EPIZOOTIOLOGICAL INVESTIGATION INTO A CASE OF SUSPICION OF INFECTIOUS SALMON ANAEMIA (ISA) IN SCOTLAND IN NOVEMBER 2004

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EPIZOOTIOLOGICAL INVESTIGATION INTO A CASE OF SUSPICION OF INFECTIOUS SALMON ANAEMIA (ISA) IN SCOTLAND IN NOVEMBER 2004

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EXECUTIVE SUMMARY

In November 2004, abnormal signs of abnormal behaviour were observed in Atlantic salmon in a marine fish farm site in Loch Sheilavaig in South Uist in the Western Isles of Scotland. The farm had been experiencing elevated mortality. Post mortem investigations – predominantly gill damage - were not typical of ISA and virus isolation tests (SHK-1 and TO cells) were negative for ISAV. However, there was evidence of the presence of ISAV using the indirect fluorescent antibody test (IFAT) and reverse transcriptase polymerase chain reaction (RT-PCR). This evidence met the criteria for suspicion of ISA as defined in part I.2.1 (c) of the Annex to Commission Decision 2003/466/EC ie reasonable evidence of the presence of ISAV from two independent laboratory tests such as RT-PCR and IFAT. Two further sites comprising the same company were also placed under official suspicion due to their having substantial epidemiological links to the first site.

Action was taken as set out in Article 5 of Directive 93/53/EEC and in accordance with the Contingency Plan for Great Britain. Movements of fish, ova and gametes into and out of the three sites were prohibited, disinfection was required at access points and controls were placed on the movement of persons, vehicles and equipment. To afford maximum guarantees for the prevention of spread of infection, a Temporary Control Zone was established in accordance with Commission Decision 2003/466/EC based on the tidal excursion model. A Surveillance Zone of overlapping tidal excursion zones was also established. No significant evidence of ISA was found during inspections carried out over a period of six months. In accordance with Commission Decision 2003/466/EC suspicion of ISA was ruled out in May 2005, controls were lifted and the Temporary Control and Surveillance Zones were revoked.

An epizootic investigation was carried out to establish, amongst other things, potential sources of infection and whether infection may have spread to other farms. No sources of infection were identified and no evidence was found of spread of infection. Molecular analysis of part of the ISAV haemagglutinin gene amplified from gill tissue revealed an identical sequence to that found previously in a wild salmon on the east coast of Scotland and a significant difference compared with sequences from ISAV isolated in the 1998 ISA outbreak in Scotland. The implications of these findings are discussed.
1. INTRODUCTION

1.1 Infectious Salmon Anaemia

Infectious salmon anaemia (ISA) is a significant viral disease of Atlantic salmon which first emerged in Norway in 1984 (Thorud and Djupvik, 1988). Between 1984 and 2004 there have been 441 outbreaks of ISA in Norway (Lyngstad et al., 2005). Only one epizootic of ISA has been recorded in Scotland and this took place in 1998-99. During that period 36 farms were suspected of being infected and of these 11 farms were confirmed to have the disease (Stagg et al., 2001). ISA has also been reported in Canada (Mullins et al., 1998), Chile (Kibbenge et al., 2001), USA (Bouchard et al., 2001), the Faroe Islands and Ireland (OIE, 2004).

1.2 The Legislative Framework


The criteria for suspicion and confirmation of ISA, and for zoning and official surveillance following suspicion or confirmation of ISA, are laid down in Part I of the Annex to Commission Decision 2003/466/EC.

The control measures to be taken in the event of suspicion of ISA are laid down in Article 5 of Council Directive 93/53/EEC and in the Diseases of Fish (Control) Regulations 1994.

2. SUSPICION OF ISA IN SCOTLAND

2.1 Chronology and Diagnostic Findings

During a routine inspection of a fish farm site - 2004/01(a) - in Loch Sheilavaig, South Uist, on 2 November 2004, fish displaying abnormal signs of abnormal behaviour and elevated mortality levels were observed. A second inspection took place on 10 November. Samples were taken for laboratory examination during both inspections. Approximately 40,000 fish, of average weight 1.3 kg, were stocked in three cages, one of which was particularly affected. Mortalities in this cage were 0.43% for the week ending 28 November. Overall mortality on the site was 0.27% for the same period.

2.1.1 Clinical signs and post mortem findings

Clinical signs included abnormal behaviour; the fish were lethargic, not shoaling and positioned near the water surface. Many of the fish had pale gills. One fish showed haemorrhaging in the pyloric caeca.

