Genomic Characterization of the Virus Causing Infectious Salmon Anemia in Atlantic Salmon (Salmo salar L.): an Orthomyxo-Like Virus in a Teleost

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The genome of infectious salmon anemia virus (ISAV), which infects farmed Atlantic salmon (Salmo salar L.), is characterized here. The virus has an RNA genome, as shown by using specific DNA virus metabolic inhibitors and radioactive in vivo labeling of ISAV nucleic acid. Electrophoresis of [14C]uridine-labeled ISAV RNA revealed that the ISAV genome is segmented. The genome consists of eight segments that range from 1.0 to 2.3 kb, with a total molecular size of approximately 14.5 kb. One ISAV-specific molecular clone, corresponding to the smallest genome segment, was obtained by cDNA cloning of mRNA from an ISAV-infected cell culture. This clone gave a positive hybridization signal on Northern blots of pelleted ISAV. Pretreatment of the ISAV pellet with RNase A resulted in the disappearance of the positive hybridization signal, demonstrating that the genome is single stranded. Reverse transcriptase PCR with primers corresponding to sequences from the molecular clone and target RNA from ISAV-infected and noninfected fish tissues gave specific positive reactions. Alignment of the molecular clone did not reveal significant homology with any other available sequence in databases. However, the data presented here, together with morphological and replicational properties previously described, indicate that ISAV has a strong resemblance to members of the Orthomyxoviridae family. This is the first thoroughly characterized orthomyxo-like virus isolated from a teleost.

Since 1985, infectious salmon anemia (ISA) has caused massive economic losses in the Atlantic salmon (Salmo salar L.) farming industry in Norway. ISA is considered to be such a threat to the fish farming industry that it is the only disease on the European Union list of the most dangerous fish diseases. The pathological findings in Atlantic salmon with ISA include severe anemia, leucopenia, ascites, and hemorrhagic liver necrosis (9, 30, 31). The etiological agent of ISA has previously been shown to be an enveloped virus (1, 5). It has been hampered by the virus does not replicate in commonly available cell lines (5). However, in 1994, a long-term cell line developed from Atlantic salmon head kidney (SHK-1) that supports the growth of ISAV was established (6). This enabled the production of ISAV antigens and subsequently the development of polyclonal and monoclonal antibodies (MAb) to ISAV, which can be used to detect viral antigens in cell cultures and tissue sections from ISAV-infected fish (10). Propagation of ISAV in SHK-1 cells has also provided materials that make characterization of the ISAV genome possible.

In this study, data for the type of nucleic acid, size, and organization of the ISAV genome are presented. Parts of the genome were cloned and sequenced, and homology alignment was performed. A reverse transcriptase PCR technique was developed for the detection of ISAV in tissues of infected fish. Based on these data, together with the properties of ISAV previously described, a tentative taxonomic allocation of ISAV is suggested.

MATERIALS AND METHODS

Viruses. Inoculation of ISAV (strain Gillewaert/290), which had been passaged seven times, was performed on SHK-1 cells as described earlier (5). In short, cell culture medium (L-15; Bio Whittaker, Verviers, Belgium) was removed and ISAV, diluted 1:3 in serum-free medium, was added. The virus was allowed to adsorb for 4 h at 15°C, followed by the addition of medium with 5% fetal calf serum (Bio Whittaker). Infection was allowed to proceed at 15°C until the cytopathic effect (CPE) was evident (5 to 7 days).

Equine herpesvirus type 1 (EHV-1), grown in RK-13 cells, and infectious pancreatic necrosis virus (IPNV) and infectious hemorrhagic necrosis virus (IHNV), both grown in SHK-1 cells, were used as nucleic acid specificity controls in this study with metabolic inhibitors.

Virus pelleting. Cell culture supernatant from ISAV-infected SHK-1 cells was cleared by low-speed centrifugation at 4,000 × g for 30 min at 4°C, and virus was pelleted by centrifugation at 104,000 × g for 2 h at 4°C.

