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Multiplication and haemadsorbing activity of infectious salmon anaemia virus in the established Atlantic salmon cell line

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Infectious salmon anaemia virus (ISAV), which previously had never been isolated in any of the commercially available established fish cell lines, was successfully propagated in the continuous cell line Atlantic salmon (AS). The yield of infectious ISAV increased with the incubation time of virus-inoculated cells, demonstrated by *in vivo* infectivity trials in groups of Atlantic salmon. Trypsin treatment of the virus was not necessary for primary infection of AS cells with salmon-grown ISAV. The infection was non-cytopathic, but it was possible to detect virus-infected cells by a haemadsorption centre assay using Atlantic salmon erythrocytes. Pleomorphic enveloped virus particles were seen by transmission electron microscopy of infected AS cells. Elongated forms were observed, but spherical particles with diameters of 90–130 nm were commonest. Growth of ISAV was inhibited by actinomycin D but not by 5-bromo-2-deoxyuridine treatment, which indicates that ISAV may be an aquatic orthomyxovirus.

Infectious salmon anaemia (ISA) is a disease of farmed Atlantic salmon (*Salmo salar* L.) in Norway. The first outbreak of ISA was recorded at the end of 1984 and reached a peak incidence in 1991. The disease is characterized by severe anaemia in the terminal stages and mortality is usually high (Thorud & Djupvik, 1988), but asymptomatic ISA virus (ISAV) infections have been demonstrated in other salmonids (Nylund *et al.*, 1994). Enveloped 100 nm virus particles have been detected in tissue from ISA-diseased Atlantic salmon by transmission electron microscopy (TEM) studies (Nylund *et al.*, 1995). ISAV has not previously been isolated in any of the commercially available continuous fish cell lines, but the establishment of a long-term cell line from Atlantic salmon head kidney which supports ISAV replication has been reported (Dannevig *et al.*, 1995). ISAV has also been isolated in cultures of head kidney macrophages. Virus infection in these primary cell cultures was non-cytopathic and was demonstrated by means of *in vivo* infectivity studies in salmon and by electron microscopy, since no reliable immunoassay was available (Sommer & Mennen, 1996). Previous studies of cytopathic changes after inoculating 34 different established fish cell lines with ISAV were negative (M. Yoshimizu, personal communication). In two of the cell lines, AS (Atlantic salmon) and CHSE-214 (Chinook salmon embryo) cells, ISAV was passaged eight times without observation of any cytopathic effects (CPE) (A.-I. Sommer, unpublished results).

In this study, we investigated the possible non-cytopathic propagation of ISAV in the only commercially available cell line derived from Atlantic salmon, called the Atlantic salmon (AS) cell line (Flow Laboratories, catalogue no. 02–776, European Cell Collection, Irvine, Scotland, UK). Many viral glycoproteins, e.g. influenza virus haemagglutin (HA), are activated by proteolytic cleavage (Klenk & Rott, 1988). This is known to make infection possible or enhance the virus yield in cell cultures by mediating multi-cycle replication (Klenk *et al.*, 1975). ISAV inoculation of the AS cells was therefore done with or without trypsin present. The yield of infectious ISAV produced was initially determined by *in vivo* infectivity trials in groups of salmon. TEM studies gave additional information about the amount of virus, its formation and morphology. A preliminary assumption that ISAV is an orthomyxovirus has previously been made based on biophysical and morphological observations (Sommer & Mennen, 1996). Influenza viruses have the ability to agglutinate erythrocytes from fowl, guinea-pig and human blood group O (Murphy & Webster, 1990). There are few reports of haemagglutinating activity among fish viruses, in contrast to viruses of mammals. Orthomyxovirus-like agents isolated from eel haemagglutinated chicken and sheep erythrocytes, but not those of human, rabbit or rainbow trout (Nagabayashi & Wolf, 1979; Neukirch, 1985). ISAV haemadsorbing ability was studied using infected AS cells and Atlantic salmon erythrocytes. In order to visualize a non-cytopathic ISAV infection in the AS cells, and to quantify the viruses, a haemadsorption centre assay was developed.

