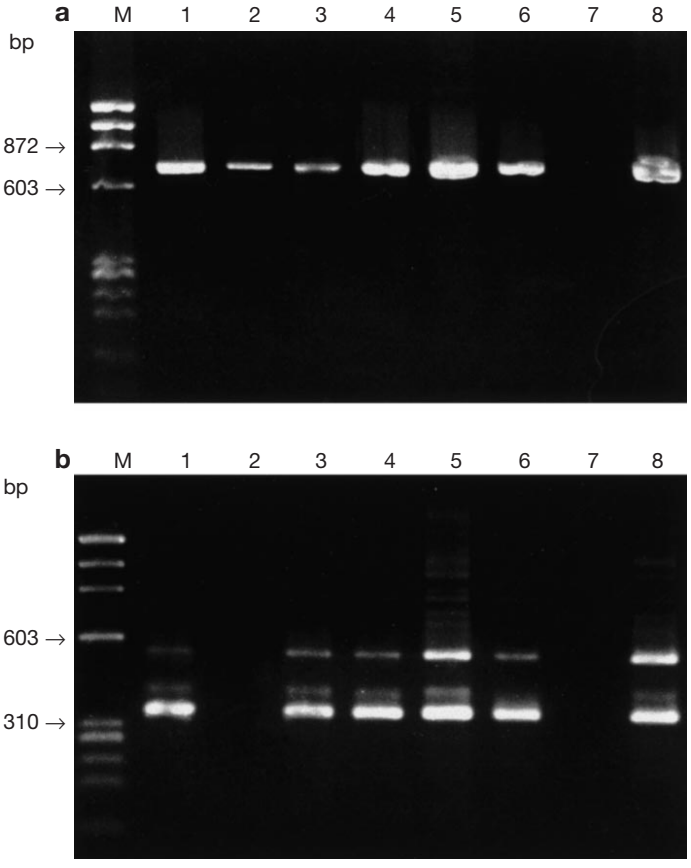

Fig. 7. PCR detection of proviral DNA of C-type retrovirus SnRV from SSN-1 and cloned cells (a) with ML1/GPOL2 primers for *pol* gene, (b) with LTR10/LTR20 primers for LTR region. M: length marker (ϕX174 *Hae*III digest); 1 to 8: cultured cells—1 (A-6), 2 (B-7), 3 (C-3), 4 (E-2), 5 (E-9), 6 (E-11), 7 (BF-2), and 8 (SSN-1)

analysis on temperature dependency among the genotypic variants strongly supports the hypothesis (Iwamoto et al. 1999) that genetic variations in piscine nodavirus come from adaptation to the normal environmental temperatures of the host fish species. The RGNNV genotype, which prefers higher optimal temperature for growth, is composed of virus isolates from warm-water fish species such as groupers (Mori et al. 1991, Nakai et al. 1994, Chua et al. 1995, Danayadol et al. 1995, Fukuda et al. 1996, Chi et al. 1997) and sea bass (Glazebrook et al. 1990, Breuil et al. 1991, Jung et al. 1996, Le Breton et al. 1997), while the most psychrophilic BFNNV genotype is mainly isolated from cold-water fish species such as Atlantic halibut *Hippoglossus hippoglossus* (Grotmol et al. 1995) and barfin flounder *Verasper moseri* (Watanabe et al. 1999). This assay system for detecting infectious agents will also be valid in epidemiological studies of VNN, especially in detection of virus carrier state in fish. The PCR technique has long been used for such a purpose in striped jack (Mori et al. 1998), but its non-quantitative nature often raised questions, e.g. PCR-positive results do not always mean that the viral agent was infectious and causal (Mushiake et al. 1994). We are now using this new assay to detect the infectious agent in a variety of broodstock fish species.

The E-11 cells, as well as the uncloned SSN-1 cells, supported virus proliferation to yields of 10^9–10^{10} $TCID_{50}$ ml^{-1} in the culture supernatant (Figs. 4b & 5). This virus productivity is equivalent to that of other fish-pathogenic viruses in fish cell lines. For example, infectious pancreatic necrosis virus (IPNV) grows to 10^8–10^9 $TCID_{50}$ ml^{-1} at 15°C in RTG-2 cells (Malsberger & Cerini 1965). Therefore, the clonal lines from SSN-1 will make it possible to prepare infectious virions at required titers for various *in vivo* and *in vitro* assays. This is particularly important for virulence assays of piscine nodavirus in fish. Every pathogenicity experiments reported so far (Mori et al. 1991, Arimoto et al. 1993, Boonyaratpalin et al. 1996, Nguyen et al. 1996, Grotmol et al. 1999, Peducasse et al. 1999, Totland et al. 1999) lacked the quantitative measurement of the inoculated dose. This lack of measurement caused difficulties in comparisons of virulence among virus strains or virus susceptibility among host fish species.

Another disadvantage of the SSN-1 cell line is that it is persistently infected by a C-type retrovirus SnRV (Hart et al. 1996). This retrovirus with high Mn^{2+}-dependent reverse transcriptase activity was originally identified as a spontaneously productive infection of the SSN-1 cell line (Frerichs et al. 1991), and the sequence analysis of the complete genome revealed that SnRV is distinguishable from all known retrovirus groups by the presence of an arginine tRNA primer binding site (Hart et al. 1996). Unfortunately it was indicated by electron microscopy and/or PCR procedures that every clone obtained in the present study carries SnRV particles or its provirus (Figs. 6 & 7). Because further procedures to obtain clonal line of SSN-1 cells that were nodavirus-permissive and SnRV-free were unsuccessful, it was deduced that possibly every SSN-1 cell carries SnRV. However, there was variation in retrovirus production among the clonal cell lines; virus particles were easily found in 2 clones, A-6 and E-9, but not in the other 4 clones.

Earlier failures to isolate piscine nodaviruses in established fish cell lines, as cited before, suggested the presence of a specific receptor for virus adhesion as deduced from its neurotropism (Munday & Nakai 1997). Therefore, successful isolation and production of the nodavirus in SSN-1 cells suggests the possibility that the receptor is induced in persistently infected SnRV. This hypothesis is countered by the results that the piscine nodavirus strain, DlEV, whose genotype is

unknown but is presumably RGNNV, was propagated in simian Cos1 and fish cells (SBL, RTG-2, and BF-2) (Delsert et al. 1997). Growth in Cos1 and the fish cells did not produce clear CPE, and no growth of the SJNag93 strain (SJNNV genotype) was observed in RTG-2 cells in our study (Fig. 5). These results indicate the possibility that the SSN-1 retrovirus plays an important role in the replication of piscine nodavirus in the cell. Future analysis on replication of piscine nodaviruses in the clonal cell lines will clarify the mechanisms of host specificity and temperature dependency of the virus.

Acknowledgements. We thank G. N. Frerichs for providing the SSN-1 cell line. This study was supported in part by a grant from the Japan Sea-Farming Association.

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Editorial responsibility: Jo-Ann Leong, Corvallis, Oregon, USA

Submitted: February 29, 2000; Accepted: June 14, 2000
Proofs received from author(s): October 27, 2000