Virus purification by ISAV MAB-coated magnetic beads. A murine MAB supernatant against ISAV (10) was conjugated to monodisperse polystyrene magnetic beads, precoated with sheep anti-mouse immunoglobulin G (Dynabeads M-280; Dynal AS, Oslo, Norway) according to the manufacturer’s instructions. In short, the beads were placed on a roller and incubated overnight with 20 μg of antibodies per mg of magnetic beads. The coated beads were washed four times for 30 min (each) in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin, resuspended, and added to debris-free virus cell culture supernatant (200 μg of magnetic beads/ml of virus supernatant). The virus suspension and beads were incubated for 2 h with slow rotation. All incubations were performed at 4°C.

Virus purification by sucrose gradient separation. Debris-cleared cell culture supernatant containing ISAV from two 175-cm² flasks was precipitated by the addition of 23.2 g of NaCl per liter and 70 g of polyethylene glycol 8000 (Sigma, St. Louis, Mo.) per liter, and the suspension was incubated at room temperature for 15 min, followed by stirring for 3 to 4 h. The precipitate was collected by centrifugation at 17,000 × g for 90 min, resuspended in 1 ml of TNE buffer (10 mM Tris, 0.1 M NaCl, 1 mM EDTA [pH 7.2]), and cleared by centrifugation at 4,500 × g for 10 min. The suspension was loaded onto a 5 to 65% continuous sucrose gradient (UltraPure sucrose; Gibco-BRL, Gaithersburg, Md.) and centrifuged at 150,000 × g for 18 h. The virus band was collected by penetration with a syringe. RNA was extracted, and electrophoresis was performed as described below. All incubations were at 4°C unless otherwise stated.
Incubation with metabolic inhibitors. The effects of DNA synthesis inhibitors 5-iodo-2-deoxyuridine (IdU; Sigma) and 5-bromo-2-deoxyuridine (BrdU; Amer- sham, Little Chalfont, Buckinghamshire, United Kingdom) on the replication of ISAV were tested by adding 50 μg of IdU or BrdU per ml to SHK-1 cell culture medium at 1 h postinfection. Control cells were cultured without the addition of inhibitors. EHV-1 (double-stranded DNA virus), grown in RK-13 cells, served as the positive DNA virus control, and IHNV (single-stranded RNA virus) and IPNV (double-stranded RNA virus), grown in SHK-1 cells, served as negative controls.

In vivo labeling of ISAV. To confirm the type of nucleic acid present in ISAV, 1 μCi of inorganic [32P]phosphate (0.5 μCi/ml; Amersham) per 175-mm2 flask was added at 5 h postinfection to SHK-1 cells cultured in phosphate-deficient medium (Sigma, St. Louis, Mo.). The medium had been removed and cells had been washed with PBS. Chloroform was added, and the suspension was spun for 50 min at 3,600 × g. The supernatant was discarded and 50 μl of diethyl pyrocarbonate (DEPC; Sigma)-treated water and used in electrophoresis (see below). The virus-containing medium was collected and the virus was purified by sucrose gradient centrifugation. Viral RNA was extracted from purified ISAV by standard protocols (2, 27) with the addition of 1 ml of an aqueous solution of RNAse A (20 U) and 1 ml of RNAse-free DNase I (14 U) prior to Northern blot hybridization. The remaining material was used as a negative control in transmission trials and from farms with no positive animals.

Reverse transcriptase PCR. RNA extracted from tissue samples of natural ISAV-infected and noninfected fish, as well as SHK-1 cells, was converted to DNA by reverse transcription. The ISAV-negative fish tissue samples were from materials used as negative controls in transmission trials and from farms with no historical reports of ISAV infection in the USA. Before addition of reverse transcriptase (RT) and 2.5 μg of RNA was denatured for 10 min at 55°C, followed by cooling on ice. The reverse transcription mix was added, giving a final volume of 20 μl with 50 mM Tris-HCl, 75 mM KCl, 5 mM MgCl2, 0.5 mM (each) deoxynucleoside triphosphates, and 0.05 U/μl units of random hexamer primers. This mixture was incubated at 95°C for 5 min and cooled on ice before the addition of 20 μl of reverse transcriptase ( Gibco-BRL). The first-strand cDNA was synthesized at 37°C for 1 h. Five microliters of cDNA was added to 45 μl of PCR mix (final concentrations, 20 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, and 0.2 mM (each) deoxyribo- nucleoside triphosphates), as well as 25 pmol (each) of the two primers and 1 U of Taq DNA polymerase (Gibco-BRL). The two primers used were 5'-GGC TAT CTF ACAA CGA ACG AAT AAC (sense) and 5'-GCC AAG TGT AAG TAG CAC TCC (antisense), which were synthesized by MedProbe, Oslo, Norway. The size of the DNA fragment amplified by these primers was calculated to be 155 bp.