2.1.2 Histopathology

Moderate to severe proliferative and degenerative lesions involving the secondary gill lamellae were recorded in all fish examined. In addition, moderate to extensive hyperplasia and fusion were apparent with lifting and patchy necrosis of the epithelium, some haemorrhage and variable-sized aneurysms (Fig. 1). *Trichodina* and a small number of cell-bound epitheliocystis-like organisms were observed.
Several foci of degeneration with pyknotic and necrotic hepatic cells were observed in the liver in one fish. Hepatic cell vacuolation and some generalised degeneration of the tissue were present in all fish. Areas of mild degeneration of the renal haematopoietic tissue and a mild to moderate congestion of the interstitium present in all fish. A mild to moderate congestion of the splenic ellipsoids was seen in all fish with loss of pulp and degeneration with evidence of haemosiderin deposition.

A mild heart inflammation-epicarditis and spongy muscle inflammation were noted with few to several areas of monocytic infiltration in the spongy myocardium of the atrium and/or ventricle. Some congestion of the compact myocardium of the ventricle was seen. One or two hypertrophic nuclei were noted in the spongy myocardium. Congestion of the pseudobranch was noted. A congestion of the lamina propria in the intestine and caeca was observed in all fish and in some fish an exudate of sloughed pyknotic cells was noted in the intestine and caeca. Mild congestion of the intestinal lamina propria was recorded.

In summary, the gill pathology was variable, but overall the hyperplastic reaction and loss of respiratory area would have compromised the fish leading to hypoxia. Proliferative gill inflammation was considered as a possible cause of the losses. There was no histological evidence of ISA.

2.1.3 Bacteriology

No bacteria were observed by light microscopy nor isolated from kidney samples using standard media.

2.1.4 Virus isolation, IFAT and RT-PCR tests

On 2 November 2004 samples were collected from six fish for laboratory tests. IFAT tests from two out of six fish sampled were positive for ISA, and a follow up inspection took place on 10 November 2005 when 150 fish were sampled. RT-PCR tests of gill and kidney tissue were positive for ISAV. The results of ISAV isolation tests on samples of gill and kidney tissue were all negative (Table 1). IPNV was isolated from one pooled sample. Tests for Atlantic salmon gill paramyxovirus were negative.

2.1.5 Molecular analysis

Kidney and gill tissues were analysed by reverse transcription - polymerase chain reaction (RT-PCR) assays to determine whether ISAV RNA was present in the sample. The genome of ISAV is composed of eight segments of RNA (Cunningham and Snow 2003). PCR amplification of part of segment 8 is commonly used as a diagnostic test for the presence of ISAV (Løvdal and Enger, 2002; Miaalund et al. 2002; Munir and Kibenge, 2004; Starkey et al. 2004). In addition, real-time PCR was used in assays to amplify parts of segments 7 and 8. Real-time PCR is more sensitive than conventional RT-PCR and offers additional specificity through the use of a probe, which confirms the identity of the PCR product.

The results of RT-PCR tests are shown in Table 2. Analysis of the cycle threshold values indicated that some samples contained very low amounts of starting material and hence, low amounts of ISAV RNA. Differences in the number of positive results from the 10 November sample reflect the low amount of viral RNA in the sample and the fact that different segments of the viral
genome are expressed at different rates. Positive results from amplification of more than one segment of ISAV provide additional evidence that the results are true positives, and sequence data from another segment adds further confirmation.

In addition to the RT-PCR assays, part of segment 6 was amplified from gill tissue and sequenced. This part of segment 6 encodes a highly polymorphic region (HPR) of the haemagglutinin of ISAV (Devoid et al., 2001; Krossøy et al., 2001; Rimstad et al., 2001; Mjaaland et al., 2002). The 300 base pair (bp) sequence obtained from 2004/01(a) was found to be identical to that of the longest HPR sequence reported to date; from the gills of a returning adult salmon caught at a netting station on the east coast of Scotland (Cunningham et al., 2002). The amino acid sequence predicted from this is shown below and the sequence is available in the EMBL nucleotide sequence database under accession number AJ440971. This sequence is not the same as that obtained from ISAV during the outbreak in Scotland in 1998-1999, being longer by 69 nucleotides (Fig. 2).

### 2.1.6 Suspicion of ISA at Loch Sheilavaig

The criteria for suspicion of ISA are laid down in the Annex to Commission Decision 2003/466/EC establishing criteria for zoning and official surveillance following suspicion or confirmation of the presence of infectious salmon anaemia (ISA). The clinical signs of disease and post mortem investigations (primarily gill pathology) at 2004/01(a) were not consistent with those described for ISA in the current edition of the *OIE Manual of Diagnostic Tests for Aquatic Animals* (OIE, 2003). However, laboratory test results on samples from 2004/01(a) Loch Sheilavaig met criterion 1.2.1(c) - “reasonable evidence of the presence of ISAV from two independent laboratory tests such as RT-PCR (Part IV) and IFAT (Part V)”.

Two further sites belonging to the Loch Sheilavaig farm – 2004/01(b) and 2004/01(c) – met criterion 1.2.1. (e) – “where an investigation reveals other substantial epidemiological links to ISA-suspected or confirmed farms” (see part 2.3.2 of this report).

