Vol. 44: 183–190, 2001 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published April 10

A salmonid cell line (TO) for production of infectious salmon anaemia virus (ISAV)

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ABSTRACT: A new cell line designated TO which provides a high yield of infectious salmon anaemia virus (ISAV) has been established. The cells originate from head kidney leukocytes isolated from Atlantic salmon and grow well at 20°C in EMEM with 5% CO_2 and without CO_2 supplement in HMEM. The cells have at present been passed more than 150 times and no changes in morphology, growth or virus production have been observed. The virus infection results in cytopathic effects (CPE) within 9 d, and the virus titre obtained from centrifuged and filtrated cell lysates, measured as $TCID_{50}$, was about $10^{9.1}$ ml^{-1}. The virus isolated from lysates of infected cells by a sucrose gradient provided purified ISAV when examined by silver stained SDS-PAGE. Salmon injected with diluted virus supernatant showed mortalities, hematocrit values and clinical signs in accordance with infectious salmon anaemia.

KEY WORDS: Cell line · Salmon · Infectious salmon anaemia virus, ISAV · Fish virus

INTRODUCTION

The infectious salmon anaemia virus (ISAV) has caused severe losses in salmonid aquaculture in Norway since late 1997 in Canada (Bouchard et al. 1999, Lovely et al. 1999) and since 1998 in Scotland (Rodger et al. 1998). The virus has been identified as an orthomyxovirus-like virus (Falk et al. 1997, Mjaaland et al. 1997).

A cell line, SHK-1, derived from head kidney cells of Atlantic salmon *Salmo salar* L. has been established to support replication of ISAV (Dannevig et al. 1995a,b). ISAV can also replicate in other cell lines like CHSE-214 derived from Chinook salmon *Oncorhynchus tshawytscha* (Bouchard et al. 1999, Lovely et al. 1999, Kibenge et al. 2000) and AS derived from visceral organs of Atlantic salmon *S. salar* L. (Sommer & Mennen 1997). It has been possible to examine samples from diseased fish by use of the SHK-1 cell line. The clinical diagnosis of ISA has been supported by using a monoclonal antibody to ISAV for indirect fluorescent staining (Falk & Dannevig 1995) or by using reverse transcriptase PCR (Mjaaland et al. 1997, Lovely et al. 1999) for identification of ISAV in infected SHK-1 cells.

Virus production from the mentioned cell lines might vary. For SHK-1 the low yield is reported to be a limiting factor for virus characterization. The virus infective titres obtained by cytopathic effects (CPE) or immunofluorescence (IF) were shown to be $10^{5.4}$ 50% Tissue Culture Infectivity Dose ($TCID_{50}$) ml^{-1}, but slow and weak cytopathic growth of ISA virus was also reported (Falk et al. 1998). Kibenge et al. (2000) reported that out of 13 isolates, 13 and 6 isolates of ISAV replicated in SHK-1 and CHSE-214 respectively and that the virus titres obtained was highest for SHK-1 showing titres of $10^{7.8}$ $TCID_{50}$ ml^{-1} and $10^{6.5}$ $TCID_{50}$ ml^{-1} for CHSE-214. There is a need for a well-growing, highly ISAV-sensitive and stable cell line with respect to virus production. In this report we describe a new cell line derived from salmon leukocytes which has a potential for use in diagnostics as well as for antigen production.

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MATERIALS AND METHODS

Primary cell culture. A head kidney was obtained from unvaccinated Atlantic salmon *Salmo salar* L. weighting 3 kg reared in the facilities of The Industrial and Aquatic Laboratory, Bergen, Norway. The fish were kept in 6500 l tanks at a temperature of 8°C and with a constant flow rate of saline water of 1.0 l kg fish^{-1} min^{-1}. The fish were fed commercial salmon pelleted food dispensed from an automatic feeder 8 h a day. The fish had no history of previous infectious diseases. The head kidney was removed aseptically, placed in 10 ml of holding medium containing RPMI 1640 (BioWhittaker), 100 µg ml^{-1} gentamicin sulphate (BioWhittaker), 2 mM L-glutamine (BioWhittaker) and 10% foetal calf serum (FCS) (Gibco BRL) and homogenized. The cell suspension was applied on ficoll gradient (Pharmacia Biotech AB) and centrifuged at 1000 × *g* for 30 min at 4°C. The isolated leukocytes were suspended in 30 ml of the holding medium and mixed well with a vortex mixer before centrifugation at 900 × *g* for 10 min at 4°C. The cell pellet was suspended in culture medium containing Eagle's MEM with Earle's BSS, without L-glutamine (EMEM) (BioWhittaker), 200 µg ml^{-1} gentamicin sulphate, 1 µg ml^{-1} fungizone (BioWhittaker), 292 µg ml^{-1} L-glutamine, 50 mM mercaptoethanol (Gibco BRL), 1% MEM Eagle Non Essential Amino Acid (NEAA) (100×) (BioWhittaker) and 10% FCS (BioWhittaker). Cells ($10^{7.9}$ ml^{-1}) were cultured in 25 cm^2 tissue culture flasks (Nunc) at 20°C in air with 5% CO_2. Non-adherent cells were removed the next day and fresh culture medium was added. Thereafter, the cells were observed daily and half of the medium was changed approximately every second week. After about 3 mo growing cell layers were observed and these were further cultivated. Cell layers were trypsinated (Trypsin Versene, BioWhittaker) and non-adherent cells were transferred to a new tissue culture flask, where some adherent cells proliferated to new layers.