RESULTS

ISAV nucleic acid. The addition of IdU and BrdU did not inhibit ISAV replication, as ISAV-infected cell cultures developed the CPE in the presence of inhibitors. In control cultures containing virus DNA, however, no CPE effect was observed in EHV-1-infected cell cultures up to 10 days after the addition of IdU and BrdU, whereas parallel control cultures without inhibitors developed the CPE within 4 days. Moreover, IdU and BrdU did not inhibit the development of the CPE in IPNV- and IHNV-infected cell cultures.

Tacing of [32P]Phosphate-labeled ISAV extract by affinity to anti-ISAV MAB-coated magnetic beads showed approximately 100-fold-higher activity by Geiger monitoring in the fraction containing virus particles compared to the activity left in the expelled supernatant. Furthermore, the radioactivity could be traced to the RNA fraction in subsequent extraction steps. Autoradiography of Northern blots from electrophoresed [32P]labeled RNA confirmed that ISAV nucleic acid consists of RNA (data not shown).

Size and organization of the ISAV genome. To separate possible genome segments, it was essential to use a 2% agarose concentration and low voltage (2.25 V/cm) and to perform electrophoresis on ice to avoid smearing. Autoradiography of Northern blots from these gels revealed seven clearly separated genome segments (Fig. 1). The autoradiographs were scanned, and the sizes of RNA segments were estimated to be in the range of 1.0 to 2.3 kb. Densitometric studies revealed that the largest (2.3-kb) band had a relative mass which was about twice that of the second largest band (Table 1). This indicates that the ISAV genome consists of eight RNA segments and that the two largest segments are apparently identical in size and not separable under the conditions used. The total molecular size of the ISAV genome, based on the sizes of individual RNA segments, was estimated to be approximately 14.5 kb. A comparison of the sizes of the ISAV genome segments with those of members of the Orthomyxoviridae family is given in Table 2.

Molecular cloning of the ISAV genome. One molecular clone (clone 14) showed repeatedly positive signals in hybridization reactions with total RNA from ISAV-infected SHK-1 cells (Fig. 2a) and no signals either from RNA of noninfected...
SHK-1 cells (Fig. 2a) or from DNA isolated from Atlantic salmon and digested with various restriction enzymes (Fig. 2b). When pelleted ISAV was used as the hybridization target, one distinct positive band, about 1 kb in size, was obtained (Fig. 2a). A comparison of the Northern blot hybridization results to the autoradiograph of ISAV genome segments revealed that clone 14 hybridized to the smallest segment. When the ISAV virus pellet was treated with RNase A before electrophoresis and blotting, the positive hybridization signal disappeared, whereas treatment with RNase-free DNase had no such effect (Fig. 3). Since RNase A attacks only single-stranded RNA (8), the ISAV genome consists of single-stranded RNA.

When lanes 1 and 3 of Fig. 2a are compared, virion RNA (pelleted ISAV) is slightly larger than is virus-encoded intracellular RNA. This indicates that the genomic segments in ISAV are not used directly as mRNA but are transcribed before transcription, i.e., they have negative sense.

The nucleotide sequence of clone 14 was obtained, and a sequence alignment was performed. However, no significant homology between this nucleotide sequence and any sequence in databases was found. Two putative open reading frames (ORFs) were found; nevertheless, the sequence was truncated in the 5' end and the starting points of the ORFs could not be determined. A homology search was performed for the translated amino acid sequence; however, no significant homology was found.