As a result of these findings, suspicion of the presence of ISA was declared on all three sites – 2004/01(a), 2004/01(b) and 2004/01(c) - comprising the Loch Sheilavaig fish farm on South Uist, on 19 November 2004.

### 2.1.7 Further inspections at Loch Sheilavaig

Site 2004/01(a) was inspected again on 23 November 2004. No clinical signs of ISA were observed and no diagnostic samples were taken. Mortalities in one cage were 2,615 since the previous inspection but a bath treatment for sea lice infestation is believed to have exacerbated mortality levels. The fish on 2004/01(a) were subsequently culled and the site was fallow on 5 December 2004.

Site 2004/01(b) was inspected on 23 November and 14 December 2004. There were no clinical signs of disease and mortality levels were low. Gill tissue from 2004/01(b) tested positive for ISAV by the conventional RT-PCR test in three out of four pools sampled on 23 November 2004. 2004/01(b) was destroyed in hurricane force winds during the second week of January 2005.

Site 2004/01(c) was inspected on 25 November. There were no clinical signs of disease and mortality levels were very low. Gill and kidney tissue from 2 of 4 pools sampled on this date

2.2 Actions Taken to Prevent the Spread of Infection

2.2.1 Restrictions on movements

Site 2004/01(a) was already subject to a Designated Area Order in respect of infectious pancreatic necrosis (IPN) at the time of the initial investigation to determine the cause of abnormal behaviour and mortalities of fish in November 2004. Thus, movements of live fish on and off the site were subject to approval by the Scottish Ministers.

Following positive IFAT and RT-PCR test results from samples of fish from 2004/01(a), in accordance with the criteria in Part I.2.1 (c) of the annex to Commission Decision 2003/466/EC, suspicion of the presence of ISA was declared on 19 November 2004. The suspicion was extended to the two other sites (2004/01(b) and 2004/01(c)) due to substantial epidemiological links with 2004/01(a), in accordance with Part I.2.1 (e) of Commission Decision 2003/466/EC.

The control measures to be taken in the event of suspicion of ISA, which are laid down in Article 5 of Council Directive 93/53/EEC, were implemented by the Official Service. Notices, made under Schedule 4 of The Diseases of Fish (Control) Regulations 1994, were served on the owners of the three sites comprising the suspect farm, prohibiting the movement of dead fish, personnel, vehicles, equipment, materials or substances liable to transmit infection on or off the farm, without the permission of the Scottish Ministers.

Designated Area Orders were made and Control Notices, made under Schedule 4 of The Diseases of Fish (Control) Regulations 1994, were served on the operators of all sites within the temporary Control and Surveillance Zones (see 2.2.2 below), prohibiting the movement of live and dead fish, respectively, without the permission of the Scottish Ministers.

2.2.2 Risk-based surveillance programme

For the purpose of establishing a risk-based official surveillance programme, a Temporary Control and Surveillance Zones were established in the vicinity of the farm suspected of infection with ISA (Appendix I). The criteria upon which the zones were based were as described in Part I.4.4.1 of Commission Decision 2003/466/EC. The Temporary Control Zone extended to one tidal excursion (a circle of radius equal to 7,012 m, based on an average current speed in the area of 1 knot, centred on the suspect farm). The Temporary Surveillance Zone comprised the area covered by overlapping tidal excursions of fish farm sites adjacent to the suspect farm.

There were five other farms within the Temporary Control Zone, of which four were stocked. These farms were subject to monthly inspections by the Official Service in accordance with Commission Decision 2003/466/EC. Samples were taken for laboratory examination if clinical signs of disease were observed. In the Temporary Surveillance Zone there were three farms, of which only two were stocked. These farms were subject to regular clinical inspections every two months. The Official Service also inspected four freshwater farms and six marine farms that were identified in the epizootic investigation, required under Council Directive 93/53/EEC, as
having an epidemiological link with the farm suspected of infection with ISA. No evidence to suspect the presence of ISA was observed on any of these farms.

2.2.3 Disposal of culled fish and mortalities

The company that owned the Loch Sheilavaig farm voluntarily culled all stock on the site that was suffering abnormal mortalities under the supervision of the Official Service and 2004/01(a) was fallowed within 16 days of the declaration of suspicion of ISA. The culled fish were ensiled in formic acid to a pH of less than 4.0 and disposed of by burial, as permitted under Regulation (EC) No 1774/2002 and Article 29 of the Animal By-Products (Scotland) Regulations 2003 for animal waste originating in remote areas.

All fish mortalities from farms within the temporary Control and Surveillance Zones were ensiled and disposed of by burial. 2004/01(b) was completely destroyed in a hurricane during the week commencing 10 January 2005 when the cage moorings broke and the cages were washed onto a reef. The fish were contained in the nets but one net was torn while recovering the dead fish and some fish were deposited on the seabed. Divers were used to recover these fish. The fish in the other two nets were contained in tarpaulins. The recovered fish were ensiled before disposal by burial.