Cell growth. The subculturing was done as described above and repeated at intervals dependent on growth rates, size of the layer and time after last subculturing. Flasks with a monolayer were split at ratios of 1:2/1:3 at intervals, and after 1 yr in culture they could be split weekly. The cell culture was termed TO. Growth of TO cells without CO_2 supplement was performed in media as described above, but Eagle's MEM with Hanks' BSS (HMEM) (BioWhittaker) or L-15 (Leibovitz) (BioWhittaker) replaced EMEM. Cells were frozen by means of a general procedure. A monolayer from a 80 cm^2 flask was trypsinated and centrifuged at 168 × *g* for 5 min at 5°C. The pellet was resuspended in freezing medium (30% FCS, 10% DMSO [dimethyl sulfoxide, Merck], 60% EMEM) which was added drop by drop until it totaled 1 ml, and then transferred to a cryo tube (1.8 ml, Nunc). The pellet and the medium were held on ice during the whole procedure. The cryo tube was slowly frozen and stored in liquid nitrogen. Thawed cells were centrifuged as described above and transferred to one flask with growth medium. The cells adhered rapidly to the plastic.

Virus strains. The virus strain ISAV HI/92 [Institute of Marine Research (HI), Bergen, Norway], was isolated from Atlantic salmon in a natural outbreak of ISA in 1992. The virus material was kindly provided by Britt Hjeltnes, at the Institute of Marine Research, as frozen whole blood from moribund fish.

Virus infection. Whole blood from moribund fish was centrifuged at 1000 × *g* for 5 min and diluted 1:10 in EMEM containing 1 µg ml^{-1} fungizone and 200 µg ml^{-1} gentamicin sulphate and then passed through a filter (0.2 µm pore size, Schleicher & Schuell). Samples (10 ml) were transferred to each flask (80 cm^2) with monolayer of TO cells and incubated at 15°C without CO_2. The next day 10 ml culture medium with a double concentration of L-glutamine (584 µg ml^{-1}), NEAA (2%) and FCS (10%) was added and further incubated until cell lysis. The cell lysate was frozen at –80°C, centrifuged at 3200 × *g* for 10 min and passed through a filter (0.2 µm pore size). Passages in cell culture were performed using the resulting virus solution later termed virus supernatant. The virus supernatant was diluted 1:100 in culture medium without L-glutamine, NEAA or FCS before incubation in 80 cm^2 flasks with a monolayer of TO cells. The flasks were incubated at 15°C without CO_2 until cell lysis.

Virus infectivity. For practical reasons TO cells were tested for growth and subsequent susceptibility and virus production without CO_2 supplement. TO cells were split 5 times in media in which HMEM or L-15 replaced EMEM before they were infected with ISAV. The infections were performed in media at pH 7 and at pH 6 using L-15 or HMEM as described for the EMEM. The effects of 15 and 20°C were tested using L-15. The virus dose used for infection was $10^{4.6}$ $TCID_{50}$ ml^{-1}. Four parallel tissue culture flasks were used, and the flasks were incubated with tightened screw caps without CO_2. At Day 7 and Day 14 two flasks were harvested and the cell lysate were mixed before 3 ml samples were centrifuged at 3200 × *g* for 10 min and filtrated through a 0.45 µm Minisart-Plus, cellulose acetate filter (Sartorius AG). The samples were used for calculating the $TCID_{50}$.