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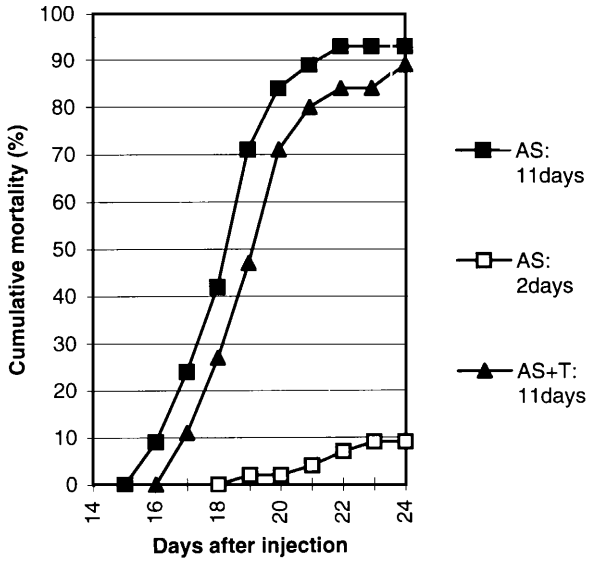

Fig. 1. Yield of infectious ISAV collected after two different periods of time from three identically virus-inoculated AS cell cultures, without trypsin added (AS: 2 days and AS: 11 days) or with trypsin (AS+T: 11 days). Infectivity is shown as cumulative ISA mortality registered in three groups of Atlantic salmon injected with harvested material.

A stock of salmon-grown virus was prepared from plasma obtained from experimentally infected ISA-diseased Atlantic salmon. The ISAV was crudely purified from the plasma pool in a discontinuous 30% over 60% (w/v) sucrose gradient by centrifugation for 3 h at 100 000 ***g*** at 4 °C. The material at the interface was collected and stored at −80 °C until used as seed virus for infection of cell cultures. Prior to use, the stock preparation was tested for infectivity at a 1:10 dilution by intraperitoneal (i.p.) injection in a group of salmon, as described below.

AS cells were seeded in six-well cell culture dishes in Eagle's minimum essential medium (EMEM) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 1% non-essential amino acids, 1% L-glutamine and 7% foetal bovine serum (FBS) and incubated with 5% CO_2 at 20 °C. When the cell layers were confluent 2 ml seed virus diluted 1:60 in EMEM, with or without 5 µg/ml trypsin (1:250, Difco), was added per well and incubated for 2 h at 12 °C. The cells were washed twice in PBS supplemented with calcium and magnesium, and fresh EMEM supplemented with 2·5% FBS was added, 4 ml per well. Because of the common carrier condition of infectious pancreatic necrosis virus (IPNV) in farmed Atlantic salmon, neutralizing amounts of IPNV-specific immune sera produced in rabbits were included in all the culture medium.

After two different periods of time ISAV was harvested from duplicate wells of identically virus-inoculated AS cultures, with or without trypsin added, as described above. Cells and medium from one set of cultures were collected after 2 days and frozen at −80 °C. From the others (with or without trypsin), medium was collected and frozen at days 2 and 7 and finally pooled together with cells and medium collected 11 days after inoculation. The three samples, each obtained from about 2×10^5 cells, were diluted to equal final volumes of 50 ml before use for *in vivo* infectivity trials. The harvested material was tested for infectivity in three groups of 45 Atlantic salmon each weighing about 40 g. The fish were anaesthetized, marked with Alcian blue and given i.p. injections of 0·5 ml each. A group of fish injected with non-infected AS cells and EMEM was always included to monitor mortality due to infection with ISAV released from injected cohabitants. All groups were kept together in one tank and given a continuous flow of brackish water of 10‰ salinity at 10 °C.

The yield of infectious ISAV increased with the incubation time of inoculated cells. This was demonstrated in the *in vivo* infectivity trials by a significantly increased cumulative mortality and the earlier start of an ISA outbreak in the group of Atlantic salmon injected with material collected 11 days after inoculating the cells, compared to the harvest at day 2 (Fig. 1). There was no significant difference between the amount of infectious ISAV produced with or without trypsin present (Fig. 1). Enhancement of influenza virus infectivity by trypsin has been reported at concentrations of 5 to 20 µg/ml, incorporated in an agar overlay (Tobita *et al*., 1975; Klenk *et al*., 1975), or down to 1 µg/ml in the culture medium (Boycott *et al*., 1994). The AS monolayer detached easily from the wells if exposed to a trypsin concentration exceeding 5 µg/ml for several hours or 1 µg/ml in the medium for several days without FBS present.