2.2.4 Processing of fish harvested from within the temporary Control and Surveillance Zones

Fish not showing clinical signs of disease were permitted to be harvested for human consumption from within the temporary Control and Surveillance Zones. All fish harvested for human consumption were eviscerated at processing plants having facilities for disinfection of the blood water and all solid waste was ensiled before disposal by burial. Fish for harvest from farms belonging to one company were transported live by well boat operating with closed valves to a killing station at Mallaig, on the mainland of Scotland, and then transported by road tanker to a processing plant having facilities for disinfection of the blood water and ensiling of viscera at Fort William. The other operator who harvested fish from the Temporary Control Zone used a processing plant located in North Uist.

2.3 Epidemiological Investigation

An epidemiological investigation was launched as soon as the results of laboratory diagnostic tests provided evidence for suspicion of ISA at 2004/01(a) in Loch Sheilavaig.

The objectives of the investigation were:

1. To establish if there were substantial epidemiological links between 2004/01(a) and any other fish farm sites.
2. To identify if any other farms may be at risk of infection.
3. To identify potential origins of suspect infection.

2.3.1 Evidence linking 2004/01(a) with other fish farm sites

Two sites in the vicinity of 2004/01(a) - 2004/01(b) and 2004/01(c) - were found to have substantial epidemiological links to 2004/01(a). The evidence consisted of:
- Close proximity to 2004/01(a) with high risk of water transfer between the three sites posing a risk of transmission of ISAV; and
- Sharing of equipment, personnel and work boats between the three sites which were operated as a single farm by the same company.

Consequently, suspicion of the presence of ISA was declared on all three sites in Loch Sheilavaig – 2004/01(a), 2004/01(b) and 2004/01(c).

No substantial epidemiological links were found with any other fish farm sites.

2.3.2 Live fish movements to and from sites in the temporary control zone

Live fish movements represent the highest risk of spreading infection between fish farm sites. There were no live fish movements from the sites in Loch Sheilavaig or other sites in the Temporary Control Zone to any other fish farm sites prior to or subsequent to the establishment of the Temporary Control Zone. Thus there was minimal risk of spread of infection via this mechanism.

2.3.3 Live fish movements on to suspect sites

The three sites under suspicion had been fallowed synchronously for five months from 26 October 2003 to 25 March 2004 prior to stocking. It is therefore unlikely that sources of infection were residing within these sites prior to being stocked in 2004.

Smolts were sourced from four freshwater smolt production sites in the stocking of the three suspect sites. None of these sites has ever used seawater at any stage of smolt production. The smolts used to stock the three suspect sites were previously inspected by the Official Service while at the freshwater sites in February 2004. No clinical signs of ISA or other diseases were observed at the time of inspection before these fish were transferred to seawater. The four smolt sites were inspected again by the Official Service in December 2004/January 2005, following suspicion of ISA at Loch Sheilavaig. The stock inspected this time were a different year class to the stock which left these smolt sites earlier in 2004. Again, there was no evidence of ISA or other diseases during these inspections.

In light of the foregoing, it appears unlikely that the origin of any ISA infection would have been from the supplying smolt sites. Furthermore, the available scientific evidence indicates that ISA does not occur in freshwater in the absence of infected seawater and that vertically transmission does not occur (Anon 2000, Stagg et al., 2001).

2.3.4 Potential links with other seawater sites through smolt transfer

Live fish movement records showed that the four smolt production sites that provided all the fish stocked on the three suspect sites also provided smolts from the same year class that were moved to a further nine seawater sites. Eight of these sites were inspected between December 2004 and January 2005. There was no evidence of ISA on any of these sites. The ninth site, a land-based research facility, had previously destroyed all of the fish of interest following completion of a trial.
2.3.5 Wellboats

Checks were made for compliance with the ISA Code of Practice for the well boat that transported smolts to the suspect sites. Records from six journeys delivering fish to the suspect and other sites showed complete compliance with the Code of Practice including Level 2 disinfection after every delivery and containment and recirculation of water within five km of any cage or farm. Following establishment of the Temporary Control and Surveillance Zones all well boat movements were subject to official control and compliance with disinfection protocols. Therefore, there was no evidence to implicate well boats in the introduction of infection.

2.3.6 Processing

A processing plant was located within the western periphery of the Temporary Surveillance Zone. Only Scottish produced salmon were used in this plant. Sources of salmon included sites within and outwith the Temporary Surveillance Zone (sites in Uist and Harris). Prior to the establishment of the Temporary Control and Surveillance Zones there was no disinfection of effluents. Following the establishment of the temporary Control and Surveillance Zones, waste water was disinfected using sodium hypochlorite and viscera were ensiled before disposal by burial. A secondary processing plant within the Surveillance Zone was supplied from processors taking fish from within and outside the Surveillance Zone at a time before suspicion was declared and from outside the zone following suspicion. Only Scottish produced salmon were used in this plant. The small amounts of solid waste from this plant were disposed of by burial. Effluent water from this plant was not disinfected. In summary, there was no evidence to implicate processing plants in the introduction of infection.