Virus isolation. For virus production 10 ml of 1:100 dilution of the virus supernatant was added to 80 cm^2 flasks and incubated until cell lysis, which took place within 7 to 9 d. Resulting virus supernatants could be used at higher dilutions for infection of TO-cells. ISAV was isolated from about 220 ml cell lysate. The cell lysate was centrifuged at 3200 × *g* for 10 min, and 36 ml

supernatant was then transferred to each centrifuge tube (25 × 89 mm, Ultra clear, Beckman) containing 1.0 ml of 65% sucrose in TNE-buffer (0.01 M Tris, 0.1 M NaCl, 0.001 M EDTA, pH 7.2). Virus was isolated from the bottom of the tube after centrifugation at 102 900 × *g* for 1 h at 4°C. A total of 34 ml of supernatant was removed from the top of the tube. The remaining 3 ml from each of 2 tubes were mixed and gently placed on top of a continuos sucrose gradient 25 to 65% in a 14 × 89 mm tube (Ultra clear, Beckman). The sucrose gradient was centrifuged at 212 100 × *g* for 20 h at 4°C. Virus material was isolated from 3 gradient tubes by pipetting 1.5 ml from the visible band in the gradient. These were mixed and suspended in TNE buffer before centrifugation at 291 000 × *g* for 1 h at 4°C. The pellets containing virus were resuspended in 600 µl PBS. This solution is later termed virus stock solution.

$TCID_{50}$. 24-well tissue culture plates (Nunclon) with a monolayer of TO-cells were used for $TCID_{50}$. Virus dilutions were made in EMEM supplied with 50 µg ml^{-1} gentamicin sulphate. Six parallels were made of each dilution and 500 µl was added to each well. Tissue culture plates were placed in open sample bags of polyethylene (Minigrip®) to ensure enough humidity. The plates were incubated at 15°C for 24 h with 5% CO_2 in air before 500 µl EMEM supplied with 584 µg ml^{-1} L-glutamine, 50 µg ml^{-1} gentamicin sulphate, 2% Eagle's MEM NEAA and 10% FCS were added to each well. Wells with CPE (e.g. vacuolization and loss of adherence) at Day 14 after infection were considered infected. $TCID_{50}$ was estimated according to Kärber (1931).

Antigen characterization. SDS-PAGE resolutions of the virus proteins and TO-cell proteins were performed according to the method of Laemmli (1970) with minor modifications as described by Tsang et al. (1983). Two ISAV stock solutions, which were purified separately (termed ISAV stock solution I and II) were diluted 1:2 or 1:5 in sample buffer before being added to the gel. TO cells from an 80 cm^2 tissue culture flask with monolayer were harvested by trypsination and washed once in HMEM prior to suspension in 500 µl distilled water. This cell solution is termed TO cell stock solution. TO cell stock solution diluted 1:32 in sample buffer was applied to 1 well. Ten µl of samples and 5 µl Low Range Protein Standard (Bio-Rad) diluted 1:100 in sample buffer were applied to respective wells in a 12.5% gel in a Mini Protean II xi slab cell (Bio-Rad). The proteins were verified by silver staining according to Switzer et al. (1979).

Challenge experiment. Non-vaccinated salmon with no previous history of infectious diseases from veterinary controls were provided by Alvøen Settefisk, Norway.

Each group consisted of 30 individuals with a weight of 58 ± 9 g (mean ± SD) and was held in a 150 l dark fibreglass tank with a constant seawater flow of 2.0 l min^{-1} kg^{-1} fish. Water temperature was 10°C and the fish were fed ad libitum twice a day. Acclimatization took place 2 wk before the experiment started. Cell lysate from ISAV-infected TO cells, harvested on Day 9 was centrifuged at 3000 × *g* for 10 min and filtered (0.2 µm pore size). Six different dilutions of ISAV supernatant ($10^{6.83}$ $TCID_{50}$ ml^{-1}), 1:10, 1:50, 1:100, 1:200, 1:500 and 1:1000, were used in the challenge and dilutions were done in EMEM. Salmon were starved for 24 h and anaesthetized in 0.03% chlorobutanol prior to intraperitoneally injection with 0.1 ml virus dilution. Two control groups received 2 different dilutions (1:10 and 1:1000) of culture supernatant from uninfected TO cells and the fish were treated as described above. Dead and moribund salmon were removed daily and samples were collected from lethargic fish for reisolation of ISAV and measuring of hematocrit. Hematocrit was determined within 30 min of sampling using heparinized haematocrit capillary tubes and centrifuged for 5 min (19 872 × *g*) in haematocrit centrifuge (Jouan A 13, ALC).

