A low-pathogenic variant of infectious salmon anemia virus (ISAV-HPR0) is highly prevalent and causes a non-clinical transient infection in farmed Atlantic salmon (Salmo salar L.) in the Faroe Islands

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Infectious salmon anemia virus (ISAV) is an orthomyxovirus responsible for a significant disease of farmed Atlantic salmon. Following and re-establishment of the Atlantic salmon farming industry in the Faroes following a recent devastating infectious salmon anaemia (ISA) disease epidemic provided a unique opportunity to study the risk of re-emergence of disease. Over 53 months, 2787 of 34 573 (8.1 %) apparently healthy Atlantic salmon analysed tested positive for ISAV by RT-PCR. Sequence analysis revealed the putative low-pathogenic ISAV-HPR0 subtype in all cases. Results demonstrated that ISAV-HPR0 appeared as a seasonal and transient infection without detectable ISA mortality or pathology. This finding, coupled to an apparent gill tropism of ISAV-HPR0, suggests ISAV-HPR0 causes a subclinical respiratory infection more like seasonal influenza, as opposed to the systemic infection and serious disease caused by highly pathogenic ISAV. The mean time before marine sites became infected was 7.7 months after transfer to seawater of the fish, suggesting a potentially unknown marine reservoir of infection. Sequence analysis identified two main subtypes of ISAV-HPR0 sequences, one of which showed close genetic association with ISAV isolates responsible for the disease outbreak in the Faroes. Thus ISAV-HPR0 might represent an ancestor of pathogenic variants and thus be a potential risk factor in the emergence of new strains of disease-causing ISAV. Our data, however, suggest that the risk of emergence of pathogenic ISAV variants from a reservoir of ISAV-HPR0 is low. This risk is probably being further reduced by practical management strategies adopted in the Faroes and aimed at reducing the potential for maintenance and adaptation of ISAV-HPR0.

INTRODUCTION

Infectious salmon anaemia (ISA) is one of the most important diseases of farmed Atlantic salmon (Salmo salar L.) and predominantly affects fish at marine grow-out sites. Following the first diagnosis in a hatchery in Norway in 1984 (Thorud & Djuvpik, 1988), the disease spread rapidly throughout the country resulting in a peak of more than 80 outbreaks per year in 1990 (Hästein et al., 1999). Disease control measures subsequently implemented by the Norwegian government in collaboration with the industry reduced the spread of ISA significantly, and the prevalence of farms experiencing ISA has since been less than 5 % per year (Johansen et al., 2008). The first ISA outbreak outside Norway was identified in New Brunswick, Canada in 1996 (Mullins et al., 1998), followed by subsequent appearances of the disease in Scotland, UK in 1998 (Rodger et al., 1998), the Faroe Islands in 2000 (Anonymous, 2000), Maine, USA in 2001 (Bouchard et al., 2001) and Chile in 2007 (Godoy et al., 2008). ISA is known to be efficiently spread horizontally, meaning that successful control is reliant on rapid containment and/or eradication of infected populations. Eradication of ISA has only been achieved in Scotland (Stagg, 2003) and the Faroe Islands. In the latter case, eradication was at the cost of an almost complete break in production in the Faroese salmon-farming industry.

ISA is caused by an aquatic orthomyxovirus possessing the major functional characteristics of this virus family including haemagglutinating, receptor destroying and
fusion activities, which are associated with the two viral surface proteins, the haemagglutinin-esterase (HE) protein and the fusion (F) protein (Falk et al., 1997, 2004; Krossøy et al., 2001; Rimstad et al., 2001; Aspehaug et al., 2005). The disease is a systemic and lethal condition, and clinical signs suggest circulatory failure with severe anaemia, ascites, congestion and enlargement of the liver and spleen (Evensen et al., 1991). By autopsy, a variable set of haemorrhages and necrosis in several organs are usually observed.