**Reverse transcriptase PCR.** Reverse transcriptase PCR showed that the amplification was specific for ISAV RNA. The size of amplified DNA segments, including primers, was approximately 155 bp, as seen on ethidium bromide-stained gels (Fig. 4), which is in accordance with the nucleotide sequence of clone 14. No amplification was observed by reverse transcriptase PCR of RNA from noninfected SHK-1 cultures (Fig. 4, lane N) or by PCR of DNA from ISAV-infected and noninfected SHK-1 cultures (data not shown). Samples from fish were tested blindly. All samples that were negative for ISAV (7 of 16) were negative by PCR. One sample that we suspected to be ISAV positive proved to be negative by reverse transcriptase PCR. Of the ISAV-positive samples, four of nine were positive after electrophoresis (Fig. 4) and two more were positive after Southern blot hybridization. The fish tissue samples used as targets in PCR had been kept at −70°C for several years without any control of eventual repeated thawing; therefore, RNA degradation was likely to have occurred in some samples.

**DISCUSSION**

The data presented here indicate that ISAV has a single-stranded RNA genome that consists of eight segments with a tentative negative polarity. These conclusions are based on the following results. (i) ISAV replication was not affected after the addition of metabolic inhibitors BrdU and IdU to the cell culture medium, (ii) radioactive RNA was adsorbed to magnetic beads in experiments with anti-ISAV antibody-coated magnetic beads after inorganic-32P labeling of ISAV, and (iii) the hybridization signal was abolished after RNase A treatment of pelleted virus nucleic acid prior to hybridization with clone 14. The segmentation was displayed by [14C]uridine labeling, and the negative-sense strandedness was indicated by the fact that the virion RNA was bigger than the intracellular virus-encoded mRNA. All of these facts are in concordance with the properties of members of the Orthomyxoviridae family.

Although titers of up to 10^7 to 10^8 50% tissue culture infectious doses/ml have been achieved for propagation of ISAV in SHK-1 cells (as determined by end point titration of SHK-1 cells by an indirect immunofluorescense technique [9a]), radiolabeling of the viral nucleic acid was necessary to visualize the genome segments in agarose gel electrophoresis. Because

![FIG. 1. The ISAV genome is segmented. Autoradiograph of a Northern blot of [14C]uridine- and in vivo-labeled ISAV nucleic acid. Molecular size (in kilobases) standards are indicated on the left.](image)

<table>
<thead>
<tr>
<th>Genome segment</th>
<th>Relative mass b</th>
<th>Molecular size (kb) a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13.6</td>
<td>2.3</td>
</tr>
<tr>
<td>2</td>
<td>7.37</td>
<td>2.2</td>
</tr>
<tr>
<td>3</td>
<td>7.11</td>
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<tr>
<td>4</td>
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<td>1.7</td>
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<td>6</td>
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</tr>
<tr>
<td>7</td>
<td>3.94</td>
<td>1.0</td>
</tr>
</tbody>
</table>

a Data are estimates of the relative mass of each genome segment after scanning of autoradiograph of Northern blot of [14C]uridine-labeled ISAV nucleic acid.

b Data are estimates of ISAV genome segment sizes compared to two RNA ladders, ranging from 0.24 to 9.4 and 0.155 to 1.770 kb.
of its small molecular size and high specific activity, inorganic 
$[^{32}\text{P}]$phosphate was chosen as the radioactive tracer for in vivo 
labeling of the nucleic acid. However, inorganic $[^{32}\text{P}]$phos-
phate was replaced with $[^{14}\text{C}]$uridine once the nucleic acid type 
was defined, since smearing of RNA on Northern blots was 
observed after labeling with inorganic $[^{32}\text{P}]$phosphate.