RESULTS

Cell culture

During the first 3 mo there were critical periods for cell growth with few live cells. Growth was slow when the cell clusters contained few cells but improved as cell numbers and number of subcultures increased. The morphology of the cells changed. In the beginning the detachment of the cells from the colony and the plastic caused by trypsination was critical due to both fragility of the cells and the low cell number. As cell numbers and subcultures increased, the cells grew faster and trypsination was easier with less cell death. Single cells or cells at the edge of the colony were enlarged, with long pseudopodial protrusions. In confluent cultures the cells do not have these characteristics but appears to be rounded. After several subcultures the trypsinated cells rapidly attached to the flask (polystyrene) and formed colonies.

More than 150 subcultures of the TO cells were grown and they could be split (1:2 or 1:3) weekly, but maintain in good shape for long periods even at high densities and rapid growth occurred after trypsination. TO cells at Days 1, 3 and 6 following subculture are shown in Fig. 1. The cells did not show any visible morphological changes after 5 subcultures in HMEM or L-15, but appeared to grow slightly faster in the HMEM medium than in the L-15 medium. Cells stored in liquid nitrogen grew rapidly after thawing.

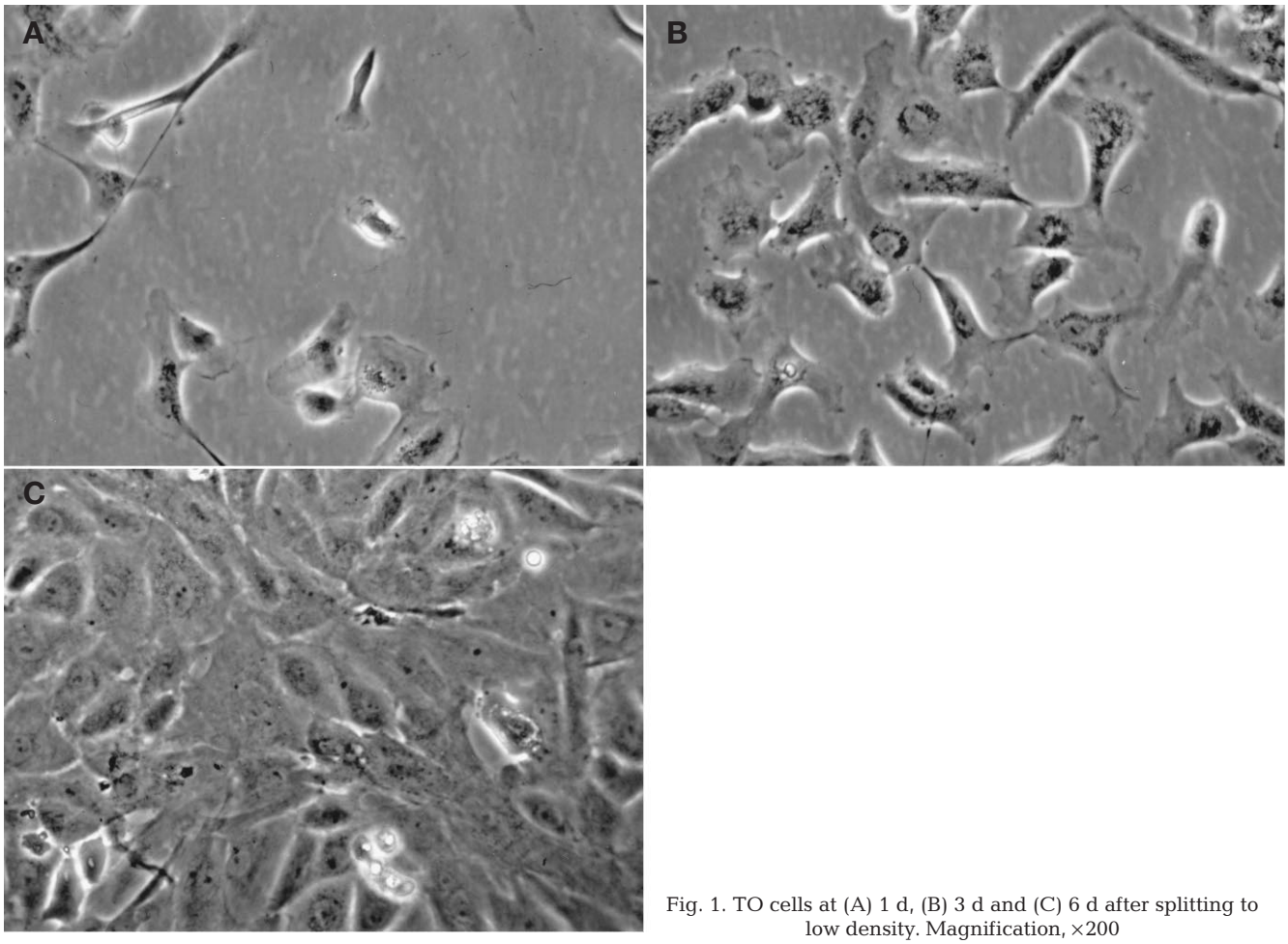

Fig. 1. TO cells at (A) 1 d, (B) 3 d and (C) 6 d after splitting to low density. Magnification, ×200

ISAV production