Salmon-grown ISAV probably contains fully cleaved spikes and is therefore not dependent on trypsin for a complete single replication cycle in AS cells. Likewise, influenza virus grown in embryonated eggs is highly infectious, while virus produced in some cell cultures has a low infectivity (Klenk *et al*., 1975; Boycott *et al*., 1994). Some avian influenza virus strains are cleaved by proteases present in practically all cells. These viruses are therefore capable of undergoing multiple replication cycles in tissue culture, and are highly pathogenic, causing systemic infection in birds (Klenk *et al*., 1975). The difficulty of finding suitable cell cultures for growing ISAV does not indicate a similar proteolytic cleavability as for the avian stains. ISAV was produced in primary infected AS cells without addition of trypsin, as demonstrated by *in vivo* infectivity trials. Further studies will show whether AS-produced ISAV requires addition of an exogenous protease for successful *in vitro*

Fig. 2. Electron micrographs of thin sections of ISAV-infected AS cells 11 days after inoculating the culture. (*a*) Virus particles in the intercellular space are indicated with arrows (×6100). (*b*) Framed area in (*a*) magnified showing virus particles of variable size and form, both elongated (arrowheads) and spherical forms (arrows). Bar, 200 nm. (*c*) Budding ISAV particles with both surface spikes and envelope visible. Bar, 50 nm.

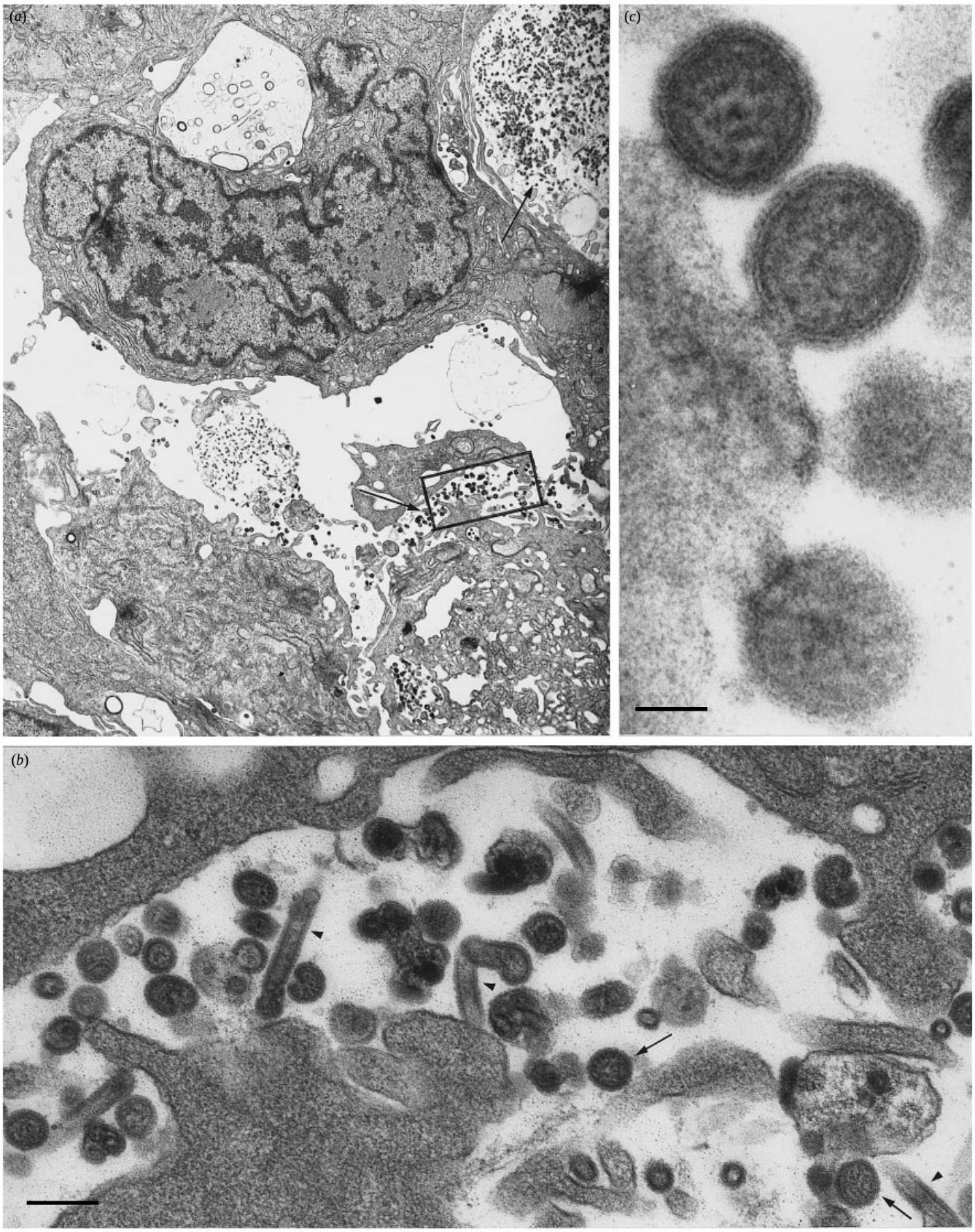

Fig. 2. For legend see facing page.