A risk factor proposed to explain new occurrences of ISA is the maintenance of putative low-pathogenic strains of infectious salmon anemia virus (ISAV) within both wild and farmed fish populations. Such strains, which have now been recognized in Norway (Nylund et al., 2007), Scotland (Cunningham et al., 2002; Anonymous, 2005; McBeath et al., 2009), Canada (Cook-Versloot et al., 2004) and Chile (Kibenge et al., 2009), are differentiated on the basis of the sequence of a highly polymorphic region (HPR) in the HE gene just upstream of the transmembrane coding region (Cunningham et al., 2002; Mjaaland et al., 2002; Nylund et al., 2003). Early comparative studies led to the suggestion that HPR subtypes associated with ISA outbreaks may have arisen within aquaculture following deletions within this gene with respect to a putative full-length ancestral progenitor, designated ISAV-HPR0 (Mjaaland et al., 2002). The subsequent first identification of such a sequence in wild fish in Scotland (Cunningham et al., 2002), in an area remote from aquaculture activity, lent credence to the hypothesis that ISAV-HPR0 may represent a wild-type form of ISAV which has been introduced and subsequently adapted to virulence as a result of selective pressures associated with intensive fish farming. In support of this requirement for adaptive change is the fact that ISAV-HPR0 viruses differ phenotypically from those responsible for disease outbreaks in that they have not been directly associated with ISA disease, have mainly been detected in gills and remain non-culturable on conventional ISAV-permissive cell lines (Nylund et al., 2007).

From analysis of ISAV sequences originating from Norway, it has been suggested that the shift in virulence from ISAV-HPR0 to a disease-causing ISAV is a stochastic event that is dependent on the replication frequency of the virus and the time available for changes in the highly polymorphic region of the HE gene to occur (Nylund et al., 2007). Reducing the capacity for maintenance of ISAV-HPR0 infection within individual farming areas thus offers a potential strategy to minimize the risk of re-emergence of pathogenic variants of ISA and the likelihood of disease emergence.

During the five year period from spring 2000, a total of 33 ISA outbreaks were recorded in the Faroes, with all but two of the 25 licensed salmon-farming areas being affected. Synchronized fallowing of all but one Faroese Atlantic salmon farming site, eradication of ISA and subsequent re-establishment of the industry has provided a unique opportunity to better understand and manage the risks of re-emergence of ISA disease within this area. Practical measures employed following restocking have included tightening of biosecurity procedures within the industry in order to safeguard against the recognized risk of import of ISA from other affected territories, reduction in production intensity, year class separation and scheduled fallowing, vaccination and comprehensive screening programme for ISAV. During a 53 month period, all farms have been regularly monitored for clinical findings and screened for the presence of ISAV using molecular detection and characterization methods. Here we present results from this comprehensive screening, demonstrating that ISAV-HPR0 is widespread, exhibits a different tissue tropism to pathogenic variants of ISAV, shows a transient and seasonal appearance and does not result in clinical ISA.

RESULTS

ISAV-HPR0 is prevalent in Atlantic salmon in the Faroes

Throughout the 53 months (August 2005–December 2009) of the study period a total of 34 573 Atlantic salmon were tested for the presence of ISAV. In total 2787 of the 34 573 (8.1 %) fish tested ISAV positive by RT-PCR (used August 2005–March 2007) or real-time RT-PCR (used April 2007–December 2009) (Table 1). The annual prevalence ranged from 0 % ISAV positive in 2005 to 15.1 % ISAV positive in 2007. The markedly lower detection of prevalence in kidneys obtained prior to 2007 (0.9 % for 2005 and 2006) compared with those obtained in gills post 2007 (10.9 % for 2008 and 2009) reflect changes in the sampling and screening and marked increase in screening the gills.

Table 1. ISAV-HPR0 prevalence in all seawater farms with production of Atlantic salmon

Total number (n) of Atlantic salmon kidneys and gills screened for ISAV and number and percentage (%) tested ISAV positive by RT-PCR (August 2005 to March 2007) or real-time RT-PCR (April 2007 to December 2009) throughout the study period from August 2005 to December 2009.