After 3 d post infection of TO cells with ISAV, vacuoles were observed in single cells or cell clusters and the cell culture was completely lysed or cells were detached within 9 d. The time from infection to CPE was constant and remained unchanged with different growth media and no unsuccessful infection took place. Frozen cells were easily grown and no changes in susceptibility or virus production were observed. ISAV-infected cell cultures at Days 3, 6 and 9 are shown in Fig. 2. The virus titre using these growth media was determined after 5 subcultures. The cells were passed about 40 times in HMEM medium and no changes with respect to growth rate, morphology or virus sensitivity were observed.

Virus supernatant harvested 12 d post infection reached an infectivity titre of $10^{9.1}$ $TCID_{50}$ ml^{-1} and, when tested after freezing, it had decreased to $10^{7.6}$ $TCID_{50}$ ml^{-1}. Both cell lysates were centrifuged at $3200 \times g$ for 10 min and filtered (0.2 µm pore size) before performing the $TCID_{50}$ test. In general, the $TCID_{50}$ did not change from Day 9 to 14 after infection, though a slight increase was sometimes observed. Some differences in virus titres ($10^{7.8}$ to $10^{9.1}$) were observed during different analyses, as the cell densities probably varied.

Virus production was reduced when the initial pH in the medium was 6 as compared to pH 7 for both cells grown in L-15 and in HMEM. The low virus titres of infected cells that were incubated at 20°C indicated that virus was not produced at this temperature. The results are summarised in Table 1, and cells grown in HMEM at pH 7 provided virus titres comparable with those obtained using EMEM.

ISAV protein profile

Protein profiles of isolated virus (stock solution I) showed 3 distinct protein bands, at molecular weights (MW) of 40 to 48 and 2 others at 53 and 71 kDa. In addition, some bands located above 53 kDa were present. Further, a distinct band at 26 kDa was found, and some low MW bands appeared between 14 and 21 kDa.