2.3.7 Importation from outside the UK

2.3.7.1 Live fish and eggs

ISA affects salmon farms in the Faroe Islands and Norway. However, there have been no live imports of salmonids from Norway or the Faroe Islands according to the records of the Official Service and there was no evidence of illegal imports. Live salmonids have been imported from Ireland but this is not considered a significant risk for ISA as the country is not currently affected by ISA.

Salmon eggs have been imported into Scotland from ISA-free zones of Norway and the USA, with double disinfection to reduce risks of disease transmission. The scientific consensus is that ISA is not vertically transmitted. The company owning the ISA suspect sites has not imported eggs from Norway. It is considered unlikely that infection was imported with live fish or egg imports.

2.3.7.2 Salmonid carcasses

Importation of salmonid carcasses for processing poses a potential risk of transfer of infection. This risk can be minimized by evisceration. Commission Decision 2003/858/EC therefore requires that processing of farmed fish from ISA-affected areas takes place at approved import centres unless the carcasses are eviscerated prior to importation into Member States. Nevertheless, muscle fillets and heads still pose some risk of infection (Thorud and Torgersen,
1994) and the possibility that infection could be introduced into the UK via importation of eviscerated carcasses cannot be ruled out.

2.3.8 Wild fish

Previous investigations have provided evidence of ISAV infection in wild salmonid fish including Atlantic salmon and sea trout (Raynard et al., 2001; Stagg et al., 2001). These surveys revealed a prevalence of infection of between 0.5 and 0.05% with a patchy distribution. However, it was not possible to conclude if wild salmonids had a role in transmission of ISA to farms. For this reason, and in view of the current state of stocks of wild salmonids on the west coast of Scotland, it was not considered appropriate to carry out a wild fish survey at this point in time in relation to a suspicion of ISA at Loch Sheilavaig.

2.4 Ruling Out Suspicion and Lifting of Controls

In accordance with Part I.2.2 of the Annex to Commission Decision 2003/466/EC, suspicion of ISA was ruled out when continued investigations involving at least one clinical inspection per month for a period of six months revealed no further significant evidence of the presence of ISA. No evidence of ISA was found during investigations involving at least one clinical inspection per month of farms in the Temporary Control zone and one clinical inspection every two months in the Surveillance zone. Controls on all farms in the Temporary Control and Surveillance Zones were lifted and the zones were revoked on 19 May 2005.

3. DISCUSSION

3.1 Grounds for Suspicion of ISA at Loch Sheilavaig

Clinical signs of disease were observed by fish health inspectors during a routine inspection of a fish farm site – 2004/01(a) - at Loch Sheilavaig in South Uist, Scotland, on 2 November 2004. Laboratory analysis of samples taken on that date and during a subsequent inspection on 10 November 2004 (Table 1) provided grounds for suspicion of ISA according to part I.2.1 (c) of the Annex to Commission Decision 2003/466/EC: “reasonable evidence of the presence of ISAV from two independent laboratory tests such as RT-PCR (Part IV) and IFAT (Part V)”.

Two further sites in Loch Sheilavaig - 2004/01(b) and 2004/01(c) - were found to have substantial epidemiological links to 2004/01(a). Consequently, suspicion of the presence of ISA was declared on all three sites in Loch Sheilavaig – 2004/01(a), 2004/01(b) and 2004/01(c).

3.2 Were There Grounds to Confirm the Presence of ISA at Loch Sheilavaig?

Virus isolation tests on samples of kidney and gills from fish on site 2004/01(a) were negative for ISA virus using two different cell lines recommended for ISAV. Furthermore, the clinical signs of disease and pathological changes observed by light microscopy (mainly gill pathology) were not consistent with those described for ISA in the OIE Manual of Diagnostic Tests for Aquatic Animals. No clinical signs of disease were observed during inspections of sites 2004/01(b) or 2004/01(c) and there were no abnormal mortalities on these sites apart from the
catastrophic losses when site 2004/01(b) was destroyed in hurricane force winds. Consequently, the criteria for confirmation of ISA according to Commission Decision 2003/466/EC were not met on any of the sites in Loch Sheilavaig.

3.3. Control Measures

The control measures to be taken in the event of suspicion of ISA, which are laid down in Article 5 of Council Directive 93/53/EEC, were implemented in full by the Official Service. These included prohibition on the movement of dead fish, personnel, vehicles, equipment, materials or substances liable to transmit infection on or off the farm which was under suspicion unless authorized by the Scottish Ministers.

To afford maximum guarantees for the prevention of spread of infection, a Temporary Control Zone was established in accordance with Commission Decision 2003/466/EC based on the tidal excursion model. A Surveillance Zone of overlapping tidal excursion zones was also established. There was a prohibition on the movement of live and dead fish onto and off all sites within the Temporary Control and Surveillance Zone, respectively, unless authorized by the Scottish Ministers.