<table>
<thead>
<tr>
<th>Year</th>
<th>Kidneys</th>
<th>Gills</th>
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<tbody>
<tr>
<td></td>
<td>Total</td>
<td>ISAV positive</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>2005</td>
<td>2 998</td>
<td>0</td>
</tr>
<tr>
<td>2006</td>
<td>7 157</td>
<td>10</td>
</tr>
<tr>
<td>2007</td>
<td>6 505*</td>
<td>142</td>
</tr>
<tr>
<td>2008</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2009</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Total</td>
<td>16 660</td>
<td>152</td>
</tr>
</tbody>
</table>

*In January and February 2007 kidneys only were collected from 1118 fish.
detection methodology rather than a genuine increase in
the prevalence of ISAV-HPR0 (Table 1). During the study
period, a total of 49 production cycles were initiated of
which 36 (73.5 %) tested ISAV positive. Considering
completed production cycles only, an overall total of 27
of 31 (87 %) tested positive for ISAV (data not shown). Of
production cycles completed in 2009, for which the more
sensitive method of gill testing using real-time RT-PCR was
employed throughout, 100 % tested ISAV-HPR0 positive,
whereas completed production cycles which commenced
prior to the implementation of this improved screening
method did not all test positive (data not shown). Thus,
the actual prevalence of ISAV-HPR0 in Atlantic salmon
production cycles completed prior to 2009 has been
underestimated.

Sequencing of the HPR of the HE gene of 1–20 selected
samples from all 36 production cycles testing positive for
ISAV RNA revealed the presence of the assumed low-
pathogenic ISAV-HPR0 subtype in all cases.

**ISAV-HPR0 exhibits a different tissue tropism to
classical ISA**

Parallel testing of kidney and gill tissues for the presence of
ISAV-HPR0 from samplings where both gills and kidneys
tested positive by real-time RT-PCR showed a significantly
higher overall detection in gill tissue compared with kidney
(601 gills ISAV HPR0 positive versus 141 positive kidneys,
P<0.0001 Fisher’s exact test (two-sided); Table 2). Only 14
fish tested kidney positive and gill negative, and the highest
median C\(t\) values were observed among these samples
(median 35.9; range 31.4–36.5), whereas the lowest median
C\(t\) values were among the gills of the 127 samples testing
both kidney and gill positive (median 28.1; range 19.0–
36.7) (Table 2). From one of the sea-site samplings in 2007,
individual gill, kidney and heart samples from 80 Atlantic
salmon were tested in parallel for the presence of ISAV
RNA by real-time RT-PCR yielding HPR0 detection
prevalences of 75, 7.5 and 5 %, respectively (data not
shown), thus emphasizing the apparently different tissue
tropism and higher detection rate in gill tissue.

**ISAV-HPR0 infection is not associated with
clinical or pathological signs of ISA disease**

Despite the high prevalence of ISAV-HPR0 in Atlantic
salmon at marine grow-out farms, intensive surveillance
revealed no clinical or gross pathological signs consistent
with ISA disease. In fact, mortality in the marine grow-out
sites with Atlantic salmon proved historically low through-
out the study period, suggesting that the presence of ISAV-
HPR0 does not result in classical ISA disease or other signs
doing. Furthermore, histological examination of 111
gills, 78 hearts and 105 kidneys from three selected cases
with very low C\(t\) values showed no lesions indicative of
ISA disease. Although the gills often had focal, moderate
hypertrophy and hyperplasia of the lamellar epithelium, no
parasites or bacteria, and no major cellular inflammatory
cell response were observed. Histopathological findings
thus also support the fact that the presence of ISAV-HPR0
itself does not lead to the development of clinical or
pathological signs consistent with ISA disease.