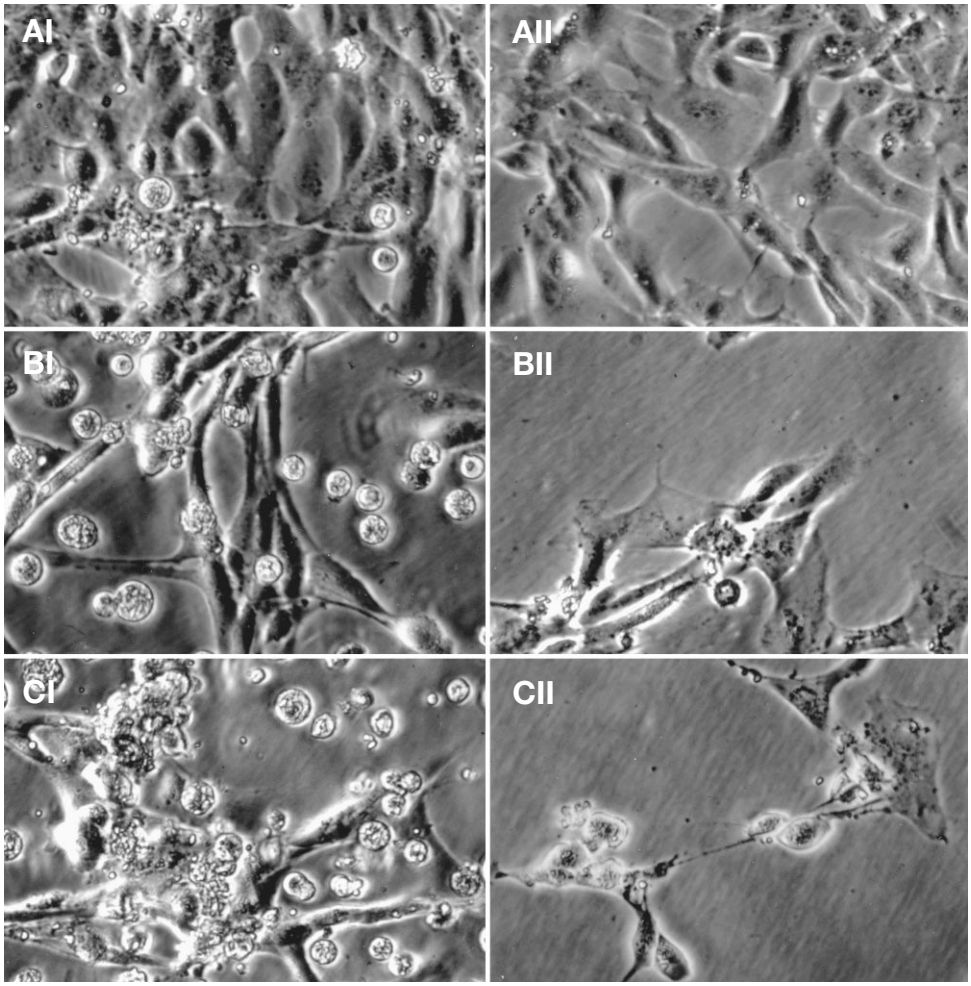

Fig. 2. TO cells after infection with virus supernatant containing ISAV. Virus supernatant had been frozen, centrifuged, filtered and diluted 1:100. (AI) 3 d after infection. Note presence of a few round cells in the medium and areas without cells. (AII) Same preparation as in (AI) but growth medium has been removed. Note presence of numerous vacuoles in neighbouring cells, the first visible sign of CPE. (BI) 6 d after infection. Note many rounded cells in the medium. (BII) Same preparation as in (BI) but growth medium has been removed. Note extended cell-free areas. (CI) 9 d after infection. Note large number of cells and amount of cell debris in the growth medium. (CII) Same preparation as in (CI), but growth medium has been removed. Magnification, ×200

The protein profile is shown in Fig. 3. The protein bands resulting from a different purification batch of the same ISAV isolate (virus stock solution II) is shown in Fig. 3B, lane 2. The protein bands in region 55 to 65 kDa are more separated, showing 2 bands, since the gel was run for a longer time. The lowest MW bands do not appear for the same reason. The protein profile of uninfected TO cells is shown in Fig. 3B, lane 1.

Challenge experiments

In the challenge experiment performed with virus supernatants, mortality started 14 d after challenge.

Table 1. The ISAV production by cells grown without CO_2 shown by $TCID_{50}$

Medium	Temperature (°C)	pH	Day 7 $TCID_{50}$ ml^{-1}	Day 14 $TCID_{50}$ ml^{-1}
L-15	15	7	$10^{-7.0}$	$10^{-7.1}$
L-15	20	7	$10^{-4.3}$ [a]	$10^{-1.0}$
L-15	15	6	$10^{-3.0}$	$10^{-3.8}$
L-15	15	7	$10^{-7.1}$	$10^{-8.0}$
HMEM	15	6	$10^{-5.5}$	$10^{-5.8}$
HMEM	15	7	$10^{-7.1}$	$10^{-7.8}$–$10^{-9.1}$ [b]

[a]CPE was not observed at this dilution, which was the lowest dilution tested
[b]This observation was made at Day 12