The absence of any further evidence of infection within or outwith the Temporary Control Zone and Surveillance Zone supports the view that the control measures that were applied were adequate to prevent the spread of any infection from the sites that were under suspicion.

3.4 Ruling Out Suspicion of ISA

No significant evidence of ISA was found during regular (monthly) inspections carried out over a period of six months on fish farm sites in the Temporary Control Zone and two-monthly inspections in the Surveillance Zone. In accordance with Commission Decision 2003/466/EC suspicion of ISA was therefore ruled out in May 2005, movement controls were lifted and the Temporary Control Zone and Surveillance Zone were revoked.

3.5 Potential Sources of Infection

The epizootiological investigation established that the three sites in Loch Sheilavaig that were placed under suspicion had been fallowed for five months prior to stocking with smolts. Thus it is unlikely that infection had been residing in those sites prior to this time. These sites had received smolts from four freshwater sources in 2004. Inspection of the freshwater smolt production units revealed no evidence of ISA. Marine farm sites that had received smolts from the same freshwater smolt production units were also inspected. No evidence of ISA was found on those sites. Therefore, there was no evidence to suggest that the freshwater sites that supplied smolts to Loch Sheilavaig had been infected.

During the 1998-99 outbreak of ISA in Scotland, well boats were implicated in the spread of infection and it was established that disinfection protocols were not always adequate (Stagg et al., 2001). Inspection of records of the well boat used to transport smolts to Loch Sheilavaig in 2004 indicated that disinfection guidelines in the ISA Code of Practice had been followed. Consequently, there was no evidence to implicate this well boat as a source or vector of infection.
Live fish movements generally pose the greatest risk of spread of infection. Movement records indicated that no live fish had been moved onto the Loch Sheilavaig farm from other marine farms or from Loch Sheilavaig to other sites prior to or subsequent to establishing the Temporary Control Zone. It is unlikely therefore that infection was transferred into Loch Sheilavaig from other marine farms or from Loch Sheilavaig to any other farms via this route.

Another potential source of infection is via import of live fish or eggs, or salmonid carcasses from infected areas. Live salmonids have been imported from Ireland. However, Ireland is believed to be free of ISA and importation of infection via this route is considered unlikely. There have been no known imports of live salmonid fish from ISA-affected countries. Atlantic salmon ova have been imported from Norway and USA, countries where ISA is present. However, all imported ova are disinfected and there is no evidence that ISAV is vertically transmitted. Therefore, it would seem unlikely that infection was imported via this route. Importation of salmonid carcasses for processing poses a potential risk but considered unlikely due to the remote location of the infected site. In the case of imports from ISA-affected countries this risk is reduced by the requirement for processing at approved importation centres or evisceration prior to importation. Nevertheless, muscle fillets and heads still pose some risk of infection (Thorud and Torgersen, 1994) and there remains the possibility that infection could be introduced into the UK via importation of eviscerated carcasses.

Previous surveys have provided evidence of ISAV infection in wild salmonid fish (Raynard et al., 2001; Stagg et al., 2001). These surveys revealed a prevalence of infection of between 0.5 and 0.05% with a patchy distribution. However, it was not possible to conclude if wild salmonids had a role in transmission of ISA to farms. For this reason, and in view of the current state of stocks of wild salmonids on the west coast of Scotland, it was not considered appropriate at the time of this investigation to carry out a wild fish survey in relation to the suspicion of ISA at Loch Sheilavaig. Nevertheless, the possibility that wild fish may be a reservoir of infection cannot be excluded, especially as the nucleotide sequence from segment 6 was identified to a wild type isolate.

### 3.6 Molecular Analysis

Evidence of ISAV RNA was detected using both conventional and real-time RT-PCR. Higher numbers of positive test results were obtained using real-time RT-PCR confirming it to be more sensitive than conventional RT-PCR. It is interesting to note that gill samples gave significantly higher numbers of positive test results for ISAV than kidney samples, both by conventional and real-time RT-PCR (Table 2). In contrast, experimental infection studies using a virulent strain of ISAV isolated during the Scottish outbreak in 1998 showed no significant difference between RT-PCR test results from kidney and gill (Snow et al., 2003).