**ISAV-HPR0 infections are transient and highly
contagious**

After the initial infection, the spread of ISAV-HPR0
throughout the population followed one of two typical
patterns. In the first pattern only one infection peak was
observed throughout the production cycle (Fig. 1a). A
second pattern was as shown in Fig. 1(b) with two infection
peaks. Looking at only the 18 completed production cycles
where gills were examined throughout production, 10
(56 %) showed one peak with a mean peak prevalence of
74 % (range 47–100 %) and six (33 %) showed two peaks
with a mean first peak prevalence of 56 % (range 30–73 %)
and a second peak prevalence of 64 % (range 29–85 %).
The mean time lag between the two peaks was 7.5 months

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**Table 2.** 2×2 table showing ISAV-HPR0 detection frequencies for 16 population samplings where at least one gill sample and one kidney sample was positive

<table>
<thead>
<tr>
<th>Gills</th>
<th>Kidneys</th>
<th></th>
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<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>480</td>
<td>14</td>
<td>494</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>35.9 [31.4–36.5]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>474</td>
<td>127</td>
<td>601</td>
<td></td>
</tr>
<tr>
<td></td>
<td>31.4 [18.6–37.9]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>954</td>
<td>141</td>
<td>1095</td>
<td></td>
</tr>
</tbody>
</table>

Sample samplings were conducted in 2007 and only include fish (n=1095) where both gills and kidneys were tested by real-time RT-PCR. Numbers in bold type are the median C\(t\) values and are followed by the range in square brackets. The threshold was set to 0.005.
(range 4–12 months). Only two production cycles (11%) showed a peak prevalence <20%. The lower peak prevalence of the cases with two peaks suggests that the second peak may indicate reinfection. Following these patterns of peaks in detection prevalence no further detections were made at the individual cage level, suggesting a transient appearance of ISAV-HPR0 in all cases with subsequent clearance of virus from the gills. Samplings with a 100% ISAV prevalence showed a mean Ct value of 28 (range 17–36) whereas samplings with a mean prevalence <20% gave a mean Ct value >34 (range 30–37) (data not shown).

**Marine production sites become infected with ISAV-HPR0 following seawater transfer**

The time period from seawater transfer of Atlantic salmon to first detection of ISAV-HPR0 infection is indicated in Fig. 1(c). ISAV-HPR0 was first detected in the marine production sites a mean of 8.5 months after seawater transfer (range 1–20 months). Considering sites where only gills were examined throughout the entire production cycle (2007–2009), the mean time to first detection was 7.7 months (range 1–13 months). Following the initial detection of ISAV-HPR0, the level of detectable virus peaked after a mean of only 2 months (range 1–5 months) with no further detection after a mean of another 2 months (range 1–4 months; data not shown). This strongly suggests a transient ISAV-HPR0 infection in most if not all production cycles of Atlantic salmon in the Faroe Islands.

**Infection with ISAV-HPR0 is seasonal**

The monthly prevalence of ISAV-HPR0 detections between 2007 and 2009 based on gill screening is indicated in Fig. 1(d). Over 35 months, prevalence of detection varied between 0 and 30% of the total number of fish tested per month. Detection of ISAV-HPR0 in gill tissue appeared to follow a seasonal pattern with the highest and lowest prevalences being recorded in the winter and summer periods, respectively.

**Different genotypes of ISAV-HPR0 are co-circulating in the Faroes**

The phylogenetic relationships between Faroese ISAV HE sequences derived from ISAV-HPR0 detection, disease outbreaks in the Faroes and those representing each of the established European subtypes (Nylund et al. 2007) are depicted in Fig. 2. These results indicate the presence of two distinct classes of ISAV-HPR0 in the Faroe Islands, classified as EU-G2-like and EU-NA-like isolates, respectively. The first group of ISAV-HPR0 Faroese sequences share a close genetic relationship with those derived from the ISAV isolates responsible for the Faroese ISA epidemic, whereas the second group is related to European-like ISAV isolated in North America.
DISCUSSION

The current study demonstrates a high prevalence of detection of the ISAV-HPR0 variant in association with Faroese Atlantic salmon aquaculture. While the presence of ISAV-HPR0 has previously been reported in other significant Atlantic salmon-growing nations including Norway (Nylund et al., 2007), Canada (Cook-Versloot et al., 2004), Scotland (Cunningham et al., 2002; McBeath et al., 2009) and Chile (Kibenge et al., 2009), this represents the first systematic and detailed study of its prevalence in Atlantic salmon aquaculture. Through combining the