The segmented nature of the ISAV genome is similar to 
those of orthomyxoviruses. These genomes are multipartite 
and, depending on the genus, consist of six to eight segments 
(4, 17, 19) (Table 2). As with influenza A, B, and C virus 
genomes, the two largest ISAV genome segments could not be 
separated according to size by electrophoresis. However, the 
sizes of individual segments vary among different genera. Ap-
proximately equimolar amounts of each of the eight segments 
are present in standard influenza virus preparations (19), as 
was found for ISAV. The total molecular size of the ISAV 
genome was estimated to be 14.5 kb, which is within the range 
of genome sizes among orthomyxoviruses, ranging from 10 to 
14.5 kb (see Table 2).

The distinct positive hybridization signal achieved with clone 
14 coincides with the smallest ISAV genome segment. Genetic 
and biochemical evidence have shown that the two smallest 
influenza A and B virus RNA segments encode at least two 
proteins each, the M1 and M2 proteins on segment 7 and the 
NS1 and NS2 proteins on segment 8 (17). An analogous ar-
rangement of unspliced and spliced mRNA transcripts has 
previously been found to encode the proteins derived from 
RNA segments 6 and 7 of influenza C virus (16). No sequence 
data are available for the smallest segments in the influenza D 
virus (24), but the sixth segment in the Dhorj virus codes for a 
protein similar to the M protein (3). The smallest segment in 
the influenza A, B, and C viruses contains two ORFs, one large 
ORF encoding the NS1 protein (26 kDa) and a smaller ORF 
coding for an NS2 protein (14 kDa) (17). The sequence data

<table>
<thead>
<tr>
<th>Genome segment</th>
<th>Influenza A virus$^a$</th>
<th>Influenza B virus$^b$</th>
<th>Influenza C virus$^b$</th>
<th>Influenza D virus (Thogoto)$^c$</th>
<th>Orthoacarivirus (Dhorj)$^d$</th>
<th>ISAV</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>2.34</td>
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<td>2.35</td>
<td>ND$^e$</td>
<td>ND$^e$</td>
<td>2.3</td>
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<tr>
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<td>2.34</td>
<td>2.38</td>
<td>2.35</td>
<td>ND$^e$</td>
<td>2.2</td>
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<tr>
<td>4</td>
<td>1.74–1.77</td>
<td>1.88</td>
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</tr>
<tr>
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<td>1.84</td>
<td>1.80</td>
<td>ND$^e$</td>
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<td>1.7</td>
</tr>
<tr>
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<td>1.40</td>
<td>1.18</td>
<td>ND$^e$</td>
<td>0.96</td>
<td>1.5</td>
</tr>
<tr>
<td>7</td>
<td>1.03</td>
<td>1.19</td>
<td>0.93</td>
<td>—$^f$</td>
<td>ND$^e$</td>
<td>1.3</td>
</tr>
<tr>
<td>8</td>
<td>0.89</td>
<td>1.09</td>
<td>—$^f$</td>
<td>—$^f$</td>
<td>—$^f$</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>13.6</strong></td>
<td><strong>14.5</strong></td>
<td><strong>12.9</strong></td>
<td><strong>10</strong></td>
<td><strong>10</strong></td>
<td><strong>14.5</strong></td>
</tr>
</tbody>
</table>

$^a$ Data are taken from references 17 and 19.
$^b$ Data are taken from reference 17.
$^c$ Data are taken from references 4 and 24.
$^d$ Data are taken from references 3, 4, 11, and 24.
$^e$ ND, no data available.
$^f$ —, no segment.

FIG. 2. Specificity of clone 14. Hybridization reactions with clone 14 as the probe. (a) Northern blot. Lanes: M, molecular size (in kilobases) ladder; 1 and 2, RNA from ISAV-infected SHK-1 cells; 3, pelleted ISAV RNA; 4, RNA from EPC cells; 5, RNA from noninfected SHK-1 cells. (b) Southern blot of total Atlantic salmon DNA treated with EcoRI (lane 1), BamHI (lane 2), or HindIII (lane 3).
FIG. 3. The ISAV genome is single stranded. Hybridization reaction with clone 14 as the probe on a Northern blot of pelleted ISAV virus RNA. Lane 1, untreated ISAV RNA; lane 2, ISAV RNA treated with DNase; lane 3, ISAV RNA pellet treated with RNase A; lane M, molecular size (in kilobases) standards.

for clone 14 indicated the presence of two ORFs coding for two putative proteins. However, it is not known whether ISAV exploits the coding strategies used by influenza viruses, e.g., splicing and translation from overlapping reading frames. Clearly, knowledge of the structures and functions of the gene products of ISAV is required before the molecular basis of the pathogenesis of ISA can be clarified.