The genome of ISAV comprises 8 RNA segments (Mjaaland et al., 1997). Part of segment 6 was amplified from gill tissue of fish from site 2004/01(a) and sequenced. This part of segment 6 encodes a highly polymorphic region (HPR) of the haemagglutinin of ISAV (Devold et al., 2001; Krossøy et al., 2001; Rimstad et al., 2001; Mjaaland et al., 2002). The 300 base pair (bp) sequence obtained from 2004/01(a) was found to be identical to that of the longest HPR sequence reported to date, from the gills of an apparently healthy returning adult salmon caught at a netting station on the east coast of Scotland (Cunningham et al., 2002). This sequence, termed HPR0, is not the same as that obtained from ISAV during the outbreak in Scotland in 1998-1999, being longer by 69 nucleotides.
The HPR0 sequence has also been found in Canada (Cook-Versloot et al., 2004) and in Norway (A. Nylund, pers. comm.). It has been hypothesized that virus containing this long HPR sequence may represent an avirulent, ancestral form of ISAV from which virulent strains are derived by deletion of amino acid segments (Cunningham et al., 2002; Mjaaland et al., 2002; Nylund et al., 2003; Cook-Versloot et al., 2004). However, HPR0 forms of ISAV have proved difficult to isolate in culture and it has yet to be established if they are indeed avirulent.

Given the significantly higher number of positive RT-PCR results from gill compared with kidney tissue in the Loch Sheilavaig investigation, the possibility that HPR0 forms of ISAV have a higher tropism towards gill tissue than shorter HPR forms merits further investigation.

### 3.7 What Was the Cause of Mortalities at Site 2004/01(a)?

Given that the criteria for confirmation of ISA were not met, what was the cause of mortalities at Loch Sheilavaig? IPNV was isolated from one sample, but there was no evidence of IPN pathology in any of the fish examined. The lack of systematic internal pathology and the severity of the gill pathology - hyperplasia, inflammation and loss of respiratory area - suggest that hypoxia would have severely compromised the fish and was probably a major contributory factor to morbidity and mortality.

Although *Trichodina* and a small number of cell-bound epitheliocystis-like organisms were observed in the gills, their numbers were not considered sufficient to be the cause of morbidity or mortality, and there was no evidence of bacterial infection. In Norway, a paramyxovirus has been isolated from the gills of Atlantic salmon post smolts that were suffering from gill disease (Kvellestad et al., 2003). However, tests for Atlantic salmon gill paramyxovirus in samples from fish in Loch Sheilavaig 2004/01(a) were negative. Evidence of ISAV RNA was detected in the gills of fish in Loch Sheilavaig. However, this does not imply that ISAV was the cause of the observed gill pathology. Indeed, gill pathology is not regarded as a characteristic feature of ISA in the most recent edition of the OIE Manual (OIE, 2003).

Thus, hypoxia was probably a major factor in morbidity and mortality at Loch Sheilavaig but the cause of the underlying gill pathology remains uncertain.

### 4. CONCLUSIONS

On the basis of laboratory tests (IFAT and RT-PCR) and diagnostic criteria laid down in Commission Decision 2003/466/EC, a fish farm site in Loch Sheilavaig was placed under official suspicion of the presence of ISA in November 2004. Although there were abnormal mortalities, the clinical signs and post mortem findings (mainly gill pathology) were not consistent with ISA. Two other sites belonging to the same company but not suffering abnormal mortalities were also placed under official suspicion due to their having substantial epidemiological links with the first site.

The control measures laid down in Article 5 of Council Directive 93/53/EEC were implemented in full by the Official Service. A Temporary Control Zone was established in accordance with Commission Decision 2003/466/EC based on the tidal excursion model, and a Temporary Surveillance Zone of overlapping tidal excursion zones was also established.
No evidence of ISA was found in other freshwater or marine fish farm sites that had direct or indirect contact with the Loch Sheilavaig farm, and no source of infection was identified.

Suspicion of ISA was ruled out when continued investigations involving at least one clinical inspection per month of all farms in the Temporary Control Zone, and bi-monthly clinical inspections of all farms in the Temporary Surveillance Zone, for a period of six months revealed no further significant evidence of the presence of ISA.

A gene sequence corresponding to the long HPR form (HPR0) of ISAV was identified in gill samples from fish on the Loch Sheilavaig farm. In Scotland, the HPR0 sequence has only previously been found in gill samples from a wild Atlantic salmon. Further studies are required to establish the significance of HPR0 forms of ISAV, their prevalence in wild and farmed fish, and whether such strains are benign or pathogenic.
5. REFERENCES


6. ACKNOWLEDGEMENTS

We would like to thank Fisheries Research Services staff for their excellent work and the cooperation from fish farmers in assisting with this investigation.
Table 1 Evidence for suspicion of the presence of ISA at 2004/01(a), Loch Sheilavaig