Fig. 2. Phylogenetic radial tree showing the relationships between 12 Faroese ISAV sequences and 25 representative ISAV sequences from each of the four established major European-like subgroups (EU-G1–G3) circulating in Europe and the European-like subgroup circulating in North American (EU-NA) previously identified by Nylund et al. (2007). The analysis is based on 951 nt (nt 61–1012) of the ORF of the HE-encoding ISAV segment 6 gene. Branch length reflects genetic distance. The North American subgroup (NA) is not included in the tree. The Faroese sequences included in the study have the following strain numbers and GenBank accession numbers, respectively for each: FO/01/01/HPR13, AJ440970; FO/02/01/HPR3, AF526253; FO/03/01/HPR4, HQ664991; FO/05/01/HPR2, AY971656; FO/01/06/HPR0, HQ664992; FO/03/06/HPR00, HQ664993; FO/01a/07/HPR0, HQ664994; FO/01b/07/HPR0, HQ664995; FO/03a/07/HPR0, HQ664996; FO/03b/07/HPR00, HQ664997; FO/08/07/HPR00, HQ664998 and FO/01a/08/HPR0, HQ664999. Bar, 0.01 substitutions per nucleotide side.
proven sensitivity of real-time PCR with an intensive and optimized sampling method focusing on assaying gill tissue from individual fish, we have detected the presence of ISAV-HPR0 in up to 15.1% of individual fish tested and in association with up to 100% of Atlantic salmon production cycles completed in a given year.

Despite the high detection frequency of ISAV-HPR0, and a previous history of ISA problems, there was no evidence of clinical ISA in the Faroe Islands during the 4.5 year study period. Indeed, no disease signs were apparent even in individual fish harbouring relatively large quantities of ISAV-HPR0 virus. The fact that mortality during this period in Faroese aquaculture was historically low, no clinical or histopathological signs were observed consistent with ISA and no endothelial infections were found by immunohistochemistry (IHC), supports the conclusion that ISAV-HPR0 is a subclinical infection that in itself does not directly lead to an outbreak of ISA. Similar findings have been observed in Scotland, where the presence of ISAV-HPR0 was demonstrated without development of ISA (Anonymous, 2005; McBeath et al., 2009). These results suggest that ISAV-HPR0 differs fundamentally and phenotypically from classical ISAV, which causes disease in Atlantic salmon farms. The fact that an ISAV-HPR0 virus has, to date, not been isolated in conventional ISAV-permissive cell lines, coupled to the apparent different tissue tropism of this virus, supports such phenotypic difference. Thus our findings support previous reports concerning the low-pathogenic nature of ISAV-HPR0 virus.

Whereas highly pathogenic ISAV has been reported to be an endotheliotropic virus with a systemic distribution, ISAV-HPR0 detection was mainly confined to gills, with significantly less frequent detections in kidney tissue. Also, C1 values in ISAV-HPR0-positive kidneys were generally much higher than those found in gills. Thus, our results suggest that ISAV-HPR0 causes a localized infection of salmon gills with low pathogenicity and occasional systemic spreading, as opposed to the systemic and severe nature of the classical ISA disease. This is further supported by failure to demonstrate the presence of ISAV-HPR0-infected endothelial cells by IHC in any tissue examined, even in gills with real-time PCR C1 values <20 (data not shown). The transient and seasonal nature of respiratory infection by ISAV-HPR0 bears a striking similarity to the pattern of seasonal influenza in mammals. In contrast, the classical ISA disease seems to mirror the progression of highly pathogenic influenza infections, which, in chickens for example, exhibit an endothelial tropism and cause systemic disease (Feldmann et al., 2000; Klenk, 2005).