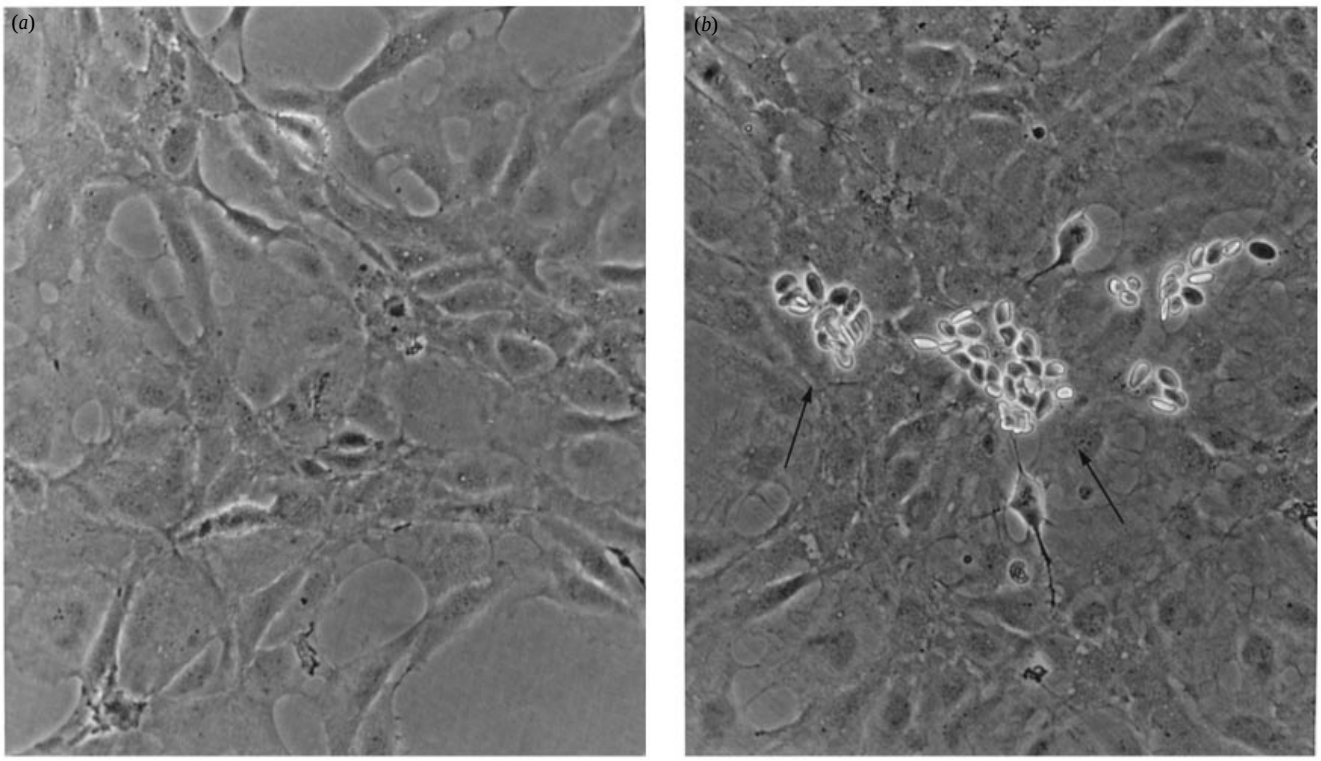

Fig. 3. HACA performed on ISAV-infected AS cells 3 h (*a*) and 6 days (*b*) after inoculation. Centres of salmon erythrocytes attached to infected cells are seen (arrows) (×850).

passages, or if the spikes are already adequately cleaved by an endogenous protease, or will be cleaved at the stage of entry.