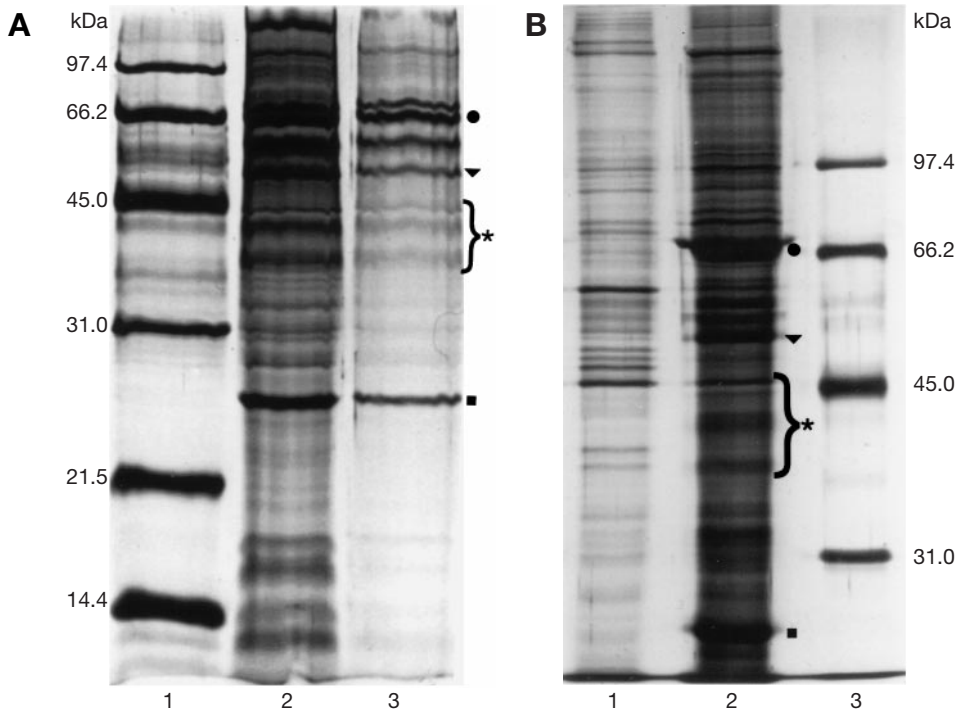

Fig. 3. Silver stained protein profile (SDS-PAGE) of purified ISAV and TO cells. (A) Molecular weight standard diluted 1:100 (5 µl) (lane 1), ISAV stock solution I diluted 1:2 (10 µl sample) (lane 2) and ISAV stock solution I diluted 1:5 (10 µl sample) (lane 3). (B) Uninfected TO cells stock solution diluted 1:32 (10 µl) (lane 1), purified ISAV stock solution II diluted 1:5 (10 µl) (lane 2) and molecular weight standard diluted 1:100 (5 µl) (lane 3). Positions of some viral proteins are indicated with symbols (26 kDa: ■; 40 to 48 kDa: ⋆; 53 kDa: ▼; and 71 kDa: ●)

Mortality rate correlated with the injected virus dilution, resulting in the highest mortality for those receiving the lowest dilutions. The mortality for all groups was between 90 and 100 % at termination of the experiment (Fig. 4). Dead and moribund salmon showed clinical signs of ISA, and hematocrit values of lethargic fish were 19.6 % ± 10.3 (mean ± SD, n = 8). Virus was reisolated from the diseased fish as diluted blood caused characteristic CPE in individual cells and cell clusters were observed from the third day on and later resulted in cell detachment and complete cell lysis.

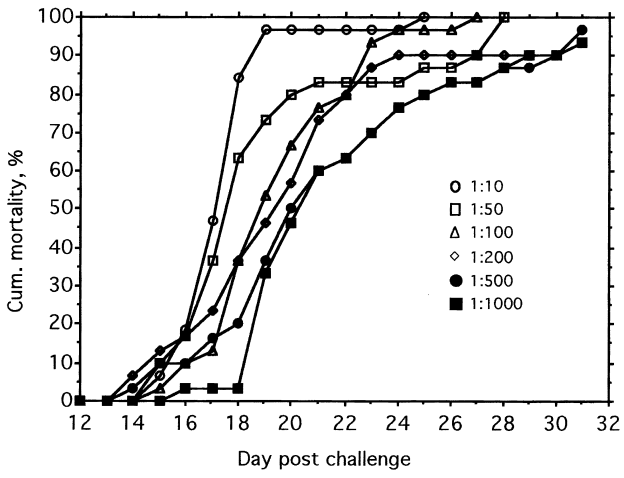

Fig. 4. Cumulative mortality of Atlantic salmon *Salmo salar* L. after injection with virus supernatant from ISAV-lysed TO cells

DISCUSSION

The growth rate of the TO cell line is relatively good, as subcultures are performed in ratios 1:2 or higher at 7 d intervals. Cell establishment and initial growth occurred in the presence of CO_2, but the growth rate did not decrease without CO_2 supplement using either HMEM or L-15 media with buffers controlling the initial pH at 7 and using 20°C for cultivation. At present the TO cells have been subcultured about 150 times. The cells grow well after freezing at –80°C. No differences in growing abilities or morphology were observed after the first 15 to 20 subcultures.