<table>
<thead>
<tr>
<th>Observations and laboratory test results</th>
<th>Date of inspection</th>
<th>Date of inspection</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macroscopic findings</strong></td>
<td>2 November 2004</td>
<td>10 November 2004</td>
</tr>
<tr>
<td>Fish in one cage behaving abnormally, congregating at surface. Variable sea lice load (3-11 per fish). Liver colour normal to slightly pale in all fish. One fish with enlarged spleen. One fish with petechial haemorrhage on pyloric caeca. No ascites in any of the fish examined.</td>
<td>Fish in one cage behaving abnormally, congregating at surface. Fish from this cage had pale gills though haematocrit was not measured. Majority of fish had high sea lice load. One fish, which had been dead for a considerable period of time, had a dark liver. One live fish was found to have a slightly dark liver and enlarged spleen at post mortem but no other significant pathology was observed. No ascites in any of the fish examined.</td>
<td></td>
</tr>
<tr>
<td><strong>Histology</strong></td>
<td>All fish were examined histologically. Moderate gill pathology with <em>Trichodina</em> infection in all fish. Mild liver, kidney, spleen, heart and intestinal pathology in most fish. Not consistent with ISA.</td>
<td>The single fish with slightly dark liver and enlarged spleen was examined histologically. Mild pathology was observed in gill, liver, kidney, spleen, heart and pancreas. Not consistent with ISA.</td>
</tr>
<tr>
<td><strong>IFAT</strong></td>
<td>2 of 6 fish positive</td>
<td></td>
</tr>
<tr>
<td><strong>Conventional RT-PCR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Gill</td>
<td>23 of 30 pools positive</td>
<td></td>
</tr>
<tr>
<td><strong>Real-Time RT-PCR, Gill</strong></td>
<td>28 of 30 pools positive</td>
<td></td>
</tr>
<tr>
<td><strong>Real Time RT-PCR, Kidney</strong></td>
<td>4 of 30 pools positive</td>
<td></td>
</tr>
<tr>
<td><strong>Virus isolation</strong></td>
<td>Kidney samples inoculated onto TO cells.</td>
<td>Kidney and gill samples inoculated onto TO cells and SHK-1 cells.</td>
</tr>
<tr>
<td></td>
<td>ISAV negative</td>
<td>ISAV negative</td>
</tr>
<tr>
<td><strong>Other diagnosis</strong></td>
<td>IPNV isolated from 1 pool.</td>
<td></td>
</tr>
<tr>
<td><strong>Mortalities</strong></td>
<td>Approximately 0.3% per week.</td>
<td>Approximately 0.3% per week.</td>
</tr>
<tr>
<td><strong>Comments</strong></td>
<td>Routine inspection visit. 6 moribund fish sampled.</td>
<td>Follow up visit. 150 fish sampled.</td>
</tr>
</tbody>
</table>
### Table 2: Conventional and Real-Time RT-PCR results – 2004/01(a)

<table>
<thead>
<tr>
<th>Date</th>
<th>Kidney</th>
<th>Gill</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conventional RT-PCR</td>
<td>Real-time PCR</td>
</tr>
<tr>
<td>2 November 2004</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>10 November 2004</td>
<td>0/30</td>
<td>4/30</td>
</tr>
</tbody>
</table>
Figure 1. Section of gill tissue showing hyperplasia and fusion of secondary lamellae and increased mucus cells. H&E stain
Figure 2. Predicted amino acid sequence of HPR from ISAV from Loch Sheilavaig and Loch Nevis samples

2004/01(a)  RNITDV KIRVDAI PPQL NQT FNT NQVEQ PA NSVL SNI FIS MGVAG
Loch Nevis 1998  RNITDV K------ ---- --- --- ----- -- TSVL SNI FIS MGVAG
Appendix I: Map showing temporary Control Zone and Surveillance Zone

South Uist Temporary Control Zone and Surveillance Zone
November 2004
### Appendix II: Schedule of accreditation of FRS inspection and testing methods for ISA

<table>
<thead>
<tr>
<th>Method</th>
<th>Accreditation Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inspection and sampling</td>
<td>ISO 17020</td>
</tr>
<tr>
<td>Histology</td>
<td>NEQUAS</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>ISO 17025</td>
</tr>
<tr>
<td>Q-PCR</td>
<td>ISO 17025</td>
</tr>
<tr>
<td>Kidney imprint IFAT</td>
<td>ISO 17025</td>
</tr>
<tr>
<td>Virus Isolation</td>
<td>ISO 17025</td>
</tr>
</tbody>
</table>
Appendix III: Report from OIE Reference Laboratory for ISA

FRS Marine Laboratory
Attn. Carey Cunningham
PO Box 101, 375 Victoria Road
Aberdeen
AB11 9DB UK

Your ref. 2005-02-1956 11.03.2005

Received 23.02.2005: 5 samples labelled: 1, 5, 10, 15, 20

The samples were submitted by dr. Carey Cunningham and contained PRC-products obtained after performing RT-PCR for ISAV from case 2004/1121

Purpose: Verification of ISAV by sequencing the PCR-products

The quality of the PCR-products was verified by gel electrophoresis of a few microliters of each sample. The product was sequenced with BigDye Terminator Sequencing kit (Applied Biosystems) on a Megabase 1000 (AME BioScience) using the same primers as was used for RT-PCR at FRS.

Results: The sequences of the PCR-products showed 100% identity with both Norwegian and Scottish ISAV isolates in the investigated area of ISAV segment 8.

Yours sincerely,

Birgit Dannevig
Senior researcher