AS cells, both non-infected and ISAV-infected, were fixed 11 days after inoculation using 1% glutaraldehyde and 4% formaldehyde in phosphate buffer, 220 mOSm, and embedded in Epon/Araldite. Thin sections of pelleted cells were stained for 1·5 h with 2% aqueous uranyl acetate solution (Nylund *et al*., 1995), and stained for 12 min with Reynold's lead citrate before examination in a JEOL JEM 1010 transmission electron microscope. Numerous virus particles somewhat variable in size and form were seen. Generally, they are spherical or ovoid with a mean diameter of 90–130 nm, but filamentous forms of similar diameter occur (Fig. 2*a*, *b*). In Fig. 2(*c*) the ISAV envelope is visible with evenly distributed surface spikes, about 10 nm long, and within the envelope lies an electron-dense matrix shell. The central zone contains amorphous material of variable density, often with a circular arrangement of distinct 'granulas' of about 10 nm. This may indicate an organized packing of the nucleocapsid. Structures resembling helical nucleocapsids of 8–10 nm in diameter have previously been detected in purified ISAV material (Sommer & Mennen, 1996).

Monolayers of AS cells grown in six-well cell culture dishes were infected as described above. After incubation at 12 °C for 3 h or 1, 2 or 6 days, a haemadsorption centre assay (HACA) was performed. A suspension of 0·05% (v/v) Atlantic salmon erythrocytes in PBS was added to the cells (1 ml per well) and incubated at room temperature for 45 min. After washing off the erythrocytes not attached to infected cells, HACA-positive cells were observed under a Leitz inverted light microscope. No HACA-positive cells were seen in non-infected cells or in cells 3 h (Fig. 3*a*) or 1 day after ISAV inoculation, but the first sign of haemadsorption was detected after 2 days of infection. After 6 days the HACA-positive areas were larger and easy to read (Fig. 3*b*). Usually, to quantify the infectious titre of a virus sample, serial 10-fold dilutions were inoculated onto monolayers of AS cells grown in 96-well cell culture dishes. After incubation for 7 days ISAV infection was demonstrated by use of HACA performed as described above, and $TCID_{50}$ titres were calculated according to the method of Reed and Muench (Dulbecco, 1980). The titre of the seed virus preparation used for primary infection of the AS cells was $10^{3·6}$ $TCID_{50}$/ml, giving an m.o.i. of about 0·05.

The HACA was used to study possible inhibition of growth by the DNA synthesis inhibitor 5-bromo-2-deoxyuridine (BrdU), a thymidine analogue, and the DNA-dependent RNA synthesis inhibitor actinomycin D (AMD). Virus replication was not inhibited in the presence of 50 μg/ml BrdU, with or without 50 μg/ml thymidine added, indicating that ISAV has an RNA genome. AMD is known to inhibit replication of influenza virus, although this is an RNA virus (Barry *et al*., 1962). Both nuclear and cytoplasmic transcription

of influenza virus were totally inhibited by 1 μg/ml AMD (Stephenson & Dimmock, 1975). Another proposed orthomyxovirus, isolated from eel, was inhibited most efficiently by 1 μg/ml AMD when treated during the first 2–3 h after infection (Nagabayashi & Wolf, 1979). When AS cells were treated with AMD for 3 h after ISAV adsorption, virus replication was almost completely inhibited by 1 μg/ml (10^4 $TCID_{50}$/ml reduced to < 10 $TCID_{50}$/ml), but not significantly by 0·1 μg/ml AMD. Replication of the double-stranded RNA virus IPNV was not affected by either inhibitor.

Similar to the orthomyxo-like eel virus, the ISAV spikes are somewhat shorter than influenza virus HA. Except for this and the typical 'granulas' ISAV is similar in size and morphology to other orthomyxoviruses (Murphy & Webster, 1990). The typical packing of the nucleocapsid is not commonly seen among orthomyxoviruses, but has been reported for paramyxoviruses such as respiratory syncytial virus (RSV) (Norrby *et al*., 1970). Nevertheless, it is unlikely that ISAV is an RSV because these viruses show no haemadsorption ability. In addition, the paramyxoviruses are nearly twice as large as ISAV and are not sensitive to AMD. So far, the most obvious differences from other known orthomyxoviruses are that ISAV is pathogenic for a poikilothermic aquatic species and has a much lower optimum growth temperature. A significant reduction in virus production when the growth temperature exceeds 18 °C has been observed (A.-I. Sommer, unpublished results). The present results, including the newly discovered haemadsorbing ability, suggest that ISAV might be an aquatic orthomyxovirus, although placement of ISAV in any specific virus family must await characterization of the viral RNA. The reported propagation of ISAV in a commercial continuous fish cell line will make it easier to complete such studies.

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