The sensitivity of TO cells for ISAV was verified by the presence of CPE in infected cell cultures. Vacuolization of individual cells and cell clusters was observed from the third day on and resulted later in cell detachment and complete cell lysis. This CPE characteristics have remained unchanged throughout our work. An infectivity titre of $10^{9.1}$ $TCID_{50}$ ml^{-1} was obtained with centrifuged and filtered fresh virus supernatant collected on 12 d post infection of cells. The titre was about 100 times higher compared with virus supernatant harvested 7 d post infection. The virus titre dropped when the virus supernatant had been frozen, but it was highly infective both for the TO cells and salmon.

The virus titres were determined in our studies by CPE to be between $10^{7.8}$ and $10^{9.1}$ $TCID_{50}$ ml^{-1} using TO cells. This was as high or higher than the titres de-

termined by IF on ISAV infected SHK-1 cells (10^6 to 10^7) (Falk et al. 1997) and in both SHK-1 ($10^{5.5}$ to $10^{7.8}$) and CHSE-214 ($10^{4.5}$ to $10^{6.5}$) determined by Kibenge et al. (2000). The virus supernatants in our studies were centrifuged and filtered to remove cell debris. Kibenge et al. (2000) did not filtrate the virus solution tested. The filtration step in our study might have reduced the virus titre, and thus the difference in titres might be greater than observed. For production of infective particles, budding from the cell membrane must be completed (Dannevig et al. 1995a), as the ISA virus has characteristics of *Orthomyxoviridae* (Falk et al. 1997). The rounding of cells and the presence of cell debris was clearly seen in the cell lysate, and thus much virus material was probably removed from the lysate by centrifugation and filtration. When ISAV was produced in CHSE-214 cells, the cell-associated virus titre was 10 times higher than cell-free virus titre (Kibenge et al. 2000), and one cannot exclude the possibility that this is also the case for TO cells.

Increased concentration of virus might be obtained using a smaller volume of medium per infected flask. Further studies could show whether changing other variables increases the outcome of virus components. Virus titres obtained by use of TO cells are higher than those obtained when a range of cell lines was used for production of sea bream iridovirus ($10^{2.5}$ to $10^{5.5}$ $TCID_{50}$ ml^{-1}) (Nakajima & Sorimachi 1994). It is also as high as titres of infectious pancreas necrosis virus (IPNV) obtained from CHSE-214 cells (10^7 to 10^8 $TCID_{50}$ ml^{-1}) (Hedrick et al. 1978). However, the amount of virus cannot be directly correlated with titres, as both cell lines and virus type varies and the number of virus particles necessary for both infection and lysis of different cell lines are seldom known.

Compared with the SHK-1 cells, TO cells produced a high yield of ISAV shown by the low volume used for isolation, and the fact that polyethylene glycol (PEG) precipitation was not necessary.

The isolation of ISAV from virus supernatants took place by first concentrating the ISAV material on a 65% sucrose layer before purification on a continuos sucrose gradient. The protein profiles in SDS-PAGE showed clear protein bands and the MW of the bands are mainly in accordance with those found by Falk et al. (1997) and Kibenge et al. (2000). One cannot exclude the possibility that the bands in region 55 to 65 kDa are of cell origin, as Kibenge et al. (2000) suggested for a band in the same region (66 kDa). This protein pattern was reproduced from different batches of isolated virus. A distinct band has always appeared at about 26 kDa. Low molecular protein bands, comparable with those observed by Kibenge et al. (2000), were present. They suggested that polypeptides of this size were of non-viral origin based on similar bands in other virus isolates and non-infected cells. For complete comparison, isolates should be analysed in the same assay and isolated by the same procedure. Variations might exist between isolates and Kibenge et al. (2000) identified minor variations in protein profiles between 2 Canadian ISAV isolates. Also, genomic sequence data have shown differences between isolates of Norwegian, Canadian and Scottish ISAV (Blake et al. 1999, Cunningham & Snow 2000). The volume of virus supernatant used to provide material for the protein profiles of purified virus indicated a high virus production from the TO cells. The ISAV-containing material from SHK-1 cells has to be concentrated by PEG before purification due to the high volume of cell lysate (Falk et al. 1997). The exact amount of cell lysate required for virus isolation from SHK-1 cells is not reported in the presented purification protocols. Further study is needed to determine the optimal time and infection procedure for harvesting cell lysate with respect to obtaining more virus antigen and infective particles. Another important aspect is sensitivity of TO cells various ISAV isolates. The amount of antigen produced by the reported procedure is completely sufficient for virus characterisation and studies of immune responses to ISAV in salmon.

Acknowledgements. This work was supported by Alpharma AS.

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

Submitted: August 21, 2000; Accepted: January 19, 2001
Proofs received from author(s): March 19, 2001