The high prevalence of ISAV-HPR0 and the absence of clinical ISA in the Faroes raises interesting questions regarding its origins. The demonstrated rapid increase in the prevalence of detection in marine farms suggests that ISAV-HPR0 is capable of rapid horizontal spread through local populations. The fact that individual sites become infected with ISAV-HPR0 a mean of 7.7 months following a endemic source during the marine phase, rather than the freshwater phase, of Atlantic salmon production. This study indicated two main patterns of the spread of infection in farms with pattern A (rapid increase in prevalence followed by a fast clearance of infection) plausibly supporting a single main input of infection from an external marine source. Pattern B might be suggestive of a second wave of infection affecting those fish that previously remained naïve. While a number of marine and salmonid fish have been reported as potential experimental carriers, including sea trout (Salmo trutta), rainbow trout (Oncorhynchus mykiss), coho salmon (Oncorhynchus kisutch), herring (Clupea harengus), saithe (Pollachius virens) and cod (Gadus morhua) (Nylund et al., 1993, 1997, 2002; Nylund & Jakobsen, 1995; Snow et al., 2001, 2002), the role of natural marine carrier species has not been exhaustively investigated.

Interestingly, ISAV-HPR0 detections have been made in freshwater and anadromous fish in Norway (Nylund et al., 2007), Scotland (Cunningham et al., 2002) and Chile (Vike et al., 2009), though these have been rare in comparison with detections in seawater-reared fish. Since vertical transmission has yet to be definitively demonstrated, the origins of these infections and thus the potential transmission pathways for ISAV-HPR0 remain uncertain. However, the time of infection post-seawater transfer, the localized infection of the gills, the transiency and seasonality of infection and the similarity to seasonal influenza in mammals all support horizontal spread, rather than vertical spread, as being the major dissemination route of ISAV-HPR0.

Arguably the most important question for fish-health managers regards the risk that the presence of low-pathogenic ISAV-HPR0 presents for the development of highly pathogenic ISAV and ISA disease. ISAV-HPR0 has been proposed to be the ancestor of highly pathogenic ISAV that is capable of causing disease in Atlantic salmon farms, and probably undergoes an adaptation event in association with intensive aquaculture. This is likely to be a consequence of the plasticity of the ISAV genome, which exhibits high mutation rates as a result of having non-proof-reading polymerases, can undergo recombination and reassortment (Markussen et al., 2008), and can also be subject to deletion events via a mechanism that is probably similar to that thought to occur in influenza viruses (Fodor et al., 2003). The virus is thus well equipped to adapt to the highly selective environment associated with aquaculture, which includes evolutionary pressures such as high host abundance and continuous availability, high rearing densities and exposure of naïve hosts to new pathogens. Such factors are known to drive the faster evolution of other fish RNA viruses within aquaculture, such as viral hemorrhagic septicemia virus (VHSV) (Einer-Jensen et al., 2004). In this case, adaptation of the virus to an alternate ‘pathogenic’ lifestyle has occurred several times within aquaculture (Einer-Jensen et al., 2004; Dale et al. 2009). In the wild, owing to a lack of available hosts, such a strategy would
probably represent an evolutionary dead end and lead to viral extinction.

This study supports the likely origins of classical disease-causing ISAV as being an endemic and low-pathogenic ISAV-HPR0 that has undergone adaptation in association with Atlantic salmon aquaculture. Indeed, phylogenetic analysis of Faroese HPR0-ISAV sequences identified a lineage with close genetic similarity to ISAV associated with the disease epidemic. Furthermore, the fact that a diverse spectrum of ISAV-HPR0 viruses have been characterized that reflect all of the major European-identified genetic types of pathogenic viruses reported to date adds support to this hypothesis.

The presence of ISAV-HPR0 on a farm probably presents some risk of the potential emergence of a pathogenic strain of ISAV. Studies in countries such as the Faroes and Scotland, where highly pathogenic ISAV is not endemic, suggest that the level of this risk is low and can be further reduced through the implementation of appropriate management regimes. Importantly, cases of ISA emergence in both 1998 and 2009 in Scotland were associated with management areas where synchronous falling had not been practised. Prior to the ISA epidemic in the Faroes, continuous and intensive production with no strict hygiene barriers was also practised, highlighting the potential importance of these factors in providing an opportunity for ISA emergence. Since the implementation of improved management policies addressing these factors during the five years since the industry was re-established in the Faroes, there has been no recurrence of ISA disease despite the continued presence of ISAV-HPR0. Widespread ISAV-specific vaccination has been employed in the Faroes but not in Scotland; therefore its role in limiting the re-emergence of ISA is unclear. Although our results suggest that vaccination did not protect against subclinical infection by ISAV-HPR0, the potential role of vaccination in protecting against newly emerging pathogenic strains is difficult to determine.