Alignments of the clone 14 nucleotide sequence did not display significant homology with any other sequence, and neither did amino acid alignment of the translated sequence. However, taking into account the evolutionary distance between the host species for ISAV and those for members of Orthomyxoviridae, i.e., a teleost compared to avian species and mammals, the lack of sequence homology between ISAV and other orthomyxoviruses was not unexpected. Pooling the genetic data for Thogoto and Dhori viruses reveals that three of the five genomic segments with available nucleotide sequences have small homologies at the amino acid level with segments of the genomes of influenza A, B, and C viruses (24); although some of the proteins are similar in size and function, they do not necessarily have any nucleotide sequence similarity (16).

Reverse transcriptase PCR performed with fish tissue samples provided evidence, in addition to Northern and Southern hybridizations, that clone 14 is ISAV specific. Notably, all the samples from ISAV-negative fish were PCR negative. These samples were considered to be negative because (i) no disease had developed when they had been used in transmission experiments or (ii) there was no history of ISAV disease on the farms from which the fish originated. Together with the positive results for 50 to 75% of the positive samples, where RNA degradation was assumed to have occurred in some samples, the reverse transcriptase PCR showed good specificity. The results are a strong indication that the described virus is the etiological cause of ISA.

The influenza viruses of the Orthomyxoviridae family depend on higher vertebrates as hosts. In contrast to types B and C, type A is a respiratory pathogen for a wide mammalian host range, including humans, pigs, horses, and sea mammals (32), and an enteric virus of many avian species worldwide (26). Transmission is by aerosol and droplets among mammals and by water among water fowl (21). In contrast, Thogoto and Dhori viruses replicate in both vertebrate and tick cells and are transmitted by tick bite (4, 24). It has been speculated that water fowl occupy a unique and important position in the epidemiology of influenza viruses, and influenza A viruses have been isolated from lake water in which ducks gathered (12–14). The factors that affect virus survival in water are complex. Dilution effects and the presence of chemical and biological antiviral agents affect virus survival unfavorably (29), though aggregation and adsorption of virus to particulate materials can be beneficial to survival. A study of the survival of influenza viruses in pond water showed that viruses could remain infectious for 1 year; there were no evidence that natural variations in water temperature or pH had any effect on the occurrence of viruses (18). Therefore, it can be speculated that orthomyxoviruses have a relatively good survival rate in water, thus making transmission between aquatic host organisms possible.

To our knowledge, this is the first thorough description of an orthomyxo-like virus isolated from a teleost. Previously, an agent isolated from eel (11, 22, 23) was described as having some orthomyxo-like properties. However, no genetic data have been presented; therefore, no taxonomic conclusions have been drawn. Many properties of ISAV have been revealed. The virus is enveloped, as concluded first by its ether and chloroform instability (30) and later by electron microscopy of the virus budding from endothelial cells (15). Furthermore, ISAV is slightly pleomorphic, with a diameter of 100 to 130 nm containing granules of 10 to 12 nm (15), with 10-nm surface projections (6). Moreover, detection of ISAV antigen in the nuclei of infected cells (10) suggests that the nucleus is involved in virus replication. Evidence that ISAV induces virus-cell fusion at an acidic pH (7) indicates that infection of SHK-1 cells by ISAV depends on a low-pH step, i.e., that ISAV utilizes the endocytic pathway to infect cells. Furthermore, it has been shown that ISAV hemagglutinates piscine, but not mammalian or avian, erythrocytes (9). However, spontaneous elution and the lack of reagglutination of hemagglutinated piscine erythrocytes suggest the presence of receptor-destroying enzyme activity. ISAV possesses acetylcholinesterase activity, but no neuraminidase activity has been detected, suggesting the
properties typical for members of the Orthomyxoviridae family.

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