In summary, this study has demonstrated that infection with high levels of ISAV-HPR0 is not associated with the development of clinical disease in marine farmed Atlantic salmon. Intensive surveillance of the Faroese industry over a five-year study period has indicated that ISAV-HPR0 is highly infectious in some circumstances and constitutes a seasonally influenced and transient infection. Current evidence based on the time between seawater transfer and infection in marine farms, the apparent efficiency of transmission within farms, the transient and seasonal nature of infection and the existence of multiple sequence variants all point towards unidentified marine sources as being significant in the introduction and maintenance of ISAV-HPR0 within marine farms. Maintenance of ISAV HPR0 infection probably constitutes a low risk for disease emergence, which has been effectively managed in both the Faroes and in Scotland through appropriate husbandry. Effective monitoring and subsequent containment of new emergences remain important supportive strategies in ensuring that ISA does not re-emerge and become established in these areas.

**METHODS**

**Study overview.** Within the Faroe Islands, 25 marine sites (epidemiological zones) with a minimum separation distance of 5 km have been defined. Following EU approval of the contingency plan for control of ISA in the Faroe Islands, ISAV-specific vaccination of all Atlantic salmon for human consumption commenced in April 2005. To monitor re-emergence of the ISA disease 4 to 6 test pens holding Atlantic salmon were defined at all marine farms producing Atlantic salmon. The study is based on samples collected from these control cages over a period of 53 months between August 2005 and December 2009.

**Sample collection.** From each of the test pens with ISAV-vaccinated and unvaccinated Atlantic salmon, individual tissue samples from randomly sampled, seemingly healthy fish were collected. For all sites a mean of 69 fish per sampling were sampled a mean of 8.6 times per year (5–12 samplings per year). In 2005 and 2006 only kidney samples were collected. Based on experiences from Norway and Scotland (A. Nylund and M. Snow, personal communication), individual kidney and gill samples were collected and examined in parallel in 2007. Following examination of these results, only gill samples were screened for the presence of ISAV in 2008 and 2009. Small tissue samples (20 mg±10 mg) from each fish were placed in 500 μl RT-Lyse buffer (Qiagen) for RNA extraction. All samples were placed on ice immediately after collection, transported to the Faroese National Reference Laboratory in refrigerated transportation boxes and either processed directly or stored at −20 °C until processed further. Scalpels and forceps were cleaned with 1.5% Virkon S (DuPont) or with Clinet 101 (Interscience) between individual samples and replaced after organ collection from five fish. Samples of gill, heart and kidney tissue were collected and fixed in 10% neutral buffered formalin.

**Histopathology.** Formalin-fixed tissue samples were processed and embedded in paraffin wax according to standard procedures. Sections (4–6 μm) were stained with haematoxylin and eosin and examined by light microscopy.

**RT-PCR and real-time RT-PCR.** Total RNA was extracted from tissue samples using an RNasey 96 mini kit (Qiagen) following minor modifications to the manufacturer’s protocol and was used directly in RT-PCR without quantification. In 2005 and 2006 a doubleplex one-step RT-PCR (Qiagen) assay was developed and was used for ISAV screening. An ISAV segment 8-specific fragment of 155 bp was amplified using primers ISAs8-1F (5’-GGCTATCTCATCATGGAAC- AATC-3’) and ISAs8-1R (5’-CCGCAAATCTGAAATGACTCACT-3’) (Mjalaand et al., 1997). In addition, the reaction mixture included the following primers MHG IIβ-1F: 5’-TCAGATTCACAGCAGC- TGCG-3’ and MHG IIβ-1R: 5’-TACATACGCCACCGAAAGAC-3’ (DNAtotechnology) for amplification of a 577 bp specific fragment of the Atlantic salmon MHG IIβ gene (GenBank accession no. X70167) as an internal control (IC). The reaction mixture was recommended (Qiagen) and included ISAV-specific primers at a final concentration of 1 μM each, IC specific primers at a final concentration of 0.1 μM each and 1 μl total RNA (approx. 25–200 ng) in a total volume of 10 μl. PCR was performed with an ABI 2700 Thermocycler using the following conditions: cDNA synthesis at 50 °C for 30 min and activation of HotStart Taq polymerase at 95 °C for 15 min followed by 40 cycles of 94 °C for 30 s, 62 °C for 30 s and 72 °C for 60 s.

A doubleplex one-step real-time RT-PCR assay was used to screen for ISAV from April 2007 onwards using a QuantiTect Probe RT-PCR kit.
The reaction mixture was as recommended by the manufacturer (Qiagen) and included IC primer and probe mixture at final concentrations of 0.04 and 0.1 μM, respectively, ISAV specific primer and probe mix at final concentrations of 1 and 0.2 μM, respectively, and 1 μl total RNA (approx. 25–200 ng) in a total volume of 10 μl. PCR was performed with a 7500 Fast real-time PCR System (Applied Biosystems) and consisted of cDNA synthesis at 50 °C for 30 min and activation of HotStart Taq polymerase at 95 °C for 15 min followed by 40 cycles of denaturation at 94 °C for 15 s and annealing/extension at 60 °C for 60 s. Primers and TaqMan probe mix at final concentrations of 1 and 0.2 μM, respectively, were transferred to the unrooted tree derived from the original data.

Sequencing. To confirm the presence of ISAV RNA the whole or only the HPR of ISAV segment 6 was amplified by one-step RT-PCR. Purified PCR products were subjected to direct cycle sequencing using a BigDye Terminator sequencing kit (Applied Biosystems), and were transferred to the unrooted tree derived from the original data. The authors are indebted to the inspectors, Anja K. Olsen, Elin Jacobsen, Guðrun K. Olsen, Ingi Joensen, Marita D. Magnussen, Maiken Neshamar, Tina Ziska, Øssur Hansen and Vinnie Fuglø, for collecting all of the samples and to the technicians Ann Siri Borg Henze, Beinta Ejdesgaard, Kristin Baldvinssdóttir and Marita Nyes, for excellent technical assistance in analysing all of the many samples.

Molecular epidemiological analysis. Sequences were imported into BioEdit version 7.0.5.3 (Hall, 1999) alongside other publicly available ISAV sequences (Nylund et al., 2007; McBeath et al., 2009) and a multiple alignment was performed using CLUSTAL X (Larkin et al., 2007). Identical sequences were identified and only a single representative of each sequence type retained in the dataset to reduce subsequent analytical bias. The final alignment consisted of 37 unique sequences spanning a region of 951 nt of the HE gene (positions 61–1012 with respect to the ORF of ISAV segment 6). The phylogenetic relationship among the ISAV isolates was inferred using a maximum-likelihood based approach implemented within PAUP* (version 4.0; Swofford, 2000) and using the PaupUP interface v1.0.3.1 (Calendini & Martin, 2005). The jModeltest 0.1.1 program (Posada, 2008) was used to identify the model that best fits the sequence data from 56 models using the Akaike information criterion (Akaike, 1974). The model selected was TIM1 + G and is defined as: assumed base frequencies A = 0.2844, C = 0.2016, G = 0.2669, T = 0.2470; Rate matrix: A–C, 1.0000; A–G, 3.9149; A–T, 0.1246; C–G, 0.1246; C–T, 5.6533; G–T, 1.0000; gamma 0.2190. An optimal unrooted maximum-likelihood tree (Fig. 2) was identified using a heuristic search implemented in PAUP* and evaluated using 100 bootstrap iterations (Felsenstein, 1985). Significant bootstrap values for the major clades were transferred to the unrooted tree derived from the